

# **XBF-2 is a transcriptional repressor that converts ectoderm into neural tissue**

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## **SUMMARY**

We have identified *Xenopus* Brain Factor 2 (XBF-2) as a potent neuralizing activity in an expression cloning screen. In ectodermal explants, XBF-2 converts cells from an epidermal to a neural fate. Such explants contain neurons with distinct axonal profiles and express both anterior and posterior central nervous system (CNS) markers. In striking contrast to X-*ngnR-1a* or X-*NeuroD*, ectopic expression of XBF-2 in *Xenopus* embryos results in an expansion of the neural plate to the ventral midline. The enlarged neural plate consists predominantly of undifferentiated neurons. XBF-2 lies downstream of the BMP antagonists *noggin*, *cerberus*, and *gremlin* since ectodermal explants expressing these molecules exhibit strong expression of XBF-2. While XBF-2 does not

upregulate the expression of secreted neural inducers, it downregulates the transcription of BMP-4, an epidermal inducer. We show that XBF-2 acts as a transcriptional repressor and that its effects can be phenocopied with either the engrailed or hairy repressor domain fused to the XBF-2 DNA-binding domain. A fusion of the DNA-binding domain to the activator domain of VP16 blocks the effects of XBF-2 and prevents neural plate development in the embryo. This provides evidence that a transcriptional repressor can affect both regional neural development and neurogenesis in vertebrates.

Key words: Winged-helix, Repressor, XBF-2, Neural specification, *Xenopus*

## **INTRODUCTION**

During development, the specification of tissues involves the integration of both cell-cell signaling and the intracellular response. Formation of the nervous system is initiated when ectoderm is exposed to signals from the organizer (Harland, 1997; Harland and Gerhart, 1997). Without organizer signals, ectoderm becomes epidermis, in part due to the activity of bone morphogenetic proteins (BMPs; Wilson and Hemmati-Brivanlou, 1995). Secreted proteins from the organizer antagonize BMP signaling, thereby suppressing the epidermal fate and promoting neural fates. A surprising variety of BMP antagonists are expressed within the organizer, including *noggin* (Smith and Harland, 1992; Zimmerman et al., 1996), *follistatin* (Hemmati-Brivanlou et al., 1994), *Xnr3* (Smith et al., 1995; Hansen et al., 1997), *chordin* (Sasai et al., 1994; Piccolo et al., 1996) and *cerberus* (Bouwmeester et al., 1996; Hsu et al., 1998). Though none has yet been found to be an essential component of the neural-inducing signal from the organizer, a working model states that they provide a redundant anti-BMP signal that allows ectoderm to adopt neural fates (Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997; Harland, 1997; Harland and Gerhart, 1997). Presumably, the initial inductive signal is then integrated within ectodermal cells by the expression of transcription factors that may suppress or activate the neural phenotype.

Neural induction is accompanied by patterning along both the anterior-posterior and mediolateral axes of the neural plate.

In addition, in both *Xenopus* and zebrafish, the process of neuronal differentiation (neurogenesis) begins at this time. A subset of neural precursors exit the mitotic cycle and begin extending axons early on to form the primary neurons (Hartenstein, 1989). The remaining cells in the neural plate provide a source of cells in the neuroepithelium and do not undergo terminal differentiation until later. Considerable progress has been made in understanding vertebrate neurogenesis because many of the genes that affect neurogenesis and lateral inhibition are conserved between organisms (Chitnis et al., 1995). These genes include homologues of the basic helix-loop-helix genes such as *achaete-scute* and *atonal*, as well as homologues of *Notch* and *Delta* (reviewed by Anderson and Jan, 1997). For example, the basic helix-loop-helix gene *neurogenin*, when expressed ectopically in *Xenopus* embryos, causes cells in the neural plate to undergo neurogenesis prematurely (Ma et al., 1996).

