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The role of Xmsx-2 in the anterior-posterior patterning of the mesoderm in *Xenopus laevis*

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Abstract Many molecules are involved in defining mesodermal patterning of the *Xenopus* embryo. In this paper, evidence is provided that a member of the *msx* family of genes, the *Xmsx-2* gene, is involved in anterior-posterior patterning of the mesoderm. A comparison of its sequence to another previously cloned *msx-2 Xenopus* homolog, *Xhox-7.1'* [45] showed that they are closely related. The *Xmsx-2* gene is first expressed at midgastrulation predominantly in the dorsal part of the embryo. It showed a complex pattern of spatial expression, consistent with a role in patterning of the anterior-posterior axis. This inference is confirmed by gain-of-function experiments in which overexpressed *msx-2* mRNA in developing *Xenopus* embryos resulted in embryos lacking anterior structures. Analysis of markers in mutant embryos showed that genes involved in ventral-posterior patterning such as *Xhox-3*, *Xwnt-8*, and *Xvent-1* were upregulated, confirming the posteriorized nature of the embryos. We believe that the *Xmsx-2* gene is involved in refining the patterning of the anterior-posterior part of the dorsal mesoderm after the initial signals determining the dorsal or ventral nature of the mesoderm have been specified.

Introduction

During development of the amphibian embryo from an egg with radial symmetry to a bilaterally symmetrical embryo with the dorsal/ventral and anterior/posterior axial polarities, a number of critical events must occur. One of the first is the movement of cytoplasmic components during the cortical rotation that occurs within 90 min after fertilization (reviewed in [11]). This movement triggers a cascade of events that leads to the formation of the Nieuwkoop center. The Nieuwkoop center induces Spemann's organizer (in the overlying cells of the dorsal marginal zone at the early blastula stage) to form mesoderm of a more dorsal kind (neural tissues, notochord and segmented muscle) whereas the rest of the vegetal hemisphere induces mesoderm of a ventral character (mesothelium, mesenchyme, blood cells).

Mesoderm inducing signals are of two kinds: dorsalizing and ventralizing (reviewed in [12, 39, 40, 42]). The initial signals dorsalize the marginal zone to form intermediate types of mesoderm [41]. Examples of dorsalizing signals include, among many others, gene products such as *noggin* [43], *chordin* [37], and follistatin [14]. The origin of the ventral-posterior mesoderm was originally believed to be the ground state mesodermal tissue acted upon by signals from the organizer [44]. There are, however, mounting data suggesting that the ventral marginal zone requires active signals for the specification of ventral-posterior state. A member of the bone morphogenetic protein family, *BMP-4* [5, 18], and *Xwnt-8* [4] were among the first molecules shown to have ventral-posteriorizing activity. Since then, a number of homeobox genes such as *Xvent-1* [10] and *Vox* [38] have also been implicated in the specification of ventral-posterior mesodermal fate. More recently, a member of the *msx* family of homeobox gene, *Xmsx-1* was characterized as one of the downstream effector molecules of *BMP-4* in defining the ventral-posterior pathway [23, 46].

In the present study, we characterized the role of another member of the *msx* family, *Xmsx-2*, in defining the anterior–posterior mesodermal pathway. We used *Xenopus* as a model system to test the hypothesis that *Xmsx-2* specifies anterior-posterior mesodermal cell types. Our results suggest that *Xmsx-2* is an important component in the anterior-posterior mesodermal pathway and that it probably functions in refining the initial anterior-posterior patterning of the dorsal mesoderm set up by other earlier acting genes.

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Methods

Library screening. A stage 17 cDNA library in λgt11 (gift of T. Sargent, NIH), was screened according to a modification of the method of Amasino [1]. Plaques (5×10^5) were grown on TB agar plates and plated using TB top agar. Duplicate filters were made. The plaques were hybridized to random-labeled probe 7.3 (gift of F. Ramirez, Mt Sinai Hospital) that contained a sequence corresponding to the first 183 bp of a partial clone of a gene believed to be the *msx2* homolog of *Xenopus* [45]. The plaques were fixed to the filters by denaturation (1.5 *M* NaCl, 0.5 *M* NaOH), neutralized in 1.5 *M* NaCl and 0.5 *M* Tris (pH 8.0) and washed in 2× SSPE. Three clones were successfully isolated after tertiary screens. Phage DNA was produced from the longest piece and digested with *EcoRI* and subcloned into pBluescript KS^+. The DNA insert contained approximately 3.3 kb of the *Xmsx-2* transcript and was sequenced using rapid florescent-tagged dideoxynucleotide sequencing chemistry. Analysis of the sequence was performed with the University of Wisconsin Genetics Computer Group sequence analysis package.

RNA preparation for Northern hybridization and RT-PCR. Embryos were collected at different time stages of development (staged according to Nieuwkoop and Faber [28]). They were then placed in Trizol solution (GibcoBRL), and RNAs extracted following manufacturer's recommendations.

RNA for microinjection. The wild-type *msx-2* and the mouse frame-shift mutant cDNA was subcloned in pBluescript SK. Both plasmids were linearized with *EcoRI* and used as templates for in vitro transcription using the mMESSAGE mMACHINE T7 transcription kit (Ambion). The green fluorescent protein (GFP) was linearized with *Asp718*, and transcribed using the Sp6 transcription kit (Ambion).

Northern and Southern hybridizations. For the Northern hybridization experiments, about 20 µg total RNA from different stages was electrophoresed on a 1% denaturing formadehyde-agarose gel [36]. For the Southern hybridization experiment, 20 µg of predigested (with *BamHI, EcoRI,* and *Pst1* restriction enzymes) *Xenopus* genomic DNA were loaded onto a 0.8% agarose gel. The RNAs and DNAs were then transferred onto Gene-Screen Plus nylon membranes (Dupont NEN) using 20× SSC (3 *M* NaCl, 300 m*M* sodium citrate, pH 7.0) and UV-crosslinked. The membranes were hybridized at 65°C to radiolabeled 7.3 overnight in Amasino buffer, which is 0.25 *M* Na^+PO_4, 0.25 *M* NaCl, 7% sodium dodecyl sulfate (SDS), 10% polyethylene glycol (PEG) 600, and 1 m*M* ethylenediaminetetraacetic acid (EDTA) [1]. The blots were washed in a buffer containing 50 m*M* Na_2HPO_4 and 0.5% SDS twice for 20 min at room temperature and 3 times for 30 min at 65°C. The Southern blots were exposed overnight to Kodak X-omat film at –80°C with one intensifying screen for the RT-PCR experiments. The genomic Southern blot was exposed for 72 h and the Northern blot was exposed to Kodak MS (Maximum Sensitivity) film and screen for 72 h.

