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Mechanisms of Development 54 (1995) 149–160

The *Xenopus laevis* homeobox gene *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm

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Received 25 August 1995; accepted 3 October 1995

Abstract

In a search for homeobox genes expressed during early *Xenopus* development, we have isolated a gene which appears to be the *Xenopus* cognate of the mouse *Gbx-2* gene. Expression of *Xgbx-2* is first detectable by in situ hybridization at the midgastrula stage when it is predominantly expressed in the dorsolateral ectoderm, with a gap in expression at the dorsal midline. By the end of gastrulation and during neurulation, *Xgbx-2* is expressed dorsolaterally in the neural ectoderm and laterally and ventrally in the epidermis with sharp anterior expression borders in both tissues. The anteriormost expression in the neural ectoderm persists throughout the early stages of development, and was mapped to the region of rhombomere 1, with an anterior expression border in the region of the midbrain–hindbrain boundary. Thus *Xgbx-2* is expressed anterior to the *Hox* genes. *Xgbx-2* expression is induced by retinoic acid (RA) in animal caps, and RA treatment of whole embryos expands and enhances *Xgbx-2* expression in the ectoderm. We suggest a role for *Xgbx-2* in establishing the midbrain–hindbrain boundary, which appears to separate early neurectodermal regions expressing genes that are positively and negatively regulated by RA.

Keywords: *Xenopus* embryogenesis; Homeobox genes; Anteroposterior patterning; *Xgbx-2*; *Gbx-2*; *CHox7*; Retinoic acid

1. Introduction

The understanding of anteroposterior (A-P) patterning in vertebrates has advanced rapidly with the identification of the vertebrate homologues of the homeobox genes contained within the homeotic gene clusters in *Drosophila*, termed the *Hox* genes (McGinnis and Krumlauf, 1992; Slack and Tannahill, 1992; Akam et al., 1994). In the mouse, the *Hox* genes are expressed in overlapping domains, with sharp anterior boundaries and generally diffuse posterior boundaries in the mesoderm and neural ectoderm, suggesting that they specify regional identities along the A-P axis in a combinatorial manner (Gaunt, 1991; Hunt and Krumlauf, 1992). It is not possible, however, to account for all A-P neural patterning in the CNS by the *Hox* genes since the *Hox* genes are not expressed anterior to rhombomere 2 in the hindbrain (Krumlauf, 1993).

Recently, it has been shown that homeobox genes only distantly related to the *Hox* genes, such as the vertebrate genes related to *Drosophila orthodenticle* (*Otx*) and *empty spiracles* (*Emx*), are expressed in central nervous system (CNS) regions anterior to the hindbrain (Simeone et al., 1992, 1993). They are expressed in nested overlapping domains, and the posteriormost expressed genes of this group, *Otx1* and *Otx2*, have a posterior expression border at the midbrain–hindbrain boundary. This suggests that the *Otx/Emx* genes specify regional identity in the head region anterior to the hindbrain in a combinatorial manner, much like the *Hox* genes in the trunk region. The region of rhombomere 1 does not express any known *Hox* or *Emx/Otx* genes, suggesting that other genes may be involved in the early patterning of this region.

The factors regulating A-P patterning in vertebrates are not known, but the vitamin A derivative all-*trans* retinoic acid (RA) or related retinoids have been suggested to play a role in this process. Addition of RA to early *Xenopus* embryos causes a truncation of the anterior CNS and a

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SSDI 0925-4773(95)00468-G

compression of the anterior hindbrain (Durston et al., 1989; Sive et al., 1990; Papalopulu et al., 1991), diminished expression of some dorso-anterior mesodermal genes like *gsc* and *pintallavis* (Cho et al., 1991; Ruiz i Altaba and Jessell, 1992) and repression of anterior ectodermal marker genes including *En-2* (Sive et al., 1990). Recently, it has also been found that RA downregulates the *Otx2* gene in cultured cells (Simeone et al., 1993, 1995) and in whole embryos of mouse (Ang et al., 1994; Simeone et al., 1995), chicken (Bally-Cuif et al., 1995) and *Xenopus* (Pannese et al., 1995). This downregulation appears to act through a *cis*-acting mechanism, suggesting that it may be direct (Simeone et al., 1995). In contrast to *Otx2*, *Hox* genes are induced by RA in cultured cells, and in some cases this activation has been shown to be direct (Boncinelli et al., 1991; Langston and Gudas, 1994). RA also increases the level and changes the pattern of *Hox* gene expression in whole embryos of mouse (Kessel and Gruss, 1991; Conlon and Rossant, 1992), chicken (Sundin and Eichele, 1992) and *Xenopus* (Cho and DeRobertis, 1990; Sive et al., 1990; Sive and Cheng, 1991; Dekker et al., 1992a,b; Leroy and DeRobertis, 1992; Lopez and Carrasco, 1992; Kolm and Sive, 1995). These and other observations have led to the view that RA may act as a posteriorizing agent in the A-P specification of the vertebrate axis, inhibiting anterior (forebrain) differentiation and enhancing differentiation of hindbrain and spinal cord levels in both the mesoderm and the ectoderm (reviewed in Yamada, 1994). It should be noted, however, that not all of the RA-induced changes in molecular markers along the A-P axis appear to be consistent with this view (reviewed in Slack and Tannahill, 1992).

We have isolated a *Xenopus* member of the *Gbx* class of homeobox genes (*Gbx* = gastrulation brain homeobox; Frohman et al., 1993), which are only distantly related to the *Hox* genes. *Xgbx-2*, which is a putative homologue of the mouse *Gbx-2* gene (Bulfone et al., 1993; Frohman et al., 1993; Chapman and Rathjen, 1995), is initially expressed during the midgastrula stage of *Xenopus* development in the presumptive neural ectoderm and epidermis, forming sharp anterior borders by the end of gastrulation. The anterior border in the neural ectoderm was followed throughout development, and shown to lie within the region of the midbrain–hindbrain boundary, which is anterior to the anteriormost expression limit of the *Hox* genes. We show that *Xgbx-2* expression is positively regulated by retinoic acid treatment, and suggest a role for *Xgbx-2* in the early establishment of the midbrain–hindbrain boundary.

2. Results

2.1. Isolation and sequence of Xgbx-2

Xgbx-2 was first identified in a search for homeobox-containing genes transcribed during the gastrula stages using a polymerase chain reaction (PCR)-based approach (Northrop and Kimelman, 1994). One of the DNA fragments isolated in our search demonstrated considerable homology to the chicken *CHox7* gene (Fainsod and Gruenbaum, 1989). *CHox7* is expressed in gastrulating chick embryos (Fainsod and Gruenbaum, 1989), although spatial localization of its expression has not been reported. To obtain the full-length sequence of our *CHox7*-like gene, we screened a *Xenopus* neurula-stage cDNA library (Kintner and Melton, 1987). The clone with the longest insert (approximately 2.4 kb) was chosen for further analysis. DNA sequence analysis revealed a 1020 bp open reading frame encoding a predicted protein of 340 amino acids (Fig. 1A).

Examination of the amino acid sequence of this gene showed that it is most similar to *Gbx-2*, a *CHox7*-related gene that was originally isolated from the mouse using the nucleotide sequence obtained from our gene (Bulfone et al., 1993; Frohman et al., 1993). We have therefore named our gene *Xgbx-2*. A fragment of the *Gbx-2* homeodomain was also isolated independently and called *MMox-A* (Murtha et al., 1991). In the homeodomain, Xgbx-2 shows 98% amino acid sequence identity to Gbx-2, and the two genes have an overall amino acid sequence identity of 80% (Fig. 1A; sequence data from Chapman and Rathjen, 1995). Xgbx-2 is less similar to CHox7 than Gbx-2 in its homeodomain (Fig. 1B; 95% amino acid identity) and C-terminus (Fig. 1C). Thus, *Xgbx-2* appears to be the *Xenopus* homologue of the mouse *Gbx-2* gene, whereas *CHox7* and human *GBX-1* (Matsui et al., 1993a) appear most similar to mouse *Gbx-1* (Fig. 1B,C). A *Gbx-2* homologue has also been found in goldfish (Levine and Schechter, 1993; E. Levine, pers. comm.). Xgbx-2 differs from the *Drosophila Antp* gene product in 27 out of the 60 amino acids of the homeodomain, showing that it is not closely related to the *Hox* genes (Fig. 1B). Recently, PCR was used to isolate a fragment of a *CHox7*-like *Xenopus* gene, *XHox7*, which codes for the same amino acid sequence as *Xgbx-2* within the homeodomain (King and Moore, 1994). This suggests that XHox7 is identical to Xgbx-2, although the sequence of regions outside the homeodomain of XHox7 has not been reported.

