Regulation of dorsal-ventral patterning: the ventralizing effects of the novel *Xenopus* homeobox gene *Vox*

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SUMMARY

The formation of the dorsal-ventral axis in *Xenopus laevis* is elicited by a signaling cascade on the dorsal side of the embryo initiated by cortical rotation. These early developmental events impart an initial axial polarity to the embryo. By the time gastrulation occurs, the embryo has established opposing dorsal and ventral regulatory regions. Through a dynamic process, the embryo acquires a definitive pattern that reflects the distribution of future cell fates. Here we present a novel homeobox gene, *Vox*, whose expression reflects this dynamic process. *Vox* is first expressed throughout the embryo and subsequently eliminated from the notochord and neural plate. Ectopic expression of *Vox* demonstrates that the normal function of this gene may be to suppress dorsal genes such as *Xnot* and *chordin*, and induce ventral and paraxial genes such as *Bmp-4* and *MyoD*. Ectopic expression of BMP-4 ventralizes embryos and positively regulates the expression of *Vox*, suggesting that these genes are components of a reciprocal regulatory network.

Key words: *Xenopus*, homeobox, dorsal/ventral patterning, organizer, BMP-4

INTRODUCTION

Fertilization of the *Xenopus* egg initiates and orients a rotation of the cortex relative to the cytoplasm, initiating a signaling cascade on the dorsal side of the embryo (reviewed by Kimelman et al., 1992; Kessler and Melton, 1994). Additional signals arising from the vegetal hemisphere induce mesoderm at the equator of the embryo. Both signaling events are believed to transpire prior to zygotic transcription, imparting a primary pattern to the blastula-stage embryo. This primary pattern appears after the onset of zygotic transcription (the mid-blastula transition; MBT), when several genes are expressed differentially in either the presumptive dorsal or ventral regions of the embryo (Cho et al., 1991; Christian et al., 1991; Jones et al., 1995; Lemaire et al., 1995; Smith et al., 1995).

Spemann and Mangold demonstrated in transplantation experiments that the organizer, the region of cells above the dorsal lip, is capable of instructing cells in the ventral ectoderm and mesoderm to adopt neural and dorsal mesodermal fates, respectively, whereas in normal development the ventral ectoderm forms epidermis and the ventral mesoderm forms mesothelium and blood (Spemann and Mangold, 1924). Thus, in the traditional view of axis formation in amphibians, the tissue above the dorsal lip was thought to hold the communicable information to pattern the entire embryo. This view has been most recently summarized in the three signal model, in which the organizer region releases a diffusible signal that dorsalizes mesodermal cells and ‘neuralizes’ ectodermal cells (Slack, 1994). Epidermis and ventral mesoderm, in this model, are default fates from which cells are redirected by active signaling from the organizer.

Recent observations suggest an alternative to this model, in which the ventral side of the embryo plays an active and vital role in patterning the embryo (Sive, 1993; Fainsod et al., 1994; Graff et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995b; Steinbeisser et al., 1995). The signaling molecule BMP-4 has received considerable attention with respect to this role. BMP-4 is expressed during gastrulation (Dale et al., 1992) in the ventral marginal zone and ectoderm (Fainsod et al., 1994; Schmidt et al., 1995b), and has been shown to be critical for the ventral patterning of the embryo (Graff et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995b; Steinbeisser et al., 1995), possibly by antagonizing dorsal signaling molecules such as chordin (Sasai et al., 1994) or noggin (Smith and Harland, 1992). Thus, in an emerging view of axis formation in amphibians, pre-MBT inductive signals establish two approximate territories, dorsal and ventral, which produce opposing dorsalizing and ventralizing signals during gastrulation. This view is borne out by experiments in which BMP-4 is ectopically expressed, causing a significant reduction in the effectiveness of the dorsal organizer (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Schmidt et al., 1995b). In addition, pattern refinement and elaboration in the embryo comes about gradually. This is evident during gastrulation in which gene expression patterns reveal the dynamic nature of regionaliza-
tion; at the beginning of gastrulation, gene expression patterns appear unrefined, and by the end of gastrulation, boundaries of gene expression within specific tissue primordia become quite distinct. For example, prior to gastrulation *Xnot* is expressed throughout the embryo, and by the end of gastrulation it is restricted to the presumptive notochord (von Dassow et al., 1993). This suggests that the combined activity of signaling molecules and regulators of gene transcription, in a dynamic process, leads eventually to a definitive pattern and to subsequent tissue differentiation within distinct regions of the embryo.

