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The initiation of *Hox* gene expression in *Xenopus laevis* is controlled by Brachyury and BMP-4

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Abstract

Hox genes encode a family of transcription factors that specify positional identities along the anterior–posterior (AP) axis during the development of vertebrate embryos. The earliest *Hox* expression in vertebrates is during gastrulation, at a position distant from the organiser or its equivalent. However, the mechanism that initiates this early expression is still not clear. Guided by the expression pattern, we identified upstream regulators in *Xenopus laevis*. The mesodermal transcription factor brachyury (*Xbra*) controls the early *Hox* expression domain in the animal–vegetal direction and the secreted growth factor BMP-4 limits it in the organiser/non-organiser direction. The overlap of these two signals, indicated by a Cartesian coordinate system, defines the initial *Hox* expression domain. We postulate that this system is a general mechanism for the activation of all *Hox* genes expressed during gastrulation.

Keywords: Brachyury; *Xbra*; BMP-4; *Hox*; Organiser mesoderm; Non-organiser mesoderm; *Xenopus*

Introduction

The anterior–posterior (AP) axis of vertebrates arises through a series of inductive events, including mesoderm induction, organiser formation, neural induction (the activation step in Nieuwkoop's, 1952, model of neural patterning) and AP patterning of the embryonic axis (including transformation in Nieuwkoop's, 1952, model). This last is closely connected to the correct expression pattern of *Hox* genes. These encode a family of transcription factors that specify positional identities along the AP axis during the development of vertebrate embryos (Hunt and Krumlauf, 1992; McGinnis and Krumlauf, 1992). A striking characteristic of *Hox* genes is their organisation in clusters on chromosomes. Interestingly, their temporal and spatial expression patterns are correlated to their positions within a cluster. 3′ -localised genes are expressed earlier during development than 5′ -localised genes (temporal colinearity, Deschamps et al., 1999; Duboule and Morata, 1994; Gaunt and Strachan, 1996; Izpisua-Belmonte et al., 1991). Furthermore, 3′-localised genes have more anterior expression domains than 5′-localised genes (spatial colinearity, Duboule and Dolle, 1989; Graham et al., 1989). Misexpressions within the spatial pattern lead to homeotic transformations, where segments of the AP axis change their fate to that of adjacent segments (Gruss and Kessel, 1991; Kessel and Gruss, 1991).

In *Xenopus laevis*, the initial *Hox* expression sequence appears during gastrulation. The *Hox* genes in this early sequence are all expressed in the same region of the embryo, but at different times. We found that not only paralogue 1 group gene expression (*Hoxd-1* and *Hoxa-1*, Kolm and Sive, 1995), but also the initial expression of other *Hox* genes is localised in the marginal zone. However, they are all excluded from the Spemann organiser (this study and unpublished observations). Dissections show that the initial expression is exclusively located in the non-organiser mesoderm.

How is *Hox* expression initiated in *Xenopus*? Several upstream regulators of *Hox* genes have been identified, including Activin (Cho and De Robertis, 1990; Green et al., 1992; Kolm and Sive, 1995), bFGF (Cho and De Robertis, 1990; Green et al., 1992; Kolm and Sive, 1995; Pownall et al., 1996, 1998), *Xcad-2* (Epstein et al., 1997), *Xcad-3* (Isaacs et al., 1998, 1999), retinoic acid (Kolm and Sive, 1995; Sive and Cheng, 1991), Wnt-8 (Kiecker and

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Niehrs, 2001) and *Hox* genes themselves (Hooiveld et al., 1999). However, these regulators have been shown to act later during development, for example, Activin and FGFs activate ectopic *Hox* gene expression at the end of gastrulation (Cho and De Robertis, 1990; Godsave et al., 1998; Green et al., 1992; Kolm and Sive, 1995) or affect only a subgroup of *Hox* genes, for example, retinoic acid activates anterior *Hox* genes (Bel-Vialar et al., 2002; Godsave et al., 1998), *Xcad* genes activate posterior *Hox* genes (Epstein et al., 1997; Pownall et al., 1996, 1998). In addition for some of these factors, it remains unknown whether they regulate mesodermal or neurectodermal *Hox* expression or both.

As opposed to the approach of identifying different activators for different *Hox* genes, we investigated whether there is a general system for the activation of *Hox* genes. As the initial expression of *Hox* genes is localised exclusively in the mesoderm, we investigated the effects of mesoderm inducers. We found that Activin and bFGF, as well as their downstream target *Xbra* (Latinkic et al., 1997; Smith et al., 1991), can expand the initial *Hox* domain. However, none of these is sufficient for the activation of early *Hox* expression.

Endogenous *Hox* expression is excluded from the organiser. One of the main functions of the organiser is the secretion of antagonists for BMP and Wnt signalling (for review, see De Robertis et al., 2000; Harland and Gerhart, 1997). We therefore asked whether these anti-organiser signals are important for the initial *Hox* expression. One of these, the ventralising and posteriorising growth factor BMP-4 (Dale et al., 1992), is necessary for the initial *Hox* expression, but not sufficient.

We found that only a combination of *Xbra* and BMP-4 signalling is necessary *and* sufficient for the activation of initial *Hox* expression. Each of the factors induced ectopic *Hox* expression exclusively within the functional domain of the other. Combined ectopic expression of both genes led to the expression of *Hox* genes all over the mesoderm and ectoderm.

We present a model based on our results. This describes the definition of the initial expression domain of early *Hox* genes in the mesoderm during gastrulation using a Cartesian coordinate system. The expression domain of *Xbra* (determined by the range of mesoderm inducing signals and transcriptional repressors) restricts the early *Hox* expression domain in the animal–vegetal direction. This *Hox* gene expression domain is further limited in the organiser/non-organiser direction by the functional domain of secreted BMP-4 protein (restricted by its range of diffusion and antagonising organiser signals). This may be the mechanism whereby a *Hox* "opening zone" (Gaunt, 2000) or a "*Hox* induction field" (Deschamps et al., 1999) is defined. These expressions describe a restricted domain for the activation of *Hox* genes early during development that is crucial for AP patterning (Gaunt, 2000; own unpublished results).

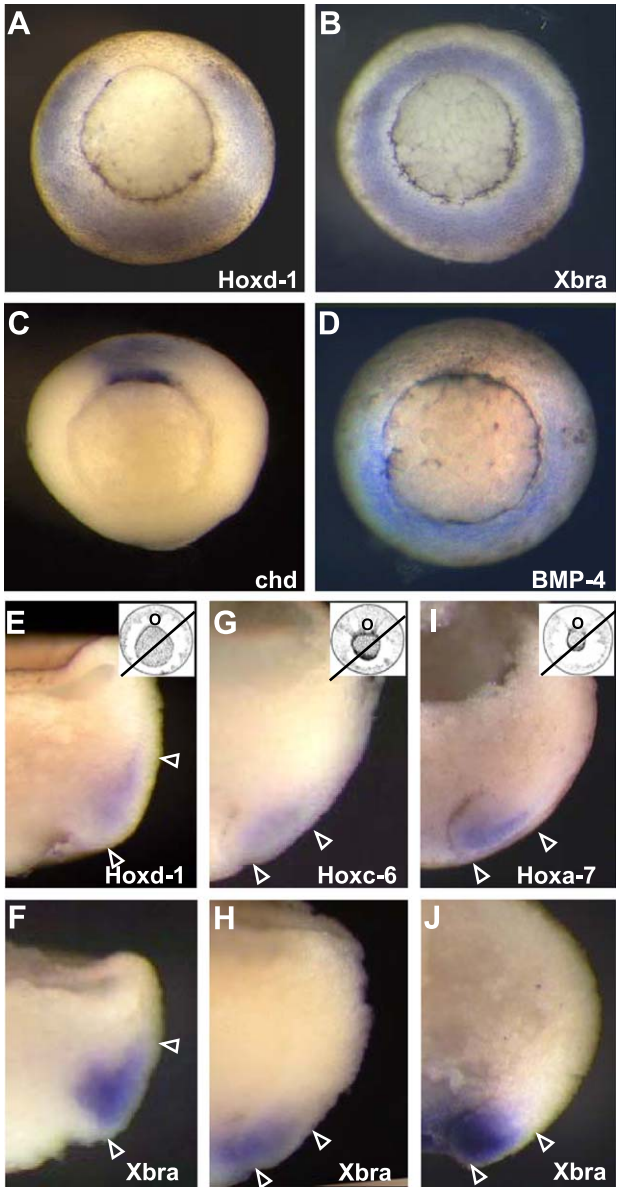


Fig. 1. The initial *Hox* expression is localised in the non-organiser mesoderm. Whole mount in situ hybridisation of *Hox* genes and mesodermal marker genes. (A–D) Vegetal view (organiser is up) of midgastrula stage embryos stained for *Hoxd-1* (A), which shows colocalisation with the mesodermal marker *Xbra* (B) with the exception of a gap in the organiser region [indicated by *chordin* (chd) expression (C)]. The expression domain of the secreted antiorganiser signal BMP-4 (i.e. the region of highest levels of secreted protein) is localised in similar embryonic regions (D). (E–J) Dissections were made across the initial *Hox* expression domain close to the organiser (O) (as indicated in the schematic drawings). In each case, one-half of an embryo shows the early expression of either *Hoxd-1* at stage 10.5 (E), *Hoxc-6* at stage 11.5 (G) or *Hoxa-7* at stage 12.5 (I), whilst the corresponding second half is stained for the mesodermal marker *Xbra* (F, H, J). The early expression of the different *Hox* genes is located within the *Xbra* domain. The arrowheads point to corresponding positions in the two half embryos.

