Suppression of head formation by Xmsx-1 through the inhibition of intracellular nodal signaling

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SUMMARY

It is well established that in Xenopus, bone morphogenetic protein (BMP) ventralizes the early embryo through the activation of several target genes encoding homeobox proteins, some of which are known to be necessary and sufficient for ventralization. Here, we used an inhibitory form of Xmsx-1, one of BMP’s targets, to examine its role in head formation. Interestingly, ventral overexpression of a dominant Xmsx-1 inhibitor induced an ectopic head with eyes and a cement gland in the ventral side of the embryo, suggesting that Xmsx-1 is normally required to suppress head formation in the ventral side. Supporting this observation, we also found that wild-type Xmsx-1 suppresses head formation through the inhibition of nodal signaling, which is known to induce head organizer genes such as cerberus, Xhex and Xdkk-1. We propose that negative regulation of the BMP/Xmsx-1 signal is involved not only in neural induction but also in head induction and formation. We further suggest that the inhibition of nodal signaling by Xmsx-1 may occur intracellularly, through interaction with Smads, at the level of the transcriptional complex, which activates the activin responsive element.

Key words: Head formation, Homeobox, BMP, MSX, Anterior endomesoderm, nodal, FAST, Xenopus laevis

INTRODUCTION

The establishment of the dorsoventral axis and subsequent pattern formation along the axis are known to be regulated by several polypeptide growth factors (PGFs) belonging to the TGF-β family (Beddington and Robertson, 1999; Heasman, 1997). In the last decade, two distinct roles for these family members have been clarified: the dorsal fate is determined by the activin/nodal/Vg1 class of ligands and the ventral fate by the bone morphogenetic protein (BMP) class of ligands (Hogan, 1996). Thus, current efforts are focused on elucidating the molecular mechanisms by which tissues are differentially induced by these signals. In particular, nodal has been shown in both zebrafish and mouse to play essential roles in endoderm and mesoderm differentiation and the subsequent cell movement during gastrulation (Conlon et al., 1994; Feldman et al., 1998; Sampath et al., 1998; Varlet et al., 1997). Recently, a rapid accumulation of knowledge from zebrafish genetics and other studies has helped clarify the role of nodal signaling in establishing left-right asymmetry. In contrast, BMPs are essential for specifying both the ventral mesoderm and cell fate in the non-neural ectoderm (Sasai and De Robertis, 1997; Weinstein and Hemmati-Brivanlou, 1999; Wilson and Hemmati-Brivanlou, 1999). It has been shown that in the absence of BMP activity, caused by the BMP antagonists noggin, chordin and follistatin that are secreted from Spemann’s organizer, the presumptive ectoderm differentiates into neuronal cells by inhibiting the epidermal fate (Iemura et al., 1998; Piccolo et al., 1996; Sasai et al., 1995; Weinstein and Hemmati-Brivanlou, 1999; Zimmerman et al., 1996). Studies of the signal transduction mechanism of BMP family ligands have identified several target genes that are necessary and sufficient for BMP signaling. Some of these are immediate early genes that respond to BMP signals without de novo protein synthesis (Ladher et al., 1996; Miyama et al., 1999; Suzuki et al., 1997). Most of the BMP target genes identified to date encode homeobox proteins, including Xmsx-1 (Suzuki et al., 1997), Xmsx-2, Xvent-1 (Gawantka et al., 1995), Xvent-2 (Onichtchouk et al., 1996), and Xdll-3 (Dlx5) (Miyama et al., 1999).

Ectopic activation of the activin/nodal/Vg1 signal or inhibition of the BMP signal induces a partial dorsal axis (Fagotto et al., 1997; Lustig et al., 1996; Suzuki et al., 1994; Thomsen et al., 1990), suggesting that the actions of these two classes of growth factors are reciprocal. The induction of a partial secondary axis is considered as a consequence of their intracellular signal transduction and target gene activation. Thus, this experimental system has been useful for studying the in vivo molecular mechanisms of dorsalization and ventralization in the early Xenopus embryo. In contrast, head formation has been thought of as a related but distinct molecular event from trunk formation, in which only TGF-β family ligands are involved (Niehrs, 1999). The role of BMP-2 and BMP-4 has been believed to be limited to suppressing
the trunk formation, because inhibiting them results in a partial dorsal axis with no head (Suzuki et al., 1994). Thus, the complete dorsal body axis has been believed to be patterned by two independent activities the ‘head organizer’ and the ‘trunk organizer’. In contrast to the action of BMP as a trunk repressor, the Wnt family of proteins has long been implicated in head formation. One of the Wnt family members, Xwnt-8, was initially reported to induce an ectopic secondary dorsal axis with a complete head when its mRNA was injected into ventral blastomers (Christian et al., 1991). It was later found, however, that Xwnt-8 is normally expressed in the ventrolateral mesoderm during the early gastrula stage and that dorsal overexpression by DNA injection ventralized the embryo, suggesting that endogenous Xwnt-8 acts as a ventralizing agent (Christian and Moon, 1993). This interpretation implied that the Wnt ligand is involved in head repression rather than head induction. Recent studies have further demonstrated that in addition to inhibition of the BMP signal, inhibition of the Wnt signal is necessary for head induction, suggesting that the default, inhibited, state of both of these signals is required for head induction (Glinka et al., 1997). Recently however, cerberus, a multiple binding protein for Wnt, nodal and BMP, was reported to be responsible for head induction (Piccolo et al., 1999). Because the late overexpression of nodal, resulting from DNA injection into the dorsal side, causes repression of the head, inhibition of nodal by cerberus may be essential for head formation. In addition, a more recent study showed that the inhibition of Wnt ligands in ventral blastomers by ECD8, an extracellular domain of the Wnt receptor Frizzled, is sufficient to induce an ectopic head (Itoh and Sokol, 1999). Therefore, the precise sequence of gene activation and protein-protein interaction events that promote head development in vivo is still unclear.