We have identified a novel homeobox-containing gene, *Vox*, which is first expressed throughout the blastula-stage embryo. During gastrulation, *Vox* is eliminated from the future notochord and neural plate. We show that ectopic expression of *Vox* on the dorsal side of embryos negatively regulates the expression of prechordal, notochordal and neural gene expression, while positively regulating the expression of the paraxial mesodermal marker *MyoD* and the ventral marker *Bmp-4*. In a reciprocal fashion, ectopic dorsal expression of *Bmp-4* maintains the expression of *Vox* on the dorsal side of the embryo, from where it is normally eliminated. Furthermore, embryos injected with *Vox* RNA, when allowed to develop further, appear completely ventralized. These results provide evidence for a network of regulation in which reciprocal interactions result in the definitive patterning of the embryo. *Vox* may play an active role in establishing the ventral fates of mesoderm and ectoderm and in limiting the extent of dorsalization.

**MATERIALS AND METHODS**

**Isolation of Xenopus homeobox-containing sequences**

*Vox* was isolated using a polymerase chain reaction (PCR)-based approach as previously described (Northrop and Kimelman, 1994). The PCR fragments were used as probes to isolate full-length cDNAs from a stage 17 phage library (Kintner and Melton, 1987), which were inserted into the EcoRI site of the Bluescript SK vector (Stratagene). The complete nucleotide sequences of the cDNAs were determined and the nucleotide sequence of the *Vox-15* cDNA was deposited in GenBank under accession number U53529 and the *Vox-1* cDNA sequence under accession number U53528. A truncated *Vox* construct was produced by PCR, which contains the *Vox* coding region from the initiation codon through the WFQNRR sequence within the homeodomain. Both the normal and truncated *Vox* cDNAs were inserted into the CS2+ expression vector (Turner and Weintraub, 1994) to produce *Vox-174* and *Vox-165*, respectively.

**Embryos and microdissections**

Fertilized *Xenopus* embryos were prepared as described previously (Newport and Kirschner, 1982). After the jelly coat was removed with 2% cysteine (pH 7.8), the eggs were washed in 0.1× MMR (1× MMR is 0.1 M NaCl, 2 mM KCl, 1.0 mM MgSO₄, 2.0 mM CaCl₂, 5.0 mM Hepes, and 0.1 mM EDTA). Embryos were incubated in 0.1× MMR at 14-23°C. Embryos were staged according to Nieuwkoop and Faber (1967). After removal of the vitelline envelope, injected and un.injected stage 12-13 embryos were placed in 1× MMR and dorsal and ventral quadrants were cut with a 90° arc centered on the dorsal or ventral midline of the embryo using a fine wire knife.

**RNA synthesis and microinjection**

RNAs were synthesized using the mMessage mMMachine kit (Ambion). The RNAs were purified by one extraction with phenol:chloroform (1:1) followed by two rounds of concentration and separation in Microcon 100 microconcentrators (Amicon) to separate the RNA from unincorporated nucleotides. RNA was microinjected as previously described (Moon and Christian, 1989). 10 nl of RNA were injected per blastomere. Embryos were injected in 2 blastomeres of 4-cell embryos. The dorsal side of four-cell embryos was identified by pigment and cell size differences between dorsal and ventral blastomeres at this stage (Nieuwkoop and Faber, 1967). Embryos were fixed in 1× MEMFA (Harland, 1991).

**In situ hybridization and probe synthesis**

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes (Knecht et al., 1995), except that glass vials were used instead of baskets.

**Histology**

After MEMFA fixation and storage in methanol, embryos were embedded in Paraplast. Embedded embryos were sectioned at 10 μm and mounted in Permount (Kelly et al., 1991). Whole-mount embryos and sections were photographed using Kodak Ektachrome 160T film.