In situ hybridization. Sections were obtained by fixing embryos at different developmental stages in MEMFA (0.1 *M* 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.3; 2 m*M* ethylene glycol-bis(β-aminoethyl ether)-N, N, N',N'-tetraacetic acid (EGTA); 1 m*M* $MgSO_4$; 3.7% formaldehyde) for 2 h and then stored overnight in methanol at 20°C. After dehydration, the embryos were embedded in paraplast and sections cut at 8 µm thickness. The sections were dewaxed and rehydrated through a series of xylene washes and graded changes of ethanol and refixed in 4% paraformaldehyde in 1× phosphate buffered saline (PBS) for 20 min. The slides were then incubated in 3 µg/ml Proteinase K in 0.1 *M* Tris pH 7.5, 10 m*M* EDTA for 30 min at 37°C. After rinsing slides in 2× SSPE (0.3 *M* NaCl, 0.02 *M* $NaH_2PO_4.H_2O$, 2 m*M* EDTA) and incubating them in 0.2 *M* HCl for 15 min at r.t., followed by rinsing again in 2× SSPE, the sections were acetylated in 0.1 *M* triethanolamine, pH 8, with 0.25% acetic anhydride (added twice while stirring). The sections were again rinsed, dipped in distilled water and prehybridized in hybridization buffer (1% blocking agent, 50% formamide, 5× SSC, 1 mg/ml torula RNA, 0.1 mg/ml heparin, 0.1% Tween 20, 0.1% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 5 m*M* EDTA) at 60°C for about 3 h in a humid chamber. Excess buffer was drained off and the sections hybridized with buffer containing 0.5–1 µg/ml of digoxigenin labeled probe at 60°C overnight. The next day, the slides were rinsed in 2× SSPE and incubated with the following at r.t.: hybridization buffer for 10 min; 50% hybridization buffer, 50% 2× SSPE, 0.3% CHAPS, 10 min; 2× SSPE, 0.3% CHAPS, 20 min; 2× SSPE, 30 min. The sections were then treated with 0.02 mg/ml RNAse A in 4× SSPE for 30 min at 37°C, then washed stringently in 50% formamide, 2× SSPE at 1 h at 50°C. The slides were drained and incubated in 2× SSPE, 0.3% CHAPS at room temperature for 10 min, afer which they were rinsed for 3×10 min in buffer 1 (0.1 *M* maleic acid, 0.15 *M* NaCl, pH 7.5), followed by incubation for 1 h with 0.5%blocking agent in buffer 1. The slides were then drained and incubated in 1:1000 dilution of preincubated anti-digoxigenin AP fragments in buffer 1 for 1 h. The slides were then rinsed in buffer 1 three times at 10 min each, followed by rinsing for 10 min in buffer 3 (0.1 *M* Tris pH 9.5, 0.1 *M* NaCl, 0.25 *M* $MgCl_2$, 0.001% Tween 20, 0.024% levamisole). Color reaction was performed by incubating the sections in BCIP/NBT in buffer 3. The sections were then fixed in MEMFA and mounted.

RT-PCR. For the RT-PCR experiments, RNAs isolated from the embryos were first digested with DNAse I and reverse-transcribed with the SuperScript Preamplification kit (Gibco BRL). The polymerase chain reaction was carried out in 50 µl reaction in a Perkin-Elmer thermal cycler. The experimental primer pair was mixed in the same reaction tube with the control, elongation factor-1 alpha (EF1-α). All reactions were carried out in the linear range of an empirically determined amplification cycle number for each set of primers. About 20 µl of the reaction products was electrophoresed on a 1% agarose gel and were subjected to Southern hybridization twice using probes purified from the PCR reaction products (experimental and control) random-radiolabeled with ^{32}P. The membranes were washed and the signal quantified by phospho-imaging. Although not shown in all cases in the Results section above, parallel control samples in which reverse transcriptase had been omitted were analyzed in all PCR assays. In these control samples gene-specific products were absent. The gene-specific primers were used: *Xmsx-2* upstream 5'-GGCAGTCTGCAACTACTA-3', downstream 5'-CTATGTCATGTCACCCTC-3'; *Xhox-3* [34], upstream 5'-ATATGATGAGCCACGCAGCAG-3', downstream 5'-CAGATGCTGCAGCTCTTTGGC-3'; *Xwnt-8* [3], upstream 5'-GAGAGAAGAAGCTGCAAGAGGC-3', downstream 5'- GGCAAACAAATCCACTGGCCCG-3'; *Xvent-1* [10], upstream 5'-TTCCCTTCAGCATGGTTCAAC-3', downstream 5'-GCATCTCCTTGGCATATTTGG-3'; *EF1-α* [20], upstream 5'-GATGCTCCTGGACACAGAGATT-3', downstream 5'-GGTAGTCTGAGAAGCTCTCCAC-3'.

Xenopus embryo manipulation and microdissection. Eggs were obtained from hormonally stimulated *Xenopus laevis* females (*Xenopus I,* Ann Arbor) and were fertilized in vitro using testis homogenates, according to Etkin et al. [8]. The embryos were first dejellied with 3% cysteine-HCl (pH 7.9) immediately following cortical rotation. The cysteine was washed away thoroughly in 1X Modified Barth's solution (MBS). The embryos then were transferred to a dish containing 1× MBS/3% (w/v) Ficoll 400. About 1.2 ηg of RNA in a volume of 4.6 ηl was injected into the animal third of each embryo. After injections, the embryos were transferred to 0.1× MBS and raised at 18°C. The embryos were allowed to grow to different stages [28] and then fixed.

