Development 121, 3121-3130 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995



# bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in *Xenopus*

**Mineko Kengaku† and Harumasa Okamoto***

Department of Neurobiology, Institute for Brain Research, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

*Author for correspondence at present address: Laboratory of Cellular Biochemistry, Department of Biomolecular Engineering, National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology MITI 1-1, Higashi, Tsukuba, Ibaraki 305, Japan
†Present address: Department of Genetics, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115, USA

## SUMMARY

**Vertebrate neural development is initiated during gastrulation by the inductive action of the dorsal mesoderm (Spemann's organizer in amphibians) on neighbouring ectoderm, which eventually gives rise to the central nervous system from forebrain to spinal cord. Here we present evidence that bFGF can mimic the organizer action by inducing *Xenopus* ectoderm cells in culture to express four position-specific neural markers (*XeNK-2, En-2, XlHbox1* and *XlHbox6*) along the anteroposterior axis. bFGF also induced the expression of a general neural marker *NCAM* but not the expression of immediate-early mesoderm markers (*goosecoid, noggin, Xbra* and *Xwnt-8*), suggesting that bFGF directly neuralized ectoderm cells without forming mesodermal cells. The bFGF dose required to induce the position-specific markers was correlated with the anteroposterior location of their expression in vivo, with lower doses eliciting more anterior markers and higher doses more posterior markers. These data indicate that bFGF or its homologue is a promising candidate for a neural morphogen for anteroposterior patterning in *Xenopus*. Further, we showed that the ability of ectoderm cells to express the anterior markers in response to bFGF was lost by mid-gastrula, before the organizer mesoderm completely underlies the anterior dorsal ectoderm. Thus, an endogenous FGF-like molecule released from the involuting organizer may initiate the formation of the anteroposterior axis of the central nervous system during the early stages of gastrulation by forming a concentration gradient within the plane of dorsal ectoderm.**

Key words: bFGF, anteroposterior axis, neural induction, morphogen, *Xenopus*

## INTRODUCTION

The development of the well-patterned vertebrate nervous system begins with the inductive differentiation of the neural plate from the dorsal side of flat ectoderm at the end of gastrulation. Transplantation experiments performed by Spemann and Mangold first demonstrated that the dorsal mesoderm is responsible for the induction of neural plate and the subsequent patterning of the nervous system (Spemann and Mangold, 1924). They showed that transplantation of the dorsal lip mesoderm of early gastrula (organizer) to the ventral side of the host embryo induced ventral ectoderm to form a secondary nervous system that displayed large scale anteroposterior (A-P) organization. Based on the detailed experiments that followed, it has long been believed that the differentiation of cells in the ectoderm into well-organized neural tissues is brought about by a two-step induction by Spemann's organizer at the appropriate developmental time (Saxen, 1989; Sive et al., 1989; Nieuwkoop and Albers, 1990; Slack and Tannahill, 1992). The initial step is a general induction of neural development that alone causes the anterior differentiation of the dorsal ectoderm, and the second step involves the further posteriorization of the induced tissue that establishes the A-P axis in the nervous system. However, in spite of intensive research on the molecular mechanism underlying the neural induction, the nature of the specific signal(s) that emanate from Spemann's organizer has not fully been understand for nearly 70 years since the discovery of the induction phenomenon (Spemann and Mangold, 1924; Warner, 1985).

Very recently, two endogenous molecules isolated from *Xenopus* embryos have been put forward as potential candidates for the neural inducers. One is the secreted protein encoded by the *noggin* gene, which is expressed in the organizer region and has neuralizing activity on ectoderm at high concentrations (Lamb et al., 1993; Smith and Harland, 1992). The other substance is follistatin, a protein that specifically inhibits the action of activin, a highly potent mesoderm inducer in *Xenopus* embryo (Hemmati-Brivanlou et al., 1994). Noggin and follistatin both separately are capable of mimicking the first-step of organizer activity that gives rise to anterior neural tissue. However, the molecular identity and mode of action of the presumptive second-step morphogen that causes regional specification of neural tissue are largely unknown to date.

We have previously demonstrated that basic fibroblast growth factor (bFGF) is another promising candidate for the neural inducer released from the organizer (Kengaku and Okamoto, 1993). Using monoclonal antibodies against various *Xenopus* embryonic tissues, we showed that physiological concentrations of bFGF changed the developmental fate of the ectoderm from epidermis to central nervous system (CNS) neurons when applied to a culture of dissociated animal cap cells from *Xenopus* early gastrula. An obvious question is whether bFGF acts solely as a first-step inducer like noggin and follistatin, or whether it can also contribute to the anteroposterior patterning of the CNS from forebrain to spinal cord, a characteristic feature of the second-step activity of Spemann's organizer. To explore this issue, we cultured animal cap ectoderm cells in the presence of various concentrations of bFGF and examined the expression of position-specific neural markers of the anteroposterior axis. Here we present evidence that bFGF can also drive the second-step in concentration-dependent manner, suggesting that it is identical to or at least can mimic the endogenous neural morphogen(s) released from Spemann's organizer. A preliminary account of this study has been presented (Kengaku and Okamoto, 1994).

## MATERIALS AND METHODS

### Microculture of *Xenopus* embryonic cells

Dissociation and culture of ectoderm cells from gastrula embryos of *Xenopus laevis* were performed as previously described (Mitani and Okamoto, 1989, 1991). Briefly, animal cap tissue was dissected from dejellied embryos in modified Barth solution (MBS; Gurdon, 1977) and dissociated by incubating in $Ca^{2+}$-, $Mg^{2+}$-deficient MBS containing 1% BSA at room temperature. The dispersed cells were suspended in standard MBS containing 1% BSA and inoculated into plastic culture wells of Terasaki plates (Nunc) at 250 cells/well. After completion of reaggregation by brief centrifugation, cells were treated with recombinant bovine bFGF (Progen Biotechnik, Heidelberg) and incubated in the presence of bFGF at 22.5°C in humidified air until control embryos reached tailbud stage (stage 25; Nieuwkoop and Faber, 1967).

### Quantitative RT-PCR assay

RNA was extracted from 20 cultures and subjected to reverse transcription with oligo$(dT)_{12\text{-}18}$ as a primer. For quantitative analysis, PCR was performed as described (Kinoshita et al., 1992) with slight modifications. In brief, one-twentieth of the reverse-transcribed mixture was used as template DNA, which was amplified in a reaction volume of 50 μl containing 10 μCi of [α-$^{32}$P]dCTP (ICN Biomedicals Inc., Costa Mesa, CA) and 5 units of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification was initiated with the primers for the marker sequence of interest. The primers for the internal control, the EF1α sequence, were added after the first 6 cycles to avoid the possible interference of large amounts of its PCR product with the amplification efficiency of the sequence of interest. After the completion of the amplification PCR products were then separated on a 4% polyacrylamide gel and radioactivity of each of the PCR products were estimated using a laser image analyzer (Fujix BAS 2000, Fuji Film). The primers used are shown in Table 1.

