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Expression and Function of *Xmsx-2B* in Dorso-Ventral Axis Formation in Gastrula Embryos

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ABSTRACT—Msx is a homeodomain-containing transcriptional factor that plays an essential role in pattern formation in vertebrata and invertebrata embryos. In *Xenopus laevis*, two *msx* genes have been identified (*Xmsx-1* and *Xmsx-2*). In the present study, we attempted to elucidate the expression and function of *Xmsx-2B* (formerly designated as *Xhox7.1*') in early embryogenesis. Whole mount *in situ* hybridization analyses showed that the expression pattern of *Xmsx-2B* at gastrula and neurula stages was very similar to that of *Xmsx-1*: the transcript of *Xmsx-2B* was observed in ventral and lateral sides of the embryo. At the tailbud stage, however, the expression pattern of *Xmsx-2B* in neural tissues was distinct from that of *Xmsx-1*. An RNA injection experiment revealed that, like *BMP-4, Xmsx-2B* has a strong ventralizing activity. In the *Xmsx-2B* -injected embryos, differentiation of axial structures such as the notochord, muscle, and neural tissue was completely suppressed, whereas *α-globin* mRNA, a blood cell marker, was highly expressed. Simultaneous injection of *Xmsx-1* and *Xmsx-2B* RNAs showed that they function in an additive manner. It was also shown that coinjection of *Xmsx-2B* with a dominant-negative *BMP-4 receptor* (*tBR*), which can induce formation of secondary axis when injected alone in ventral blastomeres, suppressed secondary axis formation. Furthermore, *Xmsx-2B* also suppressed secondary axis formation, which was induced by a dominant-negative form of *Xmsx-1* (*VP16/msx-1*). Therefore, like *Xmsx-1*, *Xmsx-2B* is a downstream nuclear factor of the BMP-4-derived ventralizing signal, and these two factors probably share the same target molecules. In conclusion, *Xmsx-1* and *Xmsx-2B* function in dorso-ventral axis formation in early *Xenopus laevis* development.

INTRODUCTION

A number of studies have indicated that BMP-4 (bone morphogenetic protein-4), a member of the TGF- β superfamily, is a key regulator in body planning, especially in dorso-ventral patterning of mesodermal tissues and in neural tissue differentiation, in the *Xenopus laevis* embryo (Dale *et al*., 1992; Jones *et al*., 1992; Fainsod *et al*., 1994; Graff *et al*., 1994; Maéno *et al*., 1994; Suzuki *et al*., 1994; Schmidt *et al*., 1995). Recently, homeodomain-containing transcriptional factors that mediate the BMP-4 signal have been isolated and analyzed: Xmsx-1 (Maeda *et al*., 1997; Suzuki *et al*., 1997), Xvent-1 (PV. 1) (Gawantka *et al*., 1995; Ault *et al*., 1996), Xvent-2 (Xbr-1, Vox, Xom) (Onichtchouk *et al*., 1996; Papalopulu and Kintner 1996; Ladher *et al*., 1996) and Xvex-1 (Shapira *et al*., 1999). We and other investigators have revealed that *Xmsx-1* is expressed in the prospective ventral ectoderm and mesoderm at mid to late gastrula stages and that it has a ventralizing activity if its RNA is injected into the prospective dorsal region (Maeda *et al*., 1997; Suzuki *et al*., 1997). In ectodermal cells, *Xmsx-1* functions as an inhibitor of neurogenesis, and it has been shown that *Xmsx-1* is a direct target gene of the BMP-4 signal (Suzuki *et al*., 1997). More recently, we have demonstrated that *Xmsx-1* is an upstream factor of Xwnt-8 in the ventralizing signal cascade and a possible direct repressor of organizer genes such as *goosecoid* (Takeda *et al*., 2000).

In comparison to the numerous studies on *Xmsx-1*, quite a few studies have been carried out on the function of Xmsx-2 (Su *et al*., 1991; Gong and Kiba, 1999). In other species, msx-2 has been studied extensively in various developing tissues such as bone, tooth, and limb bud (Monachan *et al*., 1991; Coelho *et al*., 1991; Mackenzie *et al*., 1992; Jowett *et al*., 1993). Most likely, the function of msx-2 overlaps with that of msx-1 (Semenza *et al*., 1995), but in some cases msx-2 may have distinct expression patterns and functions. For instance, expression domains of *msx-1* and *-2* overlap in some regions but are discrete along with anterior-posterior identity in chicken and mouse limb buds (Davidson *et al*., 1991; Coelho *et al*., 1991; Muneoka and Sassoon, 1992; Nohno *et al*., 1992). The BMP-4/msx-2 signal is involved in elimination of cranial neural crest cells in rhombomeres 3 and 5 (Graham *et al*., 1993; Graham *et al*., 1994; Takahashi *et al*., 1998; Farlie *et al*., 1999) and the interdigit regions of developing limbs (Zou and Niswander 1996; Ganan *et al*., 1996; Ferrari *et al*., 1998) by mediating apoptosis. Furthermore, the mechanisms of expressional control in mouse mammary development are dif-

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ferent between *msx-1* and *-2* (Friedmann *et al.*, 1996; Phippard *et al.*, 1996).