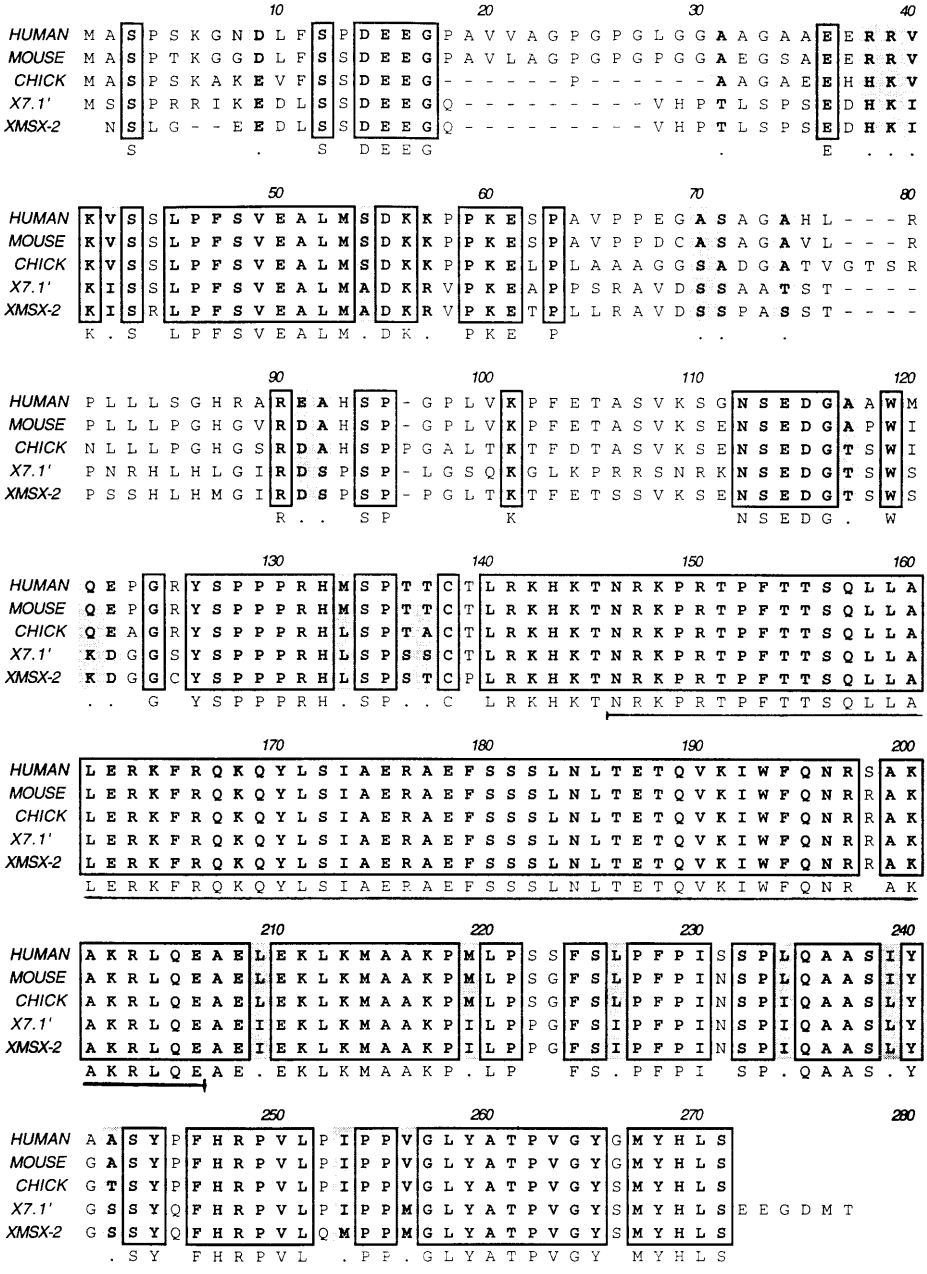


Fig. 1 Amino acid sequences of *msx-2* in human, mouse and chick are compared with the two versions of the *Xenopus msx-2* genes, *Xmsx-2* and *Xhox-7.1'*. Comparisons are based on a clustal multiple-sequence alignment of predicted amino acid sequences published in the EMBL database. The *boxed areas* indicate homology of all the members, whereas the *stippled area* indicates conservative changes. Where *Xmsx-2* and *Xhox-7.1'* differ the most, around 64 to112 a.a., *Xmsx-2* has higher conservation with the other members. The homeodomain is *underlined*. There is one amino acid residue difference in the homeodomain of the human gene compared to the rest of the members. Surrounding the homeodomain region are extensive areas of homology, as indicated by the numerous *boxed areas*. The *last row of sequence* indicates the shared homologous sequence of all the members of the genes with conserved changes indicated by a *period (.)*

Results

IIa Cloning of the *Xenopus msx2* gene

To isolate the *Xenopus* homolog of the *msx-2* gene, a stage-17 *Xenopus* cDNA library (T. Sargent, NIH) was screened under high-stringency conditions. The probe, 7.3 (F. Ramirez, Mt. Sinai Hospital), comprised a partial sequence (183 bp) of a clone, *Xhox 7.1'*, previously reported [45] to be a possible *Xenopus* homolog of the *msx-2* gene. Three clones were isolated, of which one clone, 9c, was characterized in detail. A open reading frame encoding a putative homeodomain was identified. The total length of the clone was 3.8 kb. About 3 kb of the clone was the 3' untranslated region containing the polyA tail, and 800 bp of the coding region. At the nucleotide level, comparison of the coding regions between clone *Xhox 7.1'* and clone 9c showed 91.75% identity. When the conceptual translation of this putative homeodomain protein was compared to that of *Xhox 7.1'*, there was 87% identity throughout the coding region (Fig. 1). The highest sequence conservation was seen in the putative homeobox region (100% conservation at the amino acid level with six conservative changes at the nucleotide level) and immediately downstream of this region. The major differences between the two genes at the amino acid level seemed to be clustered between amino acids 54–97 downstream of the putative translational start site of gene *Xhox 7.1'*, suggesting the possibility that the two genes might have differed in the presence of a small