## RESULTS

### Analysis of bFGF-induced expression of position-specific neural markers in ectoderm cells by quantitative PCR assay

We have previously shown that bFGF could mimic the action of Spemann's organizer to induce CNS neurons from gastrula ectoderm cells in culture by utilizing a monoclonal antibody N1 as a general marker for CNS neurons. Since it has been hypothesized that anterior and posterior neural tissues are independently induced by different inducers from the organizer (Saxen, 1989; Sive et al., 1989; Nieuwkoop and Albers, 1990; Slack and Tannahill, 1992), it is important to determine what type of neural tissue is induced by bFGF. To this end, we assayed mRNA levels of anteroposterior markers using RT-PCR as described in Fig. 1A. For quantitative analysis, PCR products were estimated within the exponential phase of amplification and were standardized against a co-amplified internal control, elongation factor 1α (EF1α; Krieg et al., 1989). As shown in Fig. 1B,C, the amplified products originated from

**Table 1. Oligonucleotide primers used in this study for RT-PCR assay**

| Markers (References) | Sequences | Sizes of products |
|---|---|---|
| *EF1α* (Krieg et al., 1989) | U 5′-GGAAAGTCCACAACAACTGG-3′<br>D 5′-GGAGCATCAATGATAGTGAC-3′ | 226 bp |
| *XeNK-2* (Saha et al., 1993) | U 5′-AGACAACGACAAGGAACTCTC-3′<br>D 5′-TTACCATCCCTTACTAGGACTG-3′ | 301 bp |
| *En-2* (Hemmati-Brivanlou et al., 1991) | U 5′-GACTTTGGAAGAGCCTCCTGC-3′<br>D 5′-ATAACAGGGAAGTGGAACCGC-3′ | 272 bp |
| *XlHbox1* (Oliver et al., 1990) | U 5′-CCCTTAATTCCACAGCCTATG-3′<br>D 5′-ATCTGAATGCTGCCTTTCTGC-3′ | 291 bp |
| *XlHbox6* (Wright et al., 1990) | U 5′-GAGGCCACAGTGTAATGTTGG-3′<br>D 5′-ATTCCGCTCTGCGCAATTCCC-3′ | 274 bp |
| *gsc* (Blumberg et al., 1991) | U 5′-GTGAATGGATCTAGGCTGGG-3′<br>D 5′-TGCAGCTAGGTTAAGCAACTGCAGCT-3′ | 245 bp |
| *noggin* (Smith and Harland, 1992) | U 5′-AGTTGCAGATGTGGCTCT-3′<br>D 5′-AGTCCAAGAGTCTGAGCA-3′ | 262 bp |
| *Xbra* (Smith et al., 1991) | U 5′-ATACTTCCAGCGGTGGTTGTC-3′<br>D 5′-TACCGGGTGGATCATCTTCTC-3′ | 270 bp |
| *Xwnt-8* (Christian et al., 1991) | U 5′-ATTCTTCACTGCCTCAGCATGG-3′<br>D 5′-TGCACTTCGAAGTCCATTGTG-3′ | 196 bp |
| *NCAM* (Kintner and Melton, 1987) | U 5′-CGTTGGAAGTGAACATTGTTCC-3′<br>D 5′-TATAGATGCCAGCATCTTGGC-3′ | 230 bp |
| *epidermal keratin* (Jonas et al., 1989) | U 5′-CTTATCGTACCAGTTACGGATC-3′<br>D 5′-CATCTAGCAAAGGTGGGCTTTGG-3′ | 291 bp |

both the marker transcript and the EF1α transcript increased exponentially until the 30th cycle. We therefore decided to quantify the amount of PCR products after the 26th cycle of amplification.

### Dose-dependent induction by bFGF of the central nervous system along the anteroposterior axis

To determine the types of neural tissue induced by bFGF, we assayed the expression of four position-specific homeobox genes in animal cap ectoderm cells from stage 10 embryos treated with various concentrations of bFGF. In intact embryos, the marker genes are expressed within specific regions along A-P axis in the developing CNS: *XeNK-2* is expressed in the anterior part of the brain (Saha et al., 1993); *En-2* at the midbrain-hindbrain boundary (Hemmati-Brivanlou et al., 1991); *XlHbox1* in the anterior spinal cord (Oliver et al., 1988), and *XlHbox6* throughout the spinal cord (Wright et al., 1990). As shown in Fig. 2, the anterior markers *XeNK-2* and *En-2* were detected at doses as low as 0.05-0.1 ng/ml of bFGF. As the bFGF dose increased, *En-2* peaked at 0.2 ng/ml and then sharply repressed, whereas *XlHbox1* and *XlHbox6* became apparent in this order. The expression of the posteriormost marker *XlHbox6* was detected at 1.0-2.0 ng/ml and peaked at 5.0-12.5 ng/ml, a concentration about 25-fold higher than the dose required for maximal *En-2* expression. *XeNK-2* and *XlHbox1* were repressed at doses above 2.0 ng/ml. Similar sets of experiments were repeated several times and we obtained essentially the same results, except for one case in which *XeNK-2* was detected at a lower concentration (0.05 ng/ml) of bFGF than *En-2* (0.1 ng/ml). From these results, we conclude that bFGF can affect the fate of ectoderm cells in culture in a dose-dependent manner. The fact that the dose-response profiles for four independent neural markers are distinct from each other within the dose range 0.05-12.5 ng/ml