Materials and methods

Embryos and explants

Embryos were staged according to Nieuwkoop and Faber (1956). Operation techniques, culture of explants and embryos and buffers (modified Barth's solution, MBS) have been described (Winklbauer, 1990).

Injection of mRNA, morpholino and growth factors

For the animal cap assay, growth factors (human recombinant Activin A, 200 nl of 200 U/ml; human recombinant bFGF, 200 nl of 200 ng/ml) were injected into the blastocoel of late-stage eight embryos. This method (introduced by Cooke and Smith, 1989) gave stronger mesoderm inducing effects (in terms of morphology, i.e., elongation of AC after Activin treatment and formation of ventral vesicles in FGF-treated AC) than incubating explants in the growth factors. Animal caps were explanted about 2–3 h later. Two individual animal caps were sandwiched together, which resulted in explants that were completely covered with an epithelial layer. These were cultivated in 10% MBS until they reached stages that were expected to show mesodermal *Hox* gene expression.

Morpholinos and mRNAs were diluted in Gurdon's buffer (15 mM Tris pH 7.5, 88 mM NaCl, 1 mM KCl) and injected at stages 1–4, depending on the experiment. The sequences of the morpholinos are as follows: BMP-4MO1: ttgacagaaaacaaggcatagaaaa; BMP-4MO2: acattccatgattcttgacagccaa; standard control MO: cctcttacctcagttacaatttata. The amount of morpholino injected was between 12 and 35 ng for BMP-4MO1, and 60 ng for BMPMO2 and control MO.

For mRNA injection, transcripts were generated from plasmids and injected at the following concentrations: tBR-

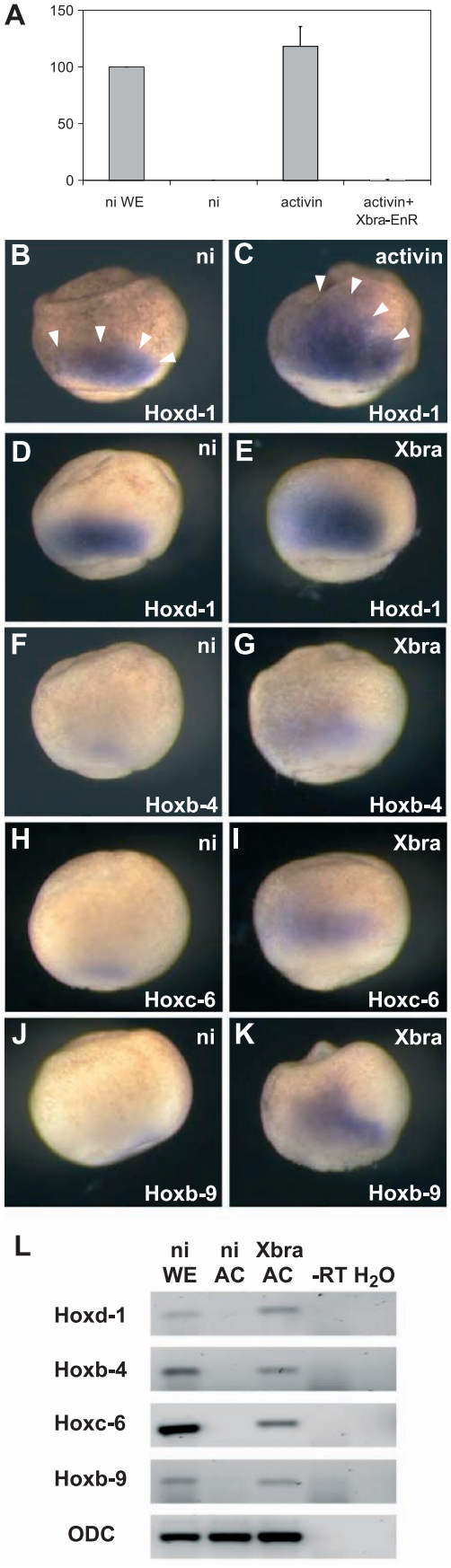


Fig. 2. The effects of mesoderm-inducing factors on early *Hox* expression. (A) Levels of *Hoxd-1* in explanted animal cap sandwiches from stage 11.5 noninjected embryos, embryos injected with Activin and embryos injected with Activin and the dominant interfering construct *Xbra-En*^{R}. Lightcycler PCR was used to quantitatively measure the levels of *Hoxd-1*, which were normalised to *ODC* levels and are shown as a percentage of the endogenous levels in whole embryos (WE). (B, C) The growth factor Activin was injected into the blastocoel of stage 8 embryos. In situ hybridisations (lateral views, organiser to the right) are shown for *Hoxd-1* at stage 11 in noninduced control (B) and Activin-injected (C) embryos. In induced embryos, the *Hoxd-1* expression is expanded in the animal direction. Arrowheads point to the animal border of *Hoxd-1* expression. (D–K) *Xbra* was ectopically expressed in the animal region. *Hox* expression was analysed by in situ hybridisation. Lateral views (organiser is to the right) of noninjected control embryos (ni) (D, F, H, J) and *Xbra*-injected embryos (E, G, I, K) stained for *Hoxd-1* at stage 11 (D, E), *Hoxb-4* at stage 11.5 (F, G), *Hoxc-6* at stage 12 (H, I) and *Hoxb-9* at stage 12.5 (J, K). Compared to corresponding controls, the expression of all analysed *Hox* genes in the *Xbra*-injected embryos is expanded in the animal direction. (L) The ability of *Xbra* to induce *Hox* genes in stage 11.5 explanted animal cap sandwiches (AC) was analysed by RT-PCR. All the *Hox* genes examined (*Hoxd-1*, *Hoxb-4*, *Hoxc-6* and *Hoxb-9*) were induced by *Xbra*. The endogenous expression in whole embryos (WE) is also shown.

64T, 1.2 ng (dominant negative BMP receptor) (Graff et al., 1994); XBMP4/pSP64T, 200 pg (BMP-4) (Nishimatsu et al., 1992); pCS2 + ALK6HA, 250 pg (constitutively active hALK6) (kind gift from Peter ten Dijke); Otx-2, 400 pg (Pannese et al., 1995); pSP73-Xbra, 800 pg-1.6 ng (Smith et al., 1991); gift from M. Sargent); pSP-gsc, 50 pg (Niehrs et al., 1994); pBSRN3-mix.1, 80 pg (Lemaire et al., 1998); pBSRN3-Xsia, 20 pg (Lemaire et al., 1995); pSP64T-Xbra-En^R, 400 pg (Conlon et al., 1996); *noggin*, 100 pg (Smith et al., 1993); pCS2Chd, 100 pg (*chordin*) (Sasai et al., 1994).

Detection of gene expression by in situ hybridisation

The whole mount in situ hybridisation protocol used has been described previously (Harland, 1991), except that the probe concentration is reduced to 40 ng/ml, hybridisation temperature is raised to 65°C and antibody incubations are done in 0.1 M Maleic acid, 0.15 M NaCl, 0.1% Tween-20, 1% blocking reagent (Roche), pH 7.5, with anti-Digoxigenin-AP, Fab fragments (Roche). Embryos were cut with a razor blade and halves used for whole mount in situ hybridisation with different probes to compare different expression patterns. For other experiments, embryos were cut after whole mount in situ hybridisation.