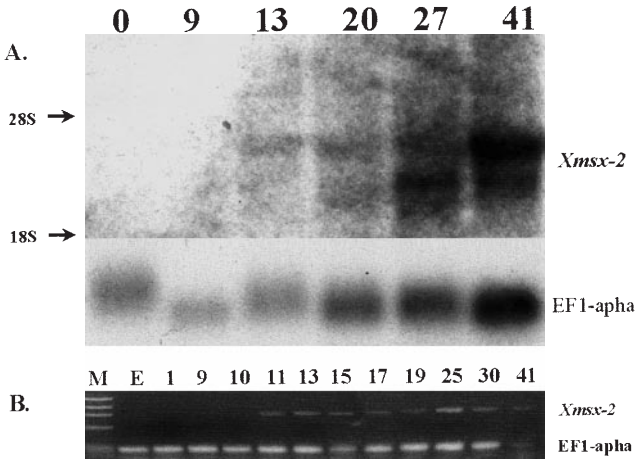


Fig. 2A,B Temporal expression profile of *Xmsx-2*. **A** Exposed film of a Northern blot analysis of *Xmsx-2* and EF1-α. Total RNAs extracted from *Xenopus* oocytes (*O*) and embryos at stages *9, 13, 20, 27* and *41* were electrophoresed on a 1% agarose formaldehye gel and transferred to a membrane that was hybridized to probe 7.3. It was rehybridized with radiolabelled EF1-α (*bottom lane*) to check for loading. The migration of the 28S and 18S ribosomal RNAs are indicated *on the left*. Signal is first detected at stage 13; two transcripts are apparent in some lanes while the larger transcript is evident first at stage 13 and gains intensity by stage 41. The slighter smaller transcript is first seen at stage 27 but seems to decrease by stage 41. **B** Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of *Xmsx-2* expression at the embryonic stages indicated (*E*, egg). Elongation factor-1 alpha (EF1-α) is used as the internal control. These experiments were performed by reverse transcribing RNAs extracted from egg and different staged embryos as indicated. Polymerse chain reactions were performed on the cDNAs with primers specific to *Xmsx-2* and EF1-α at 59°C for 27 cycles in the same reaction tube in a 50-μl volume. The reaction products were loaded and electrophoresed on a 1% agarose gel. Four hundred nanograms of ϕX*HaeIII* were also loaded as a marker (*M*). Expression of the *Xmsx-2* is first detected at stage 11 and continues to be present until stage 41

exon. Whether these two genes represent two different genes or are two different alleles of the *Xenopus msx-2* genes is unknown. Clone 9c is missing a translational start site, while *Xhox 7.1'* encodes a longer protein, with a stop codon located about 6 amino acids further downstream than in clone 9c.

A comparison was made of the putative protein encoded by our cDNA to *msx-2* homologs of human [16], mouse [27], chick [32] and *Xhox-7.1'* (Fig. 1). There is very great homology between the different members especially in the homeodomain region and in areas immediately around it. Except for one amino acid difference in the homeodomain of the human *msx-2* gene, there was 100% homology in the homeodomain region of the other members. In addition, there were numerous regions of extensive homology scattered throughout the gene, especially downstream of the homeodomain region. In the region where the two *Xenopus msx-2* clones differ the most (amino acids 64–112), the *Xmsx-2* clone has better homology with the *msx-2* genes found in the human, mouse, and chick. From this analysis, it is very likely that the newly cloned *Xmsx-2* and *xhox 7.1'* are homologs of the *msx-2* family of genes. We call our new clone *Xmsx-2* since it is likely to represent a *Xenopus* homolog of the mammalian *msx-2* gene

IIb Temporal and spatial expression of *Xmsx-2*

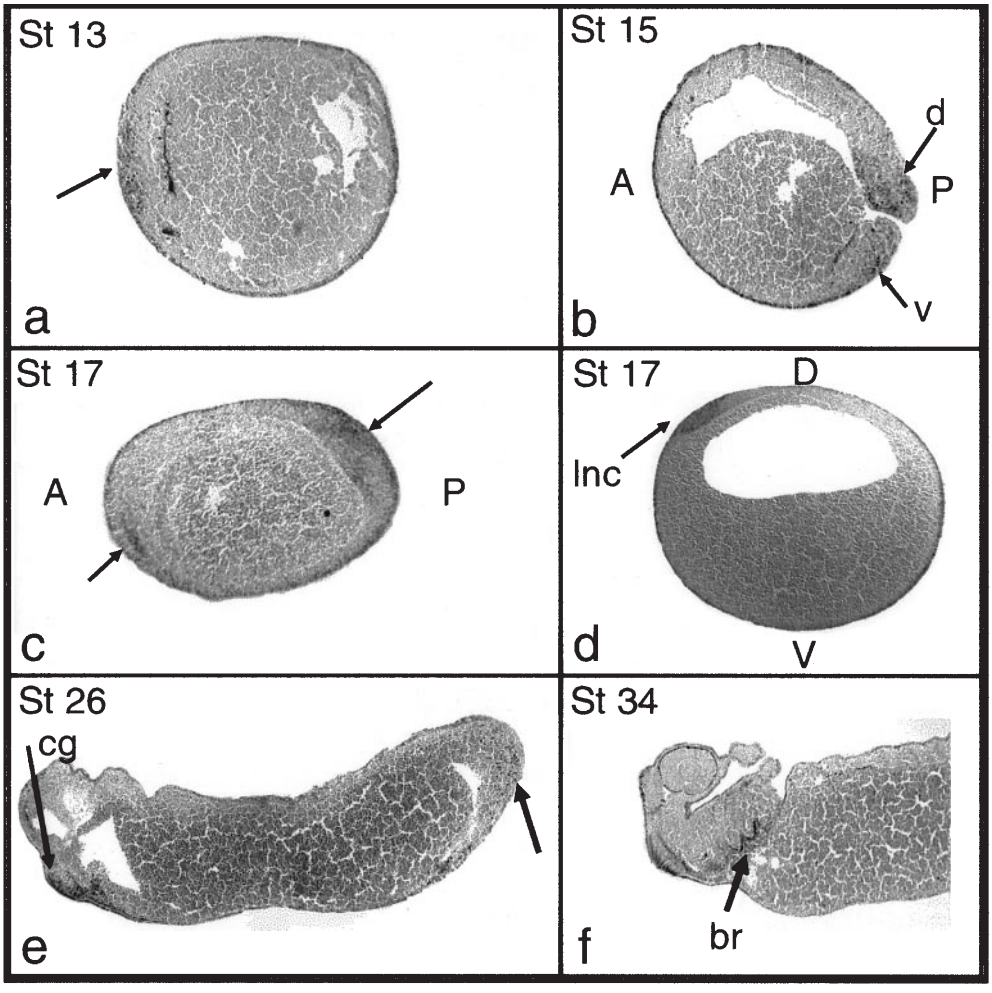
Northern-blot analysis with a probe that recognized both 7.1' and *Xmsx-2* revealed the presence of a 4.5-kb transcript at stage 13; by stage 27, another transcript of about 2.0 kb in size was also apparent (Fig. 2A). It is not known whether these two transcripts represent alternatively spliced products of the same gene or whether they are transcripts of two different genes. Further analysis with gene specific probes will answer this question.