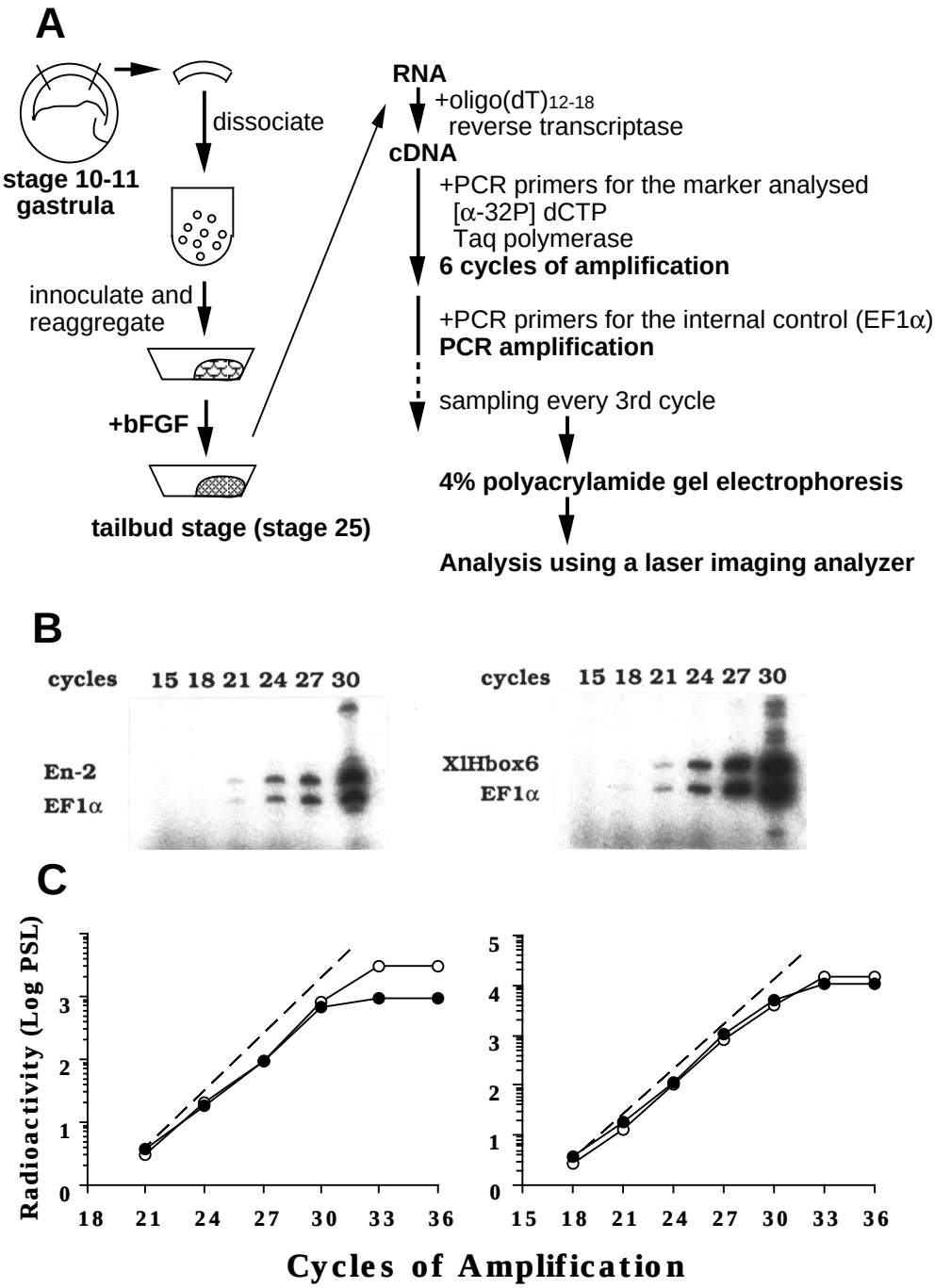


**Fig. 1.** Quantitative analysis of mRNAs for position-specific neural genes in ectoderm cells cultured in the presence of bFGF. (A) Experimental design for the primary culture of cells from *Xenopus* gastrula (left) and quantitative measurements of the expression of neural marker transcripts using RT-PCR (right). (B) Autoradiographs of PCR products for neural markers *En-2* (left) and *XlHbox6* (right) mRNA, each co-amplified with *EF1α* mRNA (internal control). During the amplification step, a 2 μl sample of the reaction mixture was removed at every 3rd cycle. Lane numbers indicate the number of cycles of PCR amplification. For comparison, 1/2 of the PCR products were applied in lane 27 and 30, respectively, and the duration of exposure was reduced to analyse the samples subjected to large numbers of amplifications. The primers and the size of PCR products are indicated in Table 1. (C) Amplification profiles for *En-2* (left, ●), *XlHbox6* (right, ●) and *EF1α* (both, ○). The intensity of photostimulated luminescence (PSL) of each band in (B) was measured with a laser image analyzer and plotted on a semilogarithmic scale against the cycle number. Three independent sets of experiments using different amount of RNA gave essentially the same results. Dashed lines represent the ideal amplification profile. Note that the actual amplification profiles are very close to the lines for the first 30 cycles.

suggests that there may be at least four states of neural cell differentiation within this dose range. The most interesting feature of these profiles is that at lower doses of bFGF the cell state tends to have an anterior characteristics, while at higher doses, the cell becomes to have more posterior characteristics. These results suggest that a concentration gradient of bFGF could contribute to A-P patterning during early neural morphogenesis.

A similar dose-dependent specification has been reported for the induction of mesodermal lineages from blastula animal cap cells by activin (Green et al., 1992). However, it has subsequently been suggested that the graded induction of mesodermal lineages by activin depends on interactions within populations of the induced cells, which initially involve only a few different cell states (Green et al., 1994). These observations raise the possibility that the graded response of gastrula ectoderm cells to bFGF is not directly induced, but rather develops as the induced cells interact with each other during later stages following induction. To test this possibility, we assayed the expression of *XeNK-2*, *En-2* and *XlHbox6* in ectoderm cells cultured in the presence of low (0.25 ng/ml) and high (5.0 ng/ml) concentrations of bFGF every 3rd hour after the start of culture (Fig. 3). When the markers were initially detected, each of them was induced within the same dose ranges observed in cultures assayed at tailbud stage (stage 25; Fig. 2): *XeNK-2* was detected after 12 hours of culture (stage 19.5) at both high and low doses; *En-2* expression was detected after 9 hours of incubation (equivalent to stage 15.5) in cultures treated with low bFGF, but was never detected in those treated with high bFGF; *XlHbox6* was first detected after 6 hours of incubation (stage 13) only in cultures treated with a high dose of bFGF. It is thus likely that bFGF determines the four different cell states at the initial stage of the induction.

### bFGF never induces differentiation of mesodermal lineages from gastrula ectoderm cells

The posterior neural markers *XlHbox1* and *XlHbox6* used here

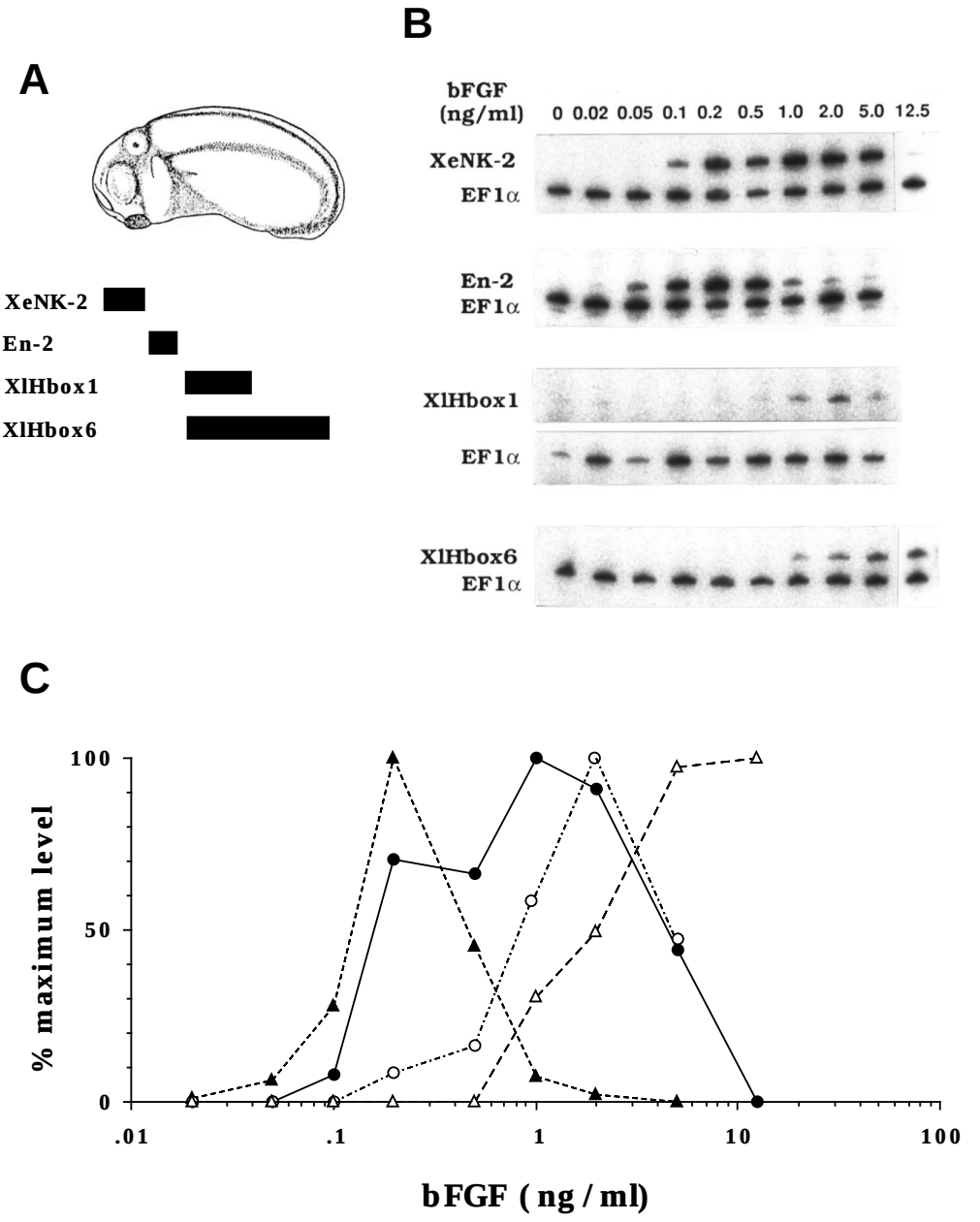


**Fig. 2.** Dose-dependent induction by bFGF of position-specific neural markers in ectoderm cells. The procedure was essentially as described in Fig. 1. (A) Spatial expression patterns of the four neural markers along the A-P axis. (B) Autoradiographs of PCR products of the marker transcripts co-amplified with *EF1α* transcript. Ectoderm cells from stage 10 gastrulae were cultured in the presence of various concentrations of bFGF (0-12.5 ng/ml) and harvested for RNA isolation at the time when control embryos reach stage 25. The entire experiment was performed four times, yielding essentially the same results. (C) A quantitative comparison of the dose-response profiles for transcript levels of four markers: ●, *XeNK-2*; ▲, *En-2*; ○, *XlHbox1*; Δ, *XlHbox6*. The ratio of the intensity of the band for the marker to the intensity of the band for *EF1α* shown in B was calculated from the respective PSL value and plotted against bFGF doses. The percentage of the maximum value of the ratio was presented in each profile. The reverse-transcribed mixture from each RNA sample was divided into four aliquots, which were then individually subjected to PCR amplification for each of four markers. PCR products were analysed after 26 cycles, which was within the exponential phase of the amplification step as shown in Fig. 1C.

