Int. J. Dev. Biol. 41: 57-65 (1997)

Original Article

Neural induction and patterning in embryos deficient in FGF signaling

SUSAN F. GODSAVE* and ANTONY J. DURSTON

Netherlands Institute for Developmental Biology, Utrecht, The Netherlands

ABSTRACT We have examined the role of FGFs in neural induction and posteriorization of the central nervous system (CNS). Using embryos micro-injected with dominant negative FGF receptor RNA *(XFD)*, we show that a patterned CNS can still develop following inhibition of FGF signaling. The most severely affected embryos developed with strong posterior defects. In these embryos, head development and expression of a marker of forebrain and midbrain, and of markers of the hindbrain, occurred relatively normally. However, the expression levels of a posterior marker, *Hoxb-9*, were considerably reduced compared to those in control embryos. The results support the idea that FGFs are involved in inducing posterior development, but they suggest that other signals are also necessary for antero-posterior patterning of the primary body axis

KEY WORDS: *neural induction, antero-posterior patterning, fibroblast growth factor, Xenopus*

Introduction

During *Xenopus* development, members of the fibroblast growth factor family, FGFs, first appear to play a role in mesoderm formation (reviewed in Kessler and Melton, 1994) and in maintaining the expression of the early response gene, *brachyury*, in the mesoderm during gastrula stages (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). Several recent reports now suggest that FGFs also play a role in inducing or posteriorizing neural tissue in the gastrula (Kengaku and Okamoto, 1993, 1995; Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; reviewed in Doniach, 1995).

The importance of the dorsal mesoderm, organizer tissue in induction of the CNS was demonstrated more than 70 years ago (Spemann and Mangold, 1924). However, it is only recently that advances have been made in understanding the molecular basis for neural induction. Several substances with anterior neural inducing properties have now been identified. Micro-injected mRNAs for noggin, follistatin and chordin are able to rescue axis formation in UV-ventralized embryos. The endogenous mRNAs for these factors are all localized in the organizer region of the *Xenopus* gastrula and all are thought to encode secreted proteins. Furthermore, noggin and chordin have also been shown to be able to bind directly to the ventralizing factor, BMP-4, and to inhibit its activity (Smith and Harland, 1992; Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996).

The specific induction of anterior neural tissue by neural inducing factors is in agreement with the two step model for neural induction and patterning proposed by Nieuwkoop and his collaborators in 1952. The differentiation of folds of ectoderm grafted into different antero-posterior regions along the neural plate of urodele embryos indicated that there is first an 'activation' step in which organizer mesoderm induces the overlying ectoderm to form neural tissue with an anterior (forebrain) specification. Secondly, posteriorizing or 'transforming' signals are released from posterior mesoderm in older gastrulae, causing the overlying neural tissue to be transformed into presumptive hindbrain and spinal cord.

Recently bFGF has been found to have both neural inducing and 'transforming' or posteriorizing activities (reviewed in Doniach, 1995). However, bFGF does not behave like the 'activation' (anterior neuralization) signal of Nieuwkoop *et al.* (1952) as it appears to be able to induce posterior neural tissue directly (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). FGF has only been found to mediate neural induction using culture conditions which favor neuralization (reviewed in Doniach, 1995), and it is therefore unclear whether this activity is of any relevance *in vivo*, although there is evidence that FGF signaling may be involved in the response to neural inducing signals (Launay *et al.*, 1996; Sasai *et al.*, 1996).

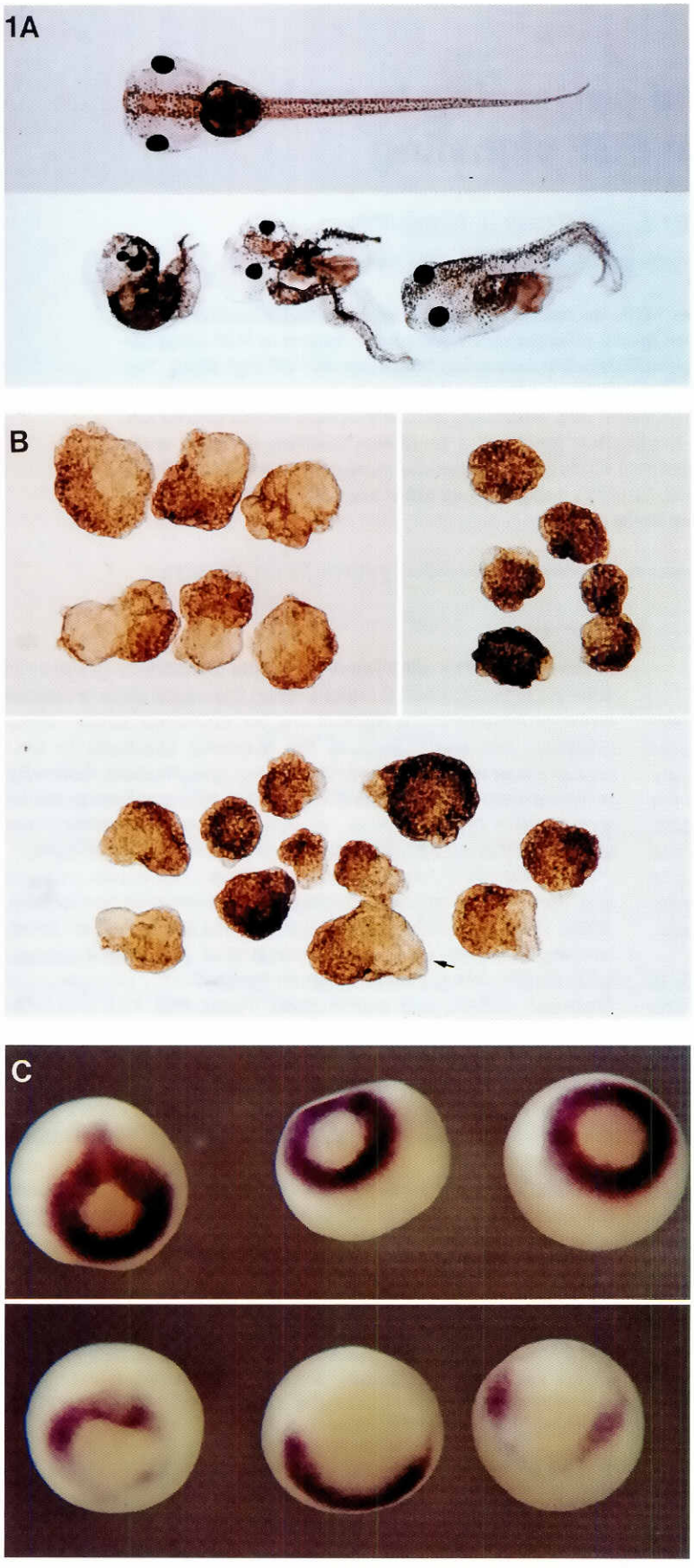
There is stronger evidence that FGFs are involved in promoting posterior development. bFGF is able to 'transform' or posteriorize anterior neural tissue from early neurula stage embryos (Cox and Hemmati-Brivanlou, 1995). RNAs for eFGF (Isaacs *et al.*, 1992,

Abbreviations used in this paper: XFD, dominant negative FGF receptor; CNS, central nervous system; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; eFGF, embryonic fibroblast growth factor; BMP, bone morphogenetic protein; TGF-ß, transforming growth factor-beta; MMR, Marc's modified Ringer; PBS, phosphate buffered saline.