These observations suggest that msx-2 could have a distinct expression pattern and function in the early development of *Xenopus laevis*. In spite of the importance of the issue, expressional and functional natures of msx-2 in *Xenopus* embryogenesis are poorly understood. *Xenopus msx-2* (*Xhox7.1*') was first isolated by Su *et al.* (1991). In their report, however, major attention was paid to the expression of *Xmsx-1*. Afterward, no report has described the expression and function of *Xmsx-2* except for a very recent one in which a related but distinct gene from *Xhox7.1*' was described (Gong and Kiba, 1999). In this study, we attempted to elucidate the expression and the function of *Xhox7.1*'. We propose designating *Xhox7.1*' as *Xmsx-2B* in order to distinguish it from *Xmsx-2*, which was isolated by Gong and Kiba (1999). Our results showed that there is a strong correlation between the expression patterns of *Xmsx-1* and *Xmsx-2B* at gastrula to neurula stages and that those two genes play a role in the establishment of the dorso-ventral axis in developing *Xenopus* embryos.

MATERIALS AND METHODS

Cloning and cDNA construction

Xmsx-2B cDNA fragments of two different sizes were amplified by polymerase chain reaction (PCR) from *Xenopus* stage10 cDNA. A short fragment including 402 N-terminal base pairs was used as a probe for *in situ* hybridization, and another fragment including the whole protein-coding region (815 bp) was used for an RNA injection assay. The primer sequences for the PCR were as follows: 5'-TC T-GCG-CCC-CTT-TAC-TCA-CCG-CA-3' and 5'-TCA-AAG-TGC-AAG-AGG-ATG-GGC-TCA-3' for the 402-bp fragment; and 5'-TCT-GCG-CCC-CTT-TAC-TCA-CCG-CA-3' and 5'- CTA-TGT-CAT-GTC-ACC-CTC-CTC-AGA-TA-3' for the 815-bp fragment (Su *et al.*, 1991). Each product was ligated into pCRTM II (*In vitro* gene) for subcloning, and the DNA sequence of the inserts was determined using an Auto Read Sequencing kit (ALF DNA Sequencer, Pharmacia Biotech). The obtained sequence was completely identical to that of *Xhox-7.1*', which was previously reported (Su *et al.*, 1991). For injection of sense RNA of *Xmsx-2*, an 815-bp fragment including an open reading frame was excised with Not I, blunt ended, and ligated into a blunt-ended pCS2+ expression vector.

Embryos, mRNA micro-injection, and DAI judgement

Xenopus laevis embryos were obtained by artificial insemination after induction of females with 250 i.u. of human chorionic gonadotropin. Fertilized embryos were dejellied with 2.5% thioglycolic acid (pH8.3) and washed several times in 5% MMR. After the embryos were transferred into 3% Ficoll/Steinberg's solution, equatorial regions of either two ventral or dorsal blastomeres at the 4-cell stage were injected with mRNAs using a Nanoject microinjector (3.7-6 nl/embryo). *pCS2*+ plasmids containing *Xmsx-1* whole cDNA (1.8 kb) or the 815-bp *Xmsx-2B* fragment were linearized with Xho I, and capped mRNA was synthesized using SP6 polymerase (Ambion). *VP16/msx-1*, a dominant-negative form of *Xmsx-1*, has been described previously (Takeda *et al.*, 2000). Injected embryos were incubated in 50% Steinberg's solution at 23°C until the sibling embryos reached stage 33/34 for determining DAI (dorso-anterior index) as described by Kao and Elinson (1988). Developmental stages were designated according to Nieuwkoop and Faber (1967).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described previously (Shain *et al.*, 1996). Digoxygenin-labeled antisense mRNA probes were synthesized from the following plasmids: *Xmsx-1*, an N-terminal fragment (EcoRI/HindIII, 440 bp) in pSP72; and *Xmsx-2B*, an N-terminal fragment (402 bp) in pCRTMII. The former plasmid was linearized with EcoRI, and RNA was synthesized by SP6 polymerase. The latter plasmid was linearized with BamHI, and RNA was synthesized by T7 polymerase. The positive signals were visualized using BM purple (Moors *et al.*, 1995). For neural marker probes, fragments of *slug* and *krox-20* were amplified by the PCR method from the cDNAs of stage18 whole embryo and of stage 27 head tissue, respectively. The primer sequences for the PCR were as follows: 5'-GAA-TCT-GGT-TGC-TGT-GTA-G-3' and 5'-CGT-TGC-TGG-ATT-GTC-TAG-GC-3' for *slug*; and 5'-CCA-ACC-GCC-CCA-GTA-AGA-CC-3' and 5'-GTG-TCA-GCC-TGT-CCT-GTT-AG-3' for *krox-20*. Both fragments were subcloned into a pCR2.1 vector (*In vitro* gene), and their DNA sequences were determined. For making an antisense RNA probe, the plasmids were cut with BamHI, and RNAs were synthesized by T7 polymerase.