2.2. Temporal and spatial expression of Xgbx-2 during early Xenopus development

Xgbx-2 transcripts were first clearly detected by the midgastrula-stage using RNase protection analysis (Fig. 2A, stage 11). Long exposures of the autoradiogram revealed a low level of *Xgbx-2* expression at the start of gastrulation (stage 10), but no maternal transcripts were detected (not shown). By the end of gastrulation, the expression level reaches a maximum (Fig. 2A; stage 13), and remains high throughout the neurula stages (Fig. 2A; stages 15, 17, and 19). Northern blot analysis of embry-

A

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                10        20        30        40        50        60        70
Xgbx-2  MSAAFQPPLMMMQRPLGSSTAFSIDSLIGNPPQPSPGHFVYTGYPMFMPYRPVVLPPPPPPPPSLSQATLQST
Gbx-2           ..................S..................................A.P..A..PA

            80        90       100       110       120       130       140
Xgbx-2  LSSAHHHHPIPSLPGGFCSSLAQGMALTSTLMATLPGGFSASTQHQEAA--RKFGAQSLHGA--FEKSDGSQSD
Gbx-2   .PP..P..Q.....T.........................P......AA...AP.P.P.GGN.D.AEAL.A.

           150       160           170       180       190       200       210
Xgbx-2  GEEGNKTYITKEGTLLPFS---ASEASL-GPVRGQGKEESGKEAEGKGKEDSYLMDSDLDYSSDDNISCQTAHK
Gbx-2   A.D.-.AFLA...S..A..AAE.VQ...V.A......D..KV.DDP....E.FSLE..V.......LPG.....

              220       230       240       250       260       270       280
Xgbx-2  EEDTP---EESPQNSNPSNNSNTSSTGKNRRRRTAFTSEQLLELEKEFHCKKYLSLTERSQIAHVLKLSEVQVK
Gbx-2   ...PGHAL..T..-.GGAAG.T.-.....................................A.........

           290       300       310       320       330       340
Xgbx-2  IWFQNRRAKWKRVKAGNTNSKTGEPSRNPKIVVPIPVHVNRFAIRSQHQQLEQARP*
Gbx-2   .................A.....................S..............*
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B

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Xgbx-2  (x)        NRRRRTAFTSEQLLELEKEFHCKKYLSLTERSQIAHVLKLSEVQVKIWFQNRRAKWKRVK
Gbx-2   (m)  98%   .....................................A.....................
GBX-2   (h)  98%   .....................................A.....................

Gbx-1   (m)                          ..............A.....................I.
GBX-1   (h)  95%   S....................................A.....................I.
CHox-7  (c)  95%   S....................................A.....................I.

Antp         55%   RK.G.QTY.RY.T........FNR..TRRR.IE...A.C.T.R.I........M...KEN
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C

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Xgbx-2  (x)        AGNTNSKTGEPSRNPKIVVPIPVHVNRFAIRSQHQQLEQ-ARP*
Gbx-2   (m)        ...A.....................S.............-...*
GBX-2   (h)        ...A.....................S.............-...*

Gbx-1   (m)        ...VS.RS...V
GBX-1   (h)        ...VS.RS...V..................V......M..G...*
CHox-7  (c)        ...VSNRS...V..................V......I..G--.
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Fig. 1. (A) Comparison of the predicted amino acid sequence of Xgbx-2 and Gbx-2. The homeodomain in Xgbx-2 is underlined. Non-identical residues in Gbx-2 are indicated. The predicted Xgbx-2 open reading frame is nine amino acids longer at the amino terminal end than is Gbx-2. (B) Comparison of the amino acid sequences of the homeodomain of different *Gbx*-class homeodomain proteins. Percentages are amino acid identities to Xgbx-2. (C) Comparison of C-terminal amino acid sequences of different *Gbx*-class homeodomain proteins. x, *Xenopus*; m, mouse (Frohman et al., 1993; Chapman and Rathjen, 1995); h, human (Matsui et al., 1993a,b); c, chick (Fainsod and Gruenbaum, 1989). Note that the high degree of amino acid similarity shown here for CHox7 extends throughout the whole C-terminal region only after deletion of one nucleotide from the published sequence of *CHox7* (Matsui et al., 1993a).

onic RNA revealed only a single *Xgbx-2* transcript during early embryonic stages (Fig. 2B).

To determine the spatial expression pattern of *Xgbx-2*, we performed whole mount in situ hybridization using a digoxygenin-labeled RNA probe (Harland, 1991). Expression of *Xgbx-2* was first detected at stage 10.5–11 (midgastrula) in the dorsolateral ectoderm at a distance from the blastopore lip and with a wide gap in expression at the dorsal midline (Fig. 3A,B). To examine whether *Xgbx-2* expression is excluded from the presumptive mesoderm, we cohybridized midgastrula stage (stage 11) embryos with antisense probes to *Xgbx-2* and *Xbra*, the *Xenopus* homologue of the *Brachyury (T)* gene (Smith et al., 1991), which in vertebrates appears to be initially expressed throughout the presumptive mesoderm (Herrmann, 1991; Schulte-Merker et al., 1992). *Xgbx-2*