Although many of the experiments on head formation in Xenopus have involved looking at the effects of extracellular ligands, in this study, we approached the problem using an inhibitory version of the homeobox protein Xmsx-1. Here we show that Xmsx-1 has an essential role in head repression that is elicited at the level of the transcriptional complex induced by nodal signaling.

**MATERIALS AND METHODS**

**Constructs**

Activating forms of Xmsx-1 were created by replacing the N-terminal 136 amino acids (aa; T1-Xmsx-1) or the first methionine (HI-Xmsx-1) of Xmsx-1 with the 81 aa activation domain of VP16 protein (VP16 AD). Xsmad1, 4ct, and 4b cDNAs were isolated by polymerase chain reaction (PCR) from a cDNA library synthesized from stage 10 embryos. A Flag epitope (DYKDDDDK) was N-terminally inserted in the inhibition of Wnt ligands in ventral blastomers by ECD8, an extracellular domain of the Wnt receptor Frizzled, is sufficient to induce an ectopic head (Itoh and Sokol, 1999). Therefore, the precise sequence of gene activation and protein-protein interaction events that promote head development in vivo is still unclear.

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**Manipulation of embryos and microinjection of synthetic mRNA**

In vitro fertilization of Xenopus eggs was performed as described previously (Suzuki et al., 1995). The fertilized embryos were dejellied using 3% cysteine hydrochloride and washed with water several times. Four-cell-stage embryos were microinjected with capped mRNAs, which were synthesized using the mMESSAGE mMACHINE sp6 kit (Ambion) and then purified by passing through a Sephadex G-50 column (Amersham Pharmacia Biotech). The injected embryos were cultured in 3% Ficoll/0.1× Steinberg’s solution until the appropriate stage for each experiment. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). The ventral marginal zone (VMZ), dorsal marginal zone (DMZ), ventral vegetal quarter, and dorsal vegetal quarter were dissected at stage 10 and cultured in 0.1% bovine serum albumin (BSA)/1× Steinberg’s solution (Asashima et al., 1990).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from the marginal zone explants and the embryos using TRizol reagent (GIBCO/BRL) according to the manufacturer’s instructions. Extracted RNA was subjected to reverse transcription with random hexameric primers. The expression of each molecular marker was detected by PCR using the following specific primers: anterior endomesodermal markers, cerberus, upstream, 5′-ATTCATCTTACGACGAGGT-3′ and downstream, 5′-CTTCTAGA- GACCACTTGAAGAC-3′; Xotx-2, upstream, 5′-GAAGCCCAAA- ACAAGTGT-3′ and downstream, 5′-GCAAGCTGATAAGGTCCA-3′; Xdkk-1, upstream, 5′-CTCTACGTTGGCACGGAGA-3′ and downstream, 5′-CCAGAATGTTTTTCTCCAGG-3′; a pan mesodermal marker, Xbra, upstream, 5′-GGATCATCCTCCACGCTGGTGA-3′ and downstream, 5′-GTGTGGTCGCCACACAAAGTCCA-3′; nodal related gene markers, Xnr-1, upstream, 5′-ACCACTTGCGA- GCCCTACTGGA-3′ and downstream, 5′-TTGTTGTGATGTT- CAGTCTC-3′; Xnr-2, upstream, 5′-GTCTCTTATATCCGAGCA- GTAAT-3′ and downstream, 5′-TTGATGAGAAAATACTG- CTGGAGC-3′; Xnr-3, upstream, 5′-GCCTCTCTTTGGATAGAAG- TTGAAGGA-3′ and downstream, 5′-CATGATCTCATCAGTTTCC-3′; Xnr-4, upstream, 5′-ACTTGGCTGCTTACCTC-3′ and downstream, 5′-CACAGGCTTGTAGTTTCC-3′; Hoxb9, upstream, 5′-TCTT- ACGGGCTTGGCTGGA-3′ and downstream, 5′-AGCGTTGAAA- CAGTGGCTGCT-3′; eFGF, upstream, 5′-TTACTGCAATGTCGG- CATCG-3′ and downstream, 5′-GCAGAACGGTCCTTTGAAAT-3′. The primer sequences for Xwnt-8, Xvent-1, Xvent-2, ventrolateral markers, and histone H4, an internal input control, were as previously described (Yamamoto et al., 2000).

**Whole-mount in situ hybridization, immunostaining and lineage tracing**

Embryos co-injected with HI-Xmsx-1 and β-galactosidase mRNA were fixed in 4% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.8), washed with PBS, and stained with 6-chloro-3-indolyl-β-D-galactoside (Nacalai tesque) for lineage labeling. Stained embryos were then refixed in MEMFA [0.1 M Mops (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% formaldehyde] and stored in methanol at −30°C before being used for whole-mount in situ hybridization. Whole-mount in situ hybridization was performed as described previously (Harland, 1991; Hemmati-Brivanlou et al., 1990) using BM Purple (Boehringer) for the color reaction. To visualize the staining in the endomesoderm the embryos were cut with a razor blade along the dorsoventral axis.