**RNA isolation and analysis by northern and RNase protection**

RNA was prepared by homogenization in an SDS-Proteinase K buffer (Cornell and Kimelman, 1994) and analyzed by the RNase protection assay (Melton et al., 1984). Probes for *Vox, Bmp-4* and *EF-1α* were prepared as previously described (Dale et al., 1992; Cornell and Kimelman, 1994). A probe for *Vox* was produced by digesting *Vox-15* with BglII and transcribing with T7 RNA polymerase. Probes were hybridized with RNA samples overnight at 45°C and then treated for 1 hour at 30°C with 40 μg/ml RNase A. Protected fragments were separated on 8% acrylamide-urea gels and exposed to film. For northern blots, poly(A) RNA was separated into a 1% agarose gel and transferred to Nylon. A *Vox* probe was produced from *Vox-15* by labeling with 32P by random priming.

**RESULTS**

**Isolation and sequence of Vox**

We originally isolated *Vox* as a novel homeobox gene by PCR amplification of animal cap RNA using degenerate oligonucleotides directed against conserved regions of the homeobox. We isolated two full-length *Vox* cDNAs from a neurula-stage cDNA library, *Vox-1* and *Vox-15* (*Vox* = *V* entral homeobox), both approximately 1.3 kb, with extensive nucleotide homology throughout the coding and untranslated regions (data not shown). Since the presence of two very similar *Vox* genes is likely to be due to the duplication of the *Xenopus* genome (Kobel and DuPasquier, 1986), we have studied only *Vox-15*, which we refer to here as *Vox*.

The *Vox* homeodomain has only limited similarity to homeodomains in previously identified homeobox-containing genes. The most closely related homeobox sequences are of the *Gbx* class; the homeodomain of the *Xenopus* gene *Xgbx-2* (von Bubnoff et al., 1996) is shown in Figure 1B and has only 56.7% amino acid identity with the *Vox* homeodomain. Other homeodomains are even less similar, and most of the shared amino acids are highly conserved among all homeodomains (Fig. 1B). *Vox* therefore represents a new family of homeobox-containing genes.

**Temporal and spatial expression of Vox during early development**

Northern blot analysis revealed the presence of a single *Vox*
transcript in the gastrula and neurula stages (Fig. 2A). The exact onset of Vox expression was determined by RNase protection analysis (Fig. 2B). Vox was very weakly detected in unfertilized eggs and early cleavage-stage embryos. At 7 hours postfertilization, strong expression of Vox was first detected. Since transcription of genes in the Xenopus embryo begins at the midblastula transition, 6 hours postfertilization, Vox is among the first genes to be zygotically expressed. The expression of Vox was maintained at a constant level throughout the gastrula and mid-neural stages, but progressively declined during the late neurula and tailbud stages (Fig. 2B and data not shown).

The spatial localization of Vox was determined using whole-mount in situ hybridization (Harland, 1991). At stage 9, prior to gastrulation, Vox is expressed throughout the animal hemisphere and equatorial region of the embryo (data not shown). As RNAs present in the vegetal hemisphere are not detected by whole-mount in situ hybridization, we analyzed the RNA levels present in animal versus vegetal hemispheres by microdissection and RNase protection. We detected equivalent levels of Vox in the animal and vegetal hemispheres at stage 10 (data not shown). Thus, Vox expression is activated uniformly at the MBT. At the onset of gastrulation (stage 10), Vox expression is cleared from a small region on the dorsal side of the embryo, this region corresponds to the animal hemisphere (Fig. 3A, B). By mid-gastrulation (stage 11), the clearing approximates both the region of the future anterior neural plate and the presumptive notochord (Fig. 3C). Near the end of gastrulation (stage 12), Vox expression is significantly cleared from the anterior dorsal region of the embryo, and the posterior paraxial expression develops distinct boundaries with the adjacent future notochord and floorplate regions (Fig. 3D). By the beginning of neurulation (stage 13-14), the posterior paraxial borders of Vox expression are sharp, and Vox expression in the anterior region is further reduced (Fig. 3E). Also in the early neurula, two new spots of expression appear in the anterior region of the embryo, which eventually becomes the dorsal side of the eye primordium in the tailbud and tadpole stages. At the end of neurulation (stage 19-20), Vox is expressed primarily on the ventral side of the embryo, and paraxial expression is limited to the posterior region of the embryo (Fig. 3F). Expression above the eye primordia is more distinct at this stage. By stage 22, posterior Vox expression is mainly limited to the tip of the tailbud flanking the notochord and floorplate (Fig. 3G). In stage 27 embryos, posterior expression is found only in the tail tip around the developing region of the notochord (Fig. 3H).