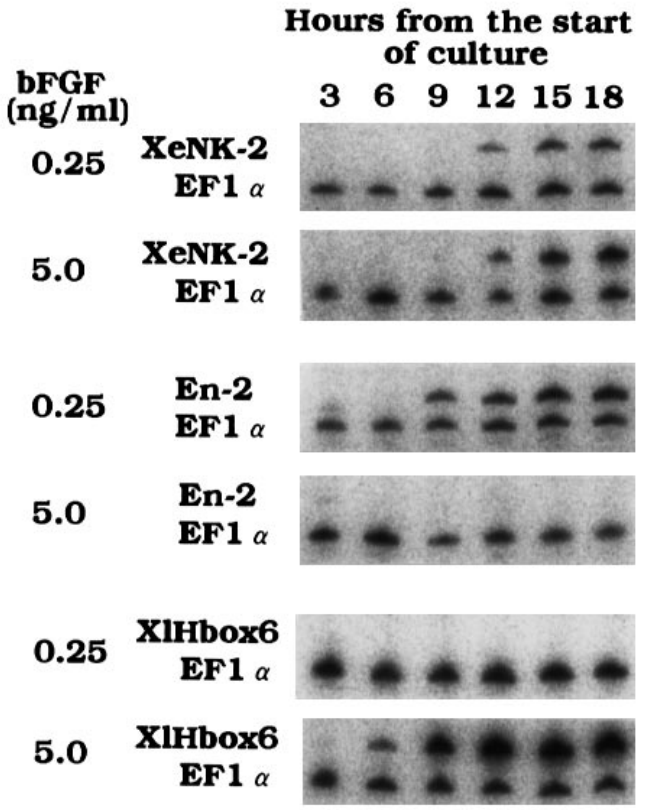


**Fig. 3.** Time course of induction by bFGF of position-specific neural markers. Animal cap ectoderm cells from stage 10 gastrula were cultured in the presence of low (0.25 ng/ml) or high (5.0 ng/ml) concentration of bFGF and harvested every 3rd hour after the start of culture. The expression patterns of *XeNK-2*, *En-2* and *XlHbox6* were analyzed by RT-PCR as in Fig. 2.

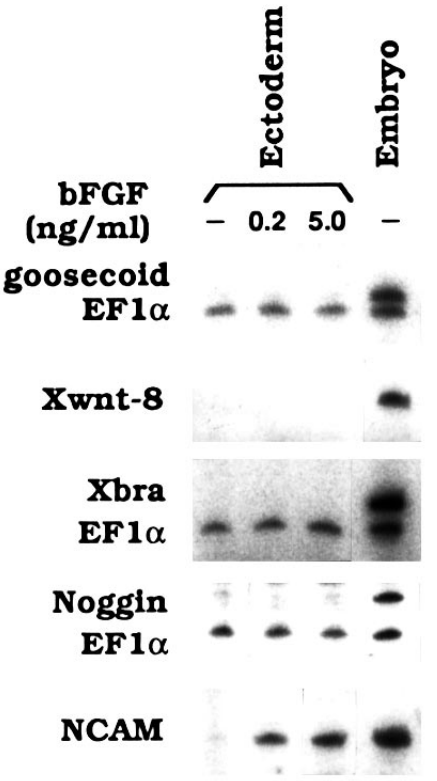


**Fig. 4.** bFGF does not induce the expression of early mesodermal markers. Animal cap ectoderm cells from stage 10 gastrula were cultured in the presence (0.2 or 5.0 ng/ml) or in the absence of bFGF and harvested at the equivalent of stage 11 control embryos for *goosecoid*, *Xwnt-8*, *Xbra* and *noggin*, and at stage 21 for *NCAM* for RT-PCR assays. Since the PCR products of *Xwnt-8* and *NCAM* are very close in size to the *EF1*α PCR product, the electrophoretic patterns of *Xwnt-8* and *NCAM* assayed without the internal *EF1*α control are shown in this figure. We confirmed that coamplification of *Xwnt-8* or *NCAM* with *EF1a* gave essentially the same conclusion.

are known to be expressed in some mesodermal derivatives in addition to neural tissue (Oliver et al., 1988; Wright et al., 1990; Green et al., 1992). It is possible that bFGF at higher doses induces the formation of posterior mesodermal cells instead of neural cells, although previous studies have shown that the competence of animal cap cells to form mesoderm in response to bFGF was lost before the stage that we examined in the present experiments (Slack et al., 1988). To test this possibility, we analyzed the expression of a neural-specific marker *NCAM* (Kintner and Melton, 1987) and four mesodermal markers that are expressed in a mesoderm-specific manner at mid-gastrula stage; both *gsc* (Blumberg et al., 1991) and *noggin* (Lamb et al., 1993) expressed in dorsal mesoderm, *Xwnt-8* (Christian et al., 1991) expressed in lateral and ventral mesoderm and *Xbra* (Smith et al., 1991) expressed throughout dorsal and ventral mesoderm. None of the four mesodermal markers were detected at a high dose of bFGF, where the posterior markers and *NCAM* were fully induced (Figs 2B,C, 4). We also examined the expression of *follistatin*, another candidate of neural inducer expressed in the dorsal mesoderm besides *noggin*. However, the endogenous expression level of *follistatin* in stage 12 embryos is very low, and we did not detect any visible bands from cultured ectoderm cells with or without bFGF (data not shown). These results are consistent with our previous observation that myocytes are never induced in stage 9+ to $10\frac{1}{2}$ gastrula ectoderm cells by bFGF, even at concentrations as high as 100 ng/ml. It is thus highly likely that the expression of posterior markers at higher doses of bFGF are not of mesodermal, but largely of neural origin.

### bFGF induces neural cells from ectoderm cells at the time of neural induction

To verify that bFGF exhibits the properties required for an authentic neural inducer, we investigated whether bFGF can induce a neural-specific marker in ectoderm cells at the time of normal neural induction. Ectoderm cells from gastrula of increasing ages were treated with various concentrations of bFGF and examined for levels of mRNA encoding *NCAM*, a cell adhesion molecule specifically expressed throughout the nervous system in intact embryos. In cultured cells from stage 10 ectoderm, *NCAM* mRNA levels increased sharply at the same low doses that increased *XeNK-2* and *En-2* mRNAs and plateaued with increasing doses (Fig. 5A, upper panel). The induction of *NCAM* mRNA levels by bFGF was correlated with the suppression of *epidermal keratin* mRNA levels (Jonas et al., 1989) in a dose-dependent manner (Fig. 5A, lower panel), which is consistent with our previous results utilizing