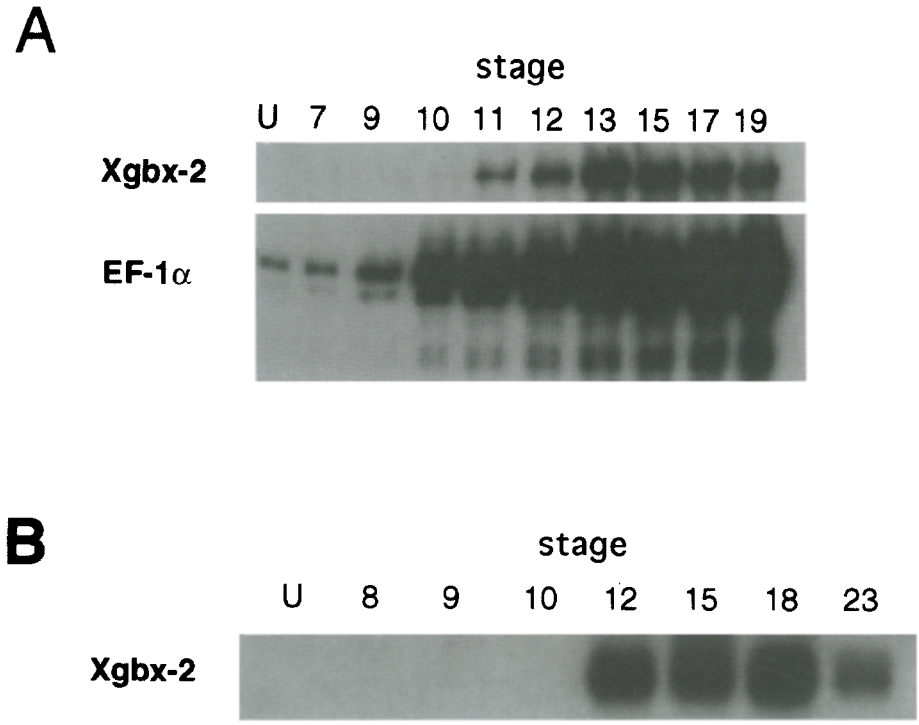


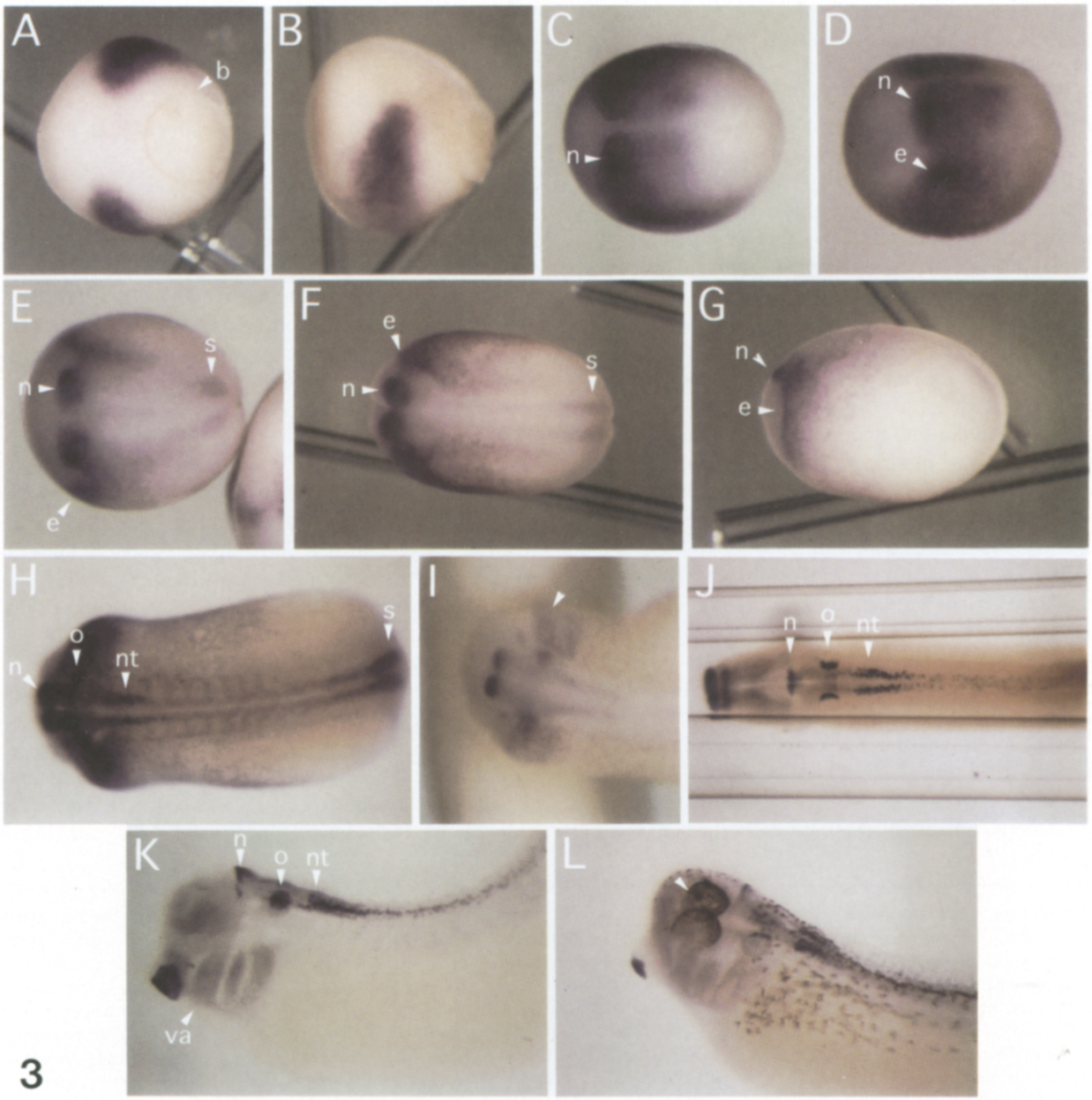
Fig. 2. (A) RNase protection analysis of the temporal expression of *Xgbx-2*. Total RNA (20 μg) from unfertilized eggs (U) and embryos at the indicated stages was analyzed by RNase protection using a mixture of *Xgbx-2* and *EF-1α* probes. Stage 7, blastula; stage 9, late blastula; stage 10, early gastrula; stage 11, mid-gastrula; stage 12, late gastrula; stage 13, early neurula; stage 15, mid-neurula; stage 17, late neurula; stage 19, late neurula. *EF-1α* is a ubiquitously expressed gene in the *Xenopus* embryo; *EF-1α* levels increase from the midblastula transition at stage 8 (Krieg et al., 1989). (B) Northern analysis of the temporal expression of *Xgbx-2*. Poly(A+) RNA was isolated from 50 unfertilized eggs (U) or 50 embryos at the indicated stages, separated on a denaturing gel, blotted, and hybridized with a ^{32}P-labeled probe from the *Xgbx-2* cDNA. Stage 8, mid-blastula; stage 9, late blastula; stage 10, early gastrula; stage 12, late gastrula; stage 15, mid-neurula; stage 18, late neurula, stage23, early tailbud.

appeared to be expressed outside of the region containing *Xbra* transcripts (Fig. 4A–C), although we cannot exclude a minor overlap in the expression of the two genes at the resolution of these experiments. The dorsal gap in *Xgbx-2* expression probably comprises at least the future floor plate of the neural tube. This is consistent with the observed lack of expression in the floor plate at later stages (Fig. 5C). The expression of *Xgbx-2* in the stage 11 embryo tapers off towards the ventral side (Fig. 3B), but can still be weakly detected at the ventral midline (not shown). Thus, at the midgastrula stage, *Xgbx-2* is expressed mainly in the presumptive ectoderm, which includes the future epidermis and neural plate.

By the end of gastrulation/start of neurulation (stage 13), the dorsal gap in *Xgbx-2* expression has become very narrow, occupying only a small strip along the dorsal

Fig. 3. Spatial expression of *Xgbx-2* during early development. The expression was analyzed by whole mount in situ hybridization of albino embryos. In all pictures, anterior is to the left. (A) Dorsal view of a stage 11 embryo. Note the wide gap at the dorsal midline and the distance of the expression from the blastopore lip (b). (B) Dorsolateral view of a stage 11 embryo. (C) Dorsal view of a stage 13–14 embryo. The gap in expression at the dorsal midline has narrowed. Note the sharp anterior expression border in the neuroectoderm (n). (D) Lateral view of a stage 13–14 embryo. *Xgbx-2* is expressed in the neuroectoderm (n) and in epidermal ectoderm (e). (E) Dorsal view of a stage 15 embryo. The neurectodermal expression is concentrated in a pair of anterior stripes (n). The epidermal expression has a sharp anterior border (e). There is additional expression in the presumptive posterior spinal cord (s). (F) Dorsal view of a stage 19–20 embryo. Labeling as in (E). (G) Lateral view of a stage 19–20 embryo. The lateral epidermal expression (e) shows a sharp anterior border and gradually tapers off towards the posterior end. Labeling as in (E). (H) Dorsal view of a cleared stage 22–23 embryo. *Xgbx-2* is expressed in the anterior neuroectoderm (n), in the otic vesicle (o), in a pair of lateral stripes anterior to the otic vesicle, in the neural tube (nt) posterior to the otic vesicle, in the posterior spinal cord (s), and in the future branchial arches (lateral to the otic vesicle). (I) Dorsal view of an uncleared stage 22–23 embryo. Expression is in the region of the neural crest (arrowhead) ventrally and posterioventrally to the otic vesicle. (J) Dorsal view of a cleared stage 31 embryo. Note the crescent-shaped expression in the dorso-medial part of the otic vesicle (o). Labeling as in (H). (K) Lateral view of a cleared stage 31 embryo. Note expression in the dorsal and ventral part of the anterior hindbrain (n). There is weak expression in the region of the visceral arches (va). Labeling as in (H). (L) Dorsolateral view of a cleared stage 31–32 embryo, showing *Xgbx-2* expression in the neural tube dorsal to the eye (arrowhead).

Fig. 4. Comparison of the expression of *Xgbx-2* to the expression of *Xbra*. All three embryos are stage 11 (midgastrula stage). The same magnification is used in all photographs. Shown are dorsovegetal views; future anterior is to the top. (A) Embryo hybridized for *Xgbx-2* alone. (B) Embryo hybridized for *Xgbx-2* and *Xbra*. (C) Embryo hybridized for *Xbra* alone.



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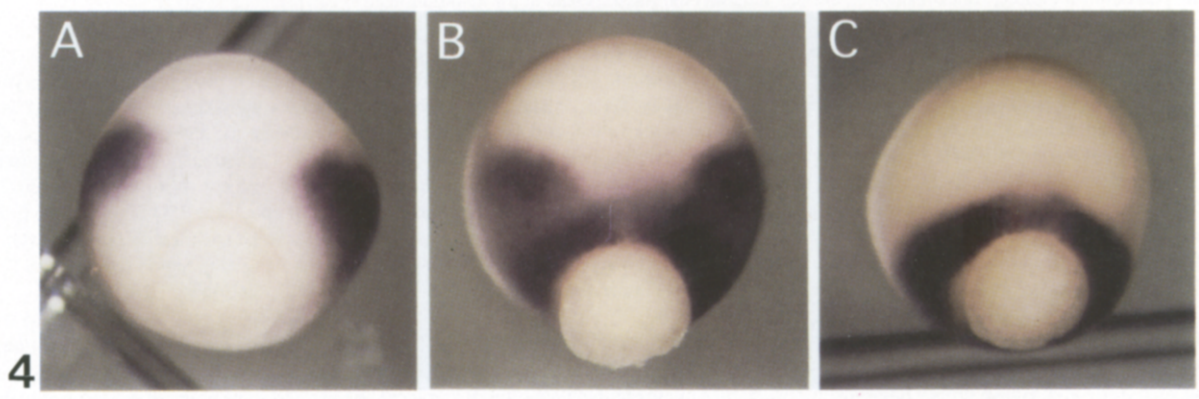

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midline (Fig. 3C). This narrowing is probably due to the convergence of the prospective neural plate (Keller et al., 1992). A lateral view of a stage 13–14 embryo (Fig. 3D) shows that, in addition to expression in the neural plate, *Xgbx-2* is now expressed in ventrolateral regions, in the presumptive epidermis. Beginning with stage 12, both the neural and the epidermal expression show a distinct anterior border at a similar anterior-posterior level (Fig. 3D). Transverse sections of stage 14–15 embryos localized the dorsal expression to the thickened neural plate and the ventral expression to the epidermis (Fig. 5B). In anterior regions, expression appeared to be restricted to the inner, sensorial layer of the epidermis (Fig. 5B). We did not detect *Xgbx-2* expression in mesodermal regions at this or later stages.