Because Vox is expressed in both the marginal zone and animal cap before gastrulation, we wished to compare the expression in the involuted versus noninvoluted cells during and after gastrulation. Sections of stage 11 embryos confirmed that Vox expression is absent at this stage in the region of the future neural plate (Fig. 4B) and along the future dorsal axis

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Fig. 1. The Xenopus Vox sequence. (A) The nucleotide and predicted amino acid sequence of Vox. The homeodomain is underlined. (B) Comparison of the Xenopus Vox homeodomain sequence to other Hox homeodomains. The dashes indicate identical amino acids. The percentage amino acid identity among the homeodomains is shown. Xgbx-2 (von Bubnoff et al., 1996), eve (MacDonald et al., 1986; Frasch et al., 1987), Xhox3 (Ruiz i Altaba and Melton, 1989a), Hox1.11 (Tan et al., 1992), and Nkx.5.1 (Bober et al., 1994).
was analyzed by RNase protection using a mixture of eggs (U) or embryos at various stages, separated on a denaturing gel, blotted and hybridized with a $^{32}$P-labeled full-length probe made from the Vox cDNA. (U) unfertilized egg; (8) stage 8, midblastula; (9) stage 9, late blastula; (10) stage 10, early gastrula; (12) stage 12, late gastrula; (15) stage 15, midneurula; (18) stage 18, midblastula; (9) stage 9, late blastula; (10) stage 10, early gastrula; (15) stage 15, midneurula; (18) stage 18, midblastula; (24) stage 24, tailbud. The blot was rehybridized with probes.

Both expressed in the dorsal region of the embryo. Both $Xnot$ and Vox are expressed throughout the embryo at stage 9. At the onset of gastrulation, however, $Xnot$ becomes restricted to the presumptive notochord, whereas Vox is eliminated from this region. This is seen clearly at stage 11 when the expression of $Xnot$ in the presumptive notochord becomes pronounced and the absence of Vox from the presumptive notochord is distinct (Fig. 5A). At this stage, chordin is expressed within the presumptive notochord and is also expressed in the anterior region of the embryo in the future neural plate (Fig. 5A). This contrasts precisely with the loss of Vox expression at this stage within the future anterior neural plate (Fig. 5A). Also within

Comparison of Vox expression with other known genes

The expression pattern of Vox bears a striking complementarity to the expression patterns of chordin (Sasai et al., 1994) and $Xnot$ (Gont et al., 1993; von Dassow et al., 1993), which are both expressed in the dorsal region of the embryo. Both $Xnot$ and Vox are expressed throughout the embryo at stage 9. At
the gastrula embryo, Vox and Bmp-4 expression overlaps; however, Vox expression extends more dorsally than that of Bmp-4, which is expressed on the ventral side of gastrula-stage embryos up to the border of the future neural plate (Fainsod et al., 1994; Schmidt et al., 1995b).

The complementarity of expression between chordin, Xnot and Vox continues into late gastrulation, and the Vox expression domain continues to overlap and extend beyond that of Bmp-4 (Figs 3 and 7; Schmidt et al., 1995b). In the early tailbud stages, chordin is expressed throughout the notochord (Fig. 5B, third embryo from top), whereas Xnot is limited to the posterior tip of the notochord and floorplate (Fig. 5B, top embryo). At this stage, the complementarity is most striking between Xnot and Vox, as Vox expression flanks the notochord and floorplate only in the posterior region of the embryo (Fig. 5B, second embryo from top). In the posterior of the embryo, Bmp-4 is restricted to a more ventral region than Vox, as well as to ectodermally derived regions of the tail fin (Fig. 5B, bottom embryo). At a slightly later tailbud stage, the complementarity between Xnot and Vox is clearly seen (Fig. 5C). By stage 31, chordin, Xnot and Vox are all restricted to the posterior tail region of the embryo and the complementarity between Vox and both of these axial genes is apparent (Fig. 5D, top three embryos). At this stage, Bmp-4 is expressed in the ventral posterior portion of the embryo as well as the tail fin of the embryo, including the tail tip (Fig. 5D, bottom embryo). Thus, not only do the expression patterns of these genes show striking complementarity, the complementarity is maintained during many stages of development.