Whole-mount immunostaining with anti-phosphoSmad1 antibody (Faure et al., 2000) and anti-MSX-1 (Babco) were performed as follows: embryos were fixed in MEMFA for 1 hour at room temperature, then washed with PBS and stored in methanol at −30°C before being used. The fixed embryos were rehydrated in PBS,
subjected to western blot analysis using an anti-HA antibody (Santa and with glutathione Sepharose 4B (Pharmacia), respectively, at 4°C analysis were performed by incubating the extracts with the M2 Flag NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF] in the presence of cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM acid, 10% glycerol, 1% Triton X-100]. For the luciferase assay, 100 µl of Luciferase Assay Substrate (Promega) were added to each extract and luciferase activity was measured using a Luminescer-30 -tetraacetic acid, N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100]. For the luciferase assay, 100 µl of Luciferase Assay Substrate (Promega) were added to each extract and luciferase activity was measured using a Luminescer-

Luciferase reporter assay
Four-cell-stage embryos were injected marginally with 50 pg of the –226gsc/Luc (Watabe et al., 1995) construct alone or in combination with the indicated capped synthesized mRNAs as described above. At stage 10.25, or 10.5, five DMZs or VMZs were isolated and lysed with 30 µl of cell lysis buffer [25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100]. For the luciferase assay, 100 µl of Luciferase Assay Substrate (Promega) were added to each extract and luciferase activity was measured using a Luminescer-PSN AB-2200 (ATTO).

Immunoprecipitation and GST pull-down analysis
293T cells were transiently transfected with the indicated constructs by the calcium phosphate method. Forty hours after transfection, the cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF] in the presence of protease inhibitors. Immunoprecipitation and the GST pull-down analysis were performed by incubating the extracts with the M2 Flag monoclonal antibody (Sigma) coupled to protein A Sepharose CL 4B and with glutathione Sepharose 4B (Pharmacia), respectively, at 4°C for 1 hour. The precipitates were then washed with lysis buffer and subjected to western blot analysis using an anti-HA antibody (Santa Cruz Biotechnology).

RESULTS
Xmsx-1 suppresses head formation
Xmsx-1 is expressed in the ventral ectoderm and mesoderm of early Xenopus embryos, suggesting a role for it as a mediator of BMP-4 signaling (Suzuki et al., 1997). In addition, Xmsx-1 activity is required downstream of BMP for the ventralization of the mesoderm and ectoderm (Yamamoto et al., 2000). Dorsal overexpression of Xmsx-1 by mRNA injection (0.25 ng of mRNA) caused a headless embryo with no significant defects in trunk formation (40.0%, n=35; Fig. 1B), as reported previously (Suzuki et al., 1997). This phenotype is similar to that caused by the dorsal overexpression of BMP-2, BMP-4 (Dale et al., 1992; Jones et al., 1992), or BMP-7 (Iemura et al., 1998). The failure of head formation by Xmsx-1 as well as by BMP-4 was found to be tightly correlated with a marked reduction in the anterior endomesodermal markers Xotx-2, Xhex and Xddk-1, all of which define head organizer activity (Fig. 1C). These results suggest that Xmsx-1, which is normally expressed in the ventral side of the embryo, may play a role in the negative regulation of head formation. Thus, the following experiments were undertaken to examine whether such suppression of head formation and release from negative regulation are part of the mechanism underlying normal head development in Xenopus.

An inhibitory form of Xmsx-1 induces an ectopic head
To examine the requirement for Xmsx-1 in Xenopus embryogenesis, we previously constructed cDNAs encoding fusion proteins of Xmsx-1 with either the VP16 transcriptional activation domain or an even-skipped-derived transcriptional repressor domain. We used these dominant-inhibitory forms to show that Xmsx-1 is an essential component of the BMP pathway and that it is involved in the ventral specification of both the mesoderm and ectoderm (Yamamoto et al., 2000). Later, we found that another version of Xmsx-1 induces an ectopic head when ventrally overexpressed. This newly constructed fusion protein, VP16-full-Xmsx-1, in which the VP16 activation domain is added to the N terminus of the full-length protein is shown in Fig. 2A, middle, and can be compared with the previously reported protein, VP16-Xmsx-1 (Fig. 2A, bottom). Although Xmsx-1 was originally found to be a transcriptional repressor for some of the BMP-target genes, such as Xvent-1, both fusion proteins inhibit the ventralizing action of wild-type Xmsx-1 when overexpressed in dorsal blastomeres. In particular, we found that VP16-full-Xmsx-1 acts as an even more potent inhibitor of Xmsx-1 and efficiently rescues the Xmsx-1-induced ventralized phenotype at lower doses of mRNA than did VP16-Xmsx-1 (data not shown). The specificities of the ability of both of the inhibitory forms to rescue the ventralized phenotype were indistinguishable, and they had no inhibitory activity against another ventralizing homeobox genes, Xdll-3, but they weakly inhibited Xmsx-2, a homeobox gene that is closely related to Xmsx-1, as reported previously (Yamamoto et al., 2000 and data not shown).

More interestingly, when 500 pg of VP16-full-Xmsx-1 mRNA was overexpressed in the ventral two blastomeres of a 4-cell-stage embryo, an ectopic head was induced (Fig. 2C), but the same or the increasing dose of VP16-Xmsx-1 mRNA never induced a head but did induce a partial secondary axis (Fig. 2B). Thus, we redesignated the new (VP16-full-Xmsx-1) and former (VP16-Xmsx-1) Xmsx-1 fusion proteins, both of which act in a dominant inhibitory way, as the ‘head-inducing’ Xmsx-1 (HI-Xmsx-1) and ‘trunk-inducing’ Xmsx-1 (TI-Xmsx-1), respectively. As shown in Fig. 2C, ventrally injected HI-Xmsx-1 induced a rather short secondary axis with an ectopic head structure that included eyes and a cement gland, which represent the most anterior tissues (Table 1). Significant truncation of the primary axis, particularly in the tail region, was also observed. Surprisingly, staining with the notochord- and otic vesicle-specific antibody MZ15, showed the presence of the notochord structure in the HI-Xmsx-1-induced short secondary axis (73%, n=11), while no notochord was induced with TI-Xmsx-1 (0%, n=10) (Fig. 2E,F). This anteriorizing phenotype was rescued by coinjecting with wild-type Xmsx-1 (Fig. 2D; Table 1).