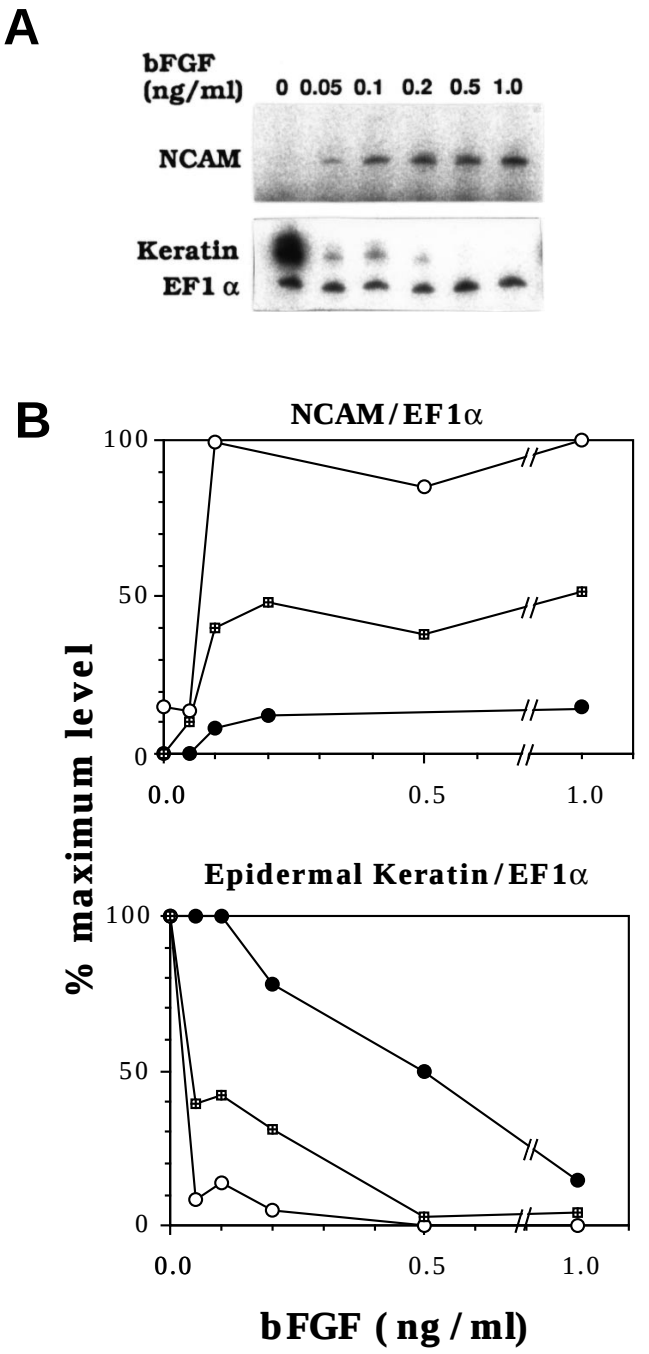


**Fig. 5.** Changes in the ability of ectoderm cells to respond to the neural inducing action of bFGF during gastrulation. (A) Dose-dependent effects of bFGF on the induction of *NCAM* and the suppression of *epidermal keratin* in ectoderm cells. Ectoderm cells from stage 10 gastrulae were cultured in the presence of various concentrations of bFGF (0-1.0 ng/ml) and harvested at stage 25 for RT-PCR assays. The entire experiment was repeated twice, with the same results. (B) Quantitative assessment of changes in the ability of ectoderm cells to respond to bFGF during gastrulation. Ectoderm cells were prepared from gastrulae of stage 10 (○), stage $10\frac{1}{4}$ (⊞) or stage $10\frac{1}{2}$ (●) and dose-response profiles for *NCAM* induction (upper) and *epidermal keratin* suppression (lower) by bFGF was constructed as in Fig. 2. PCR amplification of *NCAM* and *epidermal keratin* transcripts were carried out with aliquots of the same reverse-transcribed sample. Values were normalized to *EF1α* expression and presented as percentages of the maximum value of the ratio of each profile in the above sets of experiments.

monoclonal antibodies as markers. However, the responsiveness of ectoderm cells to bFGF in neural induction and epidermal suppression decreased as gastrula stage proceeded (Fig. 5B). At stage $10\frac{1}{2}$, there appears to be a number of cells that express neither *NCAM* nor *epidermal keratin*. These cells are thought to be differentiating into melanophores and/or possibly other neural crest derivatives (Kengaku and Okamoto, 1993). These findings agree with the observations demonstrating that the responsiveness of ectoderm cells to the action of the organizer to induce neural differentiation and suppress epidermal differentiation decreases during gastrulation (Holtfreter, 1938; Godsave and Shiurba, 1992).

### Ectodermal competence to respond to bFGF is sequentially lost in an anterior to posterior direction by mid-gastrula

To investigate whether there is a similar temporal change in the ability of ectoderm cells to differentiate into anterior and posterior neural tissues in response to bFGF, ectoderm cells from late blastula (stage 9+) and gastrulae of increasing ages (stage 10 to stage 11) were cultured in the presence of bFGF, and analyzed for the expression of *XeNK-2*, *En-2* and *XlHbox6* (Fig. 6A,C). *XeNK-2* was maximally induced in ectoderm cells isolated from stage 10 embryos, but exhibited rapidly decreased expression by stage $10\frac{1}{4}$. *En-2* expression plateaued between cells from stage 10 and stage $10\frac{1}{4}$ embryos and decreased by stage $10\frac{1}{2}$. It should be noted that the presumptive anterior neural region of the dorsal ectoderm has not yet received direct contact from the involuted organizer mesoderm at stage $10\frac{1}{2}$. By contrast, the ability to express *XlHbox6* plateaued between cells from stage 10-$10\frac{1}{2}$ embryos and remained at high levels until stage 11.

The animal cap tissue dissected from later gastrula could contain a smaller population of cells than the tissue from earlier stages because of the epiboly of animal cap cells during gastrulation. To test whether this epiboly might affect our results, we isolated ectoderm cells at stage 10 and cultured them to allow autonomous development. bFGF was added after 3 hours of preincubation and the expression of *En-2* and *XlHbox6* was assayed at the tailbud stage (Fig. 6B). The ability of ectoderm cells to express *En-2* was lost during 3 hours of preincubation, whereas the ability to express *XlHbox6* was retained, indicating that ectoderm cells first lose anterior-forming competence, then lose posterior-forming competence. A similar autonomous change of ectoderm competence was observed in the induction of neural tube and neural crest lineages (Kengaku and Okamoto, 1993). Thus, as gastrulation proceeds, the number of neural-competent cells in ectoderm decreases (Fig. 5B) and their fates become progressively restricted to more posterior neural ones (Fig. 6).

## DISCUSSION

We have presented evidence that bFGF can induce gastrula ectoderm cells to differentiate into neural tissue from forebrain to spinal cord in a dose-dependent manner (Fig. 2). Further,

bFGF induced neural differentiation from ectoderm cells at physiological low concentrations. The effective doses of bFGF for the induction of both anterior neural markers *En-2* and *XeNK-2* (2.5 pM) and the posteriormost marker *XlHbox6* (125 pM) are within the range of $K_d$ value reported for a FGF receptor in animal cap ectoderm cells of *Xenopus* blastula (Gillespie et al., 1989). We also showed that bFGF induced general neural marker *NCAM* and the position-specific markers in ectoderm cells at the time of normal neural induction (Figs 4, 6). These results indicate that bFGF or its homologue is a promising candidate for the neural morphogen derived from Spemann's organizer.

### Does bFGF directly induce the neural tissues from ectoderm cells in culture?

We previously showed that ectoderm cells of early- to mid-gastrula in culture never differentiated into myocytes, the main dorsal type derivative of mesoderm induced by bFGF, even at concentration of bFGF as high as 5 nM (Kengaku and Okamoto, 1993; Slack et al., 1987; Kimelman and Kirschner, 1987; Godsave and Slack, 1991; Kimelman and Maas, 1992). However, other mesodermal tissues could still be induced from a fraction of ectoderm cells, which in turn induce the remaining cells to differentiate into neural tissues. To address this question, we scored for the expression of several immediate early mesodermal markers in ectoderm cells treated with bFGF.

We showed that neither the organizer mesoderm, in agreement with previous observations (Green et al., 1992; Cho et al., 1991), nor the lateral and the ventral mesoderm were induced by bFGF at 250 pM, a dose where the posterior neural markers and *NCAM* were fully induced. We therefore conclude that each of the position-specific homeobox genes expressed in cultured ectoderm cells was largely of neural origin, and is directly induced by bFGF in the absence of the detectable organizer cells. In addition, the observation that bFGF does not induce the expressions of *noggin* and *follistatin* suggests that the neural inducing pathways mediated by these molecules and by bFGF are independent.

