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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 future 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 diffusable 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 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 *Eco*RI 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 using 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 $MgSO_4$, 2.0 mM $CaCl_2$, 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 uninjected stage 12-13 embryos were placed in 1× MMR and dorsal and ventral quarters 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 mMachine 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 *Bgl*II 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 on a 1% agarose gel and transferred to Nylon. A *Vox* probe was produced from *Vox-15* by labeling with ^{32}P 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 = Ventral 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-neurula 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 frequently 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 location of Spemann's organizer (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

A

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   1 CGTTTAGGTGAAGAGATGACTAAAGCTTTCTCCTCGGTTGAATGGCTTGCTCAAAGCAGCCGCAGATCTCACAGAGAGCA 80
                     M  T  K  A  F  S  S  V  E  W  L  A  Q  S  S  R  R  S  H  R  E  Q

  81 GCCAAGCAAAGTGGATCAGAGATATTCACCGTACCCCAGGCCATCCCTGCCTTCCTGGAACAGTGATGTGTCCCCTTCTT 160
     P  S  K  V  D  Q  R  Y  S  P  Y  P  R  P  S  L  P  S  W  N  S  D  V  S  P  S  S

 161 CATGGAACAGCCAACTATCTCCAGATCCAGACAGTGCCCAAGTCTCACCATGCCCTGTGAGTGCACAAGTATCTCCATAT 240
       W  N  S  Q  L  S  P  D  P  D  S  A  Q  V  S  P  C  P  V  S  A  Q  V  S  P  Y

 241 TCCTCAGACAGTGAAATATCACTGTATTCACATGAAGAAGAAGCCTCTTTCTATGGAATGGACTTTAATACATCATCATC 320
     S  S  D  S  E  I  S  L  Y  S  H  E  E  E  A  S  F  Y  G  M  D  F  N  T  S  S  S

 321 CCCTGGAGACAATGGATTGCTACACAGGGACACAACCTCATACTCCAGAGGAATGGAGGCCAGGTCGGCCAGCACTCCAG 400
     P  G  D  N  G  L  L  H  R  D  T  T  S  Y  S  R  G  M  E  A  R  S  A  S  T  P  A

 401 CAACATCACCTGTGAAAGGGGCACAACCTGTTGATTCCACCTACAGCACTAGCACTGACTCAGGCTATGAAAGTGAAACG 480
       T  S  P  V  K  G  A  Q  P  V  D  S  T  Y  S  T  S  T  D  S  G  Y  E  S  E  T

 481 AGTCGATCCAACTCTACAGCCCCTGAAGGAGAGTCCCTCCGTATCTCTGAGTCCAATGATACCTCAGATGAAGAGGGCAA 560
     S  R  S  N  S  T  A  P  E  G  E  S  L  R  I  S  E  S  N  D  T  S  D  E  E  G  K

 561 GATGGGCCGAAGGTTGAGGACGGCTTTCACCAGTGATCAGATCTCCACTCTGGAGAAGACTTTTCAGAAACACAGATACC 640
       M  G  R  R  L  R  T  A  F  T  S  D  Q  I  S  T  L  E  K  T  F  Q  K  H  R  Y  L

 641 TTGGGGCGTCTGAAAGACGGAAACTCGCAGCCAAACTCCAGCTTTCTGAAGTCCAGATTAAAACCTGGTTCCAGAACCGC 720
       G  A  S  E  R  R  K  L  A  A  K  L  Q  L  S  E  V  Q  I  K  T  W  F  Q  N  R

 721 AGGATGAAATACAAACGGGAAATCCAAGATGGCAGACCAGACTCATACCACCCAGCCCAGTTCTTTGGTGTCTACGGCTA 800
     R  M  K  Y  K  R  E  I  Q  D  G  R  P  D  S  Y  H  P  A  Q  F  F  G  V  Y  G  Y

 801 TGCACAGCAGCCCACTCCTGTATTCCAGCATGCAGTCCAACATCCCTACCCAGGTTATAACCCACTAATGGAAACCCTGC 880
       A  Q  Q  P  T  P  V  F  Q  H  A  V  Q  H  P  Y  P  G  Y  N  P  L  M  E  T  L  P

 881 CTGGTACCATGCCCTATACCATGCATCCTCCTGCCATGGACTCTATGACTCCCTTCAACTCTCAACCTTTTCAGATGCTC 960
       G  T  M  P  Y  T  M  H  P  P  A  M  D  S  M  T  P  F  N  S  Q  P  F  Q  M  L