RESULTS

Expression pattern of *Xmsx-2B* in the early embryo

To investigate the expression pattern of *Xmsx-2B* during embryogenesis and to compare it with that of *Xmsx-1*, whole-mount *in situ* hybridization analysis was performed using various stages of albino embryos. As shown in Fig.1, during gastrula and early neurula stages, the *Xmsx-2B* transcript was observed in the ventral and lateral marginal zones and animal pole area (Fig.1 B, D and F), as was also found in the case of *Xmsx-1* (Maeda *et al.*, 1997; Suzuki *et al.*, 1997; Fig.1A, C and E). At the neurula stage (stages 16–17), two parallel lines of transcript were observed at the dorsal side along the border between the prospective neural tissue and epidermis (arrowheads in Fig. 1G and H), and also strong spot signals were detected on the medial and lateral sides of the prospective neural crest (arrows in Fig. 1 G and H), with reference to the expression of *slug*, a neural crest marker (Mayor *et al.*, 1995; Fig. 1 J). We also compared the site of expression with that of *krox-20*, a marker of rhombomeres 3 and 5 (Bradley *et al.*, 1992; Fig.1 I and L), and we found that strong spot signals were located at the level of the hind brain (rhombomeres 3-5) (Fig. 1 H and K). At the early tailbud stage (stage 23), expression patterns became different between *Xmsx-1* and *Xmsx-2B* in the neural tissue (Fig.1 M, N, P and Q). *Xmsx-1* was highly expressed in the broad areas including fore-, mid- and hind-brains, according to the expression of *krox-20* (Fig.1 O and R), and spinal cord, while *Xmsx-2B* was expressed in the medial region of neural tissues. These observations suggest that *Xmsx-1* and *Xmsx-2B* have the same or similar functions in gastrula stages but have different functions after the neurula stage.

Xmsx-2B has a ventralizing activity

It has been shown in previous studies that *Xmsx-1* has ventralizing and anti-neuralizing activities in early embryogenesis (Maeda *et al.*, 1997; Suzuki *et al.*, 1997; Ishimura *et al.*, 2000; Takeda *et al.*, 2000). To examine whether *Xmsx-2B*