In contrast to mediators of neurogenesis, the intracellular mediators of neural induction are still poorly understood. One class of genes that can determine neural fates without terminal differentiation are the homologues of the genes in the *Drosophila iroquois* complex (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998). These homeobox-containing genes can induce neural plate tissue without terminal differentiation, though some members also induce neurogenesis. *Zic 3* and *Zicr-1*, genes related to the *Drosophila* gene *odd paired*, can also induce neural fates in ectodermal explants (animal cap ectoderm); overexpression also causes hyperplasia of the

neural plate, and particularly the neural crest, in whole embryos (Nakata et al., 1997; Mizuseki et al., 1998a). A POU gene, *XIPOU2*, has also been reported to induce neural tissues and can induce some neural differentiation in ectopic positions (Witta et al., 1995). Genes that may also contribute to neural fates include *Sox2*, which is expressed throughout the neural plate and predisposes cells to respond to signals such as FGF (Mizuseki et al., 1998a) and the more potent neuralizing agent *SoxD*, which can induce ectopic neural and neuronal development (Mizuseki et al., 1998b). Most of these genes are associated with specifying or patterning specific domains of the neural plate, suggesting that many factors will combine to determine the detailed pattern of the nervous system.

In order to identify genes involved in neural induction, we have screened directly for molecules with neuralizing activities. To date, none of the known neuralizing proteins have been discovered using screens designed specifically to identify proteins with neuralizing activity. Among the extracellular BMP antagonists, noggin and *Xnr3* were identified by their ability to induce dorsal mesoderm in UV-ventralized embryos (Smith and Harland, 1992; Smith et al., 1995); follistatin was identified as an activity that blocked activin-stimulated gonadotropin release from the pituitary (Nakamura et al., 1990); *chordin* and *cerberus* were identified as genes expressed in the organizer (Sasai et al., 1994; Bouwmeester et al., 1996). For intracellular mediators of neural development, vertebrate *iroquois* and *Zic-3* genes were identified by similarity to fly genes (Nakata et al., 1997; Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998), while *Zicr-1*, *sox-2* and *SoxD* were found as genes upregulated by *chordin* expression (Mizuseki et al., 1998a,b).

Here, we have used an expression cloning assay to identify a winged helix gene, *XBF-2*, as a potent mediator of neural induction. Vertebrate winged helix proteins were first identified as factors that stimulate transcription of liver-specific genes. They contain a winged helix domain that is similar to that of the *forkhead* gene of *Drosophila*. Multiple winged helix genes have been identified from a number of vertebrates, but so far only a handful have been ascribed any function (Ang and Rossant, 1994; Hatini et al., 1996; Li et al., 1995; Xuan et al., 1995; Dirksen and Jamrich, 1992; Labosky et al., 1997; Ruiz i Altaba and Jessell, 1992; reviewed in Kaufmann and Knochel, 1996). In this work, we have characterized the neuralizing activity of *XBF-2* by examining the effects of ectopic *XBF-2* expression in whole embryos and in animal cap ectoderm. We address what kind of neural tissue is formed and the mechanism of neuralization by *XBF-2* by exploiting repressor and activator fusions.

## MATERIALS AND METHODS

### Embryo manipulations and RT-PCR

*Xenopus* embryos were generated and staged as described (Nieuwkoop and Faber, 1967; Condie and Harland, 1987). For animal cap assays, 1-cell embryos were injected at the animal pole. Animal caps were explanted at stages 8-9 by conventional methods, or with the Gastromaster (Xenotek Engineering) using a 400  $\mu$ m wide tip (GYL-2), cultured in 75% NAM (Peng, 1991) and harvested at stages 12, 21-23 or 28.

For RNA expression in whole embryos, 2-cell embryos were injected in the animal pole of one blastomere. Embryos were cultured in 75% NAM and harvested at stage 12 or stage 22.

To test *XBF-2* activity, embryos were injected with synthetic RNA

made from pCS105f.1.XBF-2cDNA, pCS2XBF-2 (coding region only), or pCS2MTXBF-2 (six myc tags in frame at the N terminus). Explants neuralized by noggin were either treated with 0.5  $\mu$ g/ml human noggin in 75% NAM with 0.5% protease-free BSA or were injected with RNA from pCS2noggin. Embryos expressing NgnR-1a or NeuroD were injected with RNA generated from pCS2MTX-NgnR-1a (Ma et al., 1996) or pCS2MTNeuroD (Lee et al., 1995). RNA for mouse BF-2 and pintallavis was generated from pBSBF-2 (Hatini et al., 1996) and pSP64Tpintallavis (Ruiz i Altaba and Jessell, 1992).