 961 TACCTGCCCCAACAGCACCTTGGGCAACCTCTGGCCTATTAGGAAGAAAGGCCATTTGTTAGATATTAATCTAGAACTTA 1040
     Y  L  P  Q  Q  H  L  G  Q  P  L  A  Y

1041 TAAAAGGACTATACTAAAGGCTGGACTTTTCCATGGACTTCTGTCCTCCCGCAGGACAAACAAAATTGCACTGAATATTG 1120
1121 TTATTGACAAGATGTTTACTGAATGGATGGCTAATATTGGGCCATGTGTTGACATGATTTTATTCACATTGAATAGTGGC 1200
1201 TTGTATATTCTATGAAAAATACCATTTATATGACTAATAAATCG 1244
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B

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Antp        RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN
                :| |  :|  |   ||| |   |||    |  :|  | |:| ||| |||||||| |:|      48.3%
Vox         GRRLRTAFTSDQISTLEKTFQKHRYLGASERRKLAAKLQLSEVQIKTWFQNRRMKYKREI

Xgbx-2      N--R------e-lLE---E-HCKk--SLt--SQi-HV-K-----v-I------A-W--VK     56.7%
eve         V--Y-----R--lGR---E-Y-EN-vSRPR-CE---Q-n-P-ST--V--------D--QR     56.7%
Xhox3       M--Y-----Re--AR---E-YrEN-vSRPR-CE---A-n-P-TT--V--------D--QR     55.0%
Hox1.11     S------Y-NT-lLE---E-HFNk--CRPR-VEi--L-D-t-R-v-V--------H--QT     53.3%
Nkx5.1      KkkT--V-sRS-vFQ--S--DMK---SS---AG---S-H-t-T-v-I------N-W--Ql     51.7%
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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 Vox homeodomain sequence to other homeodomain sequences. The dashes indicate identical amino acids. The percentage amino acid identity within the homeodomain 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).

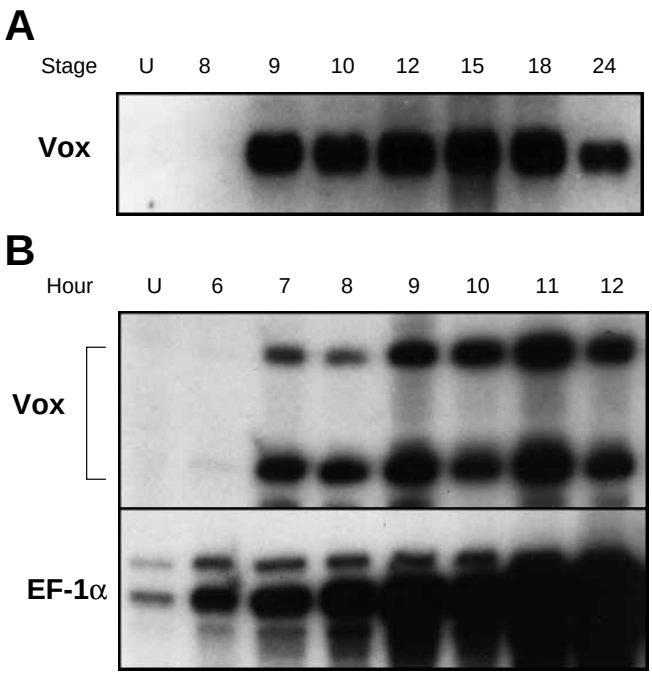


Fig. 2. Temporal expression of *Vox*. (A) Northern analysis of the developmental expression of *Vox*. Poly(A) RNA was isolated from 50 unfertilized eggs or embryos at various stages, separated on a denaturing gel, blotted and hybridized with a ^{32}P-labeled full-length probe made from the *Vox* cDNA. (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, late neurula; (24) stage 24, tailbud. The blot was rehybridized with an *EF-1α* probe (Krieg et al. 1989) to verify the loading of RNA in each lane (not shown). (B) RNase protection assay showing the temporal expression of *Vox*. RNA was isolated from five unfertilized eggs (U) or embryos at the indicated hours postfertilization. RNA was analyzed by RNase protection using a mixture of *Vox* and *EF-1α* probes. *EF-1α* is a ubiquitously expressed gene in *Xenopus* embryos and the levels of expression increase from the MBT. MBT occurs at approximately 6 hours postfertilization.