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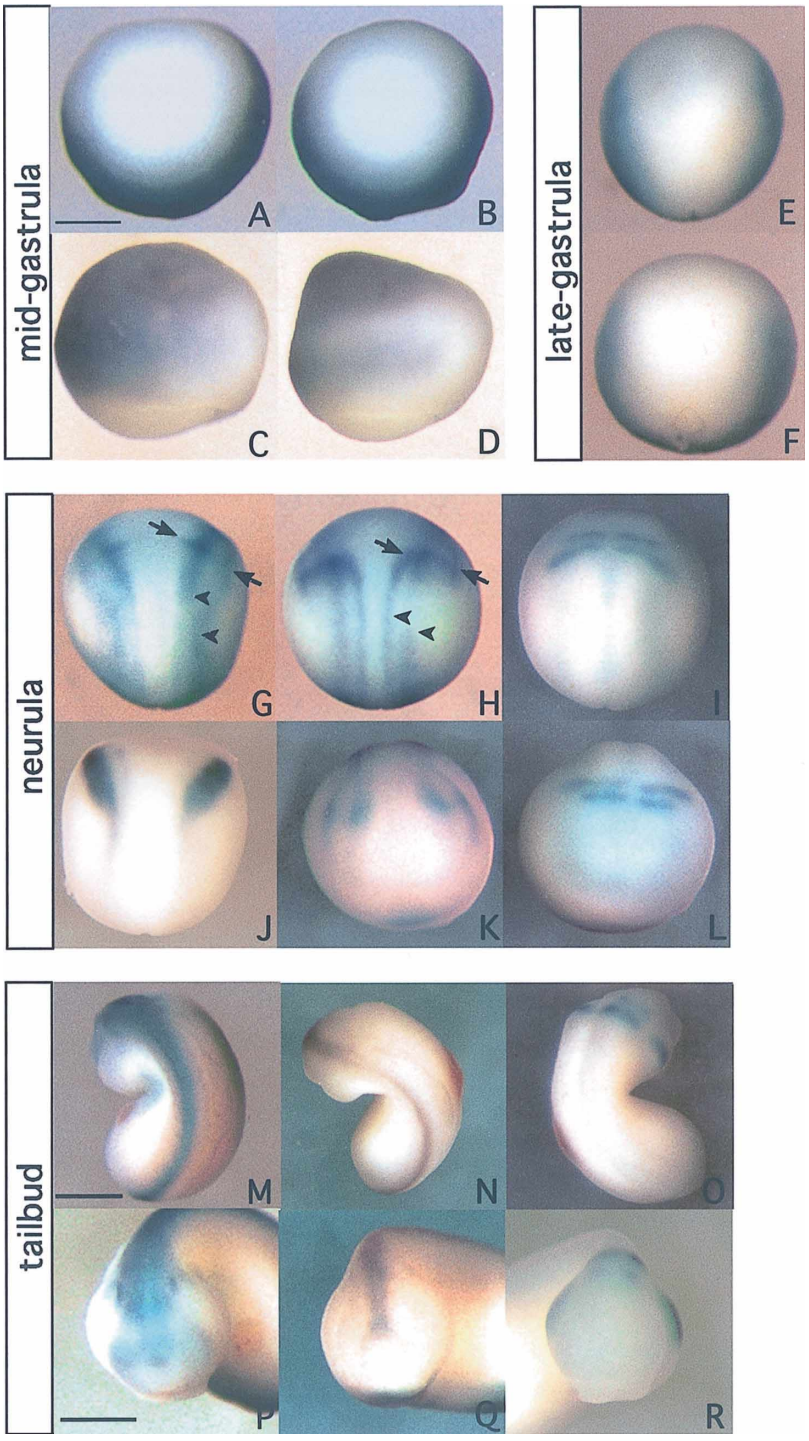
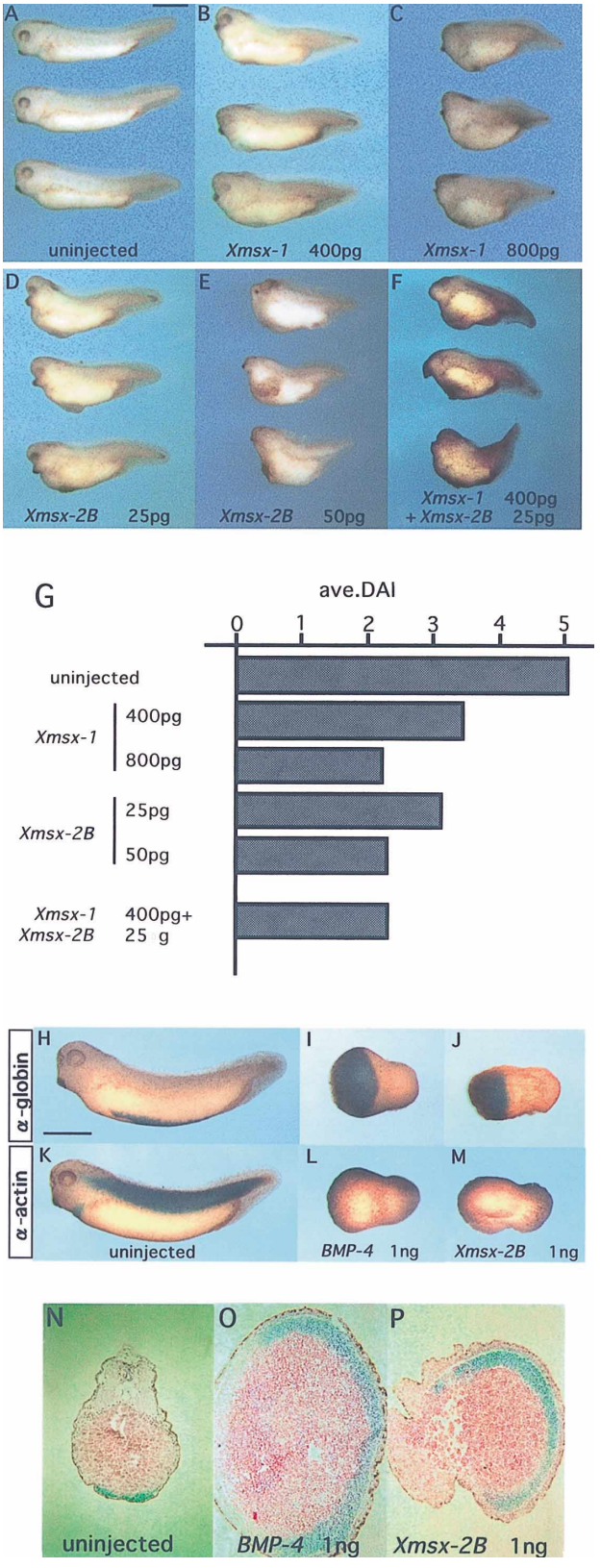