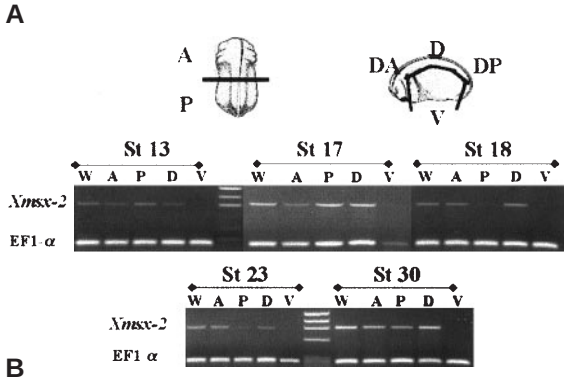
To determine more precisely the temporal pattern of expression of the *Xmsx-2* gene, RNA isolated from various staged embryos was analyzed by reverse transcriptase-PCR (RT-PCR) using primers specific to the *Xmsx-2* gene. EF1-α [21] was used as an internal control. Figure 2B shows that *Xmsx-2* was absent from oocytes and early stages of development prior to the gastrula stage. It was first detected at mid-gastrula (stage 11) and stayed relatively constant throughout the later stages examined.

The spatial pattern of expression was studied by in situ hybridization with a digoxigenin-labeled *Xmsx2*-specific probe on tissue sections obtained at different developmental stages (Fig. 3A). In stage-13 and -15 embryos, transcripts were detected around the circumporal

Fig. 3A, B Spatial expression profile of *Xmsx-2* in developing *Xenopus* embryos. **A** In situ hybridization with a digoxigenin-labeled antisense *Xmsx-2* gene-specific probe was performed on tissue sections prepared from embryos at different stages. *Xmsx-2* transcripts were present in the posterior part of the stage-13 embryo (*a* transverse section through the dorsal blastoporal lip) around the blastoporal lip region (*arrow*). At stage 15 (*b*), expression was obvious in both the ventral (*v*) and dorsal (*d*) parts of the blastoporal collar. Sections through the sagittal (*c*) and transverse (*d*) planes of stage-17 embryos revealed expression of the *Xmsx-2* gene in the anterior and posterior part of the embryo (*arrows* in *c*) and dorsal expression specifically in the lateral neural crest cells (*d, lnc*). At a later stage of development, transcripts were observed anteriorly in areas behind the cement gland (*cg*), heart primordium, and posteriorly in the tail fin area (*arrow*). The expression of the transcripts at stage 34 (*f*) seemed to be localized to the branchial arches (*br*) and in the area behind the cement gland; expression was also present in the tail fin area (not shown). **B** RT-PCR analysis of the spatial pattern of *Xmsx-2* expression. Embryos were dissected as shown on the top row of a stage-23 embryo from the dorsal view (*left*) and lateral view (*R*). A cut is made dividing the embryo into anterior (*A*) and posterior (*P*) halves. To obtain dorsal (*D*) and ventral (*V*) tissues, cuts were made along the lines as indicated. Dorsal tissues were further dissected into anterior (*DA*) and posterior (*DP*). RNAs were extracted from the different embryo parts as well as whole (*W*) embryos and RT-PCR performed as described in Methods. The PCR products were then electrophoresed in 1% agarose gels as shown above. In all stages examined, there was no expression in the ventral region. There appears to be a shift in the expression from the posterior to the anterior part of the embryo at different stages of development. Initially (stages13–17), expression seems more abundant in the posterior region, but by stages 18–23, posterior expression has declined, eventually increasing by stage 30; *M* marker, *W* whole, *A* anterior, *P* posterior, *D* dorsal, *V* ventral, *DA* dorsal anterior, *DP* dorsal posterior

collar (Fig. 3Aa, b). By stage 17, expression of the *Xmsx2* was also detected in the anterior part of the embryo (Fig. 3Ac) and in the lateral neural crest cells along the length of the embryo (Fig. 3Ad). By stage 26, transcripts were present in the cells behind the cement gland as well as the heart primordium; in addition, there was posterior expression of this gene in the tail fin area (Fig. 3Ae). This pattern of anterior and posterior expression persisted at stage 34; anteriorly the transcripts were restricted to the branchial arches. To further examine the anterior posterior distribution of the transcripts during development, RT-PCR was employed on dissected embryos. Embryos at various stages were dissected into anterior, posterior, dorsal, and ventral (Fig. 3B). RNAs were collected from the dissected tissues as well as whole embryos, and RT-PCR analysis performed using primers to the *Xmsx-2* gene and EF1-α as an internal control. Figure 3B shows that at all stages (13–30), *Xmsx-2* expression was found predominantly in the dorsal region. When the embryos were dissected into anterior and posterior at stage 13, expression was detected predominantly in the posterior region whereas at stage 17 the *Xmsx-2* gene was expressed almost equally within the anterior and posterior regions. At stages