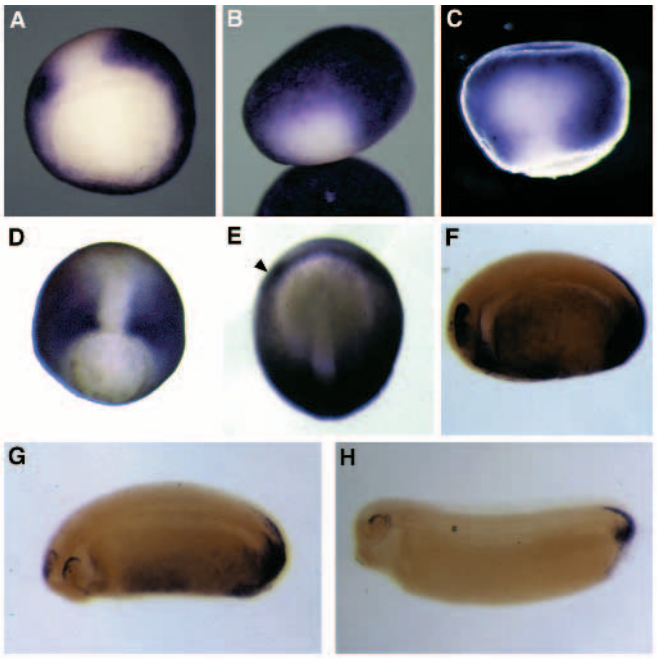


Fig. 3. Spatial expression of *Vox* transcripts during early development. *Vox* expression was analyzed by whole-mount in situ hybridization using albinos, except for the embryo in D. (A) Vegetal view of a stage 10 embryo, dorsal is upper left. *Vox* expression is cleared from the region above the blastopore lip. (B) Dorsal view of the same embryo as in A, animal pole is up. *Vox* is expressed throughout the embryo except for a small region above the dorsal lip. (C) Dorsal view of a stage 11 embryo, animal pole is up. The clearing of *Vox* expression from the embryo has expanded anteriorly and delineates the regions of the future anterior neural plate and notochord. (D) Dorsovegetal view of a stage 12 embryo, anterior is up. *Vox* expression flanking the presumptive notochord has become sharpened and the anterior dorsal clearing has broadened and extended anteriorly. (E) Dorsal view of a stage 13-14 embryo, anterior is up. *Vox* is expressed in the posterior paraxial region of the embryo. Two new spots of anterior expression are emerging (arrowhead) which mark the region dorsal to the developing eye. (F) Lateral view of a stage 19 embryo, anterior is to the left. *Vox* is expressed diffusely ventrally and with sharp borders along the posterior notochord and floorplate in the posterior paraxial region of the embryo. The staining above the future eye is more distinct at this stage. (G) Lateral view of a stage 24 embryo, anterior is to the left. Diffuse staining is present but diminishing on the ventral side of the embryo. The paraxial expression is now confined to a small region of the developing tailbud. (H) Lateral view of a stage 26 embryo, anterior is to the left. Ventral expression has diminished. *Vox* is now expressed solely in the developing tailbud flanking the notochord and floorplate and above the developing eye. The dark spot in the dorsal anterior half is a defect in this particular embryo.

of the embryo (Fig. 4A) in both involuted and noninvoluted cells. Sections of stage 12 embryos stained for *Vox* expression revealed a significantly higher level of expression in the non-involuted ventral marginal zone than in the involuted ventral mesoderm (Fig. 4C,C′,D). On the dorsal side of the embryo, sagittal sections revealed significantly diminished levels of expression along the dorsal midline, both in the involuted and noninvoluted tissues (Fig. 4C,C″). In parasagittal sections, *Vox* expression was evident in both dorsal involuted and noninvoluted marginal zone tissue, specifically in the posterior region of the embryo, near the blastopore lip (Fig. 4D,D′). Anteriorly in those same sections *Vox* expression was very weak or absent in the dorsal ectoderm and mesoderm (Fig. 4D,D′).

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 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