RT-PCR analysis was done as described in Wilson and Melton (1994). Primer sets and PCR conditions for Xbra, epidermal Keratin, EF1a, goosecoid, noggin, Muscle Actin (MA), and N-CAM are described in Wilson and Melton (1994). Other primers used were (listed here 5' to 3'): Engrailed2 (U: CGG AAT TCA TCA GGT CCG AGA TC, D: GCG GAT CCT TTG AAG TGG TCG CG); Krox20 (U: ATT CAG ATG AGC GGA GTG, D: ATG TGC TCC AGG TCA CTT); HoxB9 (U: TACTTACGGGCTTGCTGGA, D: TCAGTATTTGCAGCAGCG); Otx-2 (U: CGGGATGGATT-TGTTGCA, D: TTGAACCAGACCTGGACT); Cerberus (U: GCTGAACTATTTGATCCACC, D: ATGGCTTGATTCTGTG-GGGC), Chordin (U: CACTGAGTGATGTGGATG, D: TCTTGTTCCTGTGCAGAG).

### Fusion constructs

All fusion constructs were verified by sequence and by monitoring the size of protein produced with the TNT SP6-coupled Reticulocyte Lysate System (Promega). All fragments of *XBF-2* generated by PCR were made from the original cDNA clone template and amplified with Advantage Polymerase (Clontech). When possible, the amino or carboxy terminal location of the protein fragment in the wild-type molecule was maintained in the fusion protein. Start and stop codons were introduced where needed. Since all constructs contain myc tags, the localized expression of fusion constructs in embryos was detected by immunohistochemistry with anti-myc antibodies.

### EnR::*XBF*

The DNA-binding fragment was generated by PCR with U10: CCGCTCGAGCGGTGGGGTCCGGCAGGAGCGCG and D11: CCGCTCGAGTCAAAGCACAAAGTCCGGGACTTG, cut with *XhoI*, filled and blunt end ligated into pCS2MTenR (gift from David Turner), cut with *XhoI* and filled in.

### *XBF*::*hairly*

pCSMThairyR was constructed by cutting pCS2MT with *XbaI* and ligating in an *XbaI*-cut PCR fragment containing the hairy repressor from pBSKS-hairy from *Drosophila* (Barolo and Levine, 1997) (hU: GCTCTAGAAATGGAACAGCAGCCCCCTGTGCG, hD: GCTCTAGACTACCAGGGCCGCCAGGGCTGCTC). The DNA-binding fragment was amplified with U12: CGGAATC-AAGCGCGTTGGTGAAGCCCCC and D14: CCGCTCGAGA-GCACAAGCTCCGGGACTTG. Both were cut with *XhoI*, filled in, cut with *EcoRI* and ligated.

### *XBF*::*VP16*

pCS2MTVP16 was constructed by cutting pCS2MT with *XbaI* ligating the VP16 activator from Herpes Simplex Virus 1 amplified with vU3: GCTCTAGAAGCCCCCGACCGATGTGTCAGC and vD4: GCTCTAGACTACCCACCGTACTCGTCAATTCC from pSV40GVP (Sadowski et al., 1988). The *XBF-2* DNA-binding domain was amplified with U12 and D14. Both were cut with *XhoI*, filled in, cut with *EcoRI* and ligated.

### pCS2MTDNABD

pCS2MT was cut with *EcoRI* and *XbaI* and ligated to the *XBF-2* DNA-binding domain amplified with U12 and D13: GCTCTAGAAGCACAAAGCTCCGGGACCTG also cut with *EcoRI* and *XbaI*.

**pCS105FigGal4Cterm and pCS105FigGal4Nterm**

A *BglIII-EcoRI* fragment of 323 bp from pET-Flag-Gal4 (Thut et al., 1997) was ligated into *BamHI-EcoRI*-cut pCS105 (Hsu et al., 1998). The C-terminal and N-terminal portions of XBF-2 were amplified with U5: CGGAATTCATGACTCTGAGCTCTGACATG and D6: TTGCGGCCGCTCACCTGCCGACCCACCCGAC, U7: CGGAATTCGTCCCGGAGCTTGTGCTTAGG and D8: TTGCGGCCGCTTAGTGGTTTGTAAAGCACCG.

**Expression cloning**

Poly(A)<sup>+</sup> RNA was isolated from *Xenopus* neurulae, stage 19-22, by oligo(dT)-cellulose selection from a proteinase K/SDS lysate (Badley et al., 1988). The SuperScript cDNA cloning system (Life Technologies) was used to synthesize a cDNA library containing about 100,000 independent clones in CS105 (Hsu et al., 1998), a derivative of CS2+ (Turner and Weintraub, 1994). Pools of cDNA were made from 150 plates containing about 700 colonies each.