Antisense, Digoxigenin-labelled transcripts were prepared from the following plasmids: xHoxlab1 (*Hoxd-1*) (Sive and Cheng, 1991); a 708-bp fragment containing the complete *Hoxb-4* ORF cloned in pGEMTeasy; a 998-bp *Hoxc-6* fragment in pGEM1 containing a part of the homeodomain and extending into the 3′ UTR; Xhox-36.1 (*Hoxa-7*) (Condie and Harland, 1987); a 505-bp fragment containing the 3′ UTR of *Hoxb-8*; a 470-bp *Hoxb-9* fragment in pGEM3; pSP73-Xbra (Smith et al., 1991); pCS2Chd (Sasai et al., 1994); XBMP4 (Dale et al., 1992); *Xlim-1* (Taira et al., 1992); *Xvent-2* (Onichtchouk et al., 1996); gift from C. Niehrs).

PCR

Total RNA was extracted from animal cap sandwiches using the Tripure isolation reagent (Roche) according to the manufacturer's protocol but with an additional chloroform extraction step. cDNA was made using Superscript II M-MLV Reverse Transcriptase (GibcoBRL) and oligo dT primers. Absence of genomic DNA contamination was established by assaying samples taken through the RT procedure without the addition of reverse transcriptase. PCR was carried out either using the LightCycler System (Roche) or a normal PCR machine. For the LightCycler System the reactions consisted of 5–10 μl cDNA, 0.4–0.7 μM of each primer, 3 mM $MgCl_2$ and 2 μl DNA Master SYBR green 1 mix in a total volume of 20 μl. Reactions were cycled at 95°C, 56°C for 6 s, 72°C for 20 s, and fluorescence was acquired at 78°C. Quantification standards were included in each run. Primer sequences are as follows: *Hoxd-1* up, agggaactttgcccaactctcc; *Hoxd-1* down, gtgcag-

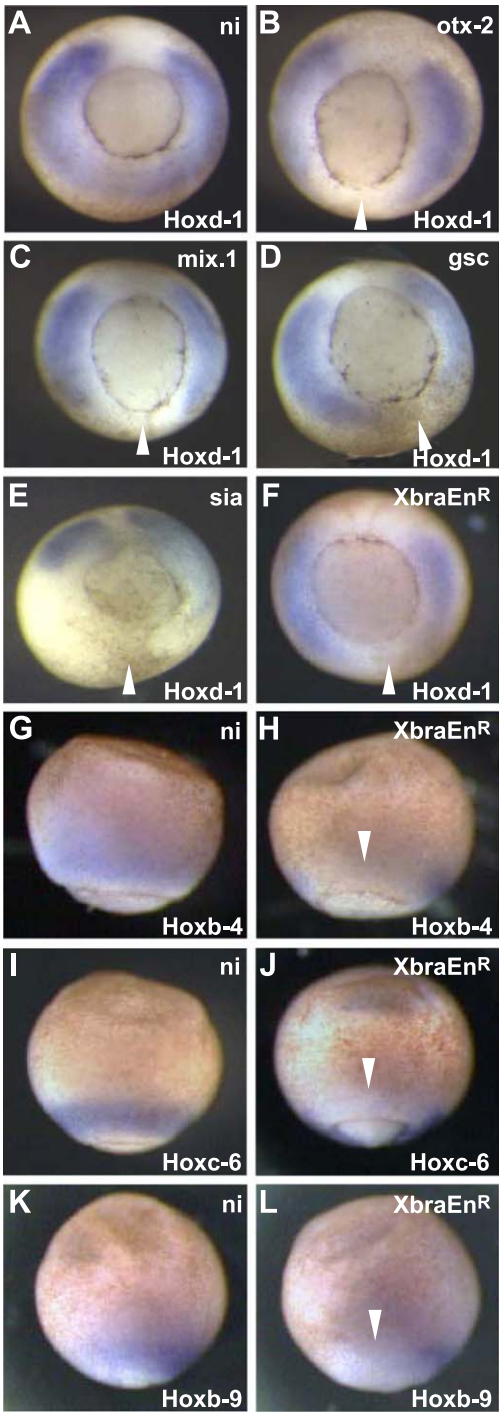


Fig. 3. Repression of *Xbra* results in a repression of *Hox* genes. Non-organiser site injection of factors that lead to a repression of *Xbra*. (A–F) Vegetal views (organiser is up) of noninjected (ni) control embryo (A) and embryos injected with *otx-2* (B), *mix.1* (C), *gsc* (D), *Sia* (E) and *Xbra-En^R* (F) mRNAs. All injected embryos show downregulation of *Hoxd-1* on the site of injection (arrowheads). (G–L) Non-organiser side views of noninjected embryos (G, I, K) and *Xbra-En^R*-injected embryos (H, J, L). Staining for *Hoxb-4* (G, H), *Hoxc-6* (I, J) and *Hoxb-9* (K, L) show that these *Hox* genes are also downregulated by repression of *Xbra* function. Arrowheads point to the side of injection.

tacatgggtgtctggc; *odc* up, gccattgtgaagactctctccatt; *odc* down, ttcgggtgattccttgccac.

For semiquantitative PCR, the reactions consisted of 5 μl cDNA, 0.15 μM of each primer, 0.33 mM dNTPs, 1.7 mM $MgCl_2$ and 0.25 μl Tfl polymerase in a total volume of 30 μl. Reactions were cycled at 95°C for 40 s, 56°C for 40 s, 72°C for 40 s. *ODC*, *Hoxd-1* and *Hoxc-6* were analysed after 25 cycles. *Hoxb-4* and *Hoxb-9* were analysed after 27 cycles. For analysis, 18 μl of the reaction was loaded on a 1.3% agarose gel containing Vistra-Green (Amersham) which was subsequently scanned and quantified with a Fluoroimager (Molecular Dynamics). The following primers were used *Hoxd-1* up: agggaactttgcccaactctcc; *Hoxd-1* down: gtgcagtacatgggtgtctggc; *Hoxb-4* up: ctgcggta-