During neurulation, the dorsal expression becomes restricted to a pair of stripes with sharp anterior boundaries (Fig. 3E–G). We have mapped this expression to the presumptive anterior hindbrain (see below). In addition, a pair of longitudinal stripes of *Xgbx-2* expression is visible by stage 15 in the presumptive posterior spinal cord (Fig. 3E). The epidermal expression domain of neurula stage embryos shows a sharp anterior border (Fig. 3E–G), and moves dorsally as the neural folds close during neurulation (compare Fig. 3D and G). The epidermal expression gradually tapers off towards the posterior end (Fig. 3G). Weak epidermal expression was also observed across the ventral midline (Fig. 5B), but the sharp anterior border of the epidermal expression was only observed in lateral regions. In horizontal sections of stage 14–15 embryos, which were cut through the lateral domain of expression, this sharp lateral anterior border of the epidermal expression was localized in the region of the boundary between the epidermis and the anterior neural plate (i.e. future midbrain and forebrain regions; Fig. 5A).

By the early tailbud stage (stage 22–23), epidermal *Xgbx-2* expression has become weaker (Fig. 3H,I). New expression is observed in a pair of lateral stripes anterior to the otic vesicle, in the otic vesicle itself and in the neural tube posterior to the otic vesicle (Fig. 3H,I, and data not shown). We also observed *Xgbx-2* expression in the region of migrating neural crest ventrally and posterioventrally to the otic vesicle (Fig. 3I).

In stage 31 embryos, expression in the epidermis and the posterior spinal cord has disappeared, but there is weak expression in the region of the visceral arches (Fig. 3K). In the anterior hindbrain, *Xgbx-2* expression is localized mainly to the dorsal neural tube (Figs. 3J,K and 5C).

In the otic vesicle, *Xgbx-2* expression becomes localized to the dorsal region at least by stage 26 (not shown), and transverse sections at stage 31 reveal that *Xgbx-2* is expressed along the medial (inner) side, where the otic epithelium abuts the rhombencephalon (Fig. 5D). Sections through the posterior hindbrain near the hindbrain/spinal cord junction of stage 31 embryos revealed *Xgbx-2* expression in two bilaterally symmetric longitudinal columns (Fig. 5E). By stage 31–32, the latest stage examined, a new area of expression was observed anterior to the hindbrain, in the neural tube, dorsal to the eyes (Fig. 3L).

Fig. 5. Histological analysis of *Xgbx-2* expression. Embryos were processed for whole-mount in situ hybridization, embedded in Paraplast, and 16 μm (A,B) or 10 μm (C–E) sections were cut. Dorsal is at the top in all sections except in (A). (A) Horizontal section of a stage 14–15 embryo which is cut anterior to the hindbrain. Note that the anterior neural plate is bent ventralwards at this stage, so that the horizontal section passes through the future forebrain and midbrain, and the lateral epidermis. Anterior is at the top. The anterior border of the epidermal expression (e) lies in the region of the posterior border of the anterior neural plate. (B) Transverse section of a stage 14–15 embryo at a level near the anterior expression border in the neural plate. The section is slightly oblique, such that the left side is more anterior than the right side. Staining is observed in the thickened neural plate (n) and in the epidermis (e). There is no expression in the dorsal midline. At this stage, the most anterior epidermal expression (left side) appeared to be restricted to the inner (sensorial) layer of the epidermis. Epidermal expression is observed across the ventral midline. (C–E) Transverse sections of the same stage 31 embryo. (C) Section at the level of the anterior hindbrain. Staining is observed in the dorsal part of the neural tube, but not at the dorsal midline. The section is slightly oblique, such that the ventrolateral expression in the anterior hindbrain (Fig. 3K) is not visible. (D) Section at the level of the otic vesicle. Expression is found along the medial (inner) side of the dorsal part of the otic epithelium. The notochord (no) is labeled. (E) Section through the posterior hindbrain. There is staining in two bilaterally symmetric spots in the neural tube.

Fig. 6. Localization of *Xgbx-2* expression in the hindbrain. Embryos were hybridized alone or in combinations with *Xgbx-2*, *Krox-20*, or *En-2*. For all embryos, a lateral view is shown with dorsal at the top and anterior to the left. The magnification is the same in all photographs. All embryos are cleared. Arrowhead in A, B, D–F: anterior border of *Xgbx-2* expression. (A–C) Stage 21. (A) Embryo hybridized with *Xgbx-2* alone. *Xgbx-2* is also expressed in the head endoderm (arrow). (B) Embryo cohybridized with *Xgbx-2* and *Krox-20*. Note the gap between the *Xgbx-2* stripe and the rhombomere 3-stripe (3) of *Krox-20*, which is a length of approximately one rhombomere. *Krox-20* is also expressed in neural crest (nc) migrating into the third visceral arch (Bradley et al., 1992). (C) Embryo hybridized with *Krox-20* alone. (D–G) Stage 31. (D) Embryo cohybridized with *Xgbx-2* and *Krox-20*. The anterior *Xgbx-2* expression border (arrowhead) lies at least 2 rhombomere lengths anterior to the rhombomere 2/3 boundary. (E) Embryo hybridized with *Xgbx-2* alone. (F) Embryo cohybridized with *En-2* and *Xgbx-2*. The *En-2* stripe does not appear to be widened compared to (G). The anterior border of *Xgbx-2* expression (arrowhead) would be predicted to lie approximately halfway into the *En-2* expressing region (compare to E). (G) Embryo hybridized with *En-2* alone. *En-2* is also expressed in the mandibular arch (arrow).

Fig. 7. Treatment with retinoic acid causes an expansion and enhancement of ectodermal *Xgbx-2* expression in whole embryos. Pigmented embryos were treated for 30 min at stage 9 with 10 μM all-*trans* RA, fixed when controls reached stage 12.5–13, and processed for whole mount in situ hybridization with *Xgbx-2* antisense probe. Embryos that were only treated with ethanol, the RA solvent, looked the same as untreated control embryos (not shown). For all embryos, anterior is to the left, and the magnification is the same. (A) Untreated control embryo, dorsal view. (B) The same control embryo, lateral view. (C) RA-treated embryo, dorsal view. (D) RA-treated embryo, lateral view.

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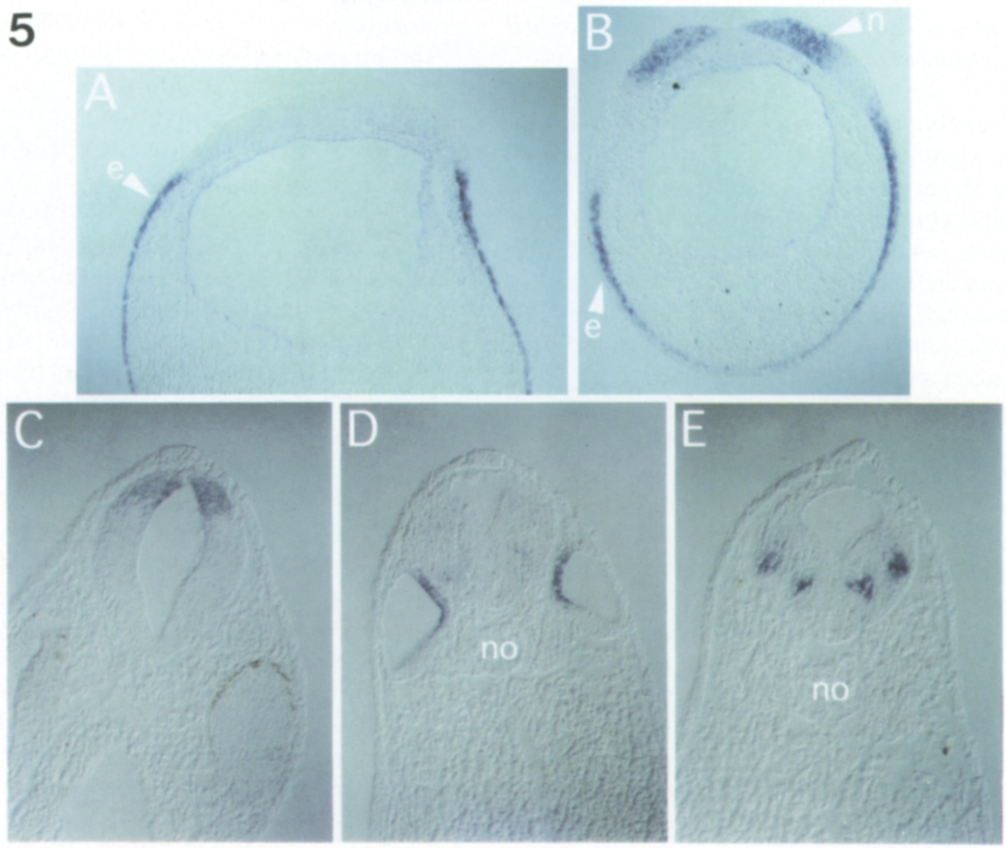