Fig. 1. Whole-mount *in situ* hybridization showing the expressions of *Xmsx-1* (A, C, E, G, M, P), *Xmsx-2B* (B, D, F, H, K, N, Q), *slug* (a neural crest marker) (J), and *krox-20* (a hindbrain marker) (I, L, O, R). At the mid gastrula stage (st.12), *Xmsx-1* and *Xmsx-2B* mRNAs are expressed in the ventral and lateral marginal zone (A–D) (A and B, vegetal view and dorsal side is toward the top; C and D, lateral view and dorsal side is to the right). At the late gastrula stage (st.13), transcripts are accumulated in the lateral regions adjacent to the presumptive neural fold (E, F). (E and F, dorsal view and anterior is toward the top). At the neurula stage (st.16–17), transcripts are detected along the border lines of prospective neural and non-neural tissues (arrowheads in G and H), and high levels of expressions are observed on the medial and lateral sides of the prospective neural crest cells (arrows in G and H), as shown by *slug* expression (J). These regions correspond to the level of the prospective hindbrain (rhombomeres 3-5), with reference to the expression of *krox-20* (I and L) (G–J, dorsal view and anterior is toward the top; K and L, anterior view and dorsal side is toward the top). At the tailbud stage (st.23), the expression patterns of *Xmsx-1* and *Xmsx-2B* in neural tissue become clearly distinct (M, N, P and Q). *Xmsx-1* is expressed in broad areas, including the fore-, mid- and hindbrains and the spinal cord, while *Xmsx-2B* is expressed in a narrower region of neural tissues. (M-O, dorsal view and anterior is toward the top; P-R, anterior view and dorsal side is toward the top). Bars in A, M and P indicate 0.5 mm.

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has the same or similar activity, we performed mRNA injection experiments. Two dorsal blastomeres of 4-cell stage embryos were injected with various concentrations of mRNAs encoding for the full-length *Xmsx-2B* protein, and the injected embryos were incubated for 2 days until the sibling embryos reached the tailbud stage. The embryos injected with *Xmsx-2B* mRNA showed ventralized phenotypes and the average DAI was reduced in dose-dependent manner (Fig. 2 D, E, G, J and M). This activity of *Xmsx-2B* seemed very strong among the known ventralizing agents, and as little as 10 pg of mRNA was enough to exert a ventralizing effect (average DAI=4.3, n=154; data not shown). Injection of 1ng RNA led to a hyperventralized phenotype completely lacking the dorso-ventral axis (average DAI=0.3, n=98; Fig. 2 J, M). The phenotype induced by *Xmsx-2B* overexpression was very similar to that obtained by the injection of *BMP-4* mRNA (average DAI=0.1, n=92; Fig. 2 I, L). In these embryos, ectodermal, mesodermal and endodermal layers were clearly distinguished, but differentiation of dorsal tissues such as the notochord, neural tube or muscle was not observed (Fig.2 O and P). Furthermore, *in situ* hybridization analysis indicated that in these hyperventralized embryos, α-*globin* mRNA was strongly expressed in the anterior region (Fig. 2 I, J) but α-*actin* was not (Fig. 2 L, M).