Ectopic expression of Vox
Restriction of Vox expression from the dorsal region of the embryo suggests that elimination of Vox from this region is important for the development and patterning of the embryo. RNA encoding Vox was injected into the dorsal region of early cleavage-stage embryos. Embryos injected dorsally at the 4-cell stage with 4 ng Vox RNA began gastrulation coincident with uninjected controls, and completed gastrulation with a slight delay in the closure of the blastopore relative to control embryos. However, as early as the late gastrula and early neurula-stages, Vox-injected embryos were morphologically distinguishable from uninjected controls and also later clearly failed to neurulate (data not shown). When allowed to develop until the tadpole stages, embryos injected dorsally with Vox RNA lacked axial structures and appeared in most cases to be completely ventralized (avg. DAI = 0.26, n=206, Fig. 6A; scored as 0 on the DAI scale where normal embryos are given a score of 5; Kao and Elinson, 1988). Lower doses of Vox RNA caused less severe effects. Ventral injections of Vox had very little or no effect on development; the predominant effect of ventral injection of Vox RNA, if any, was a slight abnormality in the ventral region of the tadpole stage embryo near the proctodeum, indicating that the dorsal side of embryos is much more sensitive to Vox RNA injections than the ventral side (avg. DAI = 4.3, n=74, Fig. 6B). As a control for these injection experiments, we made a construct encoding a truncated version of the Vox protein lacking a small portion of the C-terminal region of the homeodomain (including two DNA contacting residues thought to enhance specificity; Kissinger et al., 1990) as well as the C-terminal domain of the protein. Injection of RNA encoding this truncated form of the Vox protein into the dorsal region of early Xenopus embryos produced normal and nearly normal embryos (avg. DAI=4.2, n=81). The defective embryos in these experiments had a slight reduction in anterior development and/or a curved axis, which may have been due to an
artifact of RNA injection (Moon and Christian, 1989), or residual activity of the mutant protein.

We also injected RNA encoding the homeobox-containing protein, Xhox3, expressed from the same vector. Ectopic expression of Xhox3 has been shown to cause truncation and ventralization of Xenopus embryos (Ruiz i Altaba and Melton, 1989b). Injection of 4 ng of Xhox3 RNA into the dorsal side of early embryos caused truncation of tadpole stages as reported earlier (avg. DAI = 2.4), but we did not observe any completely ventralized embryos as we observed with Vox RNA (see above). Injection of a two-fold higher dose of Xhox3 also did not produce any completely ventralized embryos, indicating that, although ectopic expression of both genes ventralizes Xenopus embryos, the effects of misexpression of Vox are more severe.

The effects of ectopic Vox expression on gene expression in the gastrula embryo

To better understand the abnormalities observed in embryos injected dorsally with Vox RNA, we examined the expression of genes transcribed during gastrulation and patterned during and after the onset of Vox expression in gastrula-stage embryos. Since Vox is cleared from the dorsal axial region of the embryo, we reasoned that expression of Vox within this region may adversely affect the expression of dorsal axial genes during gastrulation. To examine this, embryos injected dorsally with 4 ng Vox RNA were fixed and stained at the gastrula stage for goosecoid (gsc; Cho et al., 1991; Fig. 7A) and for Xnot (Fig. 7C) expression. Expression of both of these dorsal axial genes was eliminated (Fig. 7B.D). Since Xnot expression was eliminated from injected embryos, we looked

Fig. 5. Comparison of Vox, Xnot, chordin and Bmp-4 expression in Xenopus embryos. (A) Dorsovegetal view of uncleared, stage 11 embryos stained for chordin, left, Vox, middle, and Xnot, right, dorsal is up. The expression of Vox is most visible in uncleared embryos since clearing will cause the ventral expression to be visible through the dorsal side. Vox is cleared from the regions in which chordin and Xnot are expressed. (B) Lateral view of stage 24 embryos stained for Xnot, top; Vox, second; chordin, third, and Bmp-4, bottom, anterior is to the left. At this stage, Xnot and Vox expression is limited to the posterior region of the embryo, with Xnot expressed in the posterior notochord and floorplate and Vox expression cleared from these two developing tissues. Chordin, in contrast, is expressed throughout the notochord at this stage. Bmp-4 expression at this stage is found in ventral anterior and posterior regions of the embryo and is in the developing tail fin. (C) Lateral view of the posterior region of stage 26 embryos stained for Xnot, top and Vox, bottom. Posterior is to the right. Xnot is expressed in two clear regions of expression in the developing tailbud, the developing floorplate and notochord. Vox expression is absent from these two regions in the posterior of the embryo. (D) Stage 31 embryos stained for Xnot, top; Vox, second; chordin, third and Bmp-4, bottom, anterior is to the left. At this stage, only the most posterior tip of the body axis (the tailbud) contains Xnot and chordin expression. Vox expression surrounds the domains of chordin and Xnot expression. Bmp-4 is expressed ventral to the developing tailbud region and in the surrounding tail fin.