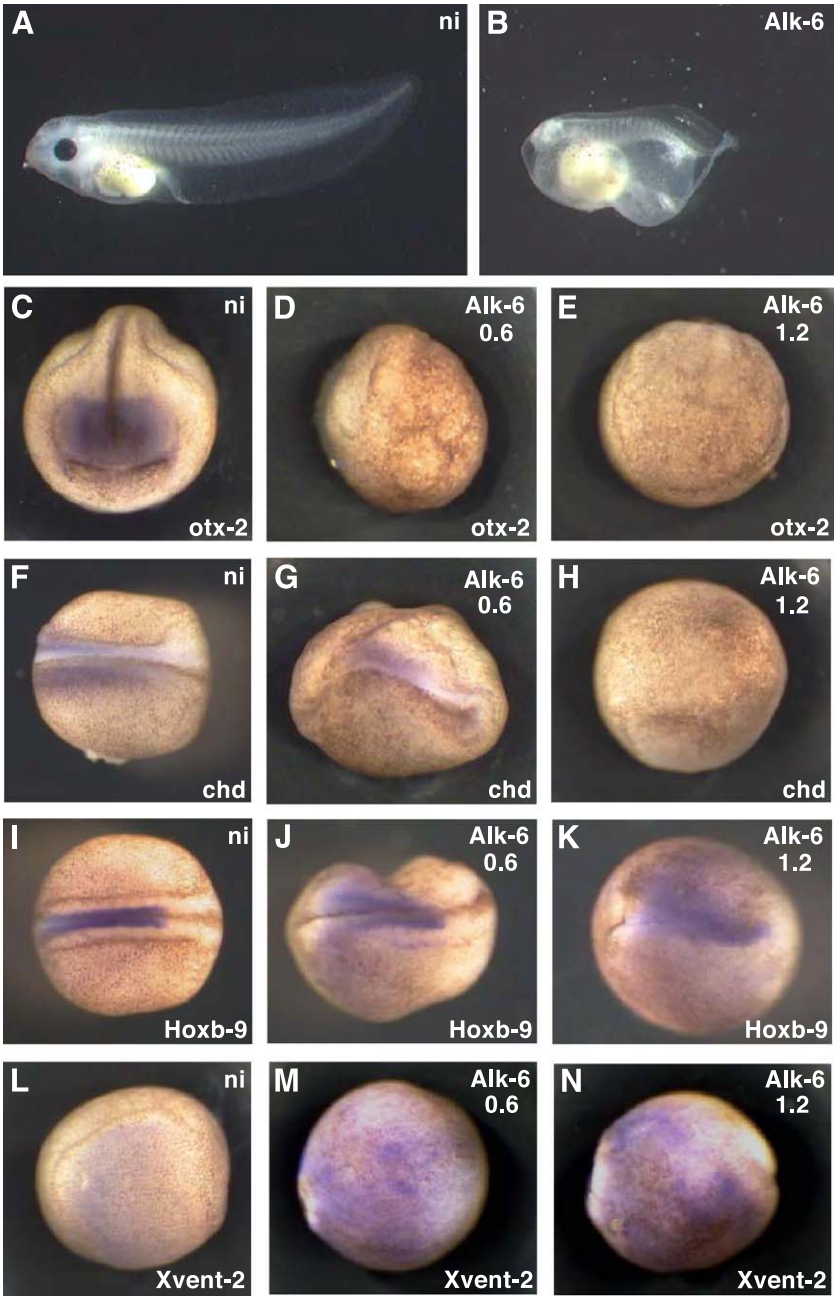


Fig. 4. The constitutively active BMP receptor, ALK-6, ventralises and posteriorises embryos. (A, B) Embryos were injected with the constitutively active human BMP receptor (Alk-6) at stage 1. Phenotypic analysis shows the expected effects of head reduction and shortened trunks in Alk-6-injected embryos (B) compared to noninjected (ni) controls (A). (C–N) Marker analysis of Alk-6-injected embryos. In situ hybridisations were performed on noninjected embryos (C, F, I, L) and embryos injected with 600 pg Alk-6 (D, G, J, M) or 1.2 ng Alk-6 (E, H, K, N) using probes for the anterior gene, *otx-2* (C, D, E), the organiser gene, *chordin* (F, G, H), the posterior gene, *Hoxb-9* (I, J, K) and the ventral gene, *Xvent-2* (L–N). Expression of *otx-2* and *chordin* was reduced, whereas expression of *Hoxb-9* and *Xvent-2* was expanded. Embryos are shown from the anterior (C–E), from the dorsal site with anterior to the right (F–K) or from the lateral side with anterior to the right (L–N).

caaaggctgaacct; *Hoxb-4* down: caggccccaaactgtgtgatc; *Hoxc-6* up: cagagccagacgtggactattcatccagg; *Hoxc-6* down: caaggtaactgtcacagtatggagatgatggc; *Hoxb-9* up: tactactacgggcttggctgga; *Hoxb-9* down: agcgtgtaaccagttggctg; *ODC* up: gtcaatgatggagtgtatggatc; *ODC* down: tccattccgctctcctgagcac.

Results

The initial expression of Hox genes is connected to mesoderm induction

We analysed the initial expression of several *Hox* genes in detail. A temporally colinear series of *Hox* genes is expressed in the marginal zone during gastrulation, starting with *Hoxd-1* (Kolm and Sive, 1995, this study and unpublished observations). The early *Hoxd-1* expression lies within the expression domain of the mesodermal marker *Xbra*, but is excluded from the Spemann organiser during gastrulation. The *Hoxd-1* expression domain is similar to the expression domain of the anti-organiser signal BMP-4, which represents the centre of the functional domain of this secreted factor (Figs. 1A–D). Similar observations were made for six other *Hox* genes analysed (*Hoxa-1*, *Hoxb-1*, *Hoxb-4*, *Hoxc-6*, *Hoxa-7*, *Hoxb-9*), whose expression is initiated at different times during gastrulation, but always in the region of *Xbra* expression and excluded from the organiser (unpublished observations). A comparison with the expression domain of *Xbra* in dissected embryos shows that the initial expression of different *Hox* genes is exclusively located in the *Xbra* domain (Figs. 1E–J). The gap between the *Xbra* domain and the blastopore (Kumano and Smith, 2000; Lemaire et al., 1998) is also free of *Hox* gene expression (Figs. 1A, B). This gap disappears in both, *Xbra* expression and *Hox* expression, during involution of the mesoderm. Later, *Hox* expression is also present in ectodermal tissue and is thus outside the *Xbra* domain, but here we want to focus on the initial expression in the mesoderm.

Based on their mesodermal localisation, and since it has been demonstrated that mesoderm inducers are able to activate later *Hox* expression (see Introduction), we investigated the effects of mesoderm-inducing factors on initial *Hox* expression. In an animal cap assay, normally *Hox*-negative animal caps (AC) were treated with Activin or bFGF. Since in situ hybridisation did not give consistent results, AC explants were analysed using lightcycler PCR. It has been described before that no early activation of *Hox* genes was detected in FGF-induced ACs and only weak expression was seen in Activin-induced ACs (Kolm and Sive, 1995). However, some modifications of the AC assay (blastocoel injection of the growth factors, sandwiched ACs, quantitative analysis using Lightcycler PCR) gave different results. Activin treatment resulted in strong activation of initial *Hox* expression (shown for *Hoxd-1*, Fig. 2A). This effect was blocked by the overexpression of the construct

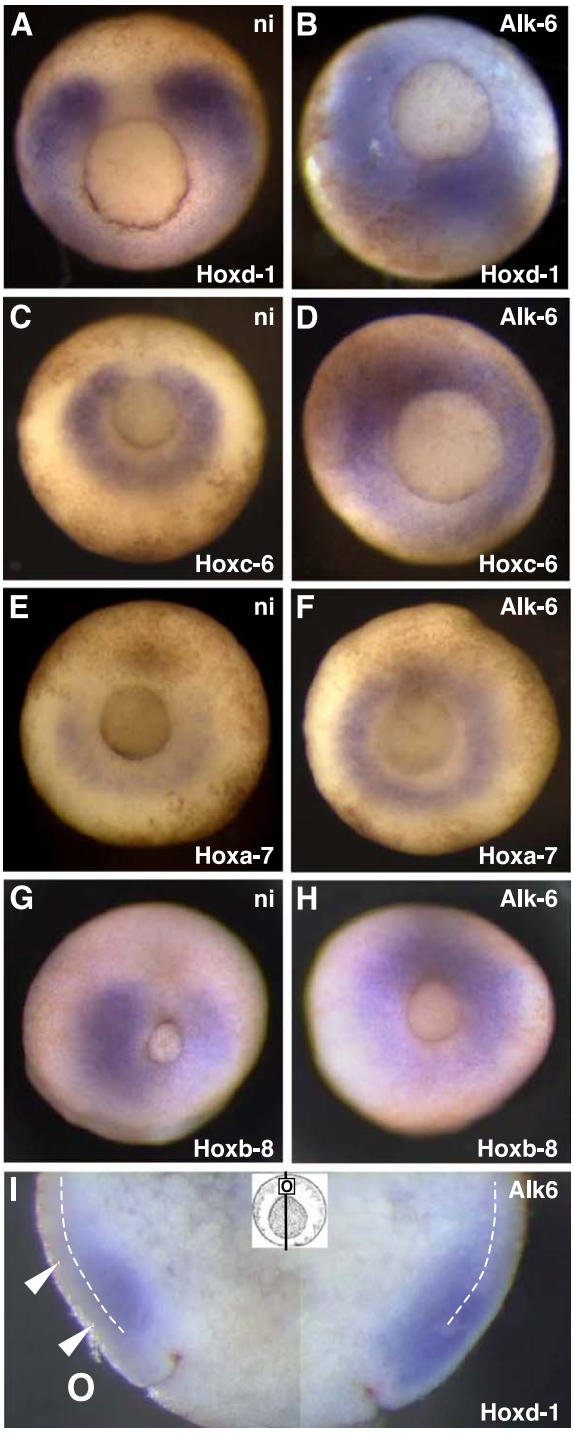


Fig. 5. Ectopic activation of the BMP pathway expands *Hox* expression to the organiser side. (A–H) Vegetal views (organiser is up) of noninjected (ni) and Alk-6-injected embryos stained for *Hoxd-1* (A, B), *Hoxc-6* (C, D), *Hoxa-7* (E, F) and *Hoxb-8* (G, H). Ectopic Alk-6 expression results in an expansion of the *Hox* expression on the organiser side. (I) A cross section (as indicated in the schematic drawing) of the marginal zone of an Alk-6-injected embryo (stage 11.5, organiser side indicated by O). Ectopic expression of *Hoxd-1* on the organiser side is present in the mesoderm, but not in the overlying ectoderm (arrowheads). The dashed lines indicate Brachet's cleft, which separates involuted mesoderm and the non-involuted tissue (i.e. neuroectoderm on the organiser side and preinvoluted mesoderm on the non-organiser side).

Xbra-En^{R}, which contains the strong engrailed repressor domain fused to the *Xbra* DNA binding domain (Conlon et al., 1996) and acts as a dominant inhibitory *Xbra* construct (Fig. 2A). Similar effects were obtained with bFGF and the combination of bFGF and *Xbra-En*^{R}, although the expression levels were much lower (not shown).