Since the phenotypes of the embryos injected with *Xmsx-2B* and *BMP-4* mRNA were similar, we examined the blood cell-inducing ability of *Xmsx-2B* in the dorsal marginal zone (DMZ) explants. After injection of several concentrations of *Xmsx-2B* mRNAs (200–800 pg/embryo) into the marginal zone

Fig. 2. Ventralizing activities of Xmsx-1 and Xmsx-2B. A-F. Typical ventralizing phenotypes obtained in the mRNA injection experiments. Two dorsal blastomeres of 4-cell stage embryos were injected with designated amounts of RNAs, and the embryos were allowed to develop until stage 33/34 to determine the DAI (dorso-anterior index; Kao and Elinson, 1988). Injection of 400 pg of *Xmsx-1* (B) and 25 pg of *Xmsx-2B* (E) led to a moderate ventralizing phenotype (average DAIs being 3.1 and 3.5, respectively). Injection of 800 pg of *Xmsx-1* (C) and 50 pg of *Xmsx-2B* (E), which are twice the amounts of B and E, resulted in a severely ventralized phenotype (average DAIs being 2.2 and 2.3, respectively). The embryos coinjected with 400 pg of *Xmsx-1* and 25 pg of *Xmsx-2B* (F) showed a similar degree of ventralization to that of C and E. G. Average DAI and SD of RNA-injected embryos are represented by a bar graph. The numbers of embryos injected in each groups were as follows: n=157 (uninjected), n=185 (*Xmsx-1* 400 pg), n=77(*Xmsx-1* 800 pg), n=122 (*Xmsx-2B* 25 pg), n=152 (*Xmsx-2B* 50 pg) , and n=140 (*Xmsx-1* 400 pg+ *Xmsx-2B* 25 pg). Note that both *Xmsx-1* and *Xmsx-2B* had ventralizing activities and that their effects were additive. H-P. Hyperventralized phenotype (DAI=0.3, n=98) induced by a high dose of *Xmsx-2B* RNA. Embryos were injected with 1ng of *Xmsx-2B* RNA (J, M) or 1ng of *BMP-4* RNA as a control (I, L), and they were allowed to develop until the sibling embryos reached st.33/34 (H, K). Whole-mount i*n situ* hybridization was performed to show the expressions of*α-globin* (H, I and J) and *α-actin* (K, L and M). The expression patterns of these markers in the embryos, which had been injected with *BMP-4* and *Xmsx-2B,* were quite similar. N, O, and P represent histological views of embryos shown in H, I and J. Note that no axial structure was observed in *BMP-4* or *Xmsx-2B* mRNA-injected embryos, and, instead, *α-globin*-positive cells were dramatically increased in comparison with those in uninjected embryos. Bars in A, H and N indicate 1 mm, 1 mm, and 0.5 mm respectively.

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Talble 1. Incidence of secondary axis formation in injected embryos

mRNA	Number of injected embryos	Secondary axis (%)
tBR	127	56
tBR+Xmsx-1	112	0
tBR+ Xmsx-2B	117	0
VP16/msx-1	95	65
VP16/msx-1+Xmsx-1	81	0
VP16/msx-1+ Xmsx-2B	118	0

The experimental protocol is described in the legend of **Fig. 3.** Note that *Xmsx-1* and *Xmsx-2B* completely suppressed formation of the secondary axis induced by *tBR* (a dominant-negative form of the BMP-4 receptor) and *VP16/msx-1* (a dominant-negative form of Xmsx-1).