Fig. 6. Phenotypic effects of Vox RNA injections. 4-5 ng of Vox RNA were injected into the dorsal (A) or ventral (B) marginal zone of 4-cell embryos. (A) The top four embryos were injected with Vox RNA, the closed blastopore is to the right. The bottom embryo is an un.injected, stage 35 control, anterior is to the left. (B) The top two embryos were injected with Vox RNA, the bottom embryo is an uninjectected control embryo, anterior is to the left. Embryos are at stage 35.
at the expression of \textit{Xbra}, which is also found within the notochord (Fig. 7E; Smith et al., 1991). In stage 12.5 embryos injected dorsally with \textit{Vox} RNA, notochordal \textit{Xbra} expression was eliminated (Fig. 7F). This may be a consequence of the elimination of \textit{Xnot} expression in \textit{Vox}-injected embryos, since \textit{floating head} (the zebrafish homologue of \textit{Xnot}) is required for the notochordal expression of \textit{no tail} (the zebrafish homologue of \textit{brachyury}), as shown by Talbot et al. (1995). We also examined the expression of \textit{MyoD}, which is first expressed during midgastrulation (Harvey, 1990; Frank and Harland, 1991; Rupp and Weintraub, 1991). \textit{MyoD} is expressed within the paraxial mesoderm and marks the presumptive somitic region of the embryo (Fig. 7G; Frank and Harland, 1991). In embryos injected dorsally with \textit{Vox} RNA, the expression of \textit{MyoD} was no longer absent from the presumptive notochordal region of the embryo, but instead circumscribed the embryo (Fig. 7H).

As embryos injected dorsally with \textit{Vox} RNA appeared in most cases completely ‘ventralized’, we examined the expression of \textit{Xwnt-8}, a marker of early lateral mesoderm
RNase protection (Fig. 8). Whereas control embryos had five-expressed, surrounding the entire blastopore (Fig. 7N). To the dorsal side of the embryo, but instead was radially gene expression, but affects gene expression selectively. does not simply promote ventralization at the expense of dorsal marginal zone of 4-cell embryos eliminated the ventral embryos. However, injection of RNA had no effect on the expression of Bmp-4 Xwnt-8 expression is absent from the dorsal side of stage 10 embryos (Fig. 7I). Dorsal injection of Bmp-4 RNA was expressed (Fig. 9B). Therefore, BMP-4 is capable of inducing longer cleared from the dorsal side but rather, was radially expression in uninjected, stage 11 embryos is fold more Bmp-4 RNA on the ventral side than on the dorsal side of the embryos, nearly equal levels of Bmp-4 RNA were found on both sides of embryos injected dorsally with Vox RNA (Fig. 8). These results suggest that ectopic expression of Vox may lead to ventralization, in part, by inducing Bmp-4 on the dorsal side of the embryo during gastrulation.

Since Vox expression is eliminated from a region approximating that of the anterior neural plate and since embryos injected dorsally with Vox RNA do not appear to neurulate, we examined genes expressed within regions of the presumptive neural plate in embryos injected dorsally with Vox RNA. chordin is normally expressed within the presumptive notochord and anterior neural plate in stage 12 embryos (Fig. 7O; Sasai et al., 1994). Neither the presumptive notochordal nor anterior neural plate expression of chordin was detected in embryos injected dorsally with Vox RNA (Fig. 7P). Xotx2, a marker of the anterior neural plate (Fig. 7Q; Blitz and Cho, 1995; Panneese et al., 1995), was also absent in embryos injected dorsally with Vox RNA (Fig. 7R). Hairy II is expressed in a stripe that borders the presumptive neural plate as well as in the floorplate in late gastrula-stage embryos (Fig. 7S; Turner and Weintraub, 1994). Hairy II expression in stage 12 embryos injected dorsally with Vox RNA was greatly reduced (Fig. 7R). Thus, the disruption of neural development in embryos injected dorsally with Vox RNA is likely due to the suppression of genes important for neural plate specification.