In whole embryos treated with Activin or bFGF in the same way and analysed with whole mount in situ hybridisation, the expression domain of *Hoxd-1* was expanded in the animal direction. This expansion was mainly found on the non-organiser side (shown for Activin in Figs. 2B, C). We conclude that mesoderm inducers do activate initial *Hox* expression. This activation is repressed when *Xbra* function is disabled.

The mesodermal transcription factor Xbra is a regulator of initial Hox expression

We performed overexpression experiments with the mesodermal transcription factor *Xbra*, which is known to be a target of both the FGF pathway and the Activin pathway (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Latinkic et al., 1997). The injection of *Xbra* mRNA resulted in an expansion of the expression domain of all the *Hox* genes examined. This expansion was towards the animal pole, whilst ectopic *Hox* expression was not observed on the organiser side (Figs. 2D–K).

Activation of *Hox* genes with *Xbra* was also obtained in an AC assay. ACs injected with *Xbra* RNA were sandwiched and cultivated for 2–3 h. RT-PCR shows that the ectopic expression of *Xbra* resulted in *Hox* gene activation (Fig. 2L).

We wanted to further test the idea that *Xbra* is necessary for the activation of early *Hox* genes. We chose a set of transcriptional regulators that are known to bind the *Xbra* promoter and to repress transcription. This included the organiser genes *otx-2* and goosecoid (*gsc*), and the vegetal gene *mix.1* (Latinkic and Smith, 1999; Latinkic et al., 1997; Lerchner et al., 2000; Papin and Smith, 2000). In addition, we used the transcriptional activator *Siamois*, which besides other effects is known to activate the repressor *gsc* (Carnac et al., 1996) and thereby should repress *Xbra* indirectly. Although it remains unknown whether all these transcription factors are Xbra regulators in vivo, they are useful tools for its manipulation. Therefore, we injected the RNA for these factors into the marginal zone opposite to the organiser and analysed how this affected *Hox* gene expression. The injection of *otx-2*, *gsc* and *mix.1* resulted in an inhibition of *Hox* gene expression creating secondary gaps in the *Hox* domain

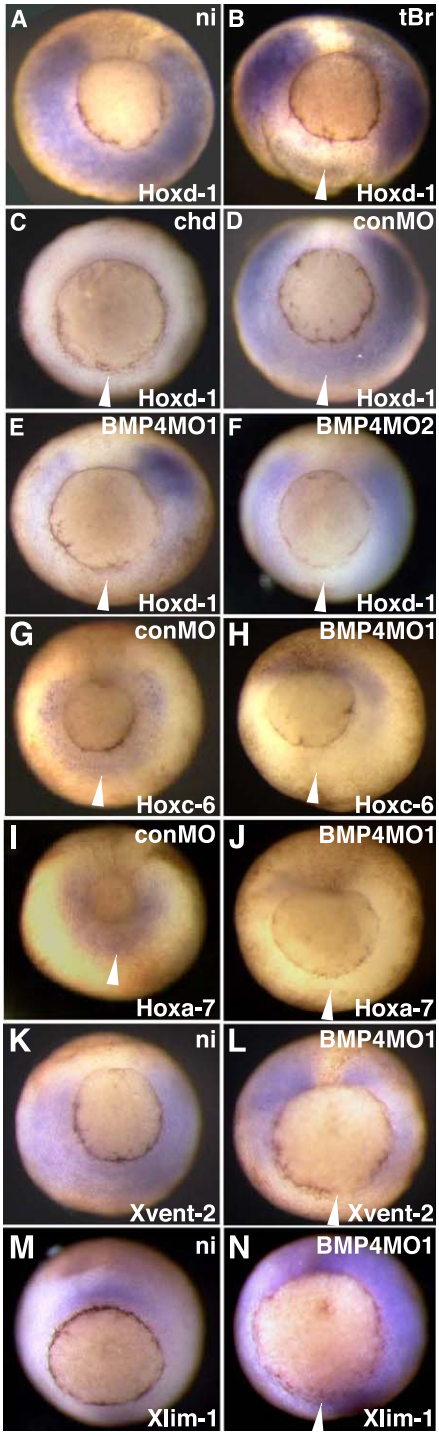


Fig. 6. Knock down of the BMP pathway results in repression of *Hox* expression. We used a dominant negative BMP receptor (tBR), a BMP antagonist [*chordin* (chd)] and two different morpholinos against *BMP-4* (BMP4MO1, BMP4MO2), to repress the BMP-4 pathway. Arrowheads point to the side of injection. (A–F) Vegetal views (organiser is up) of embryos stained for *Hoxd-1*. Noninjected (ni) embryos (A) and embryos injected with a control morpholino (conMO, D) show the characteristic horseshoe-shaped expression domain. Injection of tBR or *chordin* mRNAs on the non-organiser side resulted in a repression of *Hoxd-1* (B, C), as did the injection of the two different *BMP-4* morpholinos (E, F). (G–J) Experiments with the BMP4MO1 demonstrate that the expression of *Hoxc-6* (H) and *Hoxa-7* (J) are repressed compared to control morpholino injection (G, I). (K–N) To ensure that the *BMP-4* morpholino really affects the BMP-4 pathway, the known downstream target *Xvent-2* was analysed for changes in its expression after injection of BMP4MO1 and was seen to be downregulated (K, L). The organiser gene *Xlim-1* is upregulated (M, N).

(shown for *Hoxd-1* in Figs. 3A–D). A similar result was obtained from injections of *Siamois* (Fig. 3E).

The fact that all these different ways of *Xbra* repression have a negative effect on *Hox* gene expression is highly suggestive of a situation where *Xbra* is necessary for early *Hox* expression. However, from these experiments alone, we cannot exclude the possibility that these factors work on *Hox* genes directly or via a different route than *Xbra*. Therefore, we also injected the dominant inhibitory construct *Xbra-En*^{R} to look directly at the effect of knocking down *Xbra* function. Since the expression of *Xbra* is regulated by a feedback loop (via eFGF, Schulte-Merker and Smith, 1995), this injection also results in the reduction of the *Xbra* message itself, thus amplifying the dominant negative effect. If *Hox* genes are downstream targets of *Xbra*, their expression should be repressed. This was indeed the case for all of the *Hox* genes examined (*Hoxd-1*, *Hoxb-4*, *Hoxc-6*, *Hoxb-9*, Figs. 3F–L).

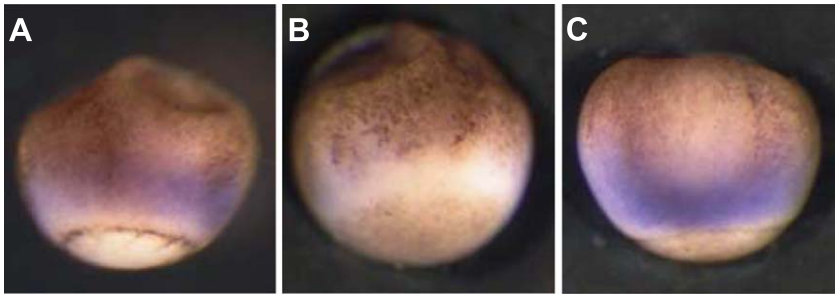
We conclude that the function of the transcription factor *Xbra* is necessary for the initial *Hox* gene expression, but not sufficient. However, the presence of the organiser gap in the endogenous expression of *Hox* genes, but not in the expression domain of *Xbra*, suggests that an additional factor required for *Hox* expression is absent from the organiser.

The secreted factor BMP-4 is a regulator of initial Hox expression

A perfect candidate for the second participating signalling molecule is BMP-4. BMP-4 is a secreted factor belonging to the TGF-β family. It is expressed from early gastrula stages in the marginal zone (Fig. 1D, compare Dale et al., 1992). The Spemann organiser secretes antagonists of BMP such as Noggin, Chordin and Follistatin (reviewed by Weinstein and Hemmati-Brivanlou, 1999; Wilson and Hemmati-Brivanlou, 1997). Thus, the endogenous BMP function is restricted to the non-organiser regions of the embryo (compare Schohl and Fagotto, 2002).