To understand the molecular basis of the ectopic head induction, we performed RT-PCR analysis to detect either induced or suppressed genes (Fig. 3A). We found that the so-called head organizer genes Xotx-2, cerberus, Xhex and Xddk-1, which are normally expressed in the anterior endomesoderm and are essential for head formation, were ectopically induced at high levels in the ventral endomesodermal zone of the HI-Xmsx-1-expressing embryos (Fig. 3A,B), suggesting that Xmsx-1 is normally required in the ventral endomesoderm to...
repress head organizer gene expression. In addition, it was noted that expression of the ventral mesodermal marker genes Xwnt-8 and Xvent-1 was significantly reduced, suggesting that the dorsal fate, including head formation, was ectopically induced at the expense of ventral specification. Xvent-2 expression was not reduced by HI-Xmsx-1 overexpression because Xvent-2 functions in a different BMP pathway from Xmsx-1 (Yamamoto et al., 2000). Also, marked suppression of a pan-mesodermal marker gene, Xenopus brachyury (Xbra), was observed, which may result in the truncation of the body axis, shown in Fig. 2C. To our surprise, all of the anterior markers tested were induced by TI-Xmsx-1 as well in early gastrula, in spite of its inability to induce an ectopic head. We found, however, that in a later stage, HoxB9, which is normally detected in posterior neural tissue, was markedly downregulated in HI-Xmsx-1-injected embryos but not in TI-Xmsx-1-injected embryos (Fig. 3). In addition, an other posterior marker eFGF, which was significantly induced by TI-Xmsx-1, was not induced in HI-Xmsx-1-injected embryo. As an anterior marker, Xotx-2 was induced in both embryos at the late stage, and it is suggested that differential regulation of the posterior marker gene expression may cause the different effects between HI- and TI-Xmsx-1.

To explain the ectopic head induction, we reasoned that a TGF-β-related ligand, nodal, might be ectopically induced in ventral blastomeres upon HI-Xmsx-1 overexpression, because nodal has been reported to be an inducer of head organizer genes (Piccolo et al., 1999; Zorn et al., 1999). The ectopic head induction may indicate that HI-Xmsx-1 blocked the ability of ventrally localized endogenous Xmsx-1 activity to inhibit the nodal action at the intracellular signaling level. To investigate this possibility, we next tested whether Xmsx-1 could inhibit not the transcription but the intracellular signaling of nodal. Fig. 6A shows a typical phenotype resulting from the ventral overexpression of nodal observed under our experimental conditions (Table 1). It was previously reported that nodal alone could not induce a complete axis formation by Xnr-1

<table>
<thead>
<tr>
<th>Ventrally injected mRNAs</th>
<th>Survivors</th>
<th>Complete ectopic axis</th>
<th>Ectopic head with eye(s) and cement gland</th>
<th>Ectopic head with cement gland</th>
<th>Incomplete ectopic trunk axis</th>
<th>Ectopic tail</th>
<th>Normal</th>
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<tbody>
<tr>
<td>(1) TI-Xmsx-1 (500 pg)</td>
<td>96.6 (28)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HI-Xmsx-1 (500 pg)</td>
<td>96.3 (30)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(2) HI-Xmsx-1 (100 pg)</td>
<td>96.7 (29)</td>
<td>-</td>
<td>30.0</td>
<td>3.3</td>
<td>63.3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HI-Xmsx-1 (100 pg)+Xmsx-1 (1 ng)</td>
<td>90.6 (29)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.4</td>
<td>62.5</td>
</tr>
<tr>
<td>(3) Xnr-1 (100 pg)</td>
<td>100.0 (32)</td>
<td>68.8</td>
<td>-</td>
<td>-</td>
<td>15.6</td>
<td>-</td>
<td>3.1</td>
</tr>
<tr>
<td>Xnr-1 (100 pg)+Xmsx-1 (250 pg)</td>
<td>100.0 (36)</td>
<td>-</td>
<td>2.8</td>
<td>-</td>
<td>91.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xnr-1 (100 pg)+Xmsx-1 (500 pg)</td>
<td>96.9 (31)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.3</td>
<td>-</td>
<td>71.9</td>
</tr>
<tr>
<td>(4) Xnr-1 (100 pg)</td>
<td>100.0 (32)</td>
<td>84.4</td>
<td>-</td>
<td>-</td>
<td>15.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xnr-1 (100 pg)+BMP-4 (200 pg)</td>
<td>100.0 (35)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.7</td>
<td>94.3</td>
<td></td>
</tr>
</tbody>
</table>

Phenotypes were determined after 60 hours of development.
Other abnormalities including dorsoposterior defect, incomplete invagination and spontaneous ventralization are not described.