***Address for reprints:** Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. FAX: 31-30-2516464. e-mail: godsave@hubrecht.nl

0214-6282/97/$05.00

Printed in Spain



1995) and Int-2 (Tannahill *et al.*, 1992) are localized around the closing blastopore of late gastrula stage *Xenopus* embryos as might be expected for a posteriorizing signal. Furthermore, inhibition of intercellular FGF signaling using overexpression of dominant negative FGF receptors, causes embryos to develop with relatively normal heads but severely reduced trunks and tails (Amaya *et al.*, 1991, 1993). The embryos do not gastrulate normally. Convergence and extension movements are affected so that blastopore closure does not occur (Amaya *et al.*, 1991; Isaacs *et al.*, 1994) and formation of the posterior dorsal mesoderm, notochord and somites, is reduced and disrupted in these embryos (Amaya *et al.*, 1993). Relatively little is known about CNS development in FGF receptor defective mutants, although brain development has been reported to occur relatively normally and disorganized neural structures have been seen in the tissue bordering the split blastopore.

The aim of the present study was to examine the role of FGFs in induction and patterning of the CNS using embryos expressing dominant negative FGF receptors.

Results

Effects of inhibiting FGF signaling using dominant negative FGF receptors

It is well established that inhibition of FGF signaling by overexpression of dominant negative FGF receptors (XFD) causes a reduction in mesoderm formation and defects in gastrulation. Head formation appears to occur normally, but embryos are considerably shortened posteriorly. The phenotypes obtained here by injection of *XFD* mRNA (Fig. 1A) were comparable to those described previously (Amaya *et al.*, 1991, 1993; Isaacs *et al.*, 1994). Embryos were microinjected with 0.75 ng *XFD* RNA into both blastomeres at the two cell stage. The survival rates were similar to those of the uninjected controls and of the control injected embryos. Of the survivors, an average of 40% of *XFD* embryos showed a strong phenotype. These embryos showed severely reduced posterior development and a splitting of the shortened CNS extending into the brain. A further 19% of

Fig. 1. Overexpression of dominant negative FGF receptors inhibits FGF signaling and causes posterior defects. (A). *Morphology of XFD embryos. (Top) Control stage 46 embryo. (Bottom) XFD mRNA injected embryos grown to stage 46. Left, phenotype of the most severely affected embryos; middle, a less severely affected phenotype with a shortened, split axis starting from the trunk region; right, mildly affected embryo with a shortened, split tail.* **(B).** *Animal caps from XFD mRNA-injected embryos do not respond to bFGF. (Top) Animal caps from control embryos: (left) induced to form mesoderm-containing vesicles by 60 ng/ml recombinant Xenopus bFGF; (right) uninduced controls which formed spheroids of atypical epidermis. (Bottom) Animal caps from XFD mRNA injected embryos treated with 60 ng/ml bFGF. Most explants failed to be induced. Only one animal cap is induced to the same extent as the caps from the control embryos (arrow).* **(C).** *Brachyury expression is reduced in XFD mRNA-injected embryos. Whole mount in situ hybridization of mid-gastrula stage embryos with an xbra anti-sense probe: (top) control embryos show a ring of strong expression around the blastopore; (bottom) XFD mRNA-injected embryos show enlarged blastopores and reduced expression of xbra transcripts.*

embryos showed a split axis starting at a more posterior level. One to two percent of embryos developed other abnormalities that were also present at the same frequency in embryos injected with control mRNAs. The remaining embryos appeared normal. Two types of control mRNA were used, wild type *FGF receptor* mRNA and *HAVNOT*. *HAVNOT* encodes a non-functional form of the FGF receptor with a 3 amino acid deletion in the extracellular domain (Byers *et al.*, 1992; Amaya *et al.*, 1993). As found previously, control injected embryos developed normally in most cases and never showed the characteristic phenotypes obtained with the *XFD* RNA (Amaya *et al.*, 1991, 1993). Injection of higher amounts of *XFD* RNA (1.5 ng/blastomere) was generally lethal.

The earliest functions of FGFs in the embryo are probably in the induction of mesoderm (reviewed by Kessler and Melton, 1994) and then in the maintenance of the mesodermal state, by maintaining the expression of the early response gene, *brachyury (xbra)* (Smith *et al.*, 1991; Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). In order to test the effectiveness of the *XFD* mRNA injections used here in inhibiting FGF signaling, two types of control experiment were carried out. First, animal caps dissected at the mid-blastula stage from XFD-injected embryos (0.75 ng RNA into each blastomere at the 2 cell stage) were tested for their ability to respond to solutions of bFGF protein by forming mesoderm. bFGF at 30-100 ng/ml caused vesicle formation in 90% (28/31) control explants from HAVNOT or non-injected control embryos. By contrast, only 24% (11/45) of animal caps from XFD-injected embryos formed any vesicles at all in response to bFGF and these were generally only small inductions (Fig. 1B). The vesicle assay is a reliable indication of the induction of ventral or ventro-lateral mesoderm formation (Slack *et al.*, 1987; Godsave *et al.*, 1988). Taken together with the approximately 60% penetrance of moderately strong to strong phenotypes for this *XFD* RNA dose, these results indicate that the *XFD* embryos which develop abnormally are deficient in responding to bFGF.