We found that ectopic activation of *Hox* genes by mesoderm inducers was restricted to the non-organiser regions of the embryo (compare Figs. 2B, C for Activin and Figs. 2D–K for *Xbra*). Therefore, we ectopically activated the BMP pathway on the organiser side of the embryo by injecting RNA for a constitutively active form of a BMP type I receptor (Alk-6, ten Dijke et al., 1994). The injection of this construct led to the same phenotype that is described for ectopic *BMP-4* expression (Dale et al., 1992; Jones et al., 1996) or the knockout of BMP antagonists in zebrafish (Hammerschmidt et al., 1996a,b). Anterior and dorsal structures were drastically reduced (Figs. 4A, B) and the expression of corresponding markers was repressed [*otx-2*, Figs. 4C–E; *chordin* (chd), Figs. 4F–H]. As expected, the expression of posterior and ventral markers was increased (*Hoxb-9*, Figs. 4I–K; *Xvent-2*, Figs. 4L–N).

Whilst *Xbra* overexpression never resulted in the ectopic activation of *Hox* genes on the organiser side, the injection of Alk-6 led to a closure of the "organiser gap" in the expression domain of several *Hox* genes (Figs. 5A–H). However, the ectopic activation of the BMP pathway did not activate *Hox* expression outside the *Xbra* domain and the closure of the organiser gap resulted from mesodermal *Hox* expression alone. The *Xbra* negative overlying ectoderm did not express *Hox* genes (Fig. 5I). This result was mimicked



D	number of analysed embryos	secondary gap in Hoxd-1 expression	% secondary gaps in Hoxd-1 expression
non-injected	38	1	3
BMP-4 MO1 30ng	21	18	86
BMP-4 MO1 30ng + BMP-4 protein 3ng	19	8	42

Fig. 7. Rescue of *BMP-4* morpholino effects on *Hox* gene expression by BMP-4 protein treatment. (A–C) In situ hybridisation of stage 11 embryos for *Hoxd-1*. Shown are ventral views of a noninjected embryo (A), an embryo ventrally injected with 30 ng of *BMP-4* morpholino (B) and an embryo ventrally coinjected with 30 ng of *BMP-4* morpholino and 3 ng of BMP-4 protein. (D) Table showing the numbers of the rescue experiment. Using the χ^2 test to compare BMP-4 morpholino injection to BMP-4 morpholino + BMP-4 protein injection, the rescue is significant at a significance level of $\alpha \leq 0.01$.

by the overexpression of full-length *BMP-4* mRNA (Fig. 9C, and data not shown).

To demonstrate that an active BMP pathway is necessary for the endogenous *Hox* expression, we knocked down the BMP signal using different approaches. The injection of mRNAs coding for tBR, a dominant inhibitory BMP receptor (Graff et al., 1994), or *chordin*, a BMP antagonist, on the non-organiser side resulted in a downregulation of *Hox* expression (shown for *Hoxd-1*, Figs. 6A–C). These two factors both produce a general block of BMP signalling (Graff et al., 1994; Piccolo et al., 1996). We also used a morpholino approach to establish whether BMP-4 itself is the key BMP factor involved in *Hox* regulation. This was shown to be the case, as when BMP-4 translation was inhibited on the non-organiser side via morpholino injection, all of the *Hox* genes examined were repressed (shown for *Hoxd-1*, *Hoxc-6* and *Hoxa-7*, Figs. 6E–J). A nonspecific control morpholino on the contrary had no effect on *Hox* gene expression (Fig. 6D). The specificity of the BMP-4 morpholino was shown by the fact that two independent, nonoverlapping BMP-4 morpholinos gave the same result (Figs. 6E, F). To further check the morpholino, we also investigated its effects on known downstream targets of BMP-4. Non-organiser side injections of the BMP-4 morpholino repressed expression of the BMP-4 target *Xvent-2* (Onichtchouk et al., 1996), whilst it led to an expansion of the expression domain of an organiser gene, *Xlim-1* (Taira et al., 1992) (Figs. 6K–N). These results indicate that the morpholino does work as would be expected.

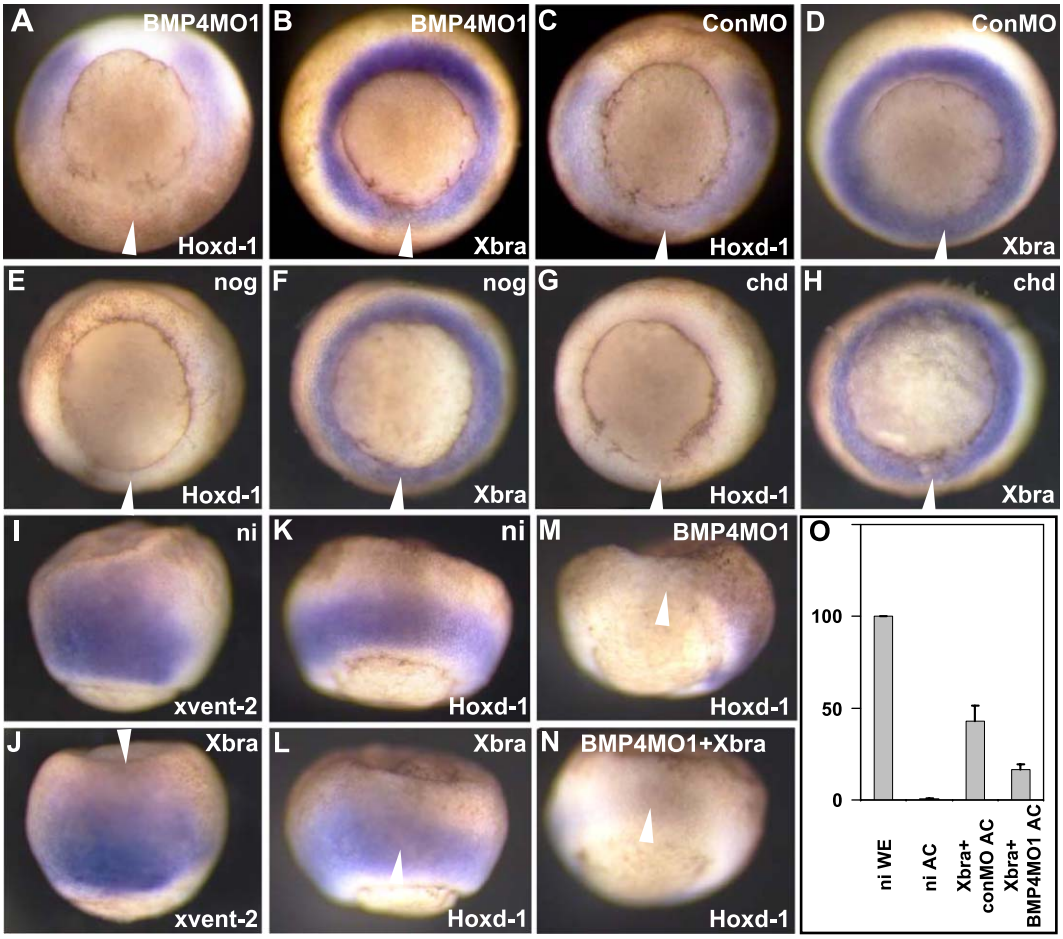


Fig. 8. BMP-4 and *Xbra* affect *Hox* expression independently but cooperatively. Arrowheads in all panels point to the site of injection. (A–D) Vegetal views (organiser is up) of gastrula stage embryos after injection of *BMP-4* morpholino (BMP4MO1) (A, B) or control morpholino (C, D). Whilst *Hoxd-1* was downregulated in BMP4MO1-injected embryos (A), *Xbra* expression was still present (B). (E–H) Vegetal views (organiser is up) of gastrula stage embryos after injection of *noggin* (nog) or *chordin* (chd) mRNA. Both of these BMP inhibitors downregulated *Hoxd-1* expression (E, G) but not *Xbra* expression (F, H). (I, J) To see whether *Xbra* had an effect on the BMP pathway, the BMP-4 target gene *Xvent-2* was analysed after animal injection of *Xbra*. The expression pattern was unchanged (I, J) (lateral views, organiser to the right). (K–N) Views of an uninjected embryo from the non-organiser side (K), an embryo injected with *Xbra* alone (L), an embryo injected with BMP4MO1 (M) and an embryo injected with BMP4MO1 and *Xbra* (N). The *Xbra* coinjection did not rescue the *Hoxd-1* downregulation by BMP4MO1. (O) Lightcycler PCR was performed to demonstrate that the upregulation of *Hoxd-1* expression by *Xbra* in animal cap sandwiches (*Xbra* + conMO AC) can be reduced by coinjection of a *BMP-4* morpholino (*Xbra* + BMP4MO1 AC). The graph shows *Hoxd-1* levels normalised to *odc* levels and expressed as a percentage of endogenous expression in whole embryos (WE). Noninjected cap sandwiches (ni AC) are also shown. Error bars indicate standard deviation ($n = 3$).