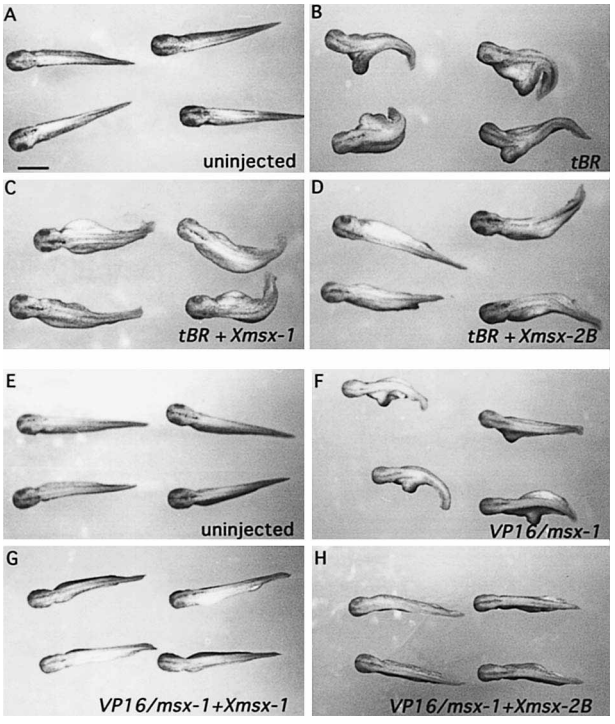


Fig. 3. A-D. Injection of *Xmsx-1* or *Xmsx-2B* mRNA suppressed the secondary axis formation induced by *tBR*, a dominant-negative form of the BMP-4 receptor. Two ventral blastomeres of 4-cell stage embryos were injected with *tBR* (500 pg) RNA (B), *tBR* (500 pg) with *Xmsx-1* (1000 pg) RNA (C), and *tBR* (500 pg) with *Xmsx-2B* (200 pg) RNA (D). The embryos were allowed to develop until st.33/34, and the existence of secondary axis was determined morphologically. E-H. Injection of *Xmsx-1* or *Xmsx-2B* mRNA suppressed the secondary axis formation induced by *VP16/msx-1*, a dominant-negative form of Xmsx-1. The embryos were injected with *VP16/msx-1* (600 pg) RNA (F), *VP16/msx-1* (600 pg) with *Xmsx-1* (200 pg) (G), *or VP16/msx-1* (600 pg) with *Xmsx-2B* (200 pg) (H). Bar in A indicates 1mm.

of dorsal blastomeres, DMZs were excised at stage10.5 and cultured until stage 39. Northern blot analysis showed that α-*globin* mRNA was not induced in the explants even though the highest amount of mRNA was injected (data not shown). Thus, we concluded that among the target genes of the BMP-4 signal, *Xmsx-1* and *Xmsx-2B* are not sufficient for blood cell differentiation.

To investigate whether *Xmsx-1* and *Xmsx-2B* act cooperatively, these RNAs were injected alone or simultaneously into two dorsal blastomeres (Fig.2 B-G). The average DAI obtained by 25 pg of *Xmsx-2B* mRNA was 3.1±1.4 (n=122), whereas that of embryos that received 400 pg of *Xmsx-1* mRNA was 3.5±1.1 (n=185). When these mRNAs were coinjected, the DAI fell to 2.3±1.0 (n=140). Since similar values of DAI were obtained in the embryos injected with 50 pg *Xmsx-2B* (DAI=2.3±1.4; n=152) or 800 pg *Xmsx-1* (DAI=2.2±1.2; n=77), we concluded that *Xmsx-1* and *Xmsx-2B* function in ventralization in an additive manner.

Xmsx-2B mediates BMP-4 signaling

It has been shown that the expression of *Xmsx-1* is regulated by the BMP-4 signal (Maeda *et al*., 1997; Suzuki *et al*., 1997). Taken together with these expression patterns of *Xmsx-2B* in the early embryo (Fig. 1), it seems that *Xmsx-2B*, like *Xmsx-1*, is regulated by the BMP-4 signal. To confirm this hypothesis, we examined whether *Xmsx-2B* could disturb the formation of the secondary axis, which was induced by *tBR* mRNA injection. Two ventral blastomeres of 4-cell stage embryos were injected with 500 pg of *tBR* mRNA alone or coinjected with 1ng of *Xmsx-1* or 200 pg of *Xmsx-2B*. As shown in Table 1 and Fig.3 B, 56 % (n=127) of *tBR*-injected embryos formed the secondary axis. In contrast, the formation of the secondary axis was completely suppressed if either *Xmsx-1* (n=112) or *Xmsx-2B* (n=117) was coinjected with *tBR* mRNA.

We also examined whether the ventralizing activity of *Xmsx-1* can be replaced by that of *Xmsx-2B*. For this purpose, we used a fusion construct of *Xmsx-1* and VP16, a dominant-negative form of *Xmsx-1*, which was shown to induce formation of the secondary axis (Takeda *et al*., 2000). The embryos injected ventrally with *VP16/msx-1* mRNA formed an incomplete secondary axis without a head structure (65%; n=95, Fig.3 F). Simultaneous injection either of *Xmsx-1* (Fig.3 G; n=81) or *Xmsx-2B* (Fig.3 H; n=118) completely suppressed the formation of the secondary axis. These results provide us with evidence that the activity of *Xmsx-1* can be replaced by that of *Xmsx-2B* and, therefore, these two transcriptional factors share the same target molecules in the ventralizing signal.

DISCUSSION

The expression of *Xmsx-2B* overlaps that of *Xmsx-1* in gastrula and neurula stages

Since Su *et al*. (1991) first reported the isolation of an *Xhox7.1*' (*Xmsx-2B*) cDNA clone in *Xenopus laevis*, there have been no reports describing the expression and function of *Xenopus msx-2*, despite the general view of importance in various vertebrate embryogenesis. Very recently, however, Gong and Kiba (1999) isolated a *msx-2* cDNA clone and emphasized its function in antero-posterior patterning of mesoderm formation. From the viewpoints of sequence similarity, expression pattern, and biological activity, *Xmsx-2B* and the cDNA isolated by Gong and Kiba (1999) are subtypes of *Xenopus msx-2*.