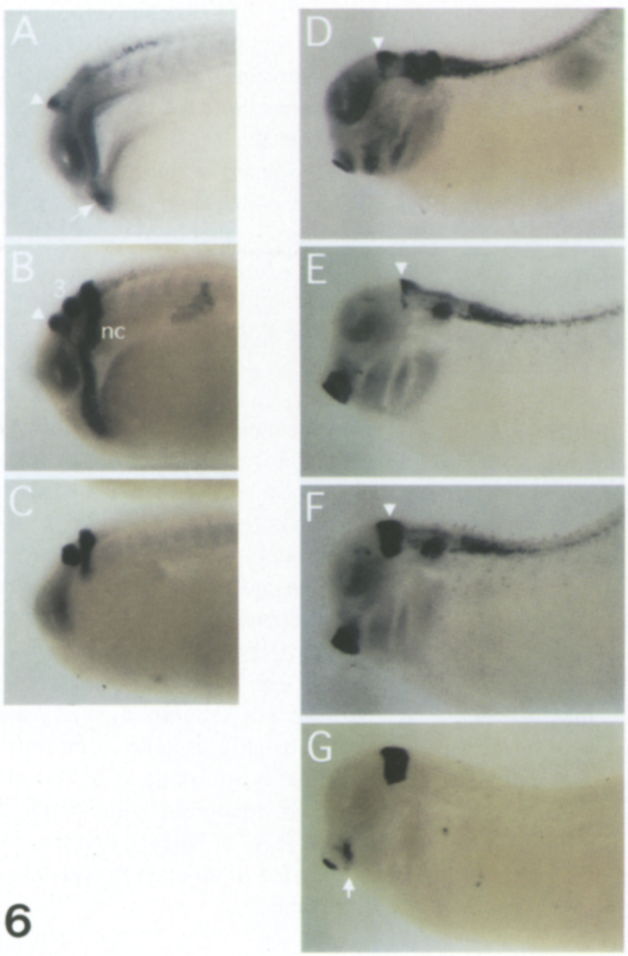

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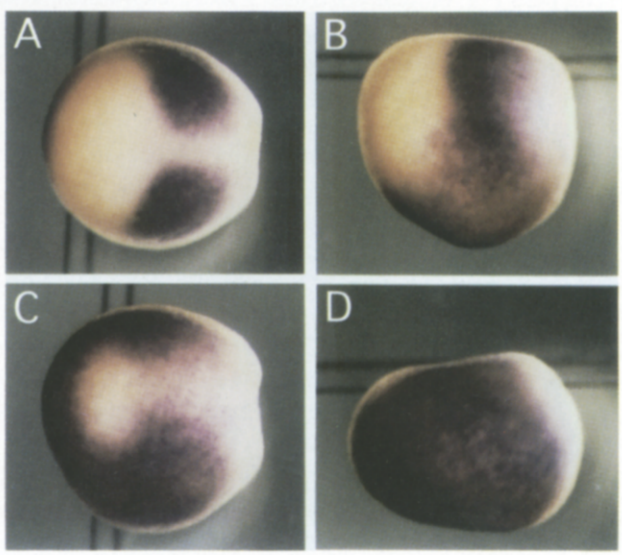

2.3. Localization of Xgbx-2 expression in the anterior and posterior hindbrain

The anterior border of *Xgbx-2* expression could be followed through successive stages of embryogenesis beginning at stage 12. By stage 15, most of the neural expression of *Xgbx-2* is concentrated in a pair of small stripes within a region of the anterior neural plate (Fig. 3E). To determine more precisely where this expression was localized, we hybridized embryos with probes for *Xgbx-2* and either *Krox-20*, which is expressed in rhombomeres 3 and 5 of the hindbrain (Bradley et al., 1992), or *Engrailed-2* (*En-2*), which, like the En-2 protein, is found in a band across the midbrain–hindbrain junction (Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1991; Bolce et al., 1992).

At stage 21, the most anterior *Xgbx-2* expression is found anterior to rhombomere 3 with a gap between *Xgbx-2* and *Krox-20* expression of approximately the same length as the rhombomere 3 staining of *Krox-20* (Fig. 6A–C). Similar results were observed at stage 26 (not shown) and at stage 31 (Fig. 6D,E). This suggests that *Xgbx-2* is expressed within the region of rhombomere 1 but only in low levels or not at all in rhombomere 2. This interpretation is consistent with the expression pattern of NCAM-PSA (the highly sialylated form of N-CAM), which has been used to distinguish individual rhombomeres in the hindbrains of stage 32–36 *Xenopus* embryos (Ruiz i Altaba and Jessell, 1991). This has shown that rhombomere 2 is of approximately the same length as rhombomere 3.

When comparing stage 31 embryos stained for *Xgbx-2* alone (Fig. 6E) and for *En-2* and *Xgbx-2* (Fig. 6F), the otic vesicle could be used as a morphological landmark. Here the anterior border of *Xgbx-2* expression is approximately halfway into the *En-2* expressing region of the costained embryos (compare Fig. 6E and F). The *En-2* stripe did not seem to be widened in the costained embryos when compared to the expression of *En-2* alone (compare Fig. 6F and G). Thus, *Xgbx-2* expression in the anterior hindbrain appears to overlap with the posterior *En-2* expression. In *Xenopus*, *En-2* is expressed in the future cerebellum (Hemmati-Brivanlou et al., 1991), and the posterior cerebellar anlage in *Xenopus* is believed to correspond to the anterior region of rhombomere 1 (Ruiz i Altaba and Jessell, 1991). This suggests that *Xgbx-2* is expressed in the presumptive posterior cerebellar anlage in rhombomere 1, with an anterior expression boundary near the anterior border of rhombomere 1, which corresponds to the midbrain–hindbrain junction.

The location of *Xgbx-2* expression in the posterior hindbrain, which appears by stage 23, can also be assessed by analyzing stage 31 embryos that have been co-stained with *Xgbx-2* and *Krox-20*. Weak *Xgbx-2* expression can be seen just posterior to the rhombomere 5 stripe of *Krox-20* expression, and strong expression begins approximately at the level of rhombomere 7 (Fig. 6D,E). This expression extends into the spinal cord and tapers off posteriorly.

2.4. Treatment with retinoic acid causes an expansion of Xgbx-2 expression in whole embryos

The anterior hindbrain is known to be a very sensitive region to RA treatment during embryonic development of vertebrates including *Xenopus* (Papalopulu et al., 1991; Morriss-Kay, 1993), and RA treatment can alter the expression of *Hox* genes in this region (Conlon and Rossant, 1992). Since *Xgbx-2*, similar to the *Hox* genes, shows a sharp anterior expression border in the neural ectoderm, we analyzed *Xgbx-2* expression in RA-treated embryos. Embryos were treated for 30 min at stage 9 with 10 μM RA and were fixed when control embryos reached stage 12.5–13. The embryos were then processed for whole mount in situ hybridization using the *Xgbx-2* probe. RA treatment of embryos under these conditions led to anterior and ventral enhancement of *Xgbx-2* expression (Fig. 7C,D). Sections of these embryos showed that RA expanded *Xgbx-2* expression in the ectodermal layer, but no expression in the mesoderm was observed (not shown). The expression areas fused anteriorly, still leaving an anteriorly widened, keyhole-shaped gap of expression in the dorsal midline. Thus, the sharp anterior borders of expression were lost in both the neural and non-neural ectodermal regions. RA-treated embryos that were allowed to develop until stage 40 showed the anterior and posterior malformations described previously (Durston et al., 1989; Sive et al., 1990; Papalopulu et al., 1991). The average dorso-anterior index (DAI; Kao and Elinson, 1988) of these embryos was approximately 2.0, corresponding to embryos that completely lacked eyes.