**Table 1. Ectopic head formation by HI-Xmsx-1 and complete axis formation by Xnr-1**

![Fig. 1. Ectopic expression of Xmsx-1 in the anterior endoderm suppresses head structure formation. Xenopus embryos were either uninjected (A and C, lanes 1, 4, 5) or injected with 500 pg of Xmsx-1 (B and C, lane 2) or 200 pg of BMP-4 (C, lane 3) mRNA in the submarginal zone of the two dorsal blastomeres at the 4-cell stage. (A,B) The embryos were cultured until stage 40 to observe the phenotypes. (C) RT-PCR analysis for the early anterior endomesodermal markers. The dorso-vegetal quarters of the embryos were dissected at stage 10.25 (lanes 1-3) and then subjected to RT-PCR analysis for the indicated markers. Lanes 4 and 5, whole embryo control with (lane 4) or without (lane 5) the RT step.**
secondary axis with anterior structures such as brain and eyes, and that only by co-injecting nodal with noggin, could a complete secondary axis with a head be induced (Lustig et al., 1996). In contrast to the report, we found nodal to be sufficient to induce a complete secondary axis with organized head structures (Fig. 5Ab) resembling the secondary axis induced by the ventral overexpression of Xwnt-8 or β-catenin mRNA (McMahon and Moon, 1989; Zeng et al., 1997). Increasing doses of Xmsx-1 gradually inhibited the formation of the head and secondary axis in a dose-dependent manner (Fig. 5Ac,d; Table 1). Changes occurred in marker gene expression which were consistent with the observed reduction of the ectopic head and secondary axis; the head organizer genes *cerberus*, *Xotx-2* and *Xdkk-1*, which were ectopically induced in the ventral endomesoderm by the nodal gene product Xnr-1, were efficiently down-regulated by Xmsx-1 (Fig. 5B). This result suggests that Xmsx-1 does not change the expression level of nodal but inhibits its signaling. This interpretation might also

**Fig. 2.** Ventral overexpression of HI-Xmsx-1 induces an ectopic head. (A) Schematic drawing of the different forms of Xmsx-1. (Top) Wild-type Xmsx-1 protein. Activating forms of Xmsx-1, HI-Xmsx-1 (middle), and TI-Xmsx-1 (bottom), were constructed by fusing the 81 amino acids of the VP16 activation domain (black box, VP16 AD) with the full-length and C-terminal 137 amino acid region of Xmsx-1 protein, respectively. The amino acid numbers of several junction sites are shown above each drawing. The homeodomain is indicated by the gray box (HD). (B–D) Phenotypes of mRNA-injected embryos. (E,F) Staining with the notochord- and otic vesicle-specific antibody, MZ15. Four-cell stage *Xenopus* embryos were ventrally injected with 500 pg of TI-Xmsx-1 (B,E) or HI-Xmsx-1 (C,F) mRNA alone or co-injected with 500 pg of HI-Xmsx-1 and 1 ng of wild-type Xmsx-1 mRNA (D). HI-Xmsx-1-injected embryos formed a secondary axis with an ectopic head containing eye(s), cement gland and short notochord in the ventral side. The ectopic head region is magnified in the right side of C. The ectopic head phenotype was rescued by wild-type Xmsx-1.

cg, cement gland; ey, eye; 2°, secondary axis.

**Fig. 3.** HI-Xmsx-1 induces head organizer marker genes in the ventral endomesoderm. Two ventral blastomeres of 4-cell-stage embryos were co-injected marginally with 100 pg of HI- or TI-Xmsx-1 mRNA and 50 pg of β-galactosidase mRNA as a lineage tracer, then processed for RT-PCR (A) and whole-mount in situ hybridization (B) at stage 10.25. (A) The ventro-vegetal quarter of the embryos injected with the indicated mRNA (top, lanes 1, 2, 3) was dissected at stage 10.25 and RNA was extracted immediately, for early stage, or after being cultured until sibling embryos reached stage 18, for late stage. RNA extracted from each explant was analyzed by RT-PCR. Lane 4 shows the expression of each marker in whole embryos and lane 5 shows the control reactions with no RT step. (B) The injected embryos were stained by red Gal for lineage tracing and then analyzed by whole-mount in situ hybridization for *Xotx-2*, *Xhex* and *cerberus* gene expression.

Anterior organizer genes were induced in the ventral endomesoderm. The dorsal blastopore lip is indicated by black arrowheads. Arrows indicate the RNA-injected ventral marginal zone, in which the nuclei of cells are stained red. AS, antisense probe; S, sense probe.
explain the marked truncation of the primary axis (Fig. 2C), because the nodal signal in early phases of development is known to be required to induce and/or maintain Xbra expression (Piccolo et al., 1999).

In addition, we next tested the effect of Xmsx-1 and HI-Xmsx-1 using a reporter gene for activin/nodal signaling. This reporter gene is derived from the activin responsive element (ARE) of the goosecoid promoter and is known to be activated by activin or nodal through a transcriptional complex that includes FAST, Smad2 and Smad4 (Chen et al., 1996; Chen et al., 1997; Watabe et al., 1995). As shown in Fig. 6A, the reporter luciferase was preferentially activated in dorsal blastomeres, most likely reflecting dorsally localized nodal signaling in the embryo, while it was only weakly activated in the ventral side of the embryo. However, the luciferase activity in the ventral side was markedly enhanced in a dose-dependent manner by the injection of HI-Xmsx-1 mRNA. The dorsal activation of the reporter was significantly suppressed by the coinjection of wild-type Xmsx-1 (Fig. 6B), suggesting that Xmsx-1 inhibits the transcription of nodal target genes. Interestingly, this suppression was restored by coinjecting Xnr-1 mRNA along with Xmsx-1. Taken together, these results strongly suggest that intracellular nodal signaling is inhibited by Xmsx-1 in vivo.

**Fig. 4.** Xmsx-1 does not suppress nodal-related gene expression. Embryos were either un.injected (lanes 1, 4, 5) or injected with 500 pg of Xmsx-1 (lane 2) or 200 pg of BMP-4 (lane 3) mRNA into the equatorial region of the two dorsal blastomeres at the 4-cell stage. Dorsal marginal zone explants (DMZ; lanes 1, 2, 3) were cultured until sibling embryos reached stage 10.5, then nodal-related gene expression was analyzed by RT-PCR.