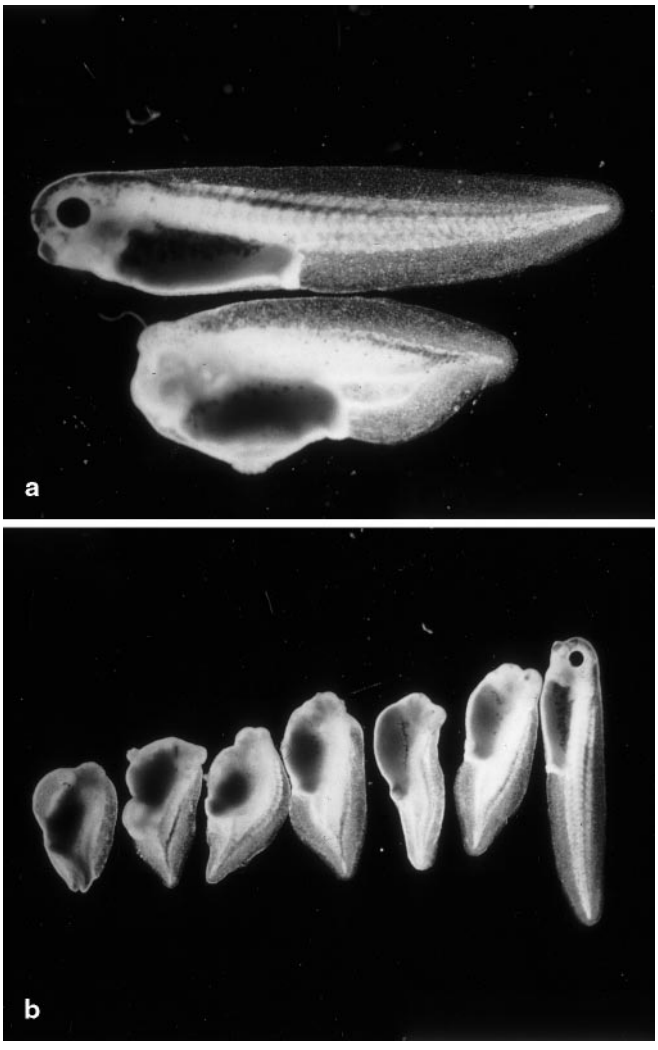


Fig. 4a, b *Xmsx-2* gain-of-function phenotype of *Xenopus* embryos. **a** One nanogram of in vitro transcribed msx-*2* mRNA was microinjected randomly into the marginal zone of newly fertilized *Xenopus* embryos. The embryos were allowed to grow and their phenotype noted. The control uninjected embryo is depicted *at the top*. Note the anterior truncation of the bottom embryo, although the overall morphology of the posterior part seems to be intact. **b** There is a range in the extent to which anterior structures are missing. The control, uninjected embryo is *on the far right*. Note the progressive decrease in the amount of anterior structures, ranging from reduced eyes and cement glands to missing head structures. The tail structure still appears intact in all the embryos

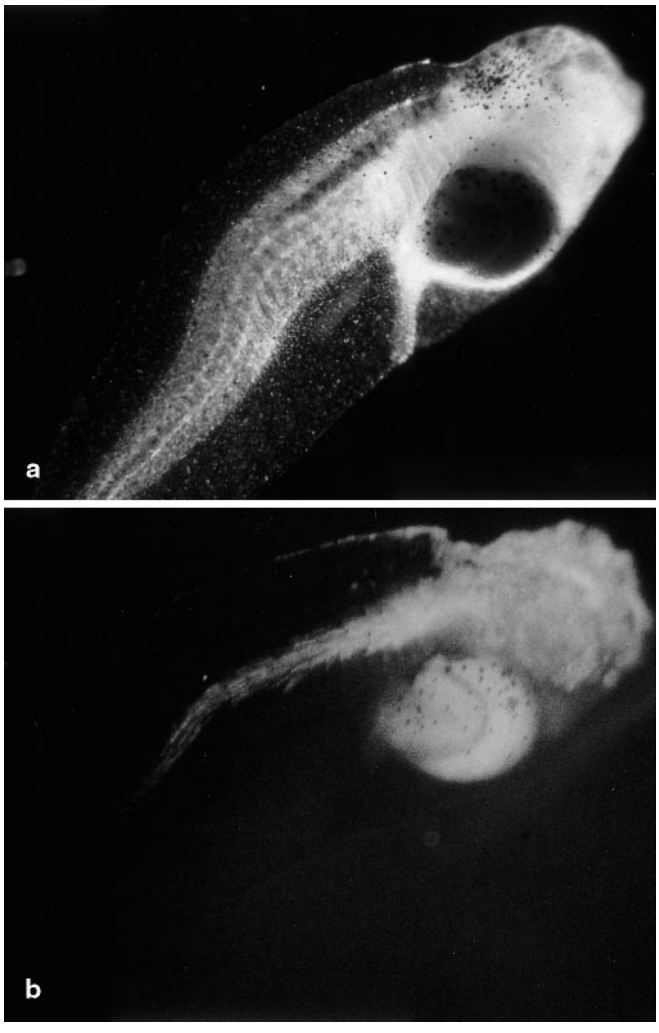


Fig. 5a, b Lineage tracing with green fluorescent protein (GFP). About 600 ρg synthetic GFP mRNA was coinjected with about 1 ηg *msx-2* mRNA into the marginal zone of one-cell *Xenopus* embryos that were then allowed to grow. **a** The embryo depicted here is approximately stage 41 and shows some phenotypic effect of the overexpression of the *msx-2* gene; the cement gland and eyes are reduced, and there is some slight bending of the body axis. **b** The same embryo under fluorescent light shows the presence of the green protein in the head and somitic region

18–23, the posterior expression was lost but was regained at stage 30. These data demonstrate that the regulation of expression of the *Xmsx-2* gene is spatially quite complex during embryogenesis, suggesting that the gene may be involved in a complex regulatory network.