Transcription templates were made by digesting cDNA pools with *AscI*, followed by digestion with 0.1 mg/ml proteinase K, 0.5% SDS, 5 mM EDTA, 50 mM Tris 7.5, 50 mM NaCl at 42°C for 1 hour, phenol/chloroform extraction and precipitation. Synthetic capped mRNA was made with the mMessage mMachine SP6 kit (Ambion). 1 ng of each mRNA pool was injected into the animal pole. Ten animal cap explants were assayed at stage 23 for the expression of MA, HoxB9 and N-CAM by RT-PCR.

**In situ hybridization, immunostaining and histology**

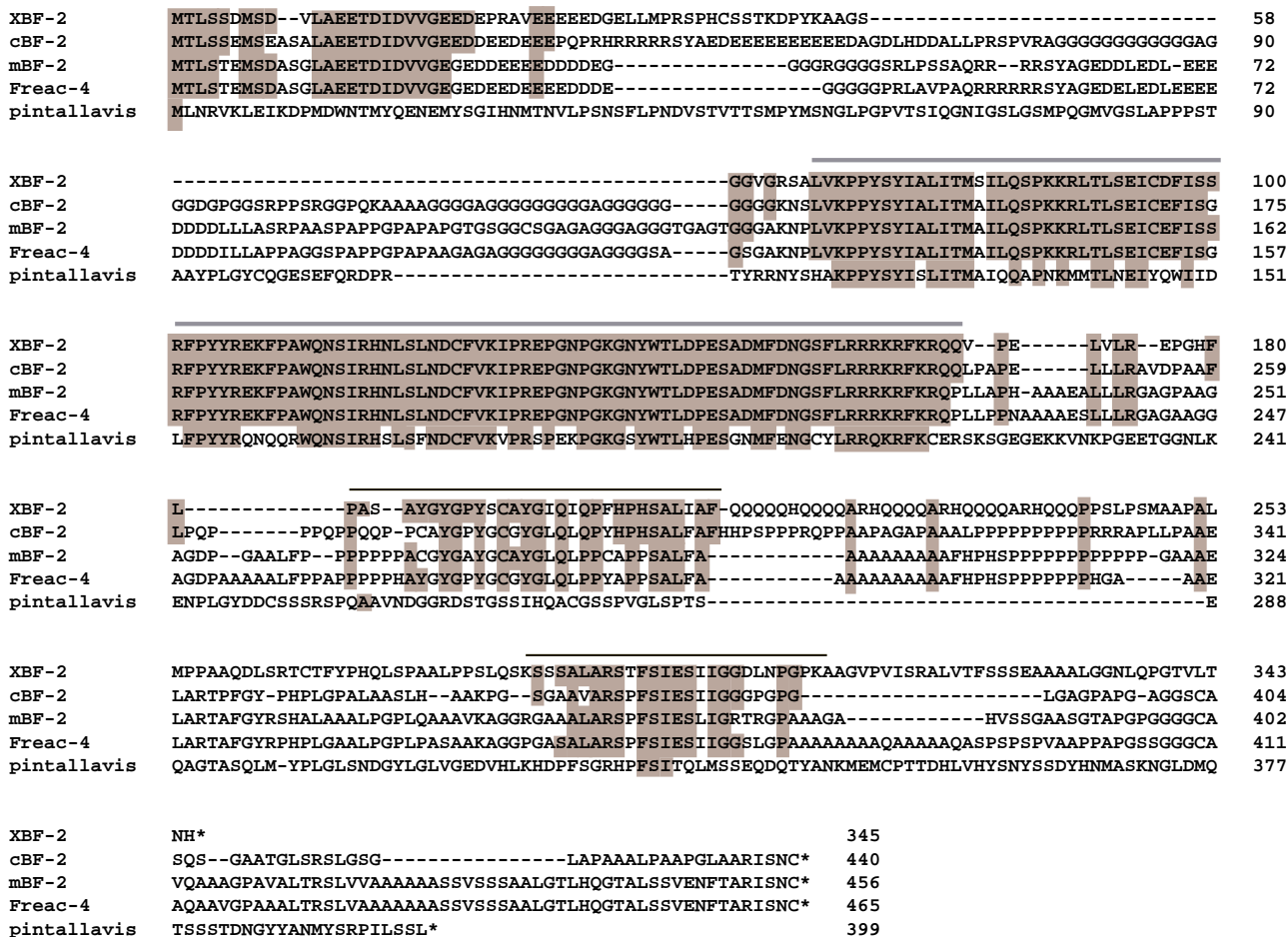
Sense and antisense probes for whole-mount in situ hybridization (Lamb et al., 1993) of XBF-2 were made from a partial cDNA construct, pCS105XBF-2ΔNsi. Other probes were: nrp1(pNPG152; Richter et al., 1990), N-CAM (pTN1; Kintner and Melton, 1987), BMP-4 (pGEM7XBMP-4; Wilson and Hemmati-Brivanlou, 1995), epidermal Keratin (pG3; Jonas et al., 1989), N-tubulin (p24-10; Good et al., 1989), Slug (pMX363Xslug, a gift from Chris Kintner; Mayor et al., 1995) and Twist (pXtwi560; Hopwood et al., 1989). After staining and fixation in MEMFA, pigmented samples were bleached (Mayor et al., 1995).