**Xmsx-1 inhibits the transcriptional complex formation of Smad2/4 and FAST-1**

How does Xmsx-1 inhibit nodal signaling? It has been shown that, following the activation of the nodal receptors ActRIA/IB and ActRII, and an EGF-CFC coreceptor (one eyed pinhead, and crypto/criptic for zebrafish and mouse, respectively), formation of a transcriptional complex is essential to activate nodal target genes. This complex includes Smad2, Smad4 and a winged helix transcription factor FAST, a protein whose binding site is the essential minimal enhancer sequence in the ARE (Watanabe and Whitman, 1999). This led us to speculate that Xmsx-1 may physically interact with the transcriptional complex triggered by nodal signaling. We tested whether Xmsx-1 protein binds the pathway-restricted Smads, Xsmad1, Xsmad2 (Baker and Harland, 1996; Graff et al., 1996), or the common Smads Xsmad4a and Xsmad4β (Howell et al., 1999; Masuyama et al., 1999). We expressed Flag-tagged Xmsx-1 protein and HA-tagged Smad proteins transiently in 293T cells, and the cell extracts were subjected to immunoprecipitation with anti-Flag antibodies, followed by western blot analysis with anti-HA antibodies. Interestingly, all of the Smads were coprecipitated with Xmsx-1, although the efficacy varied (Fig. 7A). Among them, Xsmad4β was most efficiently immunoprecipitated with Xmsx-1. This may be due to the fact that Smad4β is localized exclusively to nucleus at a higher concentration, while the other Smads are present in both the cytoplasm and the nucleus. To
investigate the possibility that the binding of Xmsx-1 to Smad4 replaces the activin/nodal-regulated Smad2, we examined whether increasing doses of Xmsx-1 could change Smad2 level in the complex (Fig. 7B). However, the level of Smad2 in the complex did not change as a result of Xmsx-1 expression. This indicates that Xmsx-1 is likely to bind to the Smad2/4 complex additively. Furthermore, in this context we tested whether the incorporation of Xmsx-1 into a Smad2/4 complex could exclude the transcription factor FAST. GST-tagged xFAST-1, HA-tagged Xmsd2 and increasing dose of HA-tagged Xmsx-1 were expressed in 293T cell in the presence or absence of Xsmad4β and subjected to GST pull down analysis (Fig. 7C). The results showed that Xmsx-1 did not bind to xFAST-1 (Fig. 7C, lane 8) but clearly inhibited xFAST-1 to bind Smad2 in a dose-dependent manner (Fig. 7C, lanes 2-5). Interestingly, this competitive inhibition depended on the presence of Xsmad4β (Fig. 7C, lanes 4 and 6). These results suggest that the binding of Xmsx-1 to Smad4 may exclude FAST from the Smad2/4 complex and this mechanism may explain how the transcriptional activity of the complex is negatively regulated.

**Endogenous BMP signaling in the endoderm**

If BMP/Xmsx-1 signaling is indeed necessary to repress head formation in vivo through the inhibition of head organizer genes, the activities of BMP and Xmsx-1 should be present in the ventral endoderm and absent in the anterior endoderm where the head organizer genes are expressed. To address this question, we localized BMP signaling using an anti-phosphoSmad1 antibody that preferentially recognizes the phosphorylated (active) form of BMP-regulated Smads, Smad1 and Smad5 (Faure et al., 2000; Kurata et al., 2000; Persson et al., 1998).

As predicted, BMP signaling represented by the anti-phosphoSmad1 staining was detected in the ventral endoderm region but not in the anterior endoderm (Fig. 8A). Consistent with this, Xmsx-1 was also detected immunohistochemically in the overlapping region with its specific antibodies (Fig. 8B). These results further support the idea that BMP/Xmsx-1 activities are necessary in the ventral endoderm to repress head formation and that both activities are absent in the corresponding dorsal region, where the head organizer is formed.

**Extensive inhibition of BMP signaling is sufficient for head formation**

We showed that a dominant-inhibitory form of a homeobox gene Xmsx-1 that faithfully reflects BMP activity alone is sufficient to induce an ectopic head. These results indicate that inhibition of BMP signaling alone should also result in head formation. This raised an interesting question as to why inhibition of BMP by tBR in the ventral side never induces a complete head but only a partial dorsal axis (Suzuki et al., 1994). As there are several BMP subfamily members such as BMP-2, BMP-4 and BMP-7, there are multiple cell surface receptors transducing their signals in *Xenopus* embryo. At least three type I receptors, BMPRIA (ALK3), BMPRIB (ALK6) and ActRIA (ALK2) and three type II receptors, ActRII, ActRIIB and BMPRII have so far been identified as receptors mediating the BMP signal (Dale and Jones, 1999). Owing to the wide range of BMP activities generated by the subtypes, we reasoned that the previous experiments inhibiting BMP signals with tBR were not completely effective. In this context, we attempted to block BMP activities as extensively as possible using several combinations of dominant-negative receptors, or BMP antagonists. We first coinjected the mRNAs for tBR and truncated BMPRII. As shown in Fig. 9A, no head structure was formed in the ectopic dorsal axis of the injected embryos. Next we coinjected a truncated ALK2 mRNA in addition to the former combination of mRNA because ALK2 is known to contribute to BMP-7 signaling. As shown in Fig. 9B, an almost complete head with eye(s) and a cement gland was induced in the injected embryos (Table 2). The head induction rate appears to be depending on the inhibitory spectrum of BMP signals. This suggests that BMP has only to be blocked extensively for head induction. To confirm this notion, we also examined the combined effect of so-called organizer factors noggin, chordin and follistatin, each of which is insufficient to induce ectopic head by ventral overexpression (Fig. 9C). As we expected, double or triple mRNA injection led to an ectopic head with eyes and a cement gland (Fig. 9D; Table 2), supporting the previous result with the dominant-negative receptors. Taking account of the results using HI-Xmsx-1, we propose here that inhibition of BMP/Xmsx-1 activities is not only necessary but sufficient to induce head formation.
Is the inhibition of BMP activity sufficient for head induction?