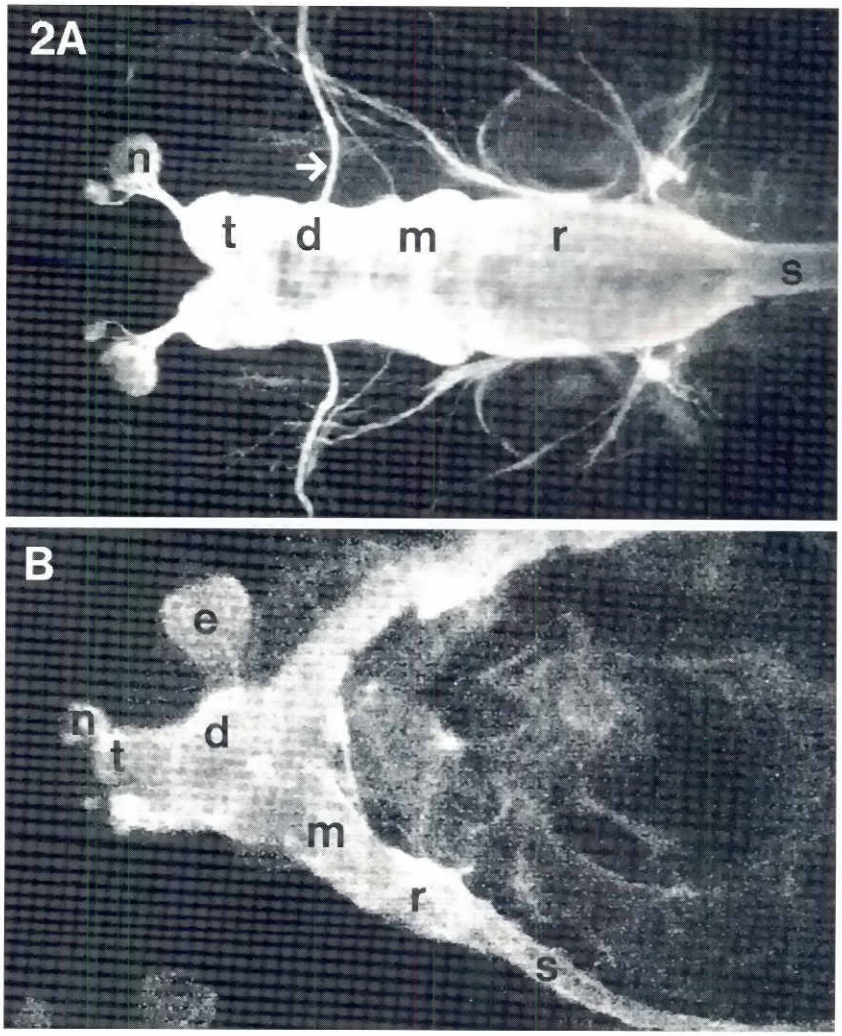


Fig. 2. Splitting of the midbrain in a severely affected *XFD* mRNA-injected embryo. *CLSM images (see Materials and Methods) of stage 46 tadpoles following whole-mount immunofluorescence using an anti-neural antibody (Xen-1).* **(A)** *Brain of a control tadpole;* **(B)** *CNS of an XFD mRNA-injected embryo. Abbreviations: n, olfactory organ; t, telencephalon; d, diencephalon; m, midbrain; r, hindbrain; s, spinal cord.*

The second type of control experiment was to test for *xbra* expression in *XFD*-injected stage 11-11.5 gastrulae using whole-mount *in situ* hybridization. As shown in Figure 1C, *xbra* mRNA is expressed in the mesoderm of control gastrulae in a ring around the blastopore. In the oldest embryos, cells on the dorsal side, which are fated to form notochord, extend anteriorly from the ring. These cells continue to express *xbra* following gastrulation when lateral and ventral mesodermal cells cease expression (Isaacs *et al.*, 1995). Considerably less *xbra* mRNA was detectable in *XFD* mRNA-injected embryos. In many embryos, detectable expression was completely eliminated from around parts of the, frequently enlarged, blastopore. Few embryos showed a complete loss of *xbra* expression. One reason for this may be that *xbra* expression has been found to be eliminated selectively from near the site of *XFD* RNA injection (Amaya *et al.*, 1993). Maximal effects on mesoderm formation and *xbra* expression should therefore be expected only following microinjection into the marginal zone. However, the aim of this study was to look at the role of FGF in neural induction and patterning, so the *XFD* mRNA was injected into the animal cap, presumptive CNS and epidermis. The animal cap mesoderm induction assay (Fig. 1B) showed that the animal cap was indeed strongly inhibited from responding to FGF in XFD embryos. A second reason for the differential loss of *xbra* expression in XFD embryos is a localized sensitivity to XFD-mediated inhibition of FGF signaling within the marginal zone. Northrop and Kimelman (1994) found that *xbra* expression was preferentially eliminated from the dorsal side of the embryo. Lateral expression was the most resistant to inhibition. Our results are in agreement with these findings.

CNS development in embryos deficient in FGF signaling

Using histology (not shown) and immunostaining with Xen-1 (Fig. 2), an antibody which recognizes the CNS (Ruiz i Altaba, 1992), we investigated brain and spinal cord development in XFD

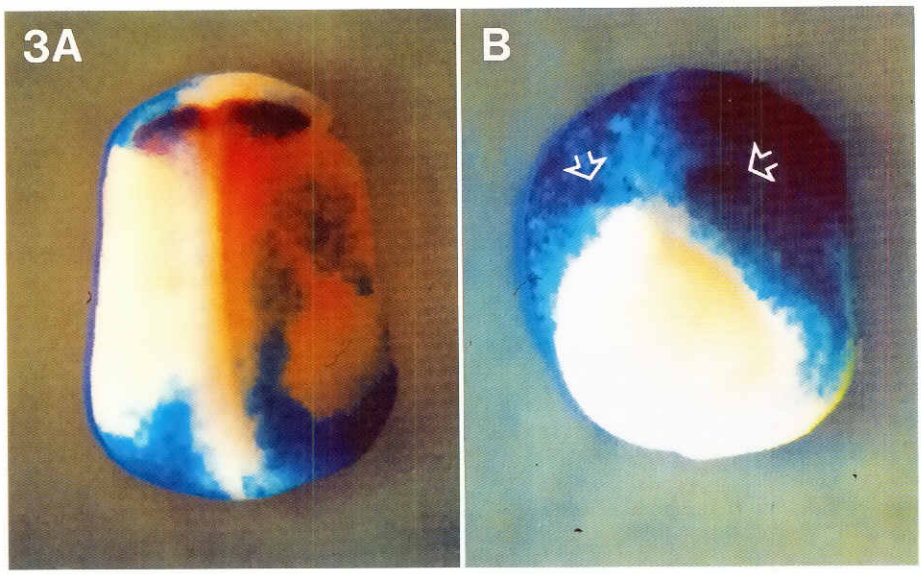


Fig. 3. Localization of injected RNA by lineage labeling. *ß-galactosidase expression (light blue) and whole-mount in situ hybridization with antisense Hoxb-3 probe (purple) in embryos coinjected with ß-galactosidase and HAVNOT RNAs* **(A)** *or ß-galactosidase and XFD RNAs* **(B)**. *Embryos are shown with anterior to the top. Injected RNA can be seen along the CNS and in epidermis in both* **A** *and* **B**. *The arrows in* **B** *indicate domains of double staining where there is Hoxb-3 expression in cells containing lineage label. Double staining is also visible in* **A**.

tadpoles (stage 26). Forebrain structure appeared relatively normal in all the XFD embryos examined, although in a number of embryos one of the eyes showed reduced development. In the most strongly affected embryos, the midbrain became expanded laterally, and then splitting of the CNS occurred in the midbrain or anterior hindbrain region and in some cases there was further splitting of the spinal cord (not shown). The spinal cord was always strongly reduced in length and volume in affected embryos.