The presence of neural tissue in embryos and explants was determined by immunostaining with the antibody 6F11 (Lamb et al., 1993), mature somites with 12/101 (Kintner and Brockes, 1984), and developing neurons with Tor 25.4 (Kushner, 1984).

For histological analysis, stained embryos were embedded in paraffin and sectioned at 10-20 μm.

**COS cell assays**

COS7 cells were plated at 3×10<sup>5</sup> in 35 mm diameter wells and cultured in Optimem (Gibco-BRL) with 10% FBS until nearly confluent. The cells were transfected with Lipofectamine (Life Technologies) over a 6 hour period in Optimem without serum. After 36 hours of culture in serum-rich media, the cells were scraped off the dish in lysis buffer. The lysate was spun briefly and the supernatant



**Fig. 1.** Alignment of XBF-2 with other winged helix transcription factors. XBF-2 is most similar to chicken BF-2 with 44% identity. XBF-2 is quite different from pintallavis sharing only 24% identity. Shaded boxes indicate amino acid residues shared in common with XBF-2. A gray line indicates the DNA-binding domain. Three regions of similarity between XBF-2 and the other highly related winged helix proteins are indicated by a black line. cBF-2, chicken BF-2; mBF-2, mouse BF-2.



was used to carry out the Dual Luciferase Assay (Promega). Luciferase activity was measured with a Turner TD-20E luminometer.

Each well received 0.01  $\mu$ g of pRL-CMV (Promega), (contains the CMV promoter driving *Renilla luciferase*) to monitor transfection efficiency. XBF-2::Gal4 fusion constructs were transfected along with the reporter plasmid pG2tklux (two Gal4-binding sites, a minimal thymidine kinase promoter driving Firefly *luciferase*) or ptklux (lacks the Gal4-binding sites; gifts from Tom O'Brian). Expression was verified in COS cell extracts by western with anti-flag antibodies (M2, IBI).

## RESULTS

### Expression cloning of XBF-2

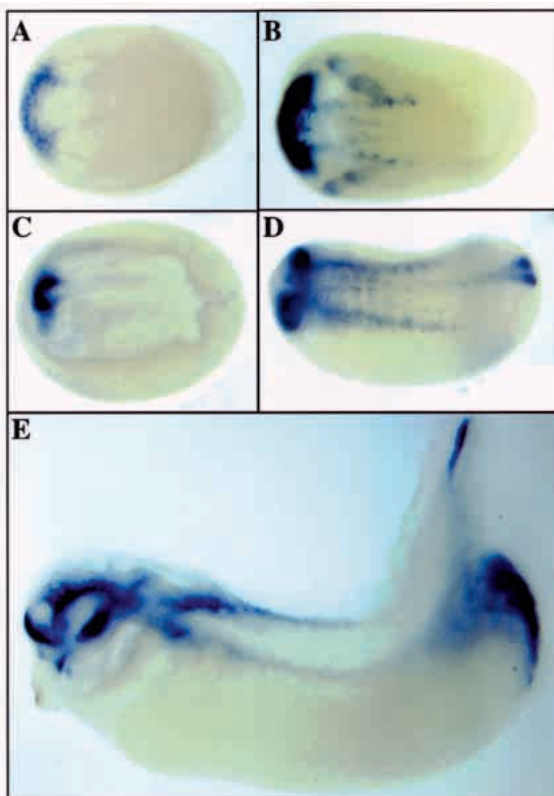
We used an animal cap assay to screen for activities in cDNA libraries that could induce the expression of various neural markers, both general and regional. This assay is similar to that used by Baker and Harland (1996) to screen for mesoderm-inducing activities. Since neural induction, patterning and neurogenesis are continuing processes, we chose to screen a late neurula library. Messenger RNA from stage 19-22 embryos was used to construct a cDNA plasmid library of 100,000 clones; this was divided into 150 pools each consisting of approximately 700 colonies. Synthetic capped mRNA from each pool was injected near the animal pole of