One of the most important observations of this report was that HI-Xmsx-1 induced an ectopic head. The general belief has been that inhibition of BMP signaling, for example, by a truncated BMP type I receptor (BMPRIA/ALK3), is not sufficient to induce a complete secondary body axis with a head structure (Suzuki et al., 1994). In addition, recent studies have suggested that inhibition of all three polypeptide growth factors, BMP, nodal and Wnts, is necessary for head formation (Glinka et al., 1997; Piccolo et al., 1999). Dkk-1, which was identified as a Wnt antagonist, is capable of inducing an ectopic head when combined with the action of the truncated BMP receptor, tBR (Glinka et al., 1998). Cerberus, originally found to be a head inducer, was later found to bind all of the above three factors to inhibit their function (Piccolo et al., 1999). In contrast, in this study, we have found that the expression of HI-Xmsx-1, an inhibitor of Xmsx-1, alone is sufficient to induce an ectopic head. Because Xmsx-1 is believed to faithfully mimic the effects of BMP in Xenopus embryos (Suzuki et al., 1997; Yamamoto et al., 2000), this observation was unexpected and somewhat puzzling, considering the results of previous studies. One possible explanation is that inhibition of the BMP/Xmsx-1 pathway by HI-Xmsx-1 might ectopically activate nodal signaling in the anterior endomesoder, which in turn could activate head organizer genes such as cerberus, Xhex and Xdik-1 to lead to head formation. Alternatively or additionally, HI-Xmsx-1 might act during later developmental stages to inhibit the BMP signal required for the maintenance of Xwnt-8 expression in the VMZ; thus, both the BMP and Xwnt-8 signals would be inhibited, conferring a condition sufficient for ectopic head formation.

Previously, the ectopic overexpression of nodal was reported to induce only a partial secondary dorsal axis without a head, and it was shown to induce a head only when cooverexpressed with noggin (Lustig et al., 1996). However, we have clearly demonstrated here that nodal alone can induce an ectopic head, and it was shown to induce a head only when cooverexpressed with noggin (Lustig et al., 1996). Therefore, the former explanation is consistent with our functional analysis of nodal in the early Xenopus embryo. It is also known that, later in development, nodal must be inhibited dorsally. This has been proposed to occur indirectly through the induction of cerberus (Piccolo et al., 1999). In addition, head induction by chordin and frz-b was also rescued by the coinjection of Xnr-1 DNA. Therefore, cerberus expressed ectopically by DNA injection may serve as an inhibitor of nodal in later stages, fulfilling the requirement for head formation. When HI-Xmsx-1 was dorsally injected, defects in head formation were observed (data not shown). This may be
due to the hyperactivation of nodal signaling, and is consistent with the above speculation.

The latter possibility, involving the down-regulation of $Xwnt-8$, is also likely. The ventral mesoderm marker, $Xwnt-8$ was significantly down-regulated as a result of the inhibition of Xmsx-1 activity. It is believed that the BMP activity, most likely through its induction of Xmsx-1 activity (Takeda et al., 2000), is necessary for the onset and maintenance of the ventral expression of $Xwnt-8$ at the early gastrula stage. Our results further confirmed that termination of the BMP signaling cascade with HI-Xmsx-1 leads to the loss of $Xwnt-8$ expression. Despite the similar inhibition of both BMP and Xwnt-8 activity, the mechanism of head induction by Dkk-1 and tBR (Glinka et al., 1998) may be slightly different because the inhibition of Wnts and BMP by Dkk-1 and tBR is thought to be solely an extracellular event, while HI-Xmsx-1 inhibits BMP signals intracellularly and $Xwnt-8$ at the transcriptional level.

To investigate the above possibilities, we expressed HI-Xmsx-1 using a DNA vector, with the intention that HI-Xmsx-1 be expressed later than it would be by mRNA injection. Interestingly, ectopic induction of the head was not observed (data not shown), indicating that early action of HI-Xmsx-1 is essential for ectopic head induction, which would support the former possibility. Also it was previously demonstrated that the blockage of BMP and anti-dorsalizing morphogenetic protein (ADMP), a member of TGF-$\beta$ superfamily expressed in the Xenopus trunk organizer, induces head formation (Dosch and Niehrs, 2000). To address whether the ectopic head induction by HI-Xmsx-1 was due to the inhibition of ADMP, we tested the effect of HI-Xmsx-1 on the ventralizing phenotype by ADMP. No antagonizing ability of HI-Xmsx-1 was observed.