IIc *Xmsx-2* affects dorsal-anterior mesoderm development

An effective way to test for the function of the *Xmsx-2* gene during development is to overexpress the gene product by injection of mRNA. To this end, in vitro transcribed mRNA was injected into the marginal zone of newly fertilized eggs. We used as a control mRNA transcribed from a construct that contained the mouse *msx-2* gene with a frame-shift mutation just upstream of the homeobox region *Δmsx-2*, resulting in a truncated protein lacking a homeodomain (gift of R.M. Maxson, USC). The microinjected eggs were allowed to develop and observed for any abnormalities during development. Development of the injected embryos proceeded normally through cleavage, blastula, and gastrula stages. By the neurula stage, however, it was evident that there was a problem in the anterior neural folds that resulted in abnormalities in the anterior head structures. In the majority of cases, no structures formed anterior to the otocyst.

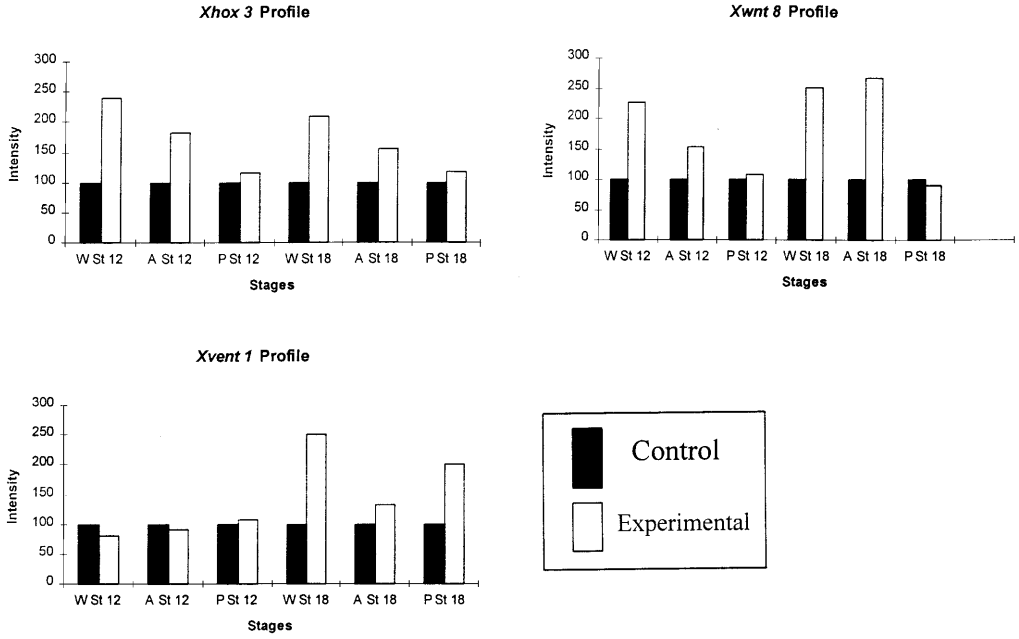


Fig. 6 Analysis of marker genes in *Xmsx-2* gain-of-function embryos. Graphs showing levels of genes normalized against those of EF1-α. RNAs were extracted from pools of about 10 stage-12 and -18 embryos that had been microinjected with the *msx-2* mRNA at the one-cell stage. The cDNAs that were reverse transcribed from these RNAs were subject to PCR reactions with primers specific to the *Xhox-3*, *Xwnt-8*, *Xvent-1*, and *Xbra* genes in combination with primers specific to the EF1-α. The PCR reactions were electrophoresed on a 1% gel and blotted onto membranes that were then hybridized to radiolabeled probes of the specific genes being tested. After hybridization, the membranes were stripped and probed with EF1-α. Levels of the genes of interest and of EF1-α, corresponding to the intensities in the graphs, were analyzed and measured with a phospho-imager. These graphs represent levels of the genes normalized against those of EF1-α. The *stippled boxes* indicate normal uninjected embryos, while the *clear boxes* indicate embryos that had been injected with the exogenous mRNA. Markers of a ventral-posterior nature, e.g. *Xhox-3*, *Xvent-1*, and *Xwnt-8* are upregulated, more in the anterior region than posteriorly

Figure 4A shows the phenotype of a stage-35 embryo microinjected at the one-cell stage compared with that of a noninjected embryo. The most conspicuous defect is the frequent truncation of head structures anterior of the otic vesicle (seen in 76% of wildtype injected embryos where *n*=88).

There seemed to be a range in the severity of loss of anterior structures. In very slightly affected embryos, defects were limited to the eyes and cement gland, which seemed smaller and less pigmented (second embryo from right in Fig. 4b). In more severely affected embryos, increasing amounts of the anterior structures were missing, and the overall size of the embryos was reduced (Fig. 4B). Embryos overexpressing the mouse truncated *msx-2* protein did not have any consistent defect (15% with nonspecific defects, e.g. gastrulation abnormalities, where *n*=55).

In order to ascertain that overexpression of the mRNA was not affecting cell viability resulting in the phenotype observed, we analyzed the expression of GFP, used here as a lineage tracer. Messenger RNA transcribed from a GFP construct [33] was co-injected with the *msx-2* mRNA into one-cell embryos. Figure 5A shows an embryo injected with both the GFP and *msx-2* mRNAs. The eyes and cement gland were reduced in size, and under UV light (Fig. 5B), we detected in this same embryo GFP protein in the affected areas. The distribution of the GFP protein suggested that the effect of the *msx-2* gene product was not through cell destruction.