Expression of antero-posterior markers in XFD embryos

As a second approach to investigating the development of the CNS in XFD embryos, *in situ* hybridization was used to analyze the expression patterns of markers for different antero-posterior (a-p) levels of the CNS in the most severely affected embryos. In some cases *XFD* or control *HAVNOT* RNA was co-injected with a lineage label to show whether a-p marker expression occurred in cells which had received injected RNA. The distribution of the lineage label was variable, but there was expression in the CNS in most embryos and this frequently coincided with a-p marker expression (Fig. 3).

otx-2 was used as a marker of anterior development. In control embryos, which had just completed neural tube closure (st. 20), *otx-2* was expressed in the forebrain and midbrain regions (Fig. 4A and Blitz and Cho, 1995; Pannese *et al.*, 1995). Expression was also seen, in an expanded, but approximately normal pattern, at the anterior end of XFD embryos (Fig. 4B). This *otx-2* expression extended into the region bordering the large blastopore, supporting the evidence from histology, that the midbrain is expanded laterally. Lateral expansion and splitting of the midbrain region in strongly affected embryos was confirmed using an *en-2* probe. The *en-2* gene is normally expressed in a stripe at the midbrain/hindbrain border (Fig. 4C and Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou *et al.*, 1991; Eizema *et al.*, 1994). Clear stripes were also visible in *XFD* embryos, bordering the enlarged blastopore (Fig. 4D). Some embryos only showed *en-2* expression on one side of the embryo, the reason for which is unclear. The *Krox-20* gene, which is normally expressed in two stripes in rhombomeres 3 and 5 in the hindbrain (Bradley *et al.*, 1992) was also expressed in 2 stripes in the CNS tissue of XFD embryos on each side of the enlarged blastopore (not shown). As with *en-2*, one or both *Krox-20* stripes were occasionally lost on one side of the embryo.

Hox genes are expressed in sequential zones along the antero-posterior (a-p) axis and can be used as markers of a-p level in the hindbrain and spinal cord (Godsave *et al.*, 1994 and reviewed in Krumlauf, 1994). Three *Hoxb* genes were used here to investigate patterning of the posterior CNS in XFD embryos.

Hoxb-3 is normally expressed in the posterior part of the hindbrain and the associated neural crest with the strongest expression occurring in a stripe across the hindbrain in rhombomeres 5 and 6, just posterior to the otic vesicles in *Xenopus* (Fig. 4E and Godsave *et al.*, 1994). *Hoxb-3* expression still occurs in most XFD embryos in a stripe on both sides of the blastopore (Fig. 4F).

In normal embryos, the strongest *Hoxb-4* expression occurs in the trunk somites. There is also *Hoxb-4* expression in the posterior hindbrain in rhombomeres 7 and 8 and in the branchial arch tissue derived from the neural crest from this region. Expression in the CNS is relatively weak (Fig. 4G and Godsave *et al.*, 1994). In XFD embryos with a strong phenotype, *Hoxb-4* expression was also weak, but was still detectable in the hindbrain region (Fig. 4H). By contrast, *Hoxb-4* expression was always highly reduced or undetectable in the somites, although in some embryos, a few *Hoxb-4*-expressing somites were detectable. Since FGF signaling is known to be required for the formation of posterior mesoderm, and somite formation is known to be disrupted in XFD embryos (Amaya *et al.*, 1991, 1993), this result was not unexpected.

Hoxb-9, the most 5' gene in the *Hoxb* cluster is normally expressed throughout the spinal cord (Fig. 4I and Dekker *et al.*, 1992a,b; Doniach *et al*, 1992; Godsave *et al.*, 1994). FGF treatment is able to induce *Hoxb-9*-expressing mesoderm from blastula stage animal cap tissue (Cho and De Robertis, 1990) and to induce neural tissue expressing *Hoxb-9* from gastrula stage ectoderm (Lamb and Harland, 1995). XFD embryos also express *Hoxb-9* in the posterior half of the split CNS in the future spinal cord, but this expression is significantly weaker than in control

embryos (Fig. 4J). This suggests that FGF signaling is required for maximal expression of posterior genes, including *Hoxb-9*.

Discussion

Several lines of evidence suggest that FGF signaling is required for normal development of the posterior part of the body axis in *Xenopus* embryos (reviewed by Doniach, 1995). This requirement was examined further here using embryos microinjected with dominant negative FGF receptor mRNA which inhibits FGF from signaling via cell surface receptors. Neural induction still occurred in the injected embryos. Posterior development of the CNS and expression of a spinal cord marker were strongly reduced, but a-p patterning of the CNS also still occurred.

Dominant negative FGF receptors have previously been shown to inhibit mesoderm formation in the marginal zone of the blastula (Amaya *et al.*, 1991, 1993; Isaacs *et al.*, 1994; Northrop and Kimelman, 1994). Since neural induction is mediated by signals from the organizer mesoderm, the signal source could be lost in XFD embryos. Several points are relevant here. The first point is that induction of the head mesoderm may be FGF-independent (Amaya *et al.*, 1991, 1993; Cornell and Kimelman, 1994). In urodeles, this tissue has been found to be the most potent source of neural inducing factors (Nieuwkoop *et al.*, 1952; Sala, 1955), and it is likely that this is responsible for at least some of the neural induction occurring in *XFD* mRNA-injected embryos. The second point is that the *XFD* mRNA injections were targeted to the animal cap ectoderm rather than the marginal zone mesoderm, in order to be maximally effective in the presumptive CNS region. The expression of the early mesodermal marker, *xbra*, in the marginal zone was strongly inhibited (Fig. 1C). However, this inhibition was rarely complete and axial mesoderm could be observed in histological sections of XFD embryos (not shown) usually asymmetrically on the two sides of the split blastopore. Neural tissue may also have been induced by this mesoderm.

It has recently been shown that bFGF is a candidate for mediating neural induction signals as it is able to induce neural differentiation in ectodermal cells from the animal caps of early gastrulae (Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995). FGF has so far also only been found to cause neural induction when the responding tissue is disaggregated or placed in a medium which prevents rapid healing. Animal cap ectoderm is now known to express inhibitors of neural induction, BMP-4 and BMP-7, members of the TGF-beta superfamily (Fainsod *et al.*, 1994; Hawley *et al.*, 1995; Schmidt *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Xu *et al.*, 1995). Previous reports