To confirm the specificity of the BMP-4 morpholino, we investigated whether coinjection with BMP-4 protein resulted in restoring the *Hox* expression. This coinjection lead to the closure of gaps in the Hox expression domain, which were observed after BMP-4 morpholino injection (Figs. 7A–C). There is a significant reduction of the BMP-4 morpholino effects on the expression of *Hoxd-1* (Fig. 7D).

Since the BMP pathway is necessary for the activation of early *Hox* expression, we wanted to examine whether the effects of BMP-4 are based on changes in *Xbra* expression. Therefore, we investigated the effects of BMP knockdown on *Xbra* expression. We found that a dose which led to a complete repression of *Hox* genes did not affect *Xbra* (12 ng, Figs. 8A–D). Only a dose about 3.5 times higher resulted in *Xbra* repression (40 ng, data not shown). In addition, the BMP inhibitors, Noggin and Chordin, whilst repressing *Hox* genes, had no effect on *Xbra* expression (Figs. 8E–H). These results agree with previous reports showing that repression of BMP signalling does not down-regulate *Xbra* expression (Northrop et al., 1995; Schmidt et al., 1995; Suzuki et al., 1994). Conversely, the injection of *Xbra* mRNA had no effect on *Xvent-2* expression, indicating that *Xbra* does not affect the BMP pathway (Figs. 8I, J). However, to ensure that the *BMP-4* morpholino effect was not due to an indirect effect on *Xbra*, we tried to rescue the

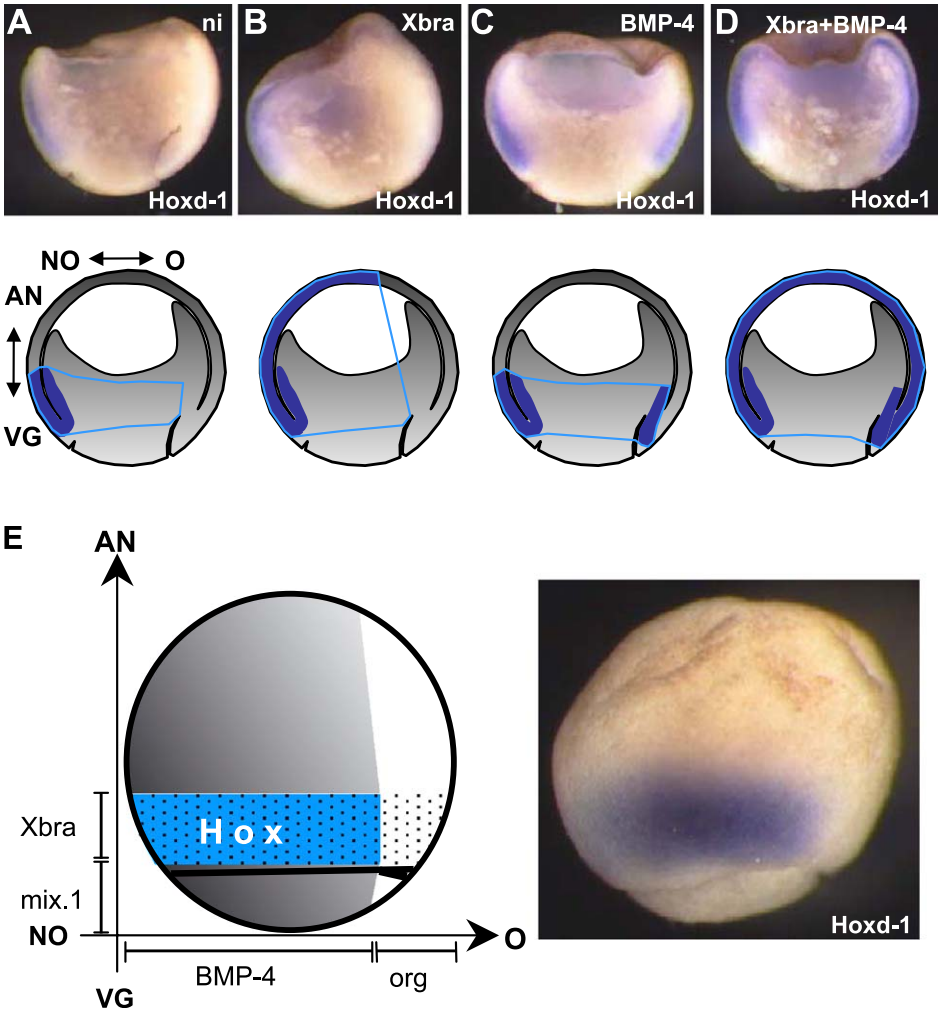


Fig. 9. Cooperation of *Xbra* and BMP-4. (A–D) In situ hybridisation of embryos dissected along the midline from stage 11 noninjected (ni) embryos (A), embryos injected with *Xbra* (B), embryos injected with *BMP-4* mRNA (C) and embryos injected with both *Xbra* and *BMP-4* (D). Pictograms indicate the localisation of *Hoxd-1* expression in the half embryos (blue colour) and projections of the expression onto the exterior of whole embryos (light blue line). In noninjected embryos (A), the normal expression in the non-organiser portion of the marginal zone is shown. No expression is present in the organiser. In embryos injected with *Xbra* (B), the *Hoxd-1* expression is expanded in the animal direction, but not to the organiser side. Expansion of the BMP-4 function by *BMP-4* mRNA injection (C) leads to ectopic *Hoxd-1* expression in organiser mesoderm, but not in animal parts of the embryo. Combination of both *Xbra* mRNA and *BMP-4* mRNA injection resulted in ectopic expression of *Hoxd-1* all over the mesoderm and ectoderm (D). O–organiser side; NO—non-organiser side; AN—animal; VG—vegetal. (E) Projection of the embryo into a Cartesian coordinate system: *Xbra* and BMP-4 restrict the *Hox* expression domain. The *Xbra* expression domain (dotted) overlaps with the functional domain of BMP-4 (grey gradient). In the overlapping region (blue), *Hox* genes are initially expressed. An actual embryo stained for *Hoxd-1* is shown in the same orientation. The expression of the *Xbra* repressor *mix.1* is also indicated, as is the presence of the organiser (org).

effects of the *BMP-4* morpholino by coinjection with *Xbra*-mRNA. The *Xbra* injection was unable to restore *Hoxd-1* expression in the morpholino-injected embryos, showing again that the BMP-4 effect is not via *Xbra* (Figs. 8K–N). These results also indicate that *Xbra* is unable to activate *Hoxd-1* in the absence of BMP-4. To test this, we used the AC assay. ACs excised from embryos injected with *Xbra*, in combination with either the control morpholino or the *BMP-4* morpholino, were sandwiched together and cultivated until stage 12. Lightcycler RT-PCR was used to quantitatively assess the levels of *Hoxd-1* in these explants. The induction of *Hoxd-1* by *Xbra* was reduced by approximately 60% when the *BMP-4* morpholino was coinjected, indicating that the endogenous BMP-4 in the animal pole (Hemmati-Brivanlou and Thomsen, 1995) is necessary for the *Xbra* induced activation of *Hoxd-1* (Fig. 8O).

We conclude that both *Xbra* function and BMP-4 signalling are necessary, but individually not sufficient, for the initial activation of *Hox* genes in the mesoderm, and that these two pathways function independently.

The combined functions of Xbra and BMP define the expression domain of early Hox genes during gastrulation

Since both *Xbra* and BMP-4 are necessary but individually not sufficient for the initial activation of *Hox* genes, we analysed how a combination of both signals affected the expression of *Hox* genes. When the expression pattern of *Hoxd-1* was examined in half embryos (Figs. 9A–D and corresponding schematic drawings), it could be seen that the *Xbra* mRNA initiated ectopic *Hox* expression only on the non-organiser side of the animal cap, that is, within the functional BMP domain (compare Schohl and Fagotto, 2002) (Fig. 9B). Conversely *BMP-4*, like Alk-6, induced ectopic *Hox* expression on the organiser side, but only in the mesoderm, that is, within the *Xbra* domain. Ectopic expression was not induced in the animal pole (Fig. 9C). However, when a combination of *Xbra* and *BMP-4* was injected, *Hoxd-1* was activated throughout the mesoderm and ectoderm, including the animal region of the organiser side that was negative for both factors individually (Fig. 9D). We do not observe ectopic expression in the vegetal cells. This could be due to the detection limits of the in situ hybridisation process. Alternatively, the vegetal cells may lack an essential cofactor normally present in mesoderm and ectoderm, or they may express a potent repressor of either *Xbra* or BMP function, or the *Hox* genes themselves.