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

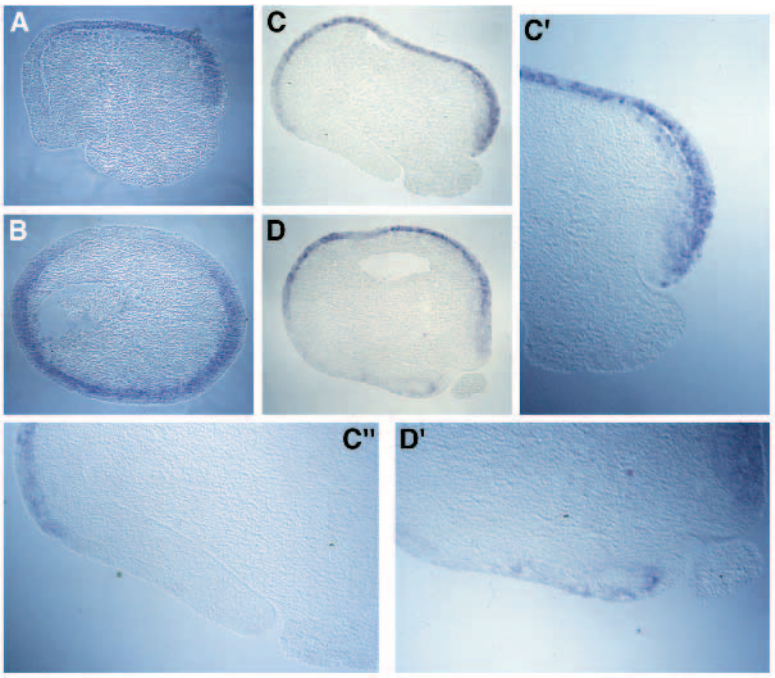


Fig. 4. Histological analysis of *Vox* expression in sections of albino embryos processed for whole-mount in situ hybridization. (A) Sagittal section of a stage 11 embryo, dorsal is to the left. *Vox* expression is absent from the dorsal side. (B) Transverse section of a stage 11 embryo, dorsal is up. *Vox* is cleared from a broad region on the dorsal side of the embryo. (C) Sagittal section of a stage 12 embryo, the blastopore is to the lower right-hand corner of the panel. Ventral is to the right. *Vox* expression is absent from the involuted and noninvoluted cells on the dorsal side of the embryo. On the ventral side, *Vox* expression is found in both the involuted mesoderm and the noninvoluted overlying ectoderm, however the expression in the noninvoluted cells appears much stronger. (C′) Higher magnification of the ventral side of the embryo shown in C, the blastopore is at the bottom of the panel. *Vox* expression is much stronger in the noninvoluted cells. (C″) Higher magnification of the dorsal side of the embryo shown in C, the blastopore at the lower right-hand corner. *Vox* expression is absent from the involuted mesoderm as well as the noninvoluted ectoderm on the dorsal side. (D) Parasagittal section of a stage 12 embryo, the blastopore is to the lower right-hand corner of the panel. Ventral is to the right. *Vox* expression is strongest in noninvoluted cells of the animal ectoderm and the ventral ectoderm, and weaker expression is found in the involuted ventral mesoderm. On the dorsal side, *Vox* expression is weak in the anterior region of the presumptive neural plate or the anterior noninvoluted cells. However, posteriorly, *Vox* is expressed in both the involuted and noninvoluted cells. (D′) Higher magnification of the dorsal side of the embryo shown in D, the blastopore is to the right. *Vox* expression is weaker in the anterior dorsal noninvoluted cells of the neuroectoderm and is stronger in the noninvoluted and just involuted cells in the posterior region of the embryo.

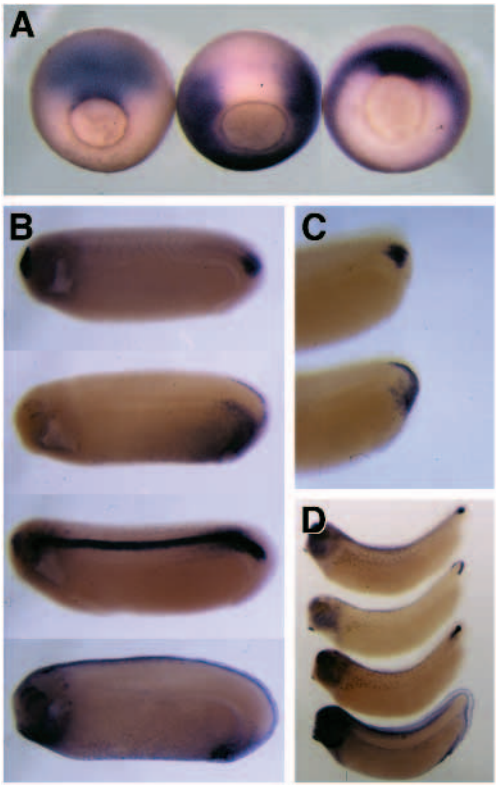
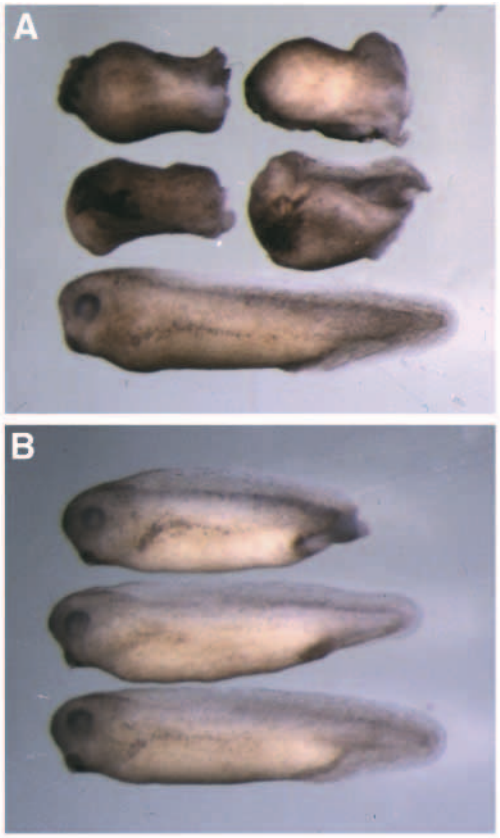