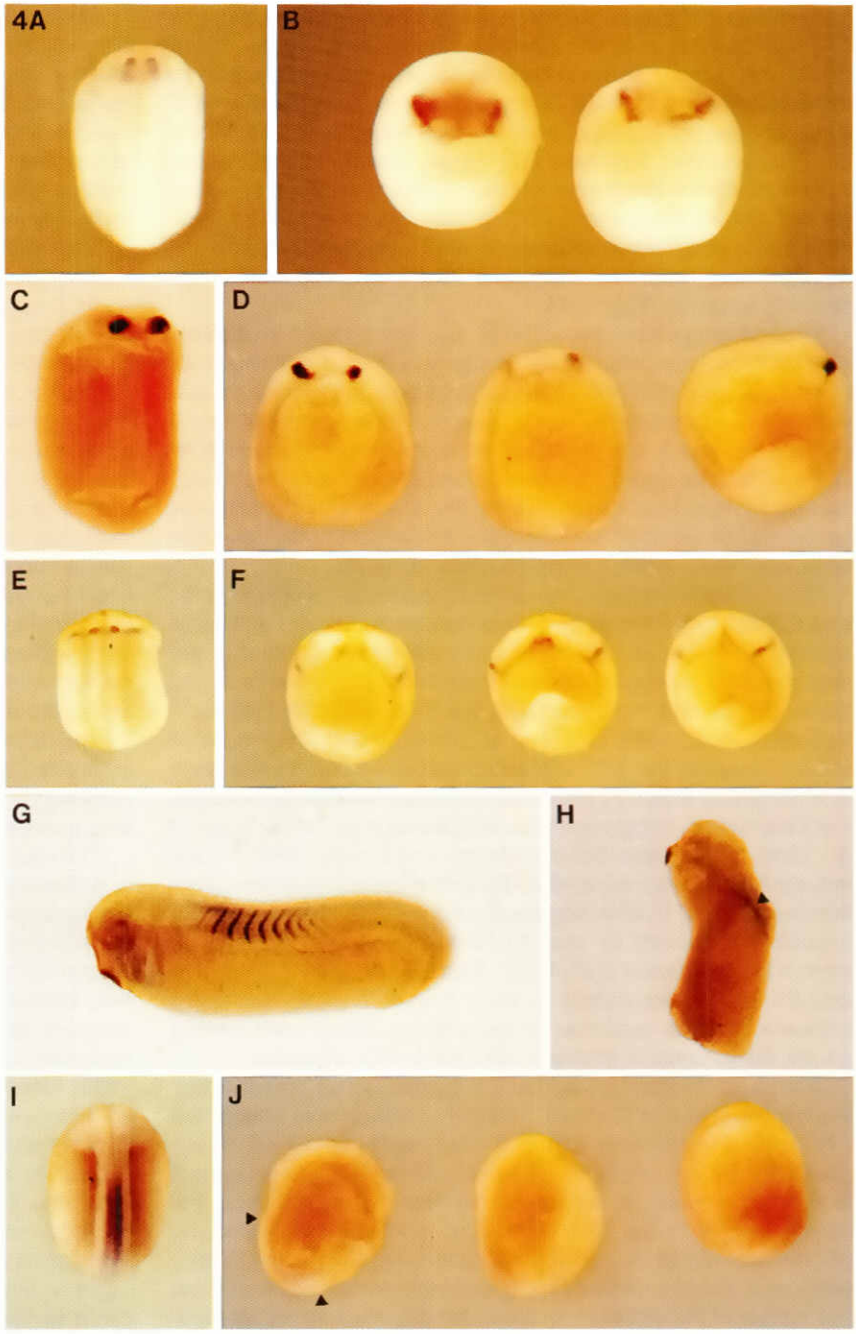


Fig. 4. Expression of antero-posterior markers in *XFD* mRNA injected embryos. *Whole-mount in situ hybridization analysis of:* **(A,C,E,I)** *stage 20 control embryos;* **(B,D,F,J)** *severely affected XFD embryos cultured until control embryos reached stage 20. Each embryo is positioned with the open blastopore in the centre, bordered by the posteriorly split CNS.* **(G)** *Stage 28 control embryo;* **(H)** *XFD embryo, stage 28.* **(A,B)** *otx-2 expression in the fore-brain and mid-brain regions;* **(C)** *en-2 expression at the midbrain/hindbrain border;* **(D)** *embryos hybridized with two probes, en-2 and the spinal cord marker, Hoxb-9. Strong stripes of en-2 expression are seen near the anterior end of the embryos (top) and very weak Hoxb-9 expression is visible in the posterior half of the CNS;* **(E,F)** *Hoxb-3 expression in the hindbrain region;* **(G)** *Hoxb-4 expression in the posterior hindbrain region and somites of a control embryo;* **(H)** *weak Hoxb-4 expression is visible in the CNS of an XFD embryo (arrowhead), and somitic expression is reduced to very low levels;* **(I,J)** *Hoxb-9 expression in the spinal cord region. In the XFD embryos, it is much weaker than in controls. Arrowheads point to the borders of Hoxb-9 expression on one side of the CNS in an XFD embryo.* **(A-F,H-J)** *Anterior is to the top;* **(G)** *anterior is to the left.*

have shown that dissociated ectodermal cells have a tendency to develop autonomously into neurons (Godsave and Slack, 1989; Grunz and Tacke, 1989) and it is likely that this is due to a reduction of the effectiveness of inhibitory proteins in open explants and in dissaggregated cells (Wilson and Hemmati-Brivanlou, 1995). These data suggest that FGF may only cause neural induction under culture conditions which favor neuralization and that FGF signaling via cell surface receptors is not the major mechanism for neural induction *in vivo*. This conclusion is further supported by the results reported here, where neural induction still occurred in XFD-expressing embryos (Figs. 2-4).

The inducing properties of bFGF are also different from those expected for the neural activation signal predicted by the activation-transformation model of neural induction and patterning proposed by Nieuwkoop *et al.* (1952), i.e. an inducer of anterior neural tissue produced in the organizer. In contrast, bFGF appears to induce posterior neural tissue preferentially, although the neural tissues formed also depend upon the timing of induction, the concentration of bFGF and the culture conditions (Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995).

Three other factors with neural inducing activity, noggin, chordin and follistatin, are much better candidates for neural activation signals. They are all secreted factors which are present in the organizer region of gastrulae, and they induce only anterior neural tissue (Smith and Harland, 1992; Lamb *et al*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994). It has recently been shown, however, that FGF signaling may be required for ectoderm to form neural tissue in response to at least two of these neural inducing factors, noggin and chordin (Launay *et al.*, 1996; Sasai *et al.*, 1996; but see also Schulte-Merker and Smith, 1995). Neural induction can be strongly inhibited in animal caps from embryos co-injected with *XFD* and *noggin* (Launay *et al.*, 1996; and our unpublished results) or *chordin* RNA, and endoderm may be formed instead (Sasai *et al.*, 1996). If neural induction by noggin or chordin is FGF-dependent *in vivo*, then it is likely that other inducers are also available in the embryo, since considerable neural tissue forms in embryos deficient in FGF signaling.

It has also been proposed that FGFs could act as 'transforming' signals (reviewed in Doniach, 1995), i.e. signals that pattern the CNS by posteriorizing newly formed anterior neural tissue (Nieuwkoop *et al.*, 1952). In XFD embryos, posterior development is strongly reduced. However, antero-posterior patterning is still clearly evident in the CNS. Even the most abnormal embryos possess cement glands and eyes and they express, in an ordered sequence: *otx-2*, a marker of fore and midbrain development; *en-2*, a marker of the midbrain/hindbrain border; *Krox-20*, *Hoxb-3* and *Hoxb-4*, genes expressed in the hindbrain; and then a spinal cord-specific gene, *Hoxb-9* (Fig. 4). Furthermore, the morphology of XFD embryos also shows that tail formation occurs. These results show that all major subdivisions of the CNS can develop in XFD embryos. However, *Hoxb-9* expression in the spinal cord is weaker than that in normal embryos, suggesting an effect of the XFD protein on spinal cord development and on *Hox* gene expression in the spinal cord. A reduced thickness of the spinal cord could contribute to the obviously reduced intensity of *Hoxb-9* staining in the XFD embryos, but there are probably also lower levels of *Hoxb-9* transcripts in *XFD* embryonic spinal cord. This result is consistent with earlier findings that FGF is important for posterior development (reviewed in Doniach, 1995).