2.5. Retinoic acid can induce Xgbx-2 expression in animal caps

Since RA led to an expansion and enhancement of ectodermal *Xgbx-2* expression in whole embryos, we tested whether *Xgbx-2* could also be induced by RA in animal caps, which are prospective ectoderm. Animal caps were excised at stage 8–9 and treated for 45 min at the equivalent of stage 9–10 with the indicated doses of all-*trans* RA (Fig. 8). The caps were analyzed for *Xgbx-2* expression when control embryos reached stage 13–14, when the expression level of *Xgbx-2* in whole embryos is maximal (Fig. 2A). We did not observe *Xgbx-2* expression in untreated animal caps (Fig. 8, lane 2) or caps incubated in the solvent dimethylsulfoxide (DMSO) alone (Fig. 8, lane 3). In contrast, treatment with 10 μM RA induced *Xgbx-2* expression (Fig. 8, lane 4), and treatment with a ten-fold higher dose led to an even higher level of *Xgbx-2* induction (Fig. 8, lane 5).

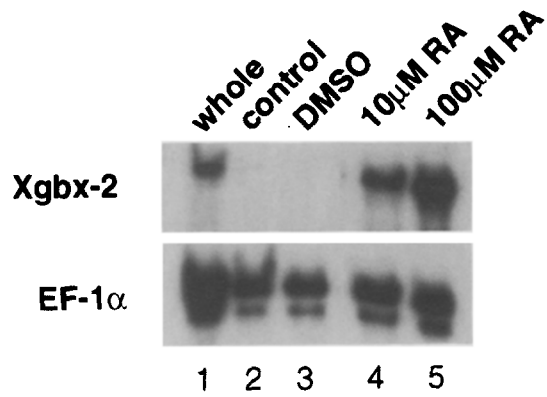

Fig. 8. Induction of *Xgbx-2* by all-*trans* RA. Embryos were cultured until stage 8–9, at which time the upper portion of the animal hemisphere (the animal cap) was removed. Animal caps were treated for 45 min at the equivalent of stage 9–10 with the indicated doses of RA. RNA was isolated at stage 13–14 of controls for RNase protection analysis. An *EF-1α* probe was included to control for RNA loading. Lane 1, RNA of one untreated control embryo was loaded. In lanes 2–5, RNA of ten animal caps was loaded per lane. Lane 2, untreated; lane 3, incubated in DMSO (the solvent of the RA stock solution) at the same concentration as with 10 μM RA; lane 4, 10 μM RA; lane 5, 100 μM RA.

3. Discussion

3.1. Xgbx-2 and Gbx-2 are likely cognate genes

We have described the isolation and expression of the *Xenopus Xgbx-2* gene, which is the likely cognate of the mouse *Gbx-2* gene (Bulfone et al, 1993; Frohman et al., 1993; Chapman and Rathjen, 1995). The expression patterns of *Xgbx-2* and *Gbx-2* are conserved in some regards, but also show differences. Similar to *Xgbx-2*, *Gbx-2* is first detected during gastrulation. During neurulation (E7.5-E8.5), *Gbx-2* is expressed in the neural ectoderm and underlying mesoderm with an anterior boundary in the region of the midbrain–hindbrain junction (M. Frohman, pers. comm.). This is similar to *Xgbx-2* expression, although by whole mount in situ hybridization, we did not detect mesodermal expression of *Xgbx-2* at all the stages analyzed. In the mouse, expression of *Gbx-2* in the epidermis has not been reported, whereas we found *Xgbx-2* expression in the epidermal ectoderm from late gastrula to early tailbud stages. By E9.5, *Gbx-2* expression is found in the hindbrain, the spinal cord, and the otic vesicle (M. Frohman, pers. comm.) and we observed similar expression sites for *Xgbx-2*. In summary, the similarities in the amino acid sequences and in most sites of expression suggest that *Xgbx-2* is the *Xenopus* cognate of the mouse *Gbx-2* gene.

3.2. Early regulation of the anteriormost Xgbx-2 expression in the neural ectoderm

Soon after *Xgbx-2* expression is activated at the mid-gastrula stage, the anterior border becomes sharply demarcated. This border can be followed through development to a position in the region of the anterior boundary of rhombomere 1, which corresponds to the midbrain–hindbrain junction. We do not know whether the expression of *Xgbx-2* is regulated by the *Hox* genes, or if it is regulated on a parallel pathway. The onset of *Xgbx-2* expression is observed at about the same time or only slightly later than the onset of expression of the earliest expressed *Hox* genes. Of these, the expression of the *Hoxd-1* (*Xhox.lab1*) gene (Sive and Cheng, 1991; Kolm and Sive, 1995) is most similar to *Xgbx-2*. By Northern analysis, *Hoxd-1* expression is first weakly detected at stage 10.5 (Kolm and Sive, 1995), which is similar to the time when we first detect *Xgbx-2* expression by RNase protection and by whole mount in situ hybridization (stage 10.5–11). Moreover, the initial expression pattern of *Hoxd-1* at stage 11 is very similar to *Xgbx-2*, in that it is also predominantly expressed in the dorsolateral ectoderm with a gap in expression at the dorsal midline (Kolm and Sive, 1995). Two other *Hox* genes, *Hoxb-1* and *Hoxb-3*, are weakly detected in early *Xenopus* embryos at the start of gastrulation by RNase-protection analysis (Dekker et al., 1992a,b), although by in situ hybridization, they are only detected by the late gastrula/early neurula stages (Godsave et al., 1994), suggesting that these genes do not regulate *Xgbx-2*. A comparison of the spatial localization of *Xgbx-2* and the *Hox* genes also suggests that the *Hox* genes do not regulate the anteriormost expression of *Xgbx-2*. The anteriormost expression of *Xgbx-2* is in rhombomere 1, which lies anterior to all *Hox* gene expression (Krumlauf, 1993).

One candidate regulator of *Xgbx-2* at its anterior expression boundary is the *Drosophila orthodenticle*-related homeobox gene, *Otx2*. In vertebrates, *Otx2* shows a posterior expression border at the presumptive midbrain–hindbrain junction, which in mouse, zebrafish and chicken becomes established during gastrulation (Simeone et al., 1992, 1993; Ang et al., 1994; Li et al., 1994; Bally-Cuif et al., 1995). In *Xenopus*, *Otx2* is expressed in presumptive neurectoderm from stage 10.25–10.5 onwards and shows a distinct posterior border of expression by stage 12 (late gastrulation; Blitz and Cho, 1995; Pannese et al., 1995). In comparison, *Xgbx-2* expression in presumptive neurectoderm is first detected at stage 10.5–11, and by stage 12, it has a distinct anterior border of expression. Thus, the first establishment of the posterior *Otx2* expression boundary in the neural ectoderm during gastrulation appears to coincide with the first establishment of the anterior *Xgbx-2* expression border. In addition, preliminary cohybridization experiments indicate that *Xgbx-2* and *Otx2* directly abut each other at the midbrain–hindbrain boundary (unpublished results). These results suggest that the midbrain–hindbrain boundary could be positioned or established by an interaction between *Xgbx-2* and *Otx2*. It is therefore interesting that, as described below, addition of RA to embryos enhances the

expression of *Xgbx-2* but decreases the expression of *Otx2*.

It should be noted that other genes are expressed in the midbrain–hindbrain boundary region, such as members of the *En-*, *Pax-* and *Wnt*-families (reviewed in Alvarado-Mallart, 1993; Fjose, 1994; Rubenstein and Puelles, 1994). Some of these have been shown to be required for the formation of this region, such as *Wnt-1* and *En-1* in mice (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994), and *Pax [zf-b]* in zebrafish (Krauss et al., 1992). However, these genes appear to be expressed after the onset of *Otx2* and *Xgbx-2* expression, suggesting that they may function at later steps in the establishment of the midbrain–hindbrain boundary.

3.3. Regulation of Xgbx-2 by retinoic acid

We have shown here that all-*trans* retinoic acid expands and enhances the expression of *Xgbx-2* in the ectoderm in whole embryos. Moreover, RA alone can induce *Xgbx-2* expression in animal caps, showing that RA can activate *Xgbx-2* expression in undifferentiated ectoderm. These results suggest that the expression of *Xgbx-2* may be regulated by endogenous retinoids. Consistent with this possibility, the expression in *Xenopus* of at least some molecules thought to mediate or modulate the effects of RA on gene regulation, such as RA receptors (RARs; Sharpe, 1992) and cellular RA binding proteins (CRABPs; Ho et al., 1994), appears to overlap with regions of *Xgbx-2* expression.