Fig. 5. Comparison of *Vox*, *Xnot*, *chordin* and *Bmp-4* expression in *Xenopus* embryos. (A) Dorsovegetal view of uncleared, stage 11 embryos stained for *chordin*, left, *Vox*, middle, and *Xnot*, right, dorsal is up. The expression of *Vox* is most visible in uncleared embryos since clearing will 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.

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



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 uninjected, stage 35 control, anterior is to the left. (B) The top two embryos were injected with *Vox* RNA, the bottom embryo is an uninjected control embryo, anterior is to the left. Embryos are at stage 35.

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

at the expression of *Xbra*, which is also found within the notochord (Fig. 7E; Smith et al., 1991). In stage 12.5 embryos injected dorsally with *Vox* RNA, notochordal *Xbra* expression was eliminated (Fig. 7F). This may be a consequence of the elimination of *Xnot* expression in *Vox*-injected embryos, since *floating head* (the zebrafish homologue of *Xnot*) is required for the notochordal expression of *no tail* (the zebrafish homologue of *brachyury*), as shown by Talbot et al. (1995). We also examined the expression of *MyoD*, which is first expressed during midgastrulation (Harvey, 1990; Frank and Harland, 1991; Rupp and Weintraub, 1991). *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 *Vox* RNA, the expression of *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 *Vox* RNA appeared in most cases completely 'ventralized', we examined the expression of *Xwnt-8*, a marker of early lateral mesoderm