At stage 20, shortly after neural tube closure, we did not find a dramatic shortening of the *Hoxb-9* expression zone. The major effect at stage 20 was on the expression level of *Hoxb-9* in the spinal cord. The subsequent shortening of the spinal cord suggests that there may be a requirement for FGF in growth and morphogenesis at later developmental stages. It is highly likely that FGFs have multiple roles during embryogenesis including a role in patterning the newly formed CNS.

The transformation signal in the 'activation-transformation' model should act in a gradient, with the highest concentration at the posterior end of the embryo inducing the most posterior structures (Nieuwkoop *et al.*, 1952). The results obtained here do not support the idea that a gradient of FGF is the sole factor involved in the generation of posterior neural structures, although FGF-mediated posteriorization of neural tissue *in vitro* has been observed to show a degree of FGF concentration dependence (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995). If FGF was acting in a concentration gradient, an inhibition of FGF signaling would be expected to result in some embryos failing to develop spinal cord, with a consequent enlargement of more anterior structures. All embryos that were examined possessed spinal cord and no increases in hindbrain development were evident, although the *otx-2* expression domain, marking the forebrain and midbrain, did appear enlarged. It is a possibility that FGFs are also able to act in neural induction and patterning via different types of receptors or directly in the nucleus. Nuclear FGF has been detected in *Xenopus* embryos (Shiurba *et al.*, 1991) as well as in other systems. However, it seems highly likely that additional signaling systems also operate.

The idea that FGFs may interact with other signaling pathways in patterning the CNS is supported by the observation that the expression of several 5' *Hox* genes is delayed in XFD embryos (Isaacs *et al.*, 1994 and our unpublished data). Retinoids have also been proposed as possible transformation signals in neural induction, involved in development of the posterior CNS. Furthermore, retinoids are probably directly involved in the activation of some *Hox* genes (Langston and Gudas, 1992; Pöpperl and Featherstone, 1993; Marshall *et al.*, 1994; Ogura and Evans, 1995). The 5', posteriorly expressed, *Hox* genes are the least sensitive to retinoids (Conlon and Rossant, 1992; Dekker *et al.*, 1992a,b). However, *Hoxb-9* is sensitive to induction by FGF (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995) and to inhibition using dominant negative FGF receptors (Fig. 4I,J). This suggests that the two signaling pathways activated by FGFs and retinoids may act together in patterning the posterior part of the embryo. We are currently investigating this idea.

During revision of this manuscript, work has been published showing the effect of transgenic expression of *XFD* in embryos after the mid blastula transition (Kroll and Amaya, 1996). These embryos show only transient expression of early mesodermal markers, but develop nervous systems which are well-patterned along the a-p axis. The data from these embryos are in good agreement with the results presented above.

Materials and Methods

Embryos and microinjection

Wild type *Xenopus* embryos were obtained by *in vitro* fertilization and dejellied as described previously (Godsave *et al.*, 1994). Two cell stage embryos were transferred into 25% MMR medium (Newport and Kirschner,

1982) containing 3% Ficoll and 25 μg/ml gentamycin for micro-injection. Capped RNA was synthesized as described previously (Amaya *et al.*, 1991) from three FGF receptor constructs: *XFD*/Xss dominant negative FGF receptor; a non-functional FGF receptor construct, *HAVNOT*, which has a three amino-acid deletion in the extracellular domain; and a wild type FGF receptor construct, *XFRΔ3′/Xss* (Amaya *et al.*, 1991, 1993). The micro-injection needle was always inserted into the animal portion of the embryo and the two blastomeres were each injected with 0.75 ng RNA dissolved in water. For lineage labeling of the injected blastomeres, an equal amount of *β-galactosidase* RNA was co-injected with the *XFD* or *HAVNOT* RNA.

Embryos were transferred into 20% MMR containing 25 μg/ml gentamycin during blastula stages and cultured until needed. Embryo staging was performed according to Nieuwkoop and Faber (1967).

Mesoderm induction assays

Vitelline membranes were removed from late stage 8 embryos using sharpened forceps. Animal cap explants were dissected in Flickinger solution (Flickinger, 1949) using electrolytically sharpened tungsten needles. Explants were cultured in 96 well plates pre-coated with 1% agarose, in Flickinger solution containing 1 mg/ml BSA, 25 μg/ml gentamycin and recombinant *Xenopus* bFGF (Kimelman *et al.*, 1988) purified by heparin Sepharose affinity chromatography.

Immunofluorescence

Stage 46 embryos were fixed for 2 h in MEMFA (Harland, 1991), rinsed with methanol and stored at -20°C until use. Bleaching was performed in 8% H_2O_2 in methanol overnight until only the eyes remained pigmented. Embryos were rehydrated in 2 ml glass vials via a methanol series and then washed 4x15 min in PBS containing 0.2% Tween 20 (PBSTw). Blocking was then carried out in 5% normal goat serum in PBSTw on a nutator for 1 h. The solution was changed for 0.15 ml fresh blocking agent containing a 1:5 dilution of Xen-1, a mouse monoclonal antibody to *Xenopus* neural tissue (Ruiz i Altaba, 1992) and the tubes were rocked vertically on a nutator overnight at 4°C. Unbound antibody was removed by 5 washes at room temperature with PBSTw for at least 30 min each. The embryos were then incubated overnight at 4°C with the second antibody, donkey anti-mouse conjugated to cy5, diluted 1:100 in PBSTw, and washed with PBSTw as before. The specimens were cleared in Murray's (2:1 benzyl alcohol:benzyl benzoate) and viewed using a Biorad MRC 600 confocal laser scanning microscope (CLSM). Composite pictures of optical sections through the anterior part of the central nervous system were obtained using the COMOS program.

In situ hybridization

Digoxigenin-labeled probes were generated by *in vitro* transcription of linearized templates, incorporating digoxigenin-11-UTP according to the manufacturers instructions (Boehringer-Mannheim). Anti-sense *xbra* probe was prepared using the template DNA made by Smith *et al.* (1991). *otx-2* probe was as described by Blitz and Cho (1995). An *en-2* probe was generated as described by Eizema *et al.* (1994). The *Hoxb-9* probe was as described by Sharpe and Gurdon (1990) for use in RNAse protection assays. The anti-sense *Hoxb-9* RNA was synthesized using Sp6 RNA polymerase. An anti-sense *Hoxb-3* probe was generated from a template containing a large 3' portion of the *Hoxb-3* coding sequence (Dekker *et al.*, 1992a). A sense *Hoxb-3* probe was used as control for non-specific *in situ* hybridization staining (results not shown). For the *Hoxb-4* probe, a 1.1kb ECoRI/BAMHI fragment of the Xhox1A clone, c1A (Harvey and Melton, 1988) containing the complete coding sequence of the *Hoxb-4* gene, was used as template. The *in situ* hybridization procedure was as described by Harland (1991) with slight modifications as follows: prehybridization steps were performed in Netwells (Costar) placed in 12 well plastic plates, then embryos were transferred into 2.5 ml glass tubes for hybridization at 62°C and subsequent washing steps; levamisole was omitted from the staining solution and the two washes with pH 9.5 Tris buffer solution (TMNT) immediately prior to staining were extended to 45 min each. Following staining and fixation in MEMFA, embryos were bleached by treatment with 0.1 M $K_2Cr_2O_7$ in 5% acetic acid for 30 min, followed by 3x10 min washes in PBSTw (PBS containing 0.1% Tween 20), and then bleaching in 4% H_2O_2 in PBSTw under a light source for 1-2 h.