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As expected, the expression pattern of *Xmsx-2B* was very similar to that of *Xmsx-1* (Maeda *et al.*, 1997; Suzuki *et al.*, 1997) during gastrula and neurula stages. Concomitant expressions of *msx-1* and *-2* have been reported in various tissues in mammalian species (Mackenzie *et al.*, 1992; Jowett *et al.*, 1993; Wang and Sassoon, 1995; Friedmann *et al.*, 1996; Phippard *et al.*, 1996) and in birds (Coelho *et al.*, 1991; Ganan *et al.*, 1996; Ganan *et al.*, 1998). At the gastrulation stage, the transcript was detected in the lateral and ventral marginal zone (Fig.1), suggesting that *Xmsx-2B* and *Xmsx-1* have the same or similar functions at this stage. At the tailbud stage, however, the expression pattern in neural tissues was clearly distinct. We suggest that the mechanism of expressional control in the two msx genes is conserved at least in early embryogenesis.

Xmsx-2B acts as a ventralizing factor

As suggested by the expression pattern of *Xmsx-2B* in gastrulating embryos, the forced expression of *Xmsx-2B* mRNA in dorsal blastomeres induced a strong ventralized phenotype (Fig. 2 J, M), and *α-globin* mRNA was strongly expressed in the embryos (Fig. 2 J). By analyzing the ventralizing activity of *Xmsx-1*, we noticed that, unlike BMP-4, *Xmsx-1* alone lacks activity to upregulate the expression of ventral genes (Maeda *et al.*, 1997). On the other hand, *Xmsx-2B* -injected embryos completely lost the body axis, while the same dose of *Xmsx-1* had a weaker ventralizing effect. We predicted, therefore, that *Xmsx-2B* may be a cofactor of *Xmsx-1* in blood cell-inducing activity. However, Northern blot analysis revealed that *Xmsx-2B* could not induce *α-globin* expression in the dorsal marginal zone explants, while BMP-4 could. Although there was no difference in the translational activities of *Xmsx-1* and *Xmsx-2B* RNAs in the reticulocyte lysate (data not shown), the stabilities of proteins in the cells were not determined. As the *Xmsx-2B* cDNA used in this study lacks the 3' UTR, there is a possibility that this 3' UTR might regulate the stability of *Xmsx-2B* mRNA. From these observations, we concluded that even though *Xmsx-2B* has a strong activity, *Xmsx-1* and *Xmsx-2B* ventralize the embryo by suppressing the expression of organizer genes (Takeda *et al.*, 2000), and in the prospective ventral cells in which the BMP-4 signal is activated, another unknown factor is required to trigger the blood cell differentiation.

Xmsx-1 and Xmsx-2B function in the dorso-ventral patterning of mesoderm

In the present study, we found that *Xmsx-1* and *Xmsx-2B* function in the ventralizing cascade in an additive manner (Fig. 2 A-G). In addition, the ability of *Xmsx-2B* to suppress the secondary axis formation induced by *VP16/msx-1* RNA (a dominant negative form of msx-1 protein) supports our conclusion that the ventralizing activity of msx-1 can be replaced by that of *Xmsx-2B* and that their target gene(s) might be common. So far, the molecular nature of how these msx proteins interact with each other is unclear, but previous reports have suggested that msx and dlx proteins can form homo- and heterodimers in various combinations (Zhang *et al.*, 1997). Since msx proteins are transcriptional repressors (Catron *et al.*, 1995) and dlx proteins are transcriptional activators (Zhang *et al.*, 1997), the biological activities in transcriptional regulation are mutually exclusive (Zhang *et al.*, 1997). Other candidates of msx's partner are Xvent-1 (PV. 1) (Gawantka *et al.*, 1995; Ault *et al.*, 1996) and Xvent-2 (Xbr-1, Vox, Xom) (Onichtchouk *et al.*, 1996; Papalopulu and Kintner, 1996; Ladher *et al.*, 1996). These factors are also homeodomain-containing proteins and possessing ventralizing activity. The similarity of expression patterns and functions between msx and vent suggests that these proteins might interact with each other, directly or indirectly, in prospective ventral cells. To elucidate the molecular mechanisms of msx's function in the ventralizing activity and the following specification of tissue distribution, isolation and analysis of the interacting molecules will be required in the future.

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