While RA positively regulates *Xgbx-2* and the *Hox* genes (Cho and DeRobertis, 1990; Sive et al., 1990; Sive and Cheng, 1991; Dekker et al., 1992a,b; Leroy and DeRobertis, 1992), the *Otx2* gene, which is expressed in the mid- and forebrain, is inhibited by exogenous RA (Simeone et al., 1993, 1995; Ang et al., 1994; Bally-Cuif et al., 1995; Pannese et al., 1995). This suggests that the midbrain–hindbrain boundary may separate regions expressing early neural genes that are positively and negatively regulated by RA.

The anterior expansion of *Xgbx-2* expression, which we observed in RA-treated embryos, may in part be responsible for the truncations of the anterior CNS which are later observed in such embryos (Durston et al., 1989; Sive et al., 1990; Papalopulu et al., 1991). In zebrafish embryos, RA treatment can lead to a specific loss of the *En-2*-expressing region around the midbrain–hindbrain boundary (Holder and Hill, 1991), and a loss of *En-2* expression is also observed in RA-treated *Xenopus* embryos (Sive et al., 1990). If *Xgbx-2* (perhaps by an interaction with *Otx2*) is involved in the positioning or establishment of the midbrain–hindbrain boundary, the loss of the sharp anterior *Xgbx-2* expression border in RA-treated embryos may lead to a failure in the formation of the midbrain–hindbrain boundary. The loss of *En-2* expression may then be a secondary consequence of a disruption of the midbrain–hindbrain boundary region; this region has been suggested to be the source of morphogenetic signals (such as Wnt-1) which appear to be required for the induction and maintenance of *En-2* expression in the adjacent neuroepithelium (reviewed in Alvarado-Mallart, 1993; Rubenstein and Puelles, 1994).

3.4. Expression of Xgbx-2 in the epidermis

The expression of *Xgbx-2* appears unique compared to most of the *Hox* genes in that it shows A-P restricted expression in the presumptive epidermis from as early as late gastrula until early tailbud stages. In *Xenopus*, only two *Hox* genes have been reported to be expressed in the epidermis. *Hoxd-1* shows expression in the presumptive epidermis during the gastrula stage, although this expression is almost lost by stage 15 (neurula stage; Kolm and Sive, 1995) and *Hoxa-9* (*Xhox.B1*) is expressed in the epidermis at stage 25–26 (Stickland et al., 1992). In the mouse, the neural crest cells are believed to activate their respective pattern of *Hox* gene expression in the overlying epidermis (Hunt et al., 1991; Krumlauf, 1993). However, the establishment of the anterior border of *Xgbx-2* expression in the epidermis precedes the beginning of the ventralward migration of the neural crest cells at stage 19 (Sadaghiani and Thiébaud, 1987). This suggests that the initial positioning of this border cannot be explained by vertical influences from underlying neural crest, and it appears unlikely that *Hox* genes expressed in the neural crest regulate or induce *Xgbx-2* expression in the surface ectoderm. It is interesting to note that *Xgbx-2* was not expressed in untreated animal caps, suggesting that the epidermal expression of *Xgbx-2* requires a positive inductive event.

It has been suggested that the ectodermal layer of the body, including the presumptive epidermis, may be anteroposteriorly subdivided into genetically defined developmental units, termed 'ectomeres', as a result of an early simultaneous specification event of neural tube, neural crest and superficial ectoderm (Couly and Le Douarin, 1990). The epidermal expression of *Xgbx-2* may reflect the establishment of an ectomere boundary during late gastrulation, before the migration of neural crest begins. It will be interesting to determine the factors that regulate the spatial distribution of *Xgbx-2* within the epidermis.

4. Experimental procedures

4.1. Embryos

Fertilized *Xenopus* embryos were obtained as previously described (Newport and Kirschner, 1982). Eggs were fertilized in 0.5× MMR (1× MMR is 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, and 0.1 mM EDTA). The jelly coat was removed with 2% cysteine in water (pH 7.8), and the eggs were rinsed in

0.1× MMR. Embryos were maintained in 0.1× MMR at 14–23°C. Staging was done according to Nieuwkoop and Faber (1956).

4.2. Animal caps

The upper portion of the animal hemisphere, corresponding to roughly one fourth of the embryo, was manually separated from stage 8–9 embryos with a fine wire knife. Care was taken to remove any adherent vegetal cells.

4.3. Retinoic acid treatment

Animal caps were cultured in 1× MBS (88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) containing 1 mg/ml BSA and 50 μg/ml gentamycin sulfate (Sigma) alone or in the dark in the presence of all-*trans* RA (Sigma), which was diluted from a 10 mM stock solution in DMSO (stored in aliquots at −80°C). Solvent controls contained only DMSO at the same concentration as with 10 μM RA (0.1% DMSO). The efficiency of the RA treatment was always tested by incubating whole embryos (using 0.1× MMR) under the same conditions, with RA taken from the same aliquot of the stock solution.

Whole embryos to be processed for whole mount in situ hybridization were treated in the dark in 0.1× MMR containing all-*trans* RA (Sigma), which in this case was diluted from a 1 mg/ml stock solution in ethanol (stored at −20°C). Solvent controls contained ethanol at the highest concentration used in the experiment (1.2% ethanol).

4.4. Isolation of Xenopus homeobox-containing sequences

Xgbx-2 was isolated in a search for homeobox-containing genes transcribed during the gastrula stages using a polymerase chain reaction (PCR)-based approach as previously described (Northrop and Kimelman, 1994). The PCR fragments were used as probes to isolate cDNAs from a stage 17 (neurula) phage library (Kintner and Melton, 1987), which were inserted into the *Eco*RI site of a Bluescript SK+ vector (Stratagene). The complete nucleotide sequence of the longest cDNA (p*Xgbx-2*) was determined and deposited in GenBank under accession number L47990.

4.5. RNA isolation and analysis

RNA was prepared by homogenization in a buffer containing proteinase K (Krieg and Melton, 1984). The ethanol precipitate was dissolved in diethylpyrocarbonate-treated water and reprecipitated overnight with an equal volume of 8 M LiCl at −20°C. RNase protection was performed with an antisense transcript from the 3′ end of the *Xgbx-2* cDNA. The p*Xgbx-2* plasmid was linearized with *Rsa*I and transcribed with T3 polymerase, producing a 433 bp protected fragment. As a control for RNA loading, a probe for the ubiquitously expressed *EF-1α* gene (Krieg et al., 1989) was synthesized at a reduced specific activity (Sargent and Bennett, 1990) and included in every hybridization reaction. Probes were hybridized with RNA samples overnight at 45°C and then treated with 1 μg/ml RNase T1 (Sigma) for 1 h at room temperature.

For Northern blot analysis, poly(A)-containing RNA from 50 unfertilized eggs or 50 embryos was isolated by oligo(dT)-cellulose chromatography, electrophoresed on a formaldehyde-agarose gel, transferred to Duralon (Stratagene) and immobilized by UV cross-linking. The filter was hybridized with an *Xgbx-2* probe labeled with ^{32}P by random priming.

4.6. In situ hybridization and probe synthesis

Whole-mount in situ hybridization was performed using digoxygenin-labeled RNA probes (Harland, 1991), with the modifications that levamisol was omitted from the alkaline phosphatase buffer, and that the RNase digestion step was omitted. Antisense probes corresponding to the complete cDNA were synthesized from *Eco*RV-digested p*Xgbx-2* DNA using T3 polymerase. As a control, sense probes were synthesized from *Bam*HI-digested p*Xgbx-2* using T7 polymerase. For the generation of antisense probes, the *Xbra* cDNA (Smith et al., 1991) was linearized with *Eco*RV and transcribed with T7 polymerase; the *Krox-20* cDNA (Bradley et al., 1992) was linearized with *Eco*RI and transcribed with T7 polymerase, and the *En-2* cDNA (Hemmati-Brivanlou et al., 1991) was linearized with *Xba*I and transcribed with T3-polymerase. For sectioning, embryos were embedded in Paraplast, sectioned at 10 or 16 μm and mounted in Permount (Kelly et al., 1991). Whole mount embryos and sections were photographed using Kodak Ektachrome 160T and 64T film, respectively.

Acknowledgements

We wish to thank D. Arendt, J. Bohrmann, R. Cassada, T. Müller, K. Nübler-Jung, E. Levine and L. Sumoy for critical reading of the manuscript, M. Frohman for helpful discussions, Adolf Maas for excellent technical assistance, and K. Cho for providing *Otx2*. A.v.B. wishes to thank L. Koschny and the laboratory of B. Christ for help with some of the sections, and P. Hausen for helpful discussions. This work was supported by a grant from the March of Dimes (1-FY94-0474) to D.K. and by the German National Scholarship Foundation to A.v.B., who was a visiting graduate student from the University of Freiburg. J.S. is a recipient of a Howard Hughes predoctoral fellowship.

References

Akam, M., Averof, M., Castelli-Gair, J., Dawes, R., Falciani, F. and Ferrier, D. (1994) Development (Suppl.), 209–215.