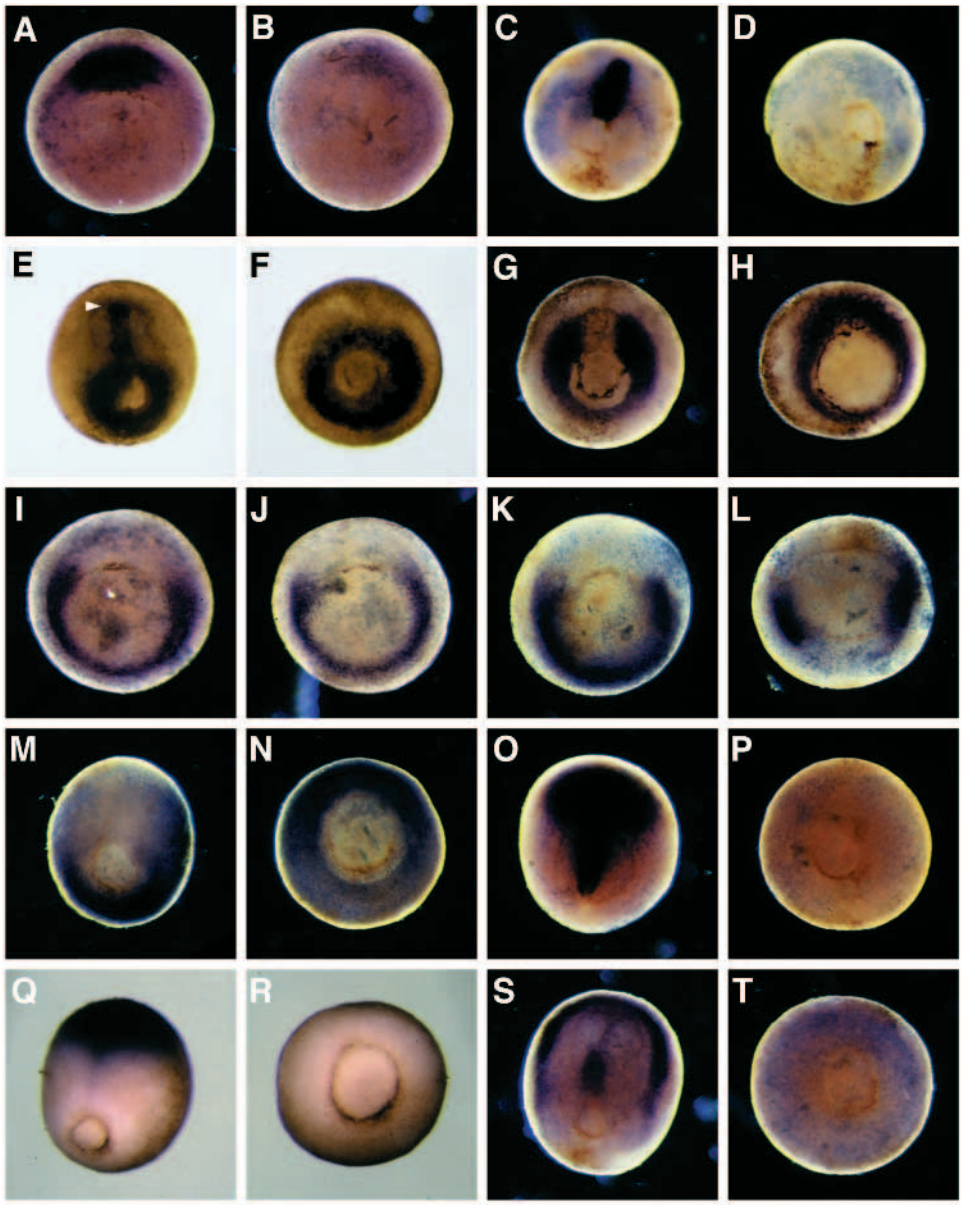


Fig. 7. Gene expression in embryos injected with *Vox* RNA. In all experiments, a percentage of injected and uninjected control embryos were allowed to develop until tadpole stages to score the late-stage phenotype in a each experiment. Injected embryos received 4-5 ng of *Vox* RNA dorsally, except in the experiment shown in L where embryos were injected ventrally. Vegetal views with dorsal up, except where noted. (A) *gsc* expression in an uninjected, stage 10.5 embryo. (B) An embryo injected dorsally with *Vox* RNA; *gsc* expression is eliminated from the dorsal side of the embryo (100% had no detectable *gsc* expression, *n*=29). (C) *Xnot* expression in an uninjected, stage 12.5 embryo. (D) An embryo injected dorsally with *Vox* RNA; *Xnot* expression is eliminated from the dorsal side of the embryo (85% had no detectable notochordal *Xnot* expression, the remainder had reduced expression, *n*=39). (E) Dorsovegetal view of *Xbra* expression in an uninjected, stage 12.5 embryo. Arrowhead indicates notochordal *Xbra* expression. (F) *Xbra* expression in a stage 12 embryo injected dorsally with *Vox* RNA; no notochordal *Xbra* expression is detectable (100% had no notochordal *Xbra* expression, *n*=10). (G) *MyoD* expression in an uninjected, stage 12 embryo. (H) *MyoD* expression in a stage 12 embryo injected dorsally with *Vox* RNA; *MyoD* is expressed along the dorsal midline (100% had radial *MyoD* expression, *n*=23). (I) *Xwnt-8* expression in an uninjected, stage 10 embryo. (J) *Xwnt-8* expression in a stage 10 embryo injected dorsally with *Vox* RNA; *Xwnt-8* expression appears unaffected (100% had normal *Xwnt-8* expression, *n*=17). (K) *Xwnt-8* expression in an uninjected, stage 10 embryo. (L) *Xwnt-8* expression in a stage 10 embryo injected ventrally with *Vox* RNA; *Xwnt-8* is eliminated from the ventral region of the embryo (100% lacked ventral expression of *Xwnt-8*, *n*=20). (M) Dorsovegetal view of *Bmp-4* expression in an uninjected, stage 12 embryo. (N) *Bmp-4* expression in a stage 12 embryo injected dorsally with *Vox* RNA; *Bmp-4* is expressed both dorsally and ventrally (95% had radial *Bmp-4* expression, *n*=21). (O) Dorsovegetal view of *chordin* expression in an uninjected, stage 12.5 embryo, dorsoanterior is up. (P) An embryo injected dorsally with *Vox* RNA; *chordin* expression is eliminated from the dorsal side of the embryo (96% had little or no detectable *chordin* expression, *n*=23). (Q) Dorsovegetal view of *Xotx2* expression in an uninjected, stage 12.5 embryo, dorsoanterior is up. (R) An embryo injected dorsally with *Vox* RNA; *Xotx2* expression is eliminated from the dorsal side of the embryo (100% had no detectable *Xotx2* expression, *n*=12). (S) Dorsovegetal view of *Hairy II* expression in an uninjected, stage 12.5 embryo, dorsoanterior is up. (T) An embryo injected dorsally with *Vox* RNA; *Hairy II* is weakly expressed on the dorsal side and no longer delineates the neural plate or floorplate (100% had faint, diffuse, dorsal *Hairy II* expression, *n*=27).