### Table 2. Extensive inhibition of BMP activity induces ectopic head structure

<table>
<thead>
<tr>
<th>Ventrally injected mRNAs</th>
<th>Survivor % (n)</th>
<th>Complete ectopic axis</th>
<th>Ectopic head with eye(s) and cement gland</th>
<th>Ectopic head with cement gland</th>
<th>Incomplete ectopic trunk axis</th>
<th>Ectopic tail</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) tBRIA (200 pg)+tBRII (500 pg)</td>
<td>93.5 (29)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>tBRIA (500 pg)+tALK2 (100 pg)</td>
<td>96.7 (29)</td>
<td>--</td>
<td>--</td>
<td>83.3</td>
<td>6.7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>tALK2 (100 pg)+tBRIA (200 pg)</td>
<td>100.0 (30)</td>
<td>--</td>
<td>--</td>
<td>93.3</td>
<td>--</td>
<td>3.3</td>
<td>--</td>
</tr>
<tr>
<td>tBRIA (200 pg)+tBRII (500 pg)+tALK2 (100 pg)</td>
<td>97.0 (32)</td>
<td>--</td>
<td>42.4</td>
<td>6.1</td>
<td>45.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(2) Chordin (300 pg)</td>
<td>100.0 (34)</td>
<td>--</td>
<td>--</td>
<td>67.6</td>
<td>29.4</td>
<td>2.9</td>
<td>--</td>
</tr>
<tr>
<td>noggin (1 pg)</td>
<td>100.0 (35)</td>
<td>--</td>
<td>--</td>
<td>94.3</td>
<td>2.9</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>follistatin (60 pg)</td>
<td>96.9 (31)</td>
<td>--</td>
<td>--</td>
<td>81.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>chordin (150 pg)+noggin (0.5 pg)</td>
<td>96.9 (31)</td>
<td>18.8</td>
<td>--</td>
<td>75.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>noggin (0.5 pg)+follistatin (30 pg)</td>
<td>96.8 (30)</td>
<td>45.2</td>
<td>--</td>
<td>32.3</td>
<td>--</td>
<td>3.2</td>
<td>--</td>
</tr>
<tr>
<td>follistatin (30 pg)+chordin (150 pg)</td>
<td>93.3 (28)</td>
<td>--</td>
<td>--</td>
<td>83.3</td>
<td>--</td>
<td>3.3</td>
<td>--</td>
</tr>
<tr>
<td>chordin (100 pg)+noggin (0.3 pg)+follistatin (20 pg)</td>
<td>93.5 (29)</td>
<td>74.2</td>
<td>--</td>
<td>19.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Phenotypes were determined after 60 hours of development.

Other abnormalities including dorsoposterior defect, incomplete invagination and spontaneous ventralization are not described.

tBRIA, truncated BMPRIA; tBRII, truncated BMPRII; tALK2, truncated ALK2.
against ADMP (data not shown). Furthermore, ADMP expression level was not repressed in DMZ, while it was induced in VMZ by HI-Xmsx-1 (data not shown). These results suggest that Xmsx-1-induced ectopic head production does not involve ADMP inhibition.

As shown in Fig. 9, we demonstrated that inhibition of an extensive spectrum of BMP activities using multiple truncated BMP receptors or three extracellular BMP antagonists, so-called organizer factors, could produce an ectopic head structure, often with eyes and a cement gland, suggesting that the incomplete secondary axis formation observed in previous studies was due to residual endogenous BMP signals. Therefore it appears that BMP/Xmsx-1 signaling play a role, not only as a trunk repressor, but also as a head repressor in vivo.

**Xmsx-1 inhibits nodal signaling**

We showed that Xmsx-1 inhibited the activation of an ARE reporter gene in the dorsal blastomeres of *Xenopus* embryos. We therefore conclude that wild-type Xmsx-1 inhibits intracellular nodal signaling. This result may in turn suggest that HI-Xmsx-1 might have activated ectopic nodal signaling when overexpressed in ventral blastomeres. In other words, potential nodal signaling may be present in the ventral side of the embryo (as it has been shown that nodal transcript is evenly distributed in early embryo; Jones et al., 1995), but is inactivated by the BMP/Xmsx-1 signal. In fact, endogenous activated Smad2 is detected in the ventral endoderm as well as in the anterior endoderm of the gastrula stage embryo (Faure et al., 2000). In this study, we hypothesized that this inhibition occurs at the intracellular signaling level. We also revealed that an increasing level of Xmsx-1 could efficiently exclude FAST protein from the Smad2/4 complex depending on the presence of Smad4. Taken together, these results suggest that through the binding to Smad2, Xmsx-1 associates with Smad2 and inhibits FAST-induced transcription. However, the exact mechanism in vivo by which nodal signaling is preferentially inhibited by the binding of Xmsx-1 to the complex remains to be investigated.

In this study, we clearly showed that Xmsx-1 inhibits nodal at the intracellular signaling level in the ventral endoderm without apparent change in the level of nodal transcripts. Recently, however, the expression of nodal was shown to be autoinduced by nodal signaling through a FAST-regulated module in the first intron of the gene (Osada et al., 2000). This indicates that the modulation of nodal signaling should lead to change in expression level of the ligand, which was not the case in this study. One possibility to explain this discrepancy is the fact that antivin/lefty, which is a member of TGF-β superfamily and the antagonist of nodal signaling acting in a negative feedback loop to suppress the maintenance of nodal ligand, is also induced by nodal signaling. By this mechanism, the expression level of nodal may be maintained at a constant level even when Xmsx-1 is overexpressed.

Finally, an important question is, how universal among species is the head repression mechanism proposed here. Targeted disruption of mouse *Msx1* and/or *Msx2* reveals no anteroposterior patterning defect, although developmental defects were found in several organs of each *Msx* gene-disrupted mice (Satokata et al., 2000; Satokata and Maas, 1994). In the mouse, three *Msx* genes were isolated, *Msx1*, *Msx2* and *Msx3*. Thus, *Msx3* or another related homeobox genes may act redundantly in the mutants, as functional redundancy was previously reported for *Msx1* and *Msx2* (Satokata et al., 2000). Alternatively, the mechanism of head repression by BMP/Msx-1 may be specific to amphibians. We have shown nuclear localization of Xmsx-1 as well as phosphorylated BMP-driven Smads in ventral endoderm but not in anterior endoderm (Fig. 8A,B), supporting the proposed mechanism of head induction (de Souza and Niehrs, 2000). However, expression of mouse *Mxs* proteins in the primitive streak and absence in anterior visceral endoderm (AVE), has not been demonstrated.

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**REFERENCES**


Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and