Alvarado-Mallart, R.-M. (1993) J. Neurobiol. 24, 1341–1355.

Ang, S.L., Conlon, R.A., Jin, O. and Rossant, J. (1994) Development 120, 2979–2989.

Bally-Cuif, L., Gulisano, M., Broccoli, V. and Boncinelli, E. (1995) Mech. Dev. 49, 49–63.

Blitz, I.L. and Cho, K.W.Y. (1995) Development 121, 993–1004.

Bolce, M.E., Hemmati-Brivanlou, A., Kushner, P.D and Harland, R.M. (1992) Development 115, 681–688.

Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F. (1991) Trends Genet. 7, 329–334.

Bradley, L.C., Snape, A., Bhatt, S. and Wilkinson, D.G. (1992) Mech. Dev. 40, 73–84.

Bulfone, A., Puelles, L., Porteus, M.H., Frohman, M.A., Martin, G.R. and Rubenstein, J.L.R. (1993) J. Neurosci. 13, 3155–3172.

Chapman, G. and Rathjen, P.D. (1995) FEBS Lett. 364, 289–292.

Cho, K.W.Y. and DeRobertis, E.M. (1990) Genes Dev. 4, 1910–1916.

Cho, K.W.Y., Blumberg, B., Steinbeisser, H. and DeRobertis, E.M. (1991) Cell 67, 1111–1120.

Conlon, R.A. and Rossant, J. (1992) Development 116, 357–368.

Couly, G. and Le Douarin, N.M. (1990) Development 108, 543–558.

Dekker, E.J., Pannese, M., Houtzager, E., Boncinelli, E. and Durston, A. (1992a) Mech. Dev. 40, 3–12.

Dekker, E.J., Pannese, M., Houtzager, E., Timmermans, A., Boncinelli, E. and Durston, A. (1992b) Development (Suppl.), 195–202.

Durston, A.J., Timmermans, J.P.M., Hage, W.J., Hendriks, H.F.J., De Vries, N.J., Heideveld, M. and Nieuwkoop, P.D. (1989) Nature 340, 140–144.

Fainsod, A. and Gruenbaum, Y. (1989) FEBS Lett. 250, 381–385.

Fjose, A. (1994) Curr. Top. Dev. Biol. 29, 65–100.

Frohman, M.A., Dickinson, M.E., Hogan, B.L.M. and Martin, G.R. (1993) Mouse Genome 91, 323–325.

Gaunt, S.J. (1991) BioEssays 13, 505–513.

Godsave, S., Dekker, E.J., Holling, T., Pannese, M., Boncinelli, E. and Durston, A. (1994) Dev. Biol. 166, 465–476.

Harland, R.M. (1991) In Kay, B.K. and Peng, H.J. (eds.), Methods in Cell Biology, Vol. 36, Academic Press, San Diego, CA, pp. 685–695.

Hemmati-Brivanlou, A. and Harland, R.M. (1989) Development 106, 611–617.

Hemmati-Brivanlou, A., de la Torre, J.R., Holt, C. and Harland, R.M. (1991) Development 111, 715–724.

Herrmann, B.G. (1991) Development 113, 913–917.

Ho, L., Mercola, M. and Gudas, L.J. (1994) Mech. Dev. 47, 53–64.

Holder, N. and Hill, J. (1991) Development 113, 1159–1170.

Hunt, P. and Krumlauf, R. (1992) Annu. Rev. Cell Biol. 8, 227–256.

Hunt, P., Whiting, J., Muchamore, I., Marshall, H. and Krumlauf, R. (1991) Development (Suppl.), 187–196.

Kao, K.R. and Elinson, R.P. (1988) Dev. Biol. 127, 64–77.

Keller, R., Shih, J. and Sater, A. (1992) Dev. Dyn. 193, 199–217.

Kelly, G.M., Eib, D.W. and Moon, R.T. (1991) In Kay, B.K. and Peng, H.J. (eds.), Methods in Cell Biology, Vol. 36, Academic Press, San Diego, CA, pp. 389–419.

Kessel, M. and Gruss, P. (1991) Cell 67, 89–104.

King, M.W. and Moore, M.J. (1994) Nucleic Acids Res. 22, 3990–3996.

Kintner, C.R. and Melton, D.A. (1987) Development 99, 311.

Kolm, P.J. and Sive, H.L. (1995) Dev. Biol. 167, 34–49.

Krauss S., Maden, M., Holder, N. and Wilson, S.W. (1992) Nature 360, 87–89.

Krieg, P. and Melton, D. (1984) Nucleic Acids Res. 12, 7057–7070.

Krieg, P.A., Varnum, S., Wormington, M.W. and Melton, D.A. (1989) Dev. Biol. 133, 93–100.

Krumlauf, R. (1993) Trends Genet. 9, 106–112.

Langston, A.W. and Gudas, L.J. (1994) Curr. Opin. Genet. Dev. 4, 550–555.

Leroy, P. and DeRobertis, E.M. (1992) Dev. Dyn. 194, 21–32.

Levine, E.M. and Schechter, N. (1993) Proc. Natl. Acad. Sci. USA 90, 2729–2733.

Li, Y, Allende, M.L., Finkelstein, R. and Weinberg, E.S. (1994) Mech. Dev. 48, 229–244.

Lopez, S.L. and Carrasco, A.E. (1992) Mech. Dev. 36, 153–164.

Matsui, T., Hirai, M., Wakita, M., Hirano, M. and Kurosawa, Y. (1993a) FEBS Lett. 322, 181–185.

Matsui, T., Hirai, M., Hirano, M. and Kurosawa, Y. (1993b) FEBS Lett. 336, 107–110.

McGinnis, W. and Krumlauf, R. (1992) Cell 68, 283–302.

McMahon, A.P. and Bradley, A. (1990) Cell 62, 1073–1085.

Morriss-Kay, G. (1993) BioEssays 15, 9–15.

Murtha, M.T., Leckman, J.F. and Ruddle, F.H. (1991) Proc. Natl. Acad. Sci. USA 88, 10711–10715.

Newport, J. and Kirschner, M.W. (1982) Cell 30, 675–686.

Nieuwkoop, P.D. and Faber, J. (1956) Normal Table of *Xenopus laevis*. North-Holland, Amsterdam, 252 pp.

Northrop, J.L. and Kimelman, D. (1994) Dev. Biol. 161, 490–503.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995) Development 121, 707–720.

Papalopulu, N., Clarke, J.D.W., Bradley, L., Wilkinson, D., Krumlauf, R. and Holder, N. (1991) Development 113, 1145–1158.

Rubenstein, J.L.R. and Puelles, L. (1994) Curr. Top. Dev. Biol. 29, 1–63.

Ruiz i Altaba, A. and Jessell, T.M. (1991) Development 112, 945–958.

Ruiz i Altaba, A. and Jessell, T.M. (1992) Development 116, 81–93.

Sadaghiani, B. and Thiébaud, C.H. (1987) Dev. Biol. 124, 91–110.

Sargent, M.G. and Bennett, M.F. (1990) Development 109, 967–973.

Schulte-Merker, S., Ho, R.K., Herrmann, B.G. and Nüsslein-Volhard, C. (1992) Development 116, 1021–1032.

Sharpe, C.R. (1992) Mech. Dev. 39, 81–93.

Simeone, A., Acampora, D., Gulisano, M., Stornaiulo, A. and Boncinelli, E. (1992) Nature 358, 687–690.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiulo, A., Rosaria D'Apice, M.R., Nigro, V. and Boncinelli, E. (1993) EMBO J. 12, 2735–2747.

Simeone, A., Avantaggiato, V., Moroni, M.C., Mavilio, F., Arra, C., Cotalli, F., Nigro, V. and Acampora, D. (1995) Mech. Dev. 51, 83–98.

Sive, H.L., Draper, B.W., Harland, R.M. and Weintraub, H. (1990) Genes Dev. 4, 932–942.

Sive, H.L. and Cheng, P.F. (1991) Genes Dev. 5, 1321–1332.

Slack, J.M.W. and Tannahill, D. (1992) Development 114, 285–302.

Smith, J.C., Price, B.M.J., Green, J.B.A., Weigel, D. and Herrmann, B.G. (1991) Cell 67, 79–87.

Stickland, J.E., Sharpe, C.R., Turner, P.C. and Hames, B.D. (1992) Gene 116, 269–273.

Sundin, O. and Eichele, G. (1992) Development 114, 841–852.

Thomas, K.R. and Capecchi, M.R. (1990) Nature 346, 847–850.

Wurst, W., Auerbach, A. and Joyner, A.L. (1994) Development 120, 2065–2075.

Yamada, T. (1994) Development 120, 3051–3062.