IId Analysis of gene expression in *msx-2* injected embryos

The expression of three genes in embryos injected with the *msx-2* mRNA was assayed by RT-PCR. We used *Xwnt-8* [4], *Xvent-1* [10], and *Xhox3* [34] as markers of postero-ventral mesoderm. Both *Xwnt-8* and *Xvent-1* have been shown to be expressed in the ventral mesoderm and when overexpressed produced phenotypes of a ventralized nature. *Xhox-3* is first expressed predominantly in mesoderm in an even pattern from anterior to posterior. It then forms a gradient at the tailbud stage, with higher levels of expression caudally than rostrally [34]. Overexpression of *Xhox3* produced a phenotype strikingly similar to that of the embryos overexpressing *msx-2* [35]. In our experiments, injected embryos were allowed to develop and collected either as whole embryos, or dissected into anterior and posterior halves at stages 12 and 18. RNA was extracted and analyzed by RT-PCR using the appropriate primers. In each PCR reaction, primers for EF1-α were added to each set of experimental primers as an internal control. From Fig. 6, it can be seen that posteriorization of cell fate was corroborated by the analyses of marker gene expression. For example, the expression of *Xhox-3* was greater in the anterior half of the

mutant injected embryos than in control embryos. A very similar pattern was seen in the expression of the *Xwnt-8* gene: There was an increase in the overall levels of the gene in the mutant whole embryo, with the increase more evident in the anterior half than in the posterior.

The expression of *Xvent-1* gene showed a somewhat different pattern. There was no apparent increase of the *Xvent-1* mRNA in stage-12 mutant embryos; however, at stage 18 a large increase in the *Xvent-1* titer was detected in mutant whole embryos. This increase was detected within both the anterior and posterior regions of the embryo. The results for the three genes, all of which are markers of a more ventral-posterior nature, suggests that *msx-2* converts mesoderm into more posterior fates. Interestingly, the character of the posteriorized mesoderm was more ventral in nature.

Discussion

The search for a *Xenopus* homolog of the *msx-2* gene was initiated in an attempt to understand better the role of the gene in *Xenopus* embryogenesis. We believe that the clone we have isolated, *Xmsx-2*, in addition to the *Xhox-7.1'* previously identified [45], is a *Xenopus* homolog of the *msx-2* family of genes.

Xhox 7.1' gene was shown previously by RNAse protection analysis to be expressed initially at stage 10 1/2 [45]. Our experiments with the *Xmsx-2* gene using RT-PCR showed no expression in oocytes and embryos until stage 11. Stage 11 corresponds to approximately the period of mid-gastrulation; this timing of expression is highly reminiscent of that reported for the mouse and quail counterparts of *msx-2* [15, 31, 47]. By in situ hybridization analysis, the spatial expression of the *Xmsx-2* has been shown to be very similar to that reported for the *Xmsx-1* gene [23, 46]. Both the *Xmsx-1* and *Xmsx-2* genes belong to the *msx* family of genes and are totally conserved in the homeodomain region and in some residues surrounding the homeodomain. In mice and chicks, *msx-1* and *msx-2* genes are coexpressed at many sites, e.g. limb [29], mandible [26] and tooth [24, 25], although *msx-2* expression is more restrictive [3]. We were able to localize the transcipts to the individual neural crest cells along the lateral border of the neural folds as well as in the branchial arches in older embryos (stage 34). The localization of the transcripts in neural crest cells and branchial arches is not surprising since there is evidence that the mouse homolog is expressed in similar tissues in the mouse [25] and this gene might possibly play a role in neural crest migration (Maxson, personal communication). There appeared to be a shift in the antero-posterior expression at different stages of development. The significance of this switch from posterior to anterior and back is unclear; however, it is possible that it is related to its role in patterning the posterior-anterior axis of the dorsal mesoderm.

The sequence conservation of the homeodomain sequences, as well as the similarity in temporal pattern of developmental expression in other species, suggest that the *msx-2* family of genes plays important roles in morphogenesis. Indeed, the gene has been implicated in numerous epithelial-mesenchymal interactive processes throughout the body (reviewed in [7]). A mutation in the homeodomain of the human *MSX2* gene has been found in a family affected with autosomal dominant craniosynostosis [17]. Moreover, mice in which both *msx-1* and *msx-2* have been disrupted by antisense oligonucleotides showed numerous craniofacial defects [9]. Embryos overexpressing the *msx-2* gene showed a range of anterior defects very similar to the phenotype obtained with overexpression experiments using the *Xhox-3* [35], *Xvent-1* [10], *Xvent-2* [30, 38] and *Xmsx-1* [23, 46]. These findings have led to the definition of a class of genes that are involved in defining posterior-ventral patterning. Zygotic overexpression of Xwnt-8 and Xvent-1 leads to anterior truncations very similar to those obtained with the msx-2 gene whereas overexpression with BMP-4 and Vox leads to a phenotype that is completely ventralized. However, lower doses of BMP-4 and Vox lead to anterior truncations very similar to those obtained with the Xmsx-2 gene, suggesting the possibility that Xmsx-2 may not be quite as potent in specifying ventral posterior mesoderm. *Xmsx-2* could presumably act after dorsal or ventral mesodermal fates have been determined by the antagonistic effects of dorsalizing and ventralizing gene products, e.g. *goosecoid* antagonizing *Xvent-1* [10] and thus can be seen as a molecule that is involved in fine-tuning the anteroposterior patterning of the dorsal mesoderm. This is suggested by the effect of *Xmsx-2* on *Xhox-3* expression in giving mesoderm a more posterior character. On the other hand, increased levels of *Xvent-1* and *Xwnt-8* transcripts suggest that *Xmsx-2* may also function either directly or indirectly in ventral patterning. Another possibility is that because the *msx-2* gene is believed to function as a repressor [2], it might act to inhibit gene activity depending on when and where during embryogenesis it is expressed.

Although bone morphogenetic protein (*BMP*)*-4* is an important molecule in setting up the ventral-posterior pathway, we did not see any upregulation of *BMP-4* in our mutant embryos (data not shown), suggesting that either *Xmsx-2* is downstream of this gene as is the case with *Xmsx-1* [23, 46] or that it is in a different pathway altogether. It would be of interest, therefore, to investigate both the other molecules that might be involved and the epistatic relationships of the genes in the ventral-posterior mesodermal pathway.

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