Discussion

We have demonstrated that the mesoderm-inducing transcription factor *Xbra* and the secreted growth factor BMP-4 are both necessary for the initial activation of a series of *Hox* genes representing paralogous groups 1–9 in the mesoderm. The expression of the later, more posterior, *Hox* genes (paralogous groups 10–13) was not analysed in this study, as they are not expressed during gastrulation (Lombardo and Slack, 2001). Neither BMP-4 nor *Xbra* alone are sufficient for the activation of *Hox* genes. In our experiments, each of these factors induced ectopic *Hox* expression only within the functional domain of the other one. A knock down of either BMP-4 or *Xbra* function prevented initial *Hox* activation. Only a combination of both signals resulted in *Hox* expression all over the animal pole. The two pathways must therefore act in a cooperative way.

Upstream regulators of Hox genes

Since the vertebrate *Hox* genes and their role in pattern formation first received attention (for review, see Kessel and Gruss, 1990; Krumlauf, 1994; McGinnis and Krumlauf, 1992), several upstream regulators of *Hox* genes have been identified. However, the general regulation, which initiates *Hox* expression and the mechanism that generates the correct temporal and spatial expression patterns that result in a correctly formed embryonic AP axis, remain mysterious. Retinoic acid (Boncinelli et al., 1991; Dekker et al., 1992; Sive and Cheng, 1991) and Krox-20 (Nonchev et al., 1996; Sham et al., 1993) are upstream regulators, but their in vivo function is restricted to the neuroectodermal *Hox* expression in the hindbrain (Chen et al., 2001; Godsave et al., 1998; Nonchev et al., 1996; Sham et al., 1993). A recent report has also described the differential effects of retinoic acid and FGF on *Hox* expression, but this again was limited to the neurectoderm (Bel-Vialar et al., 2002). *Xcad-2* (Epstein et al., 1997; Pillemer et al., 1998) and *Xcad-3* (Isaacs et al., 1998; Pownall et al., 1996, 1998) act on a subset of *Hox* genes (paralogous groups 6–9) and might affect others indirectly (Epstein et al., 1997; own unpublished observations). Indirect effects may also result from interactions among *Hox* genes, as described for *Hoxb* genes (Hooiveld et al., 1999).

Proposals for general mechanisms of *Hox* gene regulation are based on gradients that are formed in the AP direction in the developing embryo. Posteriorising gradients of FGFs (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995), retinoic acid (Durston et al., 1989; Godsave et al., 1998), Xwnt-3A (McGrew et al., 1995, 1997) or Xwnt-8 (Kiecker and Niehrs, 2001) have been postulated to pattern the embryonic AP axis in *Xenopus*. Thus, they should also create the AP *Hox* pattern. Similar suggestions have been made for other vertebrates as well (Erter et al., 2001; Gaunt, 2000). However, these gradients act only from late gastrulation, and therefore after the initial *Hox* gene activation. They are not expected to be initial activators of *Hox* gene expression in the mesoderm.

Mesoderm-inducing molecules such as Activin and FGF have been analysed for their ability to activate *Hox* expression. It was postulated that *Hox* genes are differentially activated by different mesoderm-inducing factors (Cho and De Robertis, 1990; Kolm and Sive, 1995). The activation of

posterior *Hox* genes could be via the *caudal* genes (Epstein et al., 1997; Isaacs et al., 1998; Pownall et al., 1996, 1998). It has been demonstrated that the *caudal* genes are downstream targets of the FGF pathway (Northrop and Kimelman, 1994; Northrop et al., 1995). Therefore, they are also activated by Activin (Activin activates *Xbra*, Latinkic et al., 1997; Smith et al., 1991, *Xbra* activates *eFGF* Schulte-Merker and Smith, 1995 and eFGF, activates *Xcad-3*, Pownall et al., 1996, 1998).

As BMP-4 is also an upstream regulator of *caudal* genes (Northrop et al., 1995; Pillemer et al., 1998), it was expected that this factor should have an inductive effect on the posterior *Hox* genes. Surprisingly, BMP-4 also plays a crucial role for the activation of anterior *Hox* genes. A corresponding connection was observed in *Drosophila*, albeit at a later stage, where expression of the BMP homologue *decapentaplegic* in the visceral mesoderm is necessary for *labial* (the *Hox-1* homologue) expression in the gut endoderm (Immergluck et al., 1990; Panganiban et al., 1990). This activation has been shown to be direct (Marty et al., 2001; Tremml and Bienz, 1992). With this in mind it would be interesting to know whether the BMP response and the *Xbra* response in *Xenopus* are also direct.

To address this question we used an approach combining cycloheximide treatment (inhibiting protein synthesis, Cascio and Gurdon, 1987) with BMP-4 protein and a hormone inducible *Xbra-GR* construct (Tada et al., 1997), respectively. Under these conditions, *Hox* genes should only be activated, if they are direct targets of the molecules analysed. We found that for both BMP-4 and *Xbra* the ectopic activation of *Hoxd-1* was blocked by cycloheximide treatment (not shown). This indicates intermediate steps for both regulators, the nature of which are currently under investigation.

Our results point to a mechanism independent of differential activation of *Hox* genes by different activators. We find that the early expression of a series of *Hox* genes representing anterior to posterior paralogous groups, RA-sensitive and *Xcad*-regulated *Hox* genes is, independently of the time of their initiation, affected by identical factors.

Xbra and BMP-4 define the Cartesian coordinates of the initial Hox domain

To illustrate the interaction between *Xbra* and BMP-4 in creating the early *Hox* expression domain, we have used a projection of the embryo into a two-dimensional Cartesian coordinate system (Fig. 9E). The functional domain of *Xbra* (dotted area) defines the dimensions of the initial *Hox* expression domain (blue area) in the animal to vegetal direction (i.e. the *y*-axis). *Xbra* expression is limited by known repressors such as *mix.1* in the vegetal cells (Latinkic and Smith, 1999; Lemaire et al., 1998) and *XSIP1* in presumptive neuroectodermal cells (Papin et al., 2002), or simply by the range of mesoderm-inducing signals from the vegetal hemisphere. The dimensions of the initial *Hox* expression in the organiser/non-organiser direction (i.e. the *x*-axis) are defined by the functional domain of BMP-4 (grey gradient). This is restricted by the range of diffusion of the secreted molecules and by the action of secreted antagonising molecules coming from the organiser such as Noggin and Chordin (compare functional domain in Schohl and Fagotto, 2002). The overlap of these two signals contains the initial expression domain of *Hox* genes in the presumptive mesoderm (shown for *Hoxd-1* in Fig. 9E). The knock down of one of the two functions within the overlapping domain always resulted in a downregulation of *Hox* expression. Conversely, the expansion of the overlapping domain always resulted in the expansion of the *Hox* expression domain.

The initial Hox expression pattern as a foundation for AP patterning

Recent publications (reviewed in Kumano and Smith, 2002; Lane and Sheets, 2002) indicate that the "classical" dorsal–ventral axis of *Xenopus* (i.e. the organiser/non-organiser axis) actually represents the AP axis. An obvious concept would be to connect this "new" AP polarity to the early *Hox* gene expression. *Hox* genes are then found in posterior portions of the AP axis early during development. Different subsets of *Hox* genes define different positions along this AP axis, so one could expect to find an *Hox* pattern in organiser/non-organiser direction within the *Xbra*/BMP-4 domain. However, we did not find such a spatial prepattern.

Rather, the *Xbra*/BMP-4 domain is correlated to the "opening zone" (Gaunt, 2000) or to the "*Hox* induction field" (Deschamps et al., 1999) in mouse or chick. These phrases describe a very posterior domain of the embryo, where initial activation of *Hox* genes takes place and then spreads forward along the axis to form the characteristic spatial pattern. The AP pattern arises during gastrulation (Forlani et al., 2003; Mangold, 1933; Saha and Grainger, 1992). It has been suggested that gastrulation movements and interactions between organiser and non-organiser tissue are involved in the process of AP pattern formation (Kumano and Smith, 2002). A connection between the correct timing of *Hox* genes (perhaps in an exactly defined area such as the *Xbra*/BMP-4 domain) and properly established spatial expression domains has been postulated (Duboule, 1994). In chick and in mouse, the establishment of an AP *Hox* pattern seems to be independent of morphogenetic movements (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994). However, in *Xenopus*, morphogenetic movements are involved. We are currently investigating these mechanisms identifying quite complex interactions.

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