1-cell embryos and the embryos were allowed to develop to the blastula stage. An explant was removed from the animal pole, cultured until early tailbud equivalent (stage 22-23) and then analyzed by RT-PCR for the expression of N-CAM (Neural Cell Adhesion Molecule), HoxB9 or MA (muscle actin). About 50,000 colonies have been screened (50% of the library), yielding six pools capable of inducing neural markers. Upon sib selection of one pool with potent neuralizing activity, we identified *Xenopus Brain Factor 2*, XBF-2.

### XBF-2 is a winged-helix transcription factor expressed in the nervous system

The sequence of the XBF-2 cDNA revealed an open reading frame encoding a polypeptide of 345 amino acids, containing the domain characteristic of winged-helix transcription factors and implicated in direct DNA binding (Clark et al., 1993; Fig. 1). Consistent with this, myc-tagged versions of the protein are found concentrated in the nucleus (data not shown). Typically, winged-helix transcription factors fall into classes with highly similar DNA-binding domains. Outside these domains, however, the sequences diverge widely (Kaufmann and Knochel, 1996). Since the predicted protein is most similar to chicken BF-2 (44% amino acid identity; Yuasa et al., 1996), mouse BF-2 (41%) (Hatini et al., 1996), and the human gene factor FREAC-4 (41%; Pierrou et al., 1994), and because it shares amino acid sequences with these proteins inside the DNA-binding domain (97.5%) as well as outside (see Fig. 1), it is likely to be the *Xenopus* homologue. We therefore call this gene *XBF-2*, *Xenopus Brain Factor 2* (accession #AF072889). The unrelated winged-helix protein pintallavis (Ruiz i Altaba and Jessell, 1992), for comparison, is 24% identical overall and only 60% identical within the DNA-binding domain.

We determined the spatial and temporal expression pattern of XBF-2 by in situ hybridization with a digoxigenin-labeled antisense RNA probe (Fig. 2). Localized XBF-2 transcripts first appear in the anterior neural plate at the early neurula stage (stage 12.5; Fig. 2A). At stage 14, XBF-2 is also transiently expressed in two stripes lateral to the midline, which could include cardiac and kidney precursors (Fig. 2B). This pattern is distinct from the neural crest markers slug or twist, (Hopwood et al., 1989; Mayor et al., 1995). At stage 21, embryos continue to express XBF-2 in the forebrain and expression begins in cells lateral to the rostral somites as well as in the neural tissue of the tailbud (Fig. 2D). By stage 36 (Fig. 2E), transcripts are detected in restricted regions of the forebrain and in the temporal retina. Caudally, expression continues in the neural tissue of the tailbud. At this stage, there is also strong expression at the caudal end of the embryo on the ventral side. No signal was detected with a sense control probe at any stage.