**Vox expression in embryos ventralized by BMP-4**

Ectopic expression of BMP-4 ventralizes Xenopus embryos, reducing or eliminating all axial structures (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Schmidt et al., 1995b). Since ectopic expression of Vox produces embryos that are also ventralized and lack axial structures, we examined the expression of Vox in embryos injected dorsally with Bmp-4 RNA. Vox expression in uninjected, stage 11 embryos is cleared from the dorsal side of the embryo (Fig. 9A). In embryos injected dorsally with Bmp-4 RNA, Vox was no longer cleared from the dorsal side but rather, was radially expressed (Fig. 9B). Therefore, BMP-4 is capable of inducing the expression of Vox on the dorsal side of the embryo.

**DISCUSSION**

By the late blastula stage, early gene expression patterns reveal some semblance of dorsal-ventral patterning in the embryo. During gastrulation additional genes are expressed, and as gastrulation commences, the expression patterns of these genes become increasingly defined relative to one another and relative to specific regions of the embryo. We describe here the isolation of Vox, a novel homeobox gene, which has an expression pattern that reflects the dynamic regulation of gene expression during gastrulation. Vox and the previously described gene, Xnot (von Dassow et al., 1993), are first expressed ubiquitously after the MBT and subsequently become restricted to specific regions of the embryo during gastrulation. Furthermore, the expression patterns of these genes are strikingly complementary. Xnot expression is restricted to the presumptive notochord during gastrulation, and Vox is excluded from the notochord. This complementarity suggests that these genes may function to regulate each other as well as the expression of other axial and paraxial genes. Although the
complementarity of Xnot and Vox is striking, there is an additional feature of Vox expression that is not reciprocally represented in the expression of Xnot. Not only is the expression of Vox cleared from the presumptive notochord, it is also cleared from the presumptive anterior neural plate during gastrulation. At this time, chordin is strongly expressed in both the notochord and the presumptive anterior neural plate (Sasai et al., 1994). This suggests an additional function for Vox in limiting the extent of the neural plate and a possible regulatory relationship between these genes.

The striking complementarity of the expression of these genes persists during the patterning and growth of the tail in the tailbud and tadpole stages, reflecting the patterning of the late gastrula organizing region (Gont et al., 1993; this paper). In particular, Vox and Xnot expression becomes progressively restricted to the posterior region of the tailbud embryo (e.g. stage 26). The restriction of genes expressed during gastrulation to corresponding regions in the tailbud is not unique to Xnot and Vox, but the timing of this restriction is precisely coincident for these two genes. At these stages, chordin is expressed throughout the notochord and is not yet restricted to the posterior region. However, during tadpole stages (e.g. stage 31), chordin, as well as Vox and Xnot, becomes restricted to the tip of the tail (Gont et al., 1993; von Dassow et al., 1993; Sasai et al., 1994; this work), suggesting that there may be a continuous regulatory relationship that persists well into later development.

In addition, the expression patterns of chordin and Bmp-4 are somewhat reciprocal. The complementarity of these two genes is perhaps most evident in the ectoderm, where Bmp-4 is expressed adjacent to the anterior border of the neural plate (Fainsod et al., 1994; Schmidt et al., 1995), and chordin is expressed within the anterior neural plate (Sasai et al., 1994). However, the expression of Bmp-4 within the ventral marginal zone appears quite distant from the expression of genes such as chordin within the presumptive notochord. Thus, Bmp-4 may be important in normal development for the continued expression of genes such as Vox in ventral and paraxial regions; Vox in turn may directly regulate dorsal axial genes such as chordin and Xnot.

**Fig. 9.** Ectopic BMP-4 expression prevents the dorsal clearing of Vox expression in stage 11 embryos. Vegetal views, dorsal is up. A. Vox expression in an uninjected, stage 11 embryo. B. Vox expression in a stage 11 embryo injected dorsally with 4 ng Bmp-4 RNA; Vox is radially expressed (69% had radial Vox expression, 31% had reduced dorsal clearing, n=16).