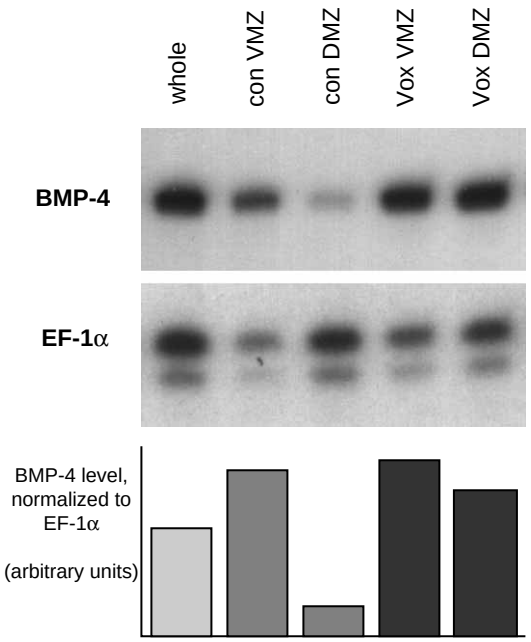


Fig. 8. RNase protection analysis of the levels of *Bmp-4* expression in dorsal and ventral quarters of uninjected stage 12.5 embryos and stage 12.5 embryos injected dorsally with 4 ng *Vox* RNA. An *EF-1α* probe was included to control for RNA loading. Except for the whole embryo control, each sample was derived from 10 dorsal or ventral quarters. The bar graph at the bottom represents levels of *Bmp-4* expression in each lane normalized to *EF-1α*. *Bmp-4* is expressed in nearly equal amounts on the dorsal and ventral sides of *Vox* RNA-injected embryos. This experiment was repeated and produced the same results (data not shown).

(Christian et al., 1991). *Xwnt-8* expression is absent from the dorsal side of stage 10 embryos (Fig. 7I). Dorsal injection of *Vox* RNA had no effect on the expression of *Xwnt-8*. The dorsal gap in expression appeared to be the same size in injected as in uninjected control embryos (Fig. 7I and J). This result demonstrated that dorsal injection of *Vox* RNA could not induce the expression of *Xwnt-8* on the dorsal side of stage 10 embryos. However, injection of *Vox* RNA into the ventral marginal zone of 4-cell embryos eliminated the ventral expression of *Xwnt-8* (Fig. 7L). These results indicate that *Vox* does not simply promote ventralization at the expense of dorsal gene expression, but affects gene expression selectively.

Because embryos injected dorsally with *Vox* RNA were ventralized, yet did not express *Xwnt-8* on the dorsal side, we examined the expression of *Bmp-4* during gastrulation in injected and uninjected embryos. In uninjected, stage 12 embryos, *Bmp-4* expression is absent from a broad region approximating that of the presumptive neural plate. Expression of *Bmp-4* appears strongest in the anterior ventral region and near the ventral side of the blastopore (Fig. 7M and Fainsod et al., 1994; Schmidt et al., 1995b). In embryos injected dorsally with *Vox* RNA, *Bmp-4* expression was no longer absent from the dorsal side of the embryo, but instead was radially expressed, surrounding the entire blastopore (Fig. 7N). To quantitate the change in *Bmp-4* expression, we dissected dorsal and ventral quarters of injected and uninjected embryos at stage 12-13, and analyzed the levels of expression of *Bmp-4* by RNase protection (Fig. 8). Whereas control embryos had five-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; Pannese 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 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., 1995b), 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*.

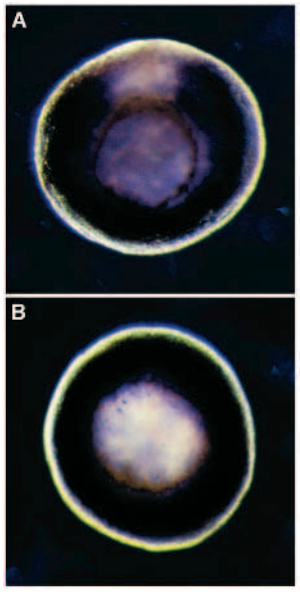


Fig. 9. Ectopic BMP-4 expression prevents the dorsal clearing of *Vox* expression in stage 11 embryos. Vegetal views, dorsal is up. A. *Vox* expression in an uninjected, stage 11 embryo. B. *Vox* expression in a stage 11 embryo injected dorsally with 4 ng *Bmp-4* RNA; *Vox* is radially expressed (69%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