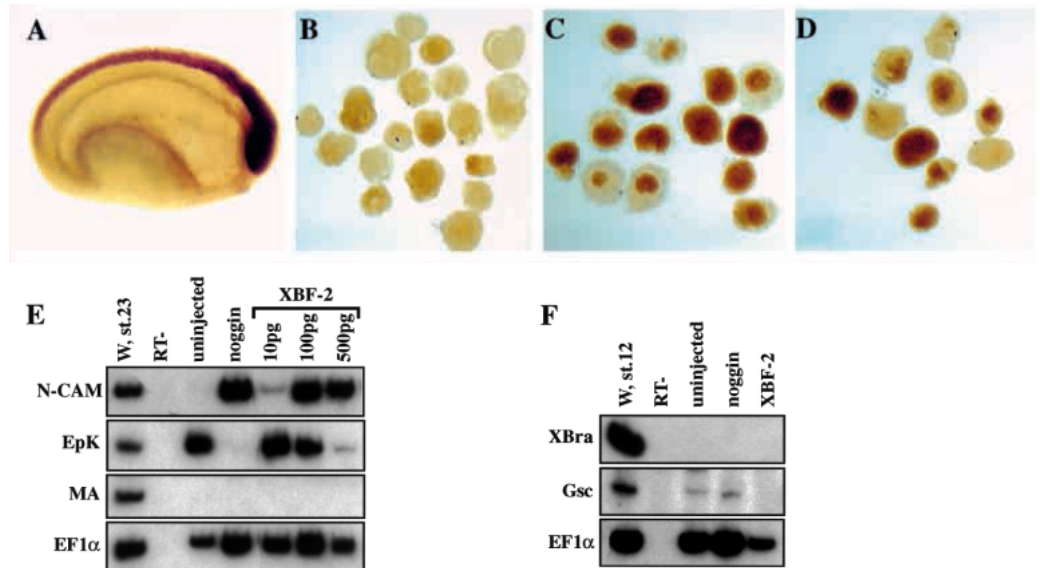
**Fig. 2.** In situ hybridization for XBF-2. (A) XBF-2 is first expressed in the anterior neural plate at stage 12.5. (B) By stage 14, XBF-2 is also expressed in cells lateral to the midline. (C) Stage 17 embryo showing the neural folds nearly closed. (D) Stage 21 embryo showing expression in the neural tissue of the tailbud and also in cells lateral to the somites. (E) Stage 36, XBF-2 is clearly expressed within the forebrain and temporal retina; there is also continued expression within the neural tissue of the tailbud and in the proctodeal region.

### XBF-2 converts ectoderm into neural tissue

Expression cloning had already indicated that XBF-2 is capable of converting ectoderm into neural tissue. In order to characterize more fully the activity of XBF-2, we examined the explants for the expression of a number of tissue-specific markers. Ectodermal explants expressing XBF-2 RNA were subjected to immunohistochemistry with the neural-specific mAb 6F11 (Fig. 3). As expected, uninjected explants are

**Fig. 3.** XBF-2 neuralizes ectoderm at the expense of epidermis.

(A) Embryo at stage 21 stained by immunohistochemistry using the neural-specific mAb 6F11. (B-D) Shows ectodermal explants fixed at the equivalent stage. As expected, uninjected explants (B) show no HRP staining while explants expressing 100 pg Noggin mRNA (C) are positive in this assay. (D) Explants expressing 1 ng mRNA from the full-length XBF-2 plasmid are strongly neuralized. (E) RT-PCR of explants expressing increasing amounts of XBF-2 RNA are diverted from the epidermal fate to the neural fate. Like explants treated with noggin protein (0.5  $\mu$ g/ml), as the explant becomes more neuralized and upregulates neural-specific N-CAM expression, the explant is epidermalized and epidermal Keratin (EpK) expression declines. The lack of Muscle Actin (MA) expression shows that neural tissue forms without a mesodermal intermediary at this stage. EF1 $\alpha$  indicates total mRNA levels assayed, W, whole embryo control. (F) In addition, explants analyzed at an earlier stage, stage 12, do not express the panmesodermal marker Xbrachyury (Xbra) nor the dorsal mesoderm marker, Goosecoid (Gsc). Explants were injected with 100 pg noggin or 1 ng full-length XBF-2 mRNA.



negative in this assay (Fig. 3B). However, like explants injected with noggin RNA (Fig. 3C; Lamb et al., 1993), those injected with XBF-2 RNA become neuralized and express the 6F11 antigen (Fig. 3D). As increasing amounts of XBF-2 RNA are injected, explants analyzed by RT-PCR show increased expression of N-CAM at the expense of epidermal Keratin (Fig. 3E). Thus, as explants are increasingly more neuralized by XBF-2, they are diverted from the epidermal fate. To address whether neural tissue was induced secondarily by mesoderm in the explant, explants expressing XBF-2 RNA were analyzed for mesodermal markers. At the gastrula stage, the explants do not express the mesoderm-specific markers goosecoid or brachyury, and at the neurula stage (stage 21), they do not express muscle-specific actin (Fig. 3E,F). Thus, XBF-2 neuralizes ectoderm without first inducing mesoderm.