It has been shown that animal cap cells from blastula- and early gastrula-stage embryos have a tendency to differentiate into neural tissues when they are kept dispersed for a considerable period (Godsave and Slack, 1991; Grunz and Tacke, 1989). However, this does not seem to be the case for animal cap cells that are immediately reaggregated prior to the start of culture (Grunz and Tacke, 1989). We also observed that gastrula ectoderm cells reproducibly differentiated into epidermal cells when cultured without bFGF (Fig. 5). Further, bFGF could

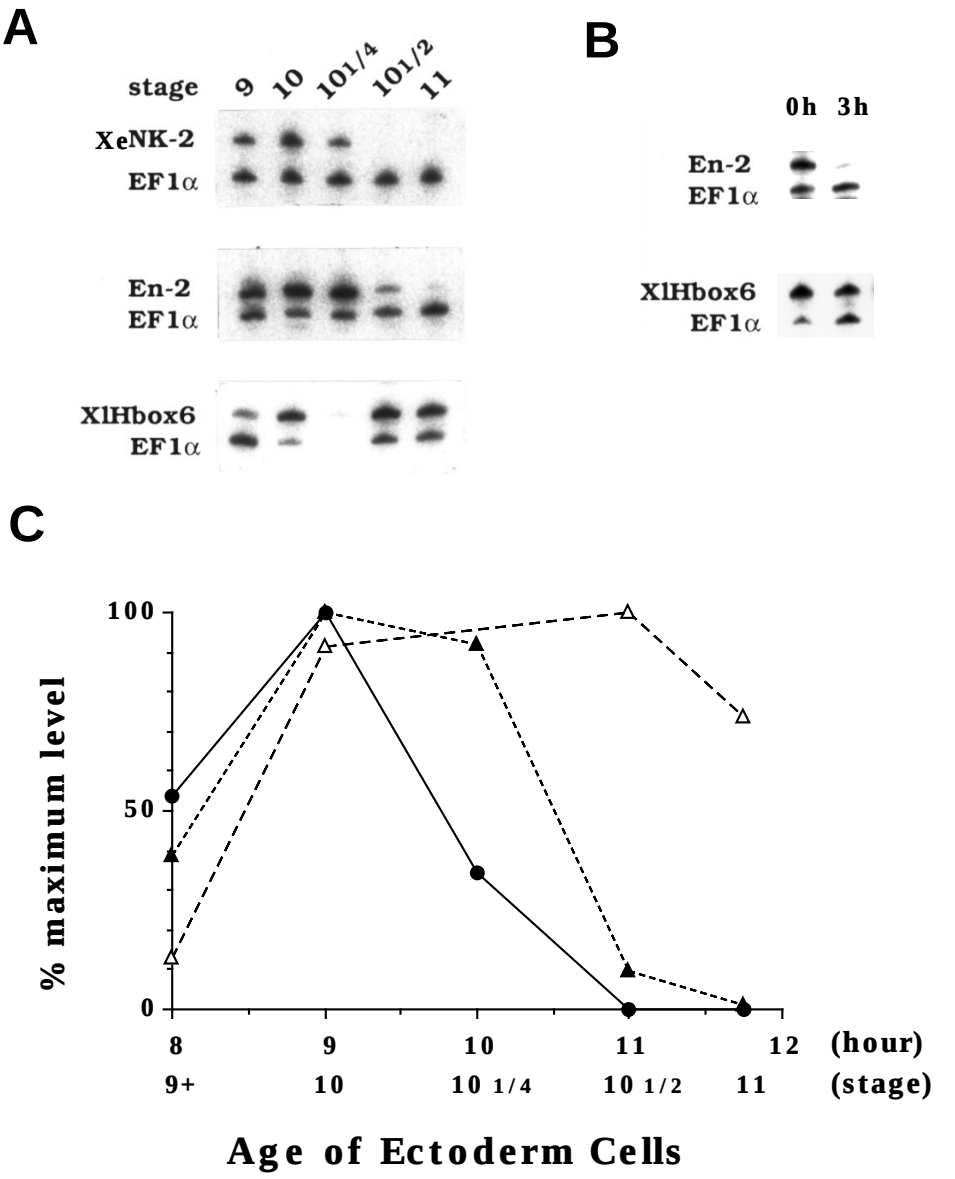


**Fig. 6.** Anterior-to-posterior progression of loss of the ability of ectoderm cells to respond to bFGF. (A) Induction of position-specific neural markers by bFGF depends on the age of the ectoderm cells. Ectoderm cells were prepared from embryos at various developmental stages indicated and treated with the optimal concentration of bFGF for induction of each marker: 0.25 ng/ml for *XeNK-2* and *En-2* and 5.0 ng/ml for *XlHbox6*. RNA was extracted at the equivalent of stage 25 for RT-PCR assays as in Figs 1 and 2. When *XeNK-2* expression was assayed using cells treated with 5.0 ng/ml of bFGF instead of 0.25 ng/ml, a similar change of competence was observed. The autographic pattern of *XeNK-2* expression represents the data from cells treated with 0.25 ng/ml bFGF. The complete experiment was repeated three times, yielding the same results. (B) Autonomous change of ectoderm competence. Stage 10 ectoderm cells were treated with bFGF at the start of culture (0h) and after 3 hours of incubation (3h), and assayed for the expression of *En-2* and *XlHbox6* as in (A). (C) Quantitative comparison of age-dependence shown in (A): ●, *XeNK-2*; ▲, *En-2*; Δ, *XlHbox6*. Values were normalized and presented as in Fig. 2.

induce neural lineages from gastrula ectoderm explants, when the outer pigmented layer was removed from the explant (Kengaku and Okamoto, 1993). It is thus unlikely that the neural inducing activity of bFGF is an artifact of the culture condition. However, we cannot exclude the possibility that the ectoderm cells in culture could be induced to form neural tissues by lower concentrations of bFGF than those present in vivo, due to the dilution of endogenous inhibitor(s) such as activin during cell dissociation process (Hemmati-Brivanlou and Melton, 1994).

### bFGF as a possible morphogen for the anteroposterior axis of the central nervous system

We showed that specific dose ranges of bFGF specify at least four different neural cell states along the anteroposterior axis of the CNS. Low doses of bFGF induced gastrula ectoderm cells to express anterior neural markers, and increasing doses of bFGF induced progressively more posterior neural markers (Fig. 2).

Induction and regionalization of the vertebrate nervous system has been thought to require two distinct inducers originating in the dorsal mesoderm. In this model, the first-step inducer is distributed uniformly along the A-P axis and induces anterior neural development. The second-step inducer distributes in an A-P gradient with a high point in the posterior mesoderm and causes posterior neural development. In contrast, our present data suggest that bFGF alone could drive both of steps by establishing a concentration gradient.

bFGF and other related molecules have been shown to be expressed at the right time and place to be the authentic neural inducer(s). bFGF is prepositioned in mesoderm-forming regions intracellularly throughout cleavage and gastrula stages, and, during the gastrula stage, it is also detected extracellularly between embryonic tissues (Shiurba et al., 1991; Godsave and Shiurba, 1992). It has been shown that XeFGF and Int-2, other members of FGF family, are expressed in the organizer region of early gastrula (Isaacs et al., 1992; Tannahill et al., 1992). It is probable that the FGF-like molecule(s) is distributed along the A-P axis with the highest point in the posteriormost (i.e., organizer) region. Such a concentration gradient could be formed within the involuted organizer mesoderm that induces the A-P pattern through a vertical action (Sharpe and Gurdon, 1990; Hemmati-Brivanlou et al., 1991; Saha and Grainger, 1992) to the overlying ectoderm. Our present results suggest, however, that bFGF is not likely to act as the vertical inducer, at least for anterior neural tissue, since ectoderm cells have almost lost their ability to respond to bFGF to form anterior neural tissue by mid-gastrula, i.e., prior to the stages at which they receive vertical contacts from the invaginated organizer mesoderm (Fig. 6). Rather, it is likely that FGF is released during early gastrula stages when the anterior-forming competence is available, forming a gradient within the plane of ectoderm with high doses inducing posterior patterns and low doses anterior patterns. Thus the action of bFGF fits the role of an initiator of planar induction, which was originally proposed by Spemann (1938) and has recently been confirmed (Kintner and Melton, 1987; Ruiz i Altaba, 1990; Doniach, 1992; Doniach et al., 1992).