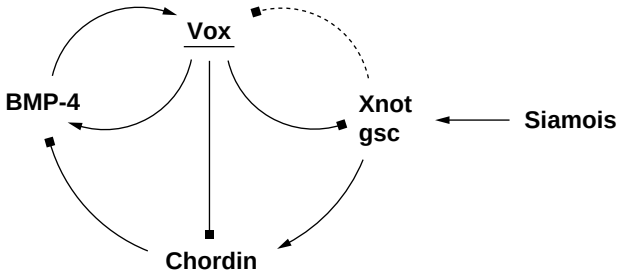


Fig. 10. A hypothetical model of the gene regulatory network active during gastrulation in *Xenopus*. The set of interactions shown in this diagram reflect the minimum necessary to account for the observations discussed here, and is not intended to be the only possible hypothesis. Arrows indicate positive 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., 1995b), 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

the ventral side of embryos resulted in the elimination of ventral *Xwnt-8* expression. This seems contradictory since both *Vox* and *Xwnt-8* are both expressed within the ventral region of the embryo. However, an examination of the localization of ventral *Vox* expression demonstrated that *Vox* is primarily expressed in the non-involuted layer, and that *Vox* expression is down-regulated once the tissue (the prospective mesoderm) involutes. *Xwnt-8*, in contrast, is expressed within the involuted layer (Christian et al., 1991). Thus, the level of *Vox* expression in the ventral mesoderm, as a result of RNA injections, may be much higher than the levels found normally in that region. Consequently, *Xwnt-8* expression is repressed in *Vox*-injected embryos, whereas in normal circumstances, *Xwnt-8* and *Vox* are coexpressed in the ventral mesoderm.

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 on the ventral side of the embryo, thereby dorsalizing the character of the tissue into which it has been introduced (Sasai et al., 1994), inhibiting BMP-4 signaling on the ventral side of embryos can also impart a dorsal character to the injected region (Graff et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995b; Steinbeisser et al., 1995). BMP-4 can eliminate all visible dorsal characters from the embryo when injected on the dorsal side (Dale et al., 1992; Jones et al., 1992), and can eliminate the expression of all axial genes tested (Schmidt et al., 1995b). Vox can eliminate *chordin* expression and induce *Bmp-4* expression when ectopically expressed on the dorsal side, and BMP-4 can induce the expression of *Vox* on the dorsal side of gastrula embryos. BMP-4 thus both regulates and is regulated by Vox either directly or indirectly; each may be required for the expression of the other on the ventral side of the embryo (Fig. 10). By extension, chordin may both regulate and be regulated by both Vox and BMP-4. In this model, chordin antagonizes BMP-4 action, thus destabilizing *Vox* expression, and relieving its own repression (Fig. 10). This reciprocal regulation may be part of the mechanism by which embryonic patterning becomes defined during gastrulation.

The positive regulatory loop between Vox and BMP-4 may establish a stable ventral regulatory region within the embryo. In order for dorsal tissue to form on the ventral side, this positive regulatory loop must be either destabilized or prevented from forming. The ability of several genes to induce complete or incomplete secondary axes may reflect the ability of these genes to prevent formation of the ventral regulatory loop. Dorsal genes capable of inducing a complete secondary axis may act in two ways, by inducing the expression of other dorsal axial genes and by inhibiting, directly or indirectly, *Vox* and/or *Bmp-4*. Dorsal genes which induce an incomplete secondary axis may interfere with the ventral regulatory loop without the induction of a sufficient set of dorsal axial genes, and dorsal genes that do not induce a secondary axis may fail to do so because they fail to interfere with the loop. *Siamois*, a homeobox-containing gene expressed very early in the organizer region, can induce a complete axis when ectopically expressed on the ventral side of the embryo (Lemaire et al., 1995). As a possible scenario, siamois may directly suppress ventral regulatory genes or may induce the expression of *chordin*. The presence of chordin may prevent formation of the ventral regulatory loop. In addition, siamois may induce the expression of dorsal axial genes such as *Xnot* and *gsc*, which may in turn stabilize *chordin* expression (Sasai et al., 1994) and elicit additional dorsal gene expression. This constitutes the establishment of a dorsal regulatory loop (Fig. 10). It seems likely that cortical rotation initiates a series of events that bias the conflict between dorsal and ventral regulatory loops toward the dorsal genes in a single region of the embryo. Subsequent gene induction and reciprocal regulation generate, maintain and refine the patterning of the embryo.

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

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