Embryos in which lineage label was co-injected with the *XFD* or *HAVNOT* RNA were fixed for 40 min at room temperature in 4% paraformaldehyde in 0.1 M potassium phosphate buffer pH 7.8 containing 4% sucrose and 0.15 M $CaCl_2$. They were then washed 2x15 min in PBS and stained for 40 min. at 37°C in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-Gal, 0.1% Triton-X100 in PBS. Stained embryos were then refixed for 1 h in MEMFA and stored for several days in methanol at -20°C before being used for *in situ* hybridization.

Acknowledgments

We are grateful to Dr. E. Amaya for the FGF receptor clones, to Dr A. Ruiz i Altaba for the Xen-1 antibody, to Dr. O. Destree for the xbra and en-2 clones, to Dr. K. Cho and Dr. C. Sharpe for the otx-2 and Hoxb-9 clones respectively and to H. Isaacs for the FGF expression clone. We would also like to thank members of the lab for assistance with maintaining clones, for help with the CLSM and for useful comments about this work. F. Vervoordeldonk and J. Heijnen assisted with the photographic work. The work was supported by the Netherlands Science Foundation (N.W.O.) via Medical Research Grant 900-582-082 and SLW grant 805-33-021 to SG and by the EEC BIOTECH program, Contract PL 920060.

References

AMAYA, E., MUSCI, T.J. and KIRSCHNER, M.W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell 66:* 257-270.

AMAYA, E., STEIN, P.A., MUSCI, T.J. and KIRSCHNER, M.W. (1993). FGF signalling in the early specification of mesoderm in *Xenopus*. *Development 118:* 477-487.

BLITZ, I.L. and CHO, K.W.Y. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development 121:* 993-1004.

BRADLEY, L.C., SNAPE, A., BHATT, S. and WILKINSON, D.G. (1992). The structure and expression of the *Xenopus Krox-20* gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech. Dev. 40:* 73-84.

BYERS, S., AMAYA, E., MUNRO, S. and BLASCHUK, O. (1992). Fibroblast growth factor receptors contain a conserved HAV region common to cadherins and influenza strain A hemagglutinins: a role in protein-protein interactions? *Dev. Biol. 152:* 411-414.

CHO, K.W.Y. and DE ROBERTIS, E.M. (1990). Differential activation of *Xenopus* homeobox genes by mesoderm-inducing growth factors and retinoic acid. *Genes Dev. 4:* 1910-1916.

CONLON, R.A. and ROSSANT, J. (1992). Exogenous retinoic acid rapidly induces anterior ectopic expression of murine Hox-2 genes *in vivo*. *Development 116:* 357-368.

CORNELL, R.A. and KIMELMAN, D. (1994). Activin-mediated mesoderm induction requires FGF. *Development 120:* 453-462.

COX, W.G. and HEMMATI-BRIVANLOU, A. (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development 121:* 4349-4358.

DEKKER, E-J., PANNESE, M., HOUTZAGER, E., BONCINELLI, E. and DURSTON, A. (1992a). Colinearity in the *Xenopus laevis Hox-2* complex. *Mech. Dev. 40:* 3-12.

DEKKER, E-J., PANNESE, M., HOUTZAGER, E., TIMMERMANS, A., BONCINELLI, E. and DURSTON, A. (1992b). *Xenopus Hox-2* genes are expressed sequentially after the onset of gastrulation and are differentially inducible by retinoic acid. *Development (Suppl.):* 195-202.

DONIACH, T. (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell 83:* 1067-1070.

DONIACH, T., PHILLIPS, C.R. and GERHART, J.C. (1992). Planar induction of anteroposterior pattern in the developing central nervous system of *Xenopus laevis*. *Science 257:* 542-545.

EIZEMA, K., KOSTER, J.G., STEGEMAN, B.I., BAARENDS, W.M., LANSER, P.H. and DESTREE, O.H.J. (1994). Comparative analysis of *engrailed-1* and *Wnt-1* expression in the developing central nervous system of *Xenopus laevis*. *Int. J. Dev. Biol. 38:* 623-632.

FAINSOD, A., STEINBEISSER, H. and DE ROBERTIS, E.M. (1994). On the function of BMP-4 in patterning the marginal zone of the *Xenopus* embryo. *EMBO J. 13:* 5015-5025.

FLICKINGER, R.A. (1949). A study of the metabolism of amphibian neural crest cells during their migration and pigmentation *in vitro*. *J. Exp. Zool. 112:* 465-484.

GODSAVE, S.F. and SLACK, J.M.W. (1989). Clonal analysis of mesoderm induction in *Xenopus laevis*. *Dev. Biol. 134:* 486-490.

GODSAVE, S.F., DEKKER, E-J., HOLLING, T., PANNESE, M., BONCINELLI, E. and DURSTON, A. (1994). Expression patterns of *Hoxb* genes in the *Xenopus* embryo suggest roles in anteroposterior specification of the hindbrain and in dorsoventral patterning of the mesoderm. *Dev. Biol. 166:* 465-476.

GODSAVE, S.F., ISAACS, H.V. and SLACK, J.M.W. (1988). Mesoderm-inducing factors: a small class of molecules. *Development 102:* 555-566.

GRUNZ, H. and TACKE, L. (1989). Neural differentiation of *Xenopus-laevis* ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Differ. Dev. 28:* 211-218.

HARLAND, R. (1991). *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biol. 36:* 685-695.

HARVEY, R.P. and MELTON, D.A. (1988). Microinjection of synthetic *Xhox-1A* homeobox mRNA disrupts somite formation in developing *Xenopus* embryos. *Cell 53:* 687-697.

HAWLEY, S.H.B., WUNNENBERG-STAPLETON, K., HASHIMOTO, C., LAURENT, M.N., WATABE, T., BLUMBERG, B.W. and CHO, K.W.Y. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev. 9:* 2923-2935.

HEMMATI-BRIVANLOU, A. and HARLAND, R.M. (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development 106:* 611-617.

HEMMATI-BRIVANLOU, A., DE LA TORRE, J.R., HOLT, C. and HARLAND, R.M. (1991). Cephalic expression and molecular characterization of *Xenopus en-2*. *Development 111:* 715-724.

HEMMATI-BRIVANLOU, A., KELLY, O. G. and MELTON, D.A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell 77:* 283-295.