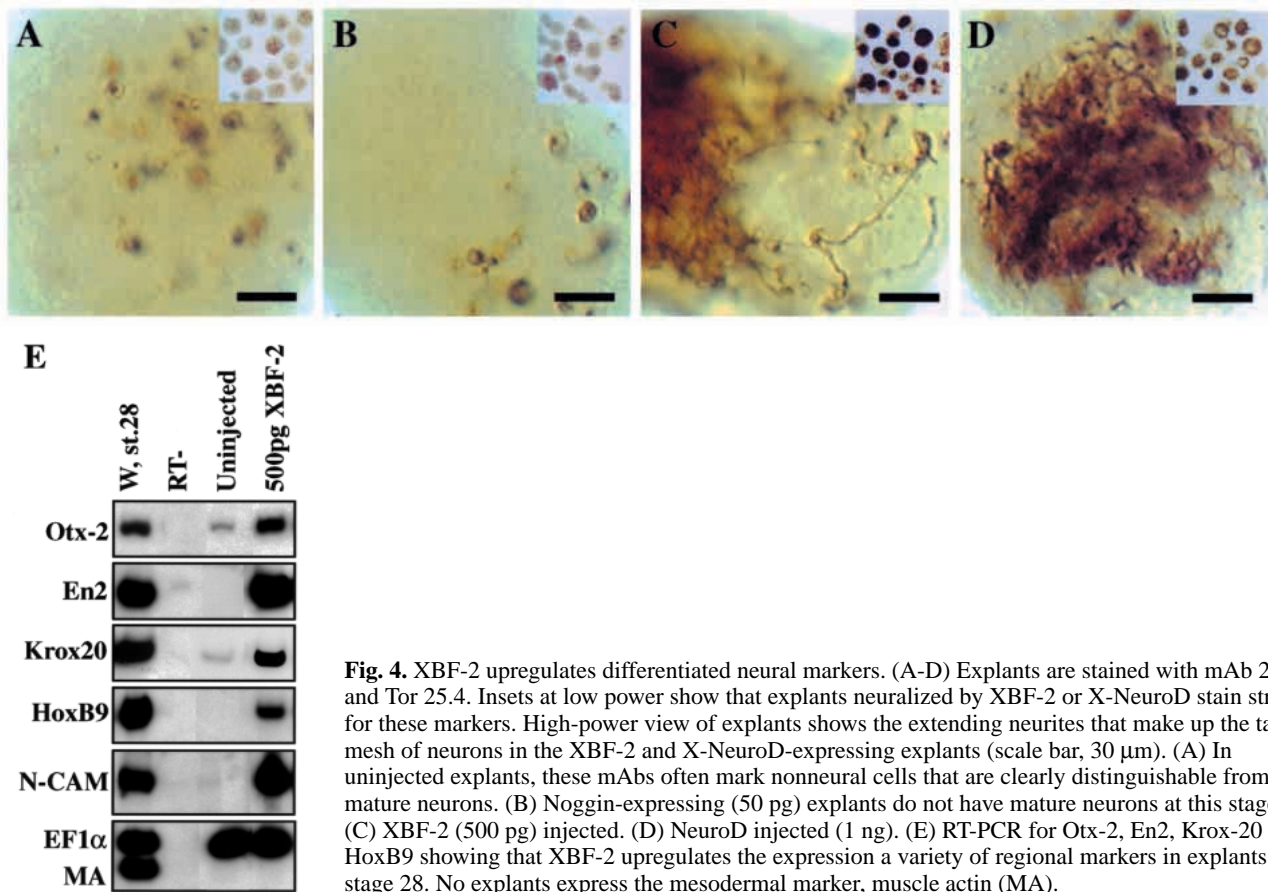
The observation that XBF-2 is expressed in the presumptive CNS and has neuralizing activity led us to test whether XBF-2 is sufficient to cause neuronal differentiation. At the late neurula stage, mAb Tor25.4 (Kushner, 1984) recognizes an epitope on the surface of axons that are in the process of outgrowth (A. Hemmati Brivanlou, F. V. M., R. M. H., unpublished observations). Unlike explants neuralized by noggin (Fig. 4B), XBF-2-injected explants contain cells with distinct axonal profiles characteristic of differentiated neurons (