Induction of the anteriormost marker *XeNK-2* within a broad dose range does not necessarily conform to the prediction of the above model, which suggests that *XeNK-2* should peak at the lowest dose of bFGF (Fig. 2). This apparent contradiction may partly be explained by the fact that *XeNK-2* is expressed in the anterior neural crest lineages (Saha et al., 1993). We have previously shown that neural crest-derived melanophores were induced by high doses of bFGF (Kengaku and Okamoto, 1993), suggesting that *XeNK-2* induced by higher doses of bFGF may originate from neural crest lineages. It is thus plausible that *XeNK-2* expression in the CNS peaks and declines at lower doses of bFGF than *En-2* expression.

Alternatively, differential regulation of anterior markers may be accomplished by factors other than FGF-like molecules at later stages, possibly through vertical transmission. If this were the case, our model should be modified as follows: general anterior induction would occur at low doses of FGF driven by planar transmission and with early-receding ectoderm competence. Then posterior respecification, for which late ectoderm competence is available, would be driven by a gradient of higher doses of FGF transmitted through planar and/or vertical contact.

Our previous study showed that bFGF induced melanophores from late-gastrula ectoderm cells when they began to lose their competence to form posterior neural tissue. Melanophores originate from the lateral edges of the neural plate (neural fold) which simultaneously gives rise to the peripheral nervous system. Taken together with the present results, it is strongly suggested that bFGF or other related molecules can contribute to the two-dimensional patterning of the nervous system along the anteroposterior and the mediolateral axes in both dose- and time-dependent manners. It is shown that two distinct types of FGF receptor named XFGFR-1 (Musci et al., 1990; Friesel and Dawid, 1991) and XFGFR-2 (Gillespie et al., 1989; Friesel and Brown, 1992) are expressed in *Xenopus* embryo. Further, we have recently cloned three new FGF receptor cDNAs from *Xenopus* gastrula, which we designated XFGFR-3, XFGFR-4 and XFGFR-4′ based on their homology to the human FGF receptors FGFR-3 and FGFR-4, respectively (XFGFR-4′ is highly homologous, but distinct from XFGFR-4). It might be that these receptor molecules with differential affinities to FGF molecules mediate the neural patterning by their appropriate spatiotemporal expressions in the dorsal ectoderm. Detailed studies using the dominant-negative molecule for each of those five types of FGF receptors are in progress to reveal the precise role of FGF signalling in neural induction.

### Other candidate for the neural-inducing morphogens

Our present data do not necessarily exclude the contribution of FGF to other early developmental processes such as mesoderm induction (Amaya et al., 1991, 1993; Cornell and Kimelman, 1994; LaBonne and Whitman, 1994), nor do they exclude a contribution to early neural development from other molecules. There is some evidence suggesting that animal cap cells of late blastula have received an endogenous signal from vegetal blastomeres, which cooperates with Xwnt-8 in mesoderm induction (Sokol, 1993; Doniach et al., 1992). This raises the possibility that gastrula animal cap cells used in this study had already been prepatterned anteriorly by a similar endogenous signal, although those cells does not include the presumptive neural region according to Keller's fate map (Keller, 1976). Low doses of bFGF might cooperate with the signal to form anterior neural tissue. It should be noted, however, that increasing doses of bFGF changed the cell fate to progressively

posterior ones, suggesting that FGF acted as a A-P patterning signal of the nervous system.

Recent studies have shown that noggin (Lamb et al., 1993) and follistatin (Hemmati-Brivanlou et al., 1994), secreted proteins expressed in organizer, are potential candidates for anterior neural inducers. These molecules might cooperate with FGF to accomplish anterior neural induction and A-P patterning in the CNS. Both the gain-of-function and the loss-of-function studies of FGF signalling appear to agree with this idea. Isaacs et al. (1994) have recently demonstrated that overexpression of XeFGF during gastrulation suppressed anterior head development and instead produced expanded posterior tissues derived from ectoderm. Their observation is consistent with our idea that ectoderm cells are induced to form posterior neural tissues by concentrated FGF emanated from the organizer mesoderm. In contrast, Amaya et al. (1993) have shown that inhibition of the normal function of a fraction of FGF receptors in *Xenopus* early embryos by overexpression of a dominant-negative form of XFGFR-1 disrupted development of trunk axial structures such as notochord and spinal cord, but not of anterior head structures. Lack of axial mesoderm in the mutant embryos may indicate some important role of FGF in mesoderm induction, but it is not inconsistent with the idea that an FGF-like molecule also acts as an endogenous inducer for posterior neural development. Rather, their results may mean that the anterior neural tissue is induced by either noggin or follistatin, or both, in the dominant-negative mutant embryos. However, this idea does not exclude the possible involvement of FGF-like molecules in anterior neural development via parallel complementary pathway with those of noggin and follistatin.

Alternatively, it is also possible that a concentration gradient of FGF-like molecule roughly determines the A-P polarity during early gastrulation stages and that some other molecules from the dorsal mesoderm bring about additional fine tuning of the A-P axis formation. Retinoic acid (RA) has been put forward as a potential candidate for the second-step inducer for the anteroposterior axis of the developing CNS (a 'neural morphogen' in Wolpert model reviewed by Green and Smith, 1991). RA can transform anterior embryonic axial tissue containing both neurectoderm and mesoderm to a more posterior specification in a dose-dependent manner (Boncinelli et al., 1991; Durston et al., 1989; Dekker et al., 1992; Ruiz i Altaba and Jessell, 1991).

In conclusion, although many aspects of the function of FGFs in neural induction remain to be elucidated, the dose-dependent induction of neural markers by bFGF suggests that gradients of FGF-like molecules may be important for A-P neural patterning and other morphogeneses during early vertebrate development.

We are grateful to Ikuko Hongo and Susumu Ijuin for help with PCR reactions. We thank Prof. Kunitaro Takahashi for advice and discussions and Dr David W. Saffen for critical reading of the manuscript. This work is supported by a grant-in-aid for science research from the Ministry of Education, Science and Culture, Japan and by a fellowship from Japan Society for the Promotion of Science to M. K.

## 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.

**Blumberg, B., Wright, C. V. E., De Robertis, E. M. and Cho, K. W. Y.** (1991). Organizer-specific homeobox genes in *Xenopus* laevis embryos. *Science* **253**, 194-196.

**Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F.** (1991). *HOX* gene activation by retinoic acid. *Trends Genet.* **7**, 329-334.

**Cho, K. W. Y. Blumberg, B., Steinbeisser, H. and De Robertis, E. M.** (1991). Molecular nature of Spemann's organizer: the role of *Xenopus* homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

**Christian, J. L., McMahon, J. A., McMahon A. P. and Moon, R. T.** (1991). Xwnt-8, a Xenopus Wnt-1/int-1 related gene responsive to mesoderm inducing factors may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-1056.

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

**Dekker, E. J., Pannese, M., Houtzager, E., Timmermans, A., Boncinelli, E and Durston, A.** (1992). Xenopus Hox-2 genes are expressed sequentially after the onset of gastrulation and are differentially inducible by retinoic acid. *Development* **1992 Supplement**, 195-202.

**Doniach, T.** (1992). Induction of anteroposterior neural pattern in *Xenopus* by planar signals. *Development* **1992 Supplement**, 183-193.

**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.

**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). Retinoic acid causes an anteroposterior transformation an the developing central nervous system. *Nature* **340**, 140-144.