**Fig. 10.** A hypothetical model of the gene regulatory network active during gastrulation in Xenopus. The set of interactions shown in this diagram reflect the minimum necessary to account for the observations discussed here, and is not intended to be the only possible hypothesis. Arrows indicate positive regulation, boxes indicate negative regulation; the interactions between the molecules shown may be either direct or indirect, transcriptional or posttranscriptional. As shown in this work, BMP-4 and Vox promote each other’s expression. Chordin, meanwhile, inhibits Bmp-4 function, but is itself repressed by Vox. The positive loop between Vox and BMP-4 is self-perpetuating. Vox may either directly repress chordin transcription, or function indirectly by regulating Xnot and gsc. Siamois may provide an early bias that distinguishes the dorsal from the ventral side of the embryo.

After submission of this manuscript, another Xenopus homeobox gene, Xvent-1, was described, which is very similar in sequence to Vox within the homeodomain and also has ventralizing properties when ectopically expressed (Gawantka et al., 1995). Since the sequences of Xvent-1 outside of the homeobox share very little similarity with Vox, it may be that the ventral region of the embryo is under the control of a set of genes with similar DNA-binding characteristics.

**Vox and the regulation of gene expression in the embryo**

Heretofore a number of genes have been found that are expressed on the dorsal side of the early embryo, and are therefore putative regulators of organizer specification. Some of these genes induce the formation of a secondary axis when expressed on the ventral side of the embryo. Relatively few genes have been described thus far that promote the development of ventral tissues; Vox and Bmp-4 are among these few. BMP-4 ventralizes embryos when ectopically expressed on the dorsal side (Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995), and Vox, when expressed ectopically on the dorsal side, also ventralizes embryos such that little or no axial development is apparent. This indicates that elimination of Vox expression from the neural plate, notochord and organizer is critical for the formation of axial structures.

Closer examination of Vox-injected embryos by in situ hybridization reveals that the ventralization takes place during gastrulation as a result of the loss of expression of axial genes such as gsc and Xnot, and the induction of non-axial genes such as MyoD and Bmp-4 on the dorsal side of the embryo. The induction of MyoD in Vox-injected embryos suggests that Vox is important for establishing MyoD expression in the paraxial mesoderm. Vox may achieve this either by restricting the expression of axial genes from the paraxial region, thereby allowing the expression of MyoD, or by positively regulating the expression of MyoD in the paraxial mesoderm.

An unexpected result was that ectopic Vox expression on
Ectopic expression of Vox on the dorsal side of the embryo also eliminates or greatly reduces the expression of neural genes such as chordin, Hairy II and Xotx2. This suggests that the clearing of Vox expression from the neural plate is important for the development of the neuroectoderm. Ectopic expression of BMP-4 similarly eliminates or greatly reduces the expression of neural plate markers (Schmidt et al., 1995b). What relationship do these antagonists of neural induction have to one another? Ectopic Vox expression in early embryos induces Bmp-4 such that it is expressed in nearly equal amounts on both the dorsal and ventral sides of the embryo. In addition, ectopic expression of BMP-4 radializes Vox expression, eliminating the dorsal clearing. Vox expression normally precedes that of Bmp-4 (Dale et al., 1992) and therefore Vox might initially activate Bmp-4. However, Vox is eliminated from the organizer region at approximately the same time as Bmp-4 is first expressed, and Bmp-4 expression is absent from a broader dorsal domain than Vox. At this stage the ventral expression of Vox may become dependent on BMP-4 signaling for its continued expression. A previous study showed that ectopic expression of exogenous Bmp-4 in whole embryos enhanced the expression of endogenous Bmp-4 (Jones et al., 1992). Our results suggest that this may have occurred by the activation of endogenous Vox expression.

Evidence for reciprocal regulatory interactions in the gastrula embryo

Patterning of the embryo during gastrulation is, not surprisingly, a complex phenomenon. Searching for regulatory hierarchies and linear relationships between genes expressed during early development may not be sufficient to explain embryonic patterning. Previous studies have shown that chordin and BMP-4 may be functional antagonists in the early embryo (Sasai et al., 1995). While chordin can induce a secondary axis when ectopically expressed on the ventral side of the embryo (Lemaire et al., 1995), the presence of chordin may prevent formation of the ventral regulatory loop. In addition, siamois may directly suppress ventral regulatory genes or may induce the expression of chordin expression when ectopically expressed on the ventral side of the embryo.

REFERENCES


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Note added in proof

Vox was also isolated as Xbr-1 in a screen for homeobox genes expressed in the eye.