ISAACS, H.V., POWNALL, M.E. and SLACK, J.M.W. (1994). eFGF regulates *xbra* expression during *Xenopus* gastrulation. *EMBO J. 19:* 4469-4481.

ISAACS, H.V., POWNALL, M.E. and SLACK, J.M.W. (1995). eFGF is expressed in the dorsal midline of *Xenopus laevis*. *Int. J. Dev. Biol. 39:* 575-579.

ISAACS, H. V., TANNAHILL, D. and SLACK, J.M.W. (1992). Expression of a novel FGF in the *Xenopus* embryo – a new candidate inducing factor for mesoderm formation and anteroposterior specification, *Development 114:* 711-720.

KENGAKU, M. and OKAMOTO, H. (1993). Basic fibroblast growth factor induces differentiation of neural tube and neural crest lineages of cultured ectoderm cells from *Xenopus* gastrula. *Development 119:* 1067-1078.

KENGAKU, M. and OKAMOTO, H. (1995). bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in *Xenopus*. *Development 121:* 3121-3130.

KESSLER, D.S. and MELTON, D.A. (1994). Vertebrate embryonic induction: mesodermal and neural patterning. *Science 266:* 596-604.

KIMELMAN, D., ABRAHAM, J.A., HAAPARANTA, T., PALISI, T.M. and KIRSCHNER, M.W. (1988). The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. *Science 242:* 1053-1056.

KROLL, K.L. and AMAYA, E. (1996). Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development 122:* 3173-3183.

KRUMLAUF, R. (1994). *Hox* genes in vertebrate development. *Cell 78:* 191-201.

LAMB, T.M. and HARLAND, R.M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development 121:* 3627-3636.

LAMB, T.M., KNECHT, A.K., SMITH, W.C., STACHEL, S.E., ECONOMIDES, A.N., STAHL, N., YANCOPOULOS, G.D. and HARLAND, R.M. (1993). Neural induction by the secreted polypeptide noggin. *Science 262:* 713-718.

LANGSTON, A.W. and GUDAS, L.J. (1992). Identification of a retinoic acid responsive enhancer 3' of the murine homeobox gene Hox-1.6. *Mech. Dev. 38:* 217-228

LAUNAY, C., FROMENTOUX, V., SHI, D-L. and BOUCAUT, J-C. (1996). A truncated receptor blocks neural induction by endogenous *Xenopus* inducers. *Development 122:* 869-880.

MARSHALL, H., STUDER, M., POPPERL, H., APARICIO, S., KUROIWA, A., BRENNER, S. and KRUMLAUF, R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature 370:* 567-571.

NEWPORT, J. and KIRSCHNER, M.W. (1982). A major developmental transition in early *Xenopus* embryos. I. Characterization and timing of cellular changes at the midblastula stage. *Cell 30:* 675-686.

NIEUWKOOP, P.D. and FABER, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. North Holland, Amsterdam.

NIEUWKOOP, P.D., BOTERENBROOD, E.C., KREMER, A., BLOEMSMA, F.F.S.N., HOESSELS, E.L.M.J., MEYER, G. and VERHEYEN, F.J. (1952). Activation and organization of the central nervous system in Amphibians. Part I. Induction and activation. Part II. Differentiation and organization. Part III. Synthesis of a new working hypothesis. *J. Exp. Zool. 120:* 1-108.

NORTHROP, J.L. and KIMELMAN, D. (1994). Dorsal-ventral differences in xcad-3 expression in response to FGF-mediated induction in *Xenopus*. *Dev. Biol. 161:* 490-503.

OGURA, T. and EVANS, R.M. (1995). Evidence for two distinct retinoic acid response pathways for *HOXB1* gene regulation. *Proc. Natl. Acad. Sci. USA 92:* 392-396.

PANNESE, M., POLO, C., ANDREAZZOLI, M., VIGNALI, R., KABLAR, B., BARSACCHI, G. and BONCINELLI, E. (1995). The *Xenopus* homologue of *otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development 121:* 707-720.

PICCOLO, S., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell 86:* 589-598.

PÖPPERL, H. and FEATHERSTONE, M.S. (1993). Identification of a retinoic acid response element upstream of the murine *Hox-4.2* gene. *Mol. Cell. Biol. 13:* 257-265.

RUIZ I ALTABA, A. (1992). Planar and vertical signals in the induction and patterning of the *Xenopus* nervous system. *Development 115:* 67-80.

SALA, M. (1955). Distribution of activating and transforming influences in the archenteron roof during the induction of the nervous system in amphibians. *Proc. Kon. Ned. Akad. Wet. Serie C, 58:* 635-647.

SASAI, Y., LU, B., PICCOLO, S. and DE ROBERTIS, E.M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in *Xenopus* animal caps. *EMBO J. 15:* 4547-4555.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L.K. and DE ROBERTIS, E.M. (1994). *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell 79:* 779-790.

SCHMIDT, J.E., SUZUKI, A., UENO, N. and KIMELMAN, D. (1995). Localized BMP-4 mediates dorsal ventral patterning in the early *Xenopus* embryo. *Dev. Biol. 169:* 37-50.

SCHULTE-MERKER, S. and SMITH, J.C. (1995). Mesoderm formation in response to *Brachyury* requires FGF signalling. *Curr. Biol. 5:* 62-67.

SHARPE, C.R. and GURDON, J.B. (1990). The induction of anterior and posterior neural genes in *Xenopus laevis*. *Development 109:* 765-774.

SHIURBA, R.A., JING, N., SAKAKURA, T. and GODSAVE, S.F. (1991). Nuclear translocation of fibroblast growth factor during *Xenopus* mesoderm induction. *Development 113:* 487-493.

SLACK, J.M.W., DARLINGTON, B.G., HEATH, J.K. and GODSAVE, S.F. (1987). Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature 326:* 197-200.

SMITH, J.C., PRICE, B.M.J., GREEN, J.B.A., WEIGEL, D. and HERMANN, B.G. (1991). Expression of a *Xenopus* homolog of *brachyury* (t) is an immediate-early response to mesoderm induction. *Cell 67:* 79-87.

SMITH, W.C. and HARLAND, R.M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell 70:* 829-840.

SPEMANN, H. and MANGOLD, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Arch. Mikrosk. Anat. Entw.Mech. 100:* 599-638.

TANNAHILL, D., ISAACS, H.V., CLOSE, M.J., PETERS, G. and SLACK, J.M.W. (1992). Developmental expression of the *Xenopus int-2* (FGF-3) gene: activation by mesodermal and neural induction. *Development 115:* 695-702.

WILSON, P.A. and HEMMATI-BRIVANLOU, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature 376:* 331-336.

XU, R-H., KIM, J., TAIRA, M., ZHAN, S., SREDNI, H. and KUNG, H. (1995). A dominant negative bone morphogenetic protein 4 receptor causes neuralization in *Xenopus* ectoderm. *Biochem. Biophys. Res. Commun. 212:* 212-219.

ZIMMERMAN, L.B., DE JESUS-ESCOBAR, J.M. and HARLAND, R.M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell 86:* 599-606.

Received: September 1996
Accepted for publication: October 1996