**Friesel, R. and Dawid, I. B.** (1991). cDNA cloning and developmental expression of fibroblast growth factor receptors from Xenopus laevis. *Mol. Cell. Biol.* **11**, 2481-2488.

**Friesel, R. and Brown, S. A. N.** (1992). Spatially restricted expression of fibroblast growth factor receptor-2 during Xenopus development. *Development* **116**, 1051-1058.

**Gillespie, L. L., Paterno, G. D. and Slack, J. M. W.** (1989). Analysis of competence: receptors for fibroblast growth factor in early *Xenopus* embryos. *Development* **106**, 203-208.

**Godsave, S. F. and Slack, J. M. W.** (1991). Single cell analysis of mesoderm formation in the *Xenopus* embryo. *Development* **111**, 523-530.

**Godsave, S. F. and Shiurba, R. A.** (1992). *Xenopus* blastulae show regional differences in competence for mesoderm induction; correlation with endogenous basic fibroblast growth factor levels. *Dev Biol*. **151**, 506-515.

**Green, J. B. A. and Smith, J. C.** (1991). Growth factors as morphogens: do gradients and thresholds establish body plan? *Trends in Genetics* **7**, 245-250.

**Green, J. B. A., New H. V. and Smith, J. C.** (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of mesoderm. *Cell* **71**, 731-739.

**Green, J. B. A., Smith, J. C. and Gerhalt, J. C.** (1994). Slow emergence of a multithreshold response to activin requires cell-contact-dependent sharpening but not prepattern. *Development* **120**, 2271-2278.

**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**, 211-217.

**Gurdon, J. B.** (1977). Methods for nuclear transplantation in Amphibia. *Meth. Cell Biol.* **16**, 125-139.

**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.

**Hemmati-Brivanlou, A. and Melton, D. A.** (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77**, 273-281.

**Holtfreter, J.** (1938). Veränderungen der Reaktionsweise im alternden isolierten Gastrulaektoderm. *Wilhelm Roux Arch. EntwMech. Org.* **138**, 163-196.

**Isaacs, H. V., Tannahill, D. and Slack, J. M. W.** (1992). Expression of a novel FGF in *Xenopus* embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* **114**, 711-720.

**Isaacs, H. V., Pownall, M.E. and Slack, J. M. W.** (1994). eFGF regulates *Xbra* expression during Xenopus gastrulation. *EMBO J.* **13**, 4469-4481.

**Jonas, E. A., Snape, A. M. and Sargent, T. D.** (1989). Transcriptional regulation of a *Xenopus* embryonic epidermal keratin gene. *Development* **106**, 399-405.

**Keller, R. E.** (1976). Vital dye mapping of the gastrula and neurula of Xenopus laevis. II. Prospective areas and morphogenetic movements of the deep layer. *Dev. Biol.* **51**, 118-137.

**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.** (1994). Morphogenetic action of basic FGF in determining anteroposterior axis in the developing central nervous system of *Xenopus laevis*. *Society for Neurosci. Abstracts* **20**, 254.

**Kimelman, D. and Kirschner, M.** (1987). Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for FGF in early *Xenopus* embryo. *Cell* **51**, 869-877.

**Kimelman, D. and Maas, A.** (1992). Induction of dorsal and ventral mesoderm by ectopically expressed *Xenopus* basic fibroblast growth factor. *Development* **114**, 261-269.

**Kinoshita, T., Imamura, J., Nagai, H and Shimotohno, K.** (1992). Quantification of gene expression over a wide range by polymerase chain reaction. *Anal. Biochem.* **206,** 231-235.

**Kintner, C. R. and Melton, D. A.** (1987). Expression of *Xenopus* N-CAM RNA is an early response to neural induction. *Development* **99**, 311-325.

**Krieg, P. A., Varnum, S. M., Wormington, W. M. and Melton, D. A.** (1989). The mRNA encoding elongation factor 1α (EF1α) is a major transcript at the mid-blastula transition in *Xenopus*. *Dev. Biol.* **133**, 93-100.

**LaBonne, C and Whitman, M.** (1994). Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development* **120**, 463-472.

**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.

**Mitani, S. and Okamoto, H.** (1989). Embryonic development of *Xenopus* studied in a cell culture system with tissue specific monoclonal antibodies. *Development* **105**, 53-59.

**Mitani, S. and Okamoto, H.** (1991). Inductive differentiation of two neural lineages reconstituted in a microculture system from *Xenopus* early gastrula cells. *Development* **112**, 21-31.

**Musci, T. J., Amaya, E. and Kirschner, M. W.** (1990). Regulation of the fibroblast growth factor receptor in early *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **87**, 8365-8369.

**Nieuwkoop, P. D. and Farber, J.** (1967). *Normal Table of Xenopus laevis* (*Daudin*). Amsterdam: North-Holland Publishing Company.

**Nieuwkoop, P. D. and Albers, B.** (1990). The role of competence in the cranio-caudal segregation of the central nervous system. *Dev. Growth and Differ.* **32,** 23-31.

**Oliver, G., Wright, C. V. E., Hardwicke J. and De Robertis, E. M.** (1988). Differential anteroposterior expression of two proteins encoded by a homeobox gene in *Xenopus* and mouse embryos. *EMBO J.* **7**, 3199-3209.

**Ruiz i Altaba, A.** (1990). Neural expression of the Xenopus homeobox gene Xhox3: evidence for a patterning neural signal that spreads through the ectoderm. *Development* **108**, 595-604.

**Ruiz i Altaba, A. and Jessell, T. M.** (1991). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **112**, 945-958.

**Saha, M. S. and Grainger, R. M.** (1992). A labile period in the determination of the anterior-posterior axis during early neural development in Xenopus. *Neuron* **8**, 1003-1014.

**Saha, M. S., Michel, R. B., Gulding, K. M. and Grainger, R. M.** (1993). A *Xenopus* homeobox gene defines dorsal-ventral domains in the developing brain. *Development* **118**, 193-202.

**Saxen, L.** (1989). Neural induction. *International Journal of Developmental Biology* **33**, 21-48.

**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.

**Sive, H. L., Hattori, K. and Weintraub, H.** (1989). Progressive determination during formation of the anteroposterior axis in Xenopus laevis. *Cell* **58**, 171-180.

**Slack, J. M. W., Darlington, B. G., Heath, J. K. and Godsave, S. F.** (1987). Mesoderm induction by early *Xenopus* embryos by heparin-binding growth factors. *Nature* **326**, 197-200.

**Slack, J. M. W., Isaacs, H. V. and Darlington, B. G.** (1988). Inductive effects of fibroblast growth factor and lithium ion on *Xenopus* blastula ectoderm. *Development* **103**, 581-590.

**Slack, J. M. W. and Tannahill, D.** (1992). Mechanism of anteroposterior axis specification in vertebrates: Lessons from the amphibians. *Development* **114**, 285-302.

**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 Induction von Embryonalanlagen durch Implantation artfremder Organisation. *Arch. mikrosk. Anat. EntwMech.* **100**, 599-638.

**Spemann, H.** (1938). *Embryonic Development and Induction.* New Heaven: Yale University Press; reprinted by Garland, New York, 1988.

**Sokol, S. Y.** (1993). Mesoderm formation in *Xenopus* ectodermal explants overexpressing Xwnt8: evidence for a cooperating signal reaching the animal pole by gastrulation. *Development* **116**, 1335-1342.

**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.

**Warner, A.E.** (1985). Factors controlling the early development of the nervous system. *In Molecular Bases of Development* (ed. G. M. Edelman, W. E. Gall and W. M. Cowan), pp. 11-34.

**Wright, C. V. E., Morita, E. A., Wilkin, D. J. and De Robertis, E. M.** (1990). The Xenopus XlHbox6 homeo protein, a marker of posterior neural induction, is expressed in proliferating neurons. *Development* **109**, 225-234.

(Accepted 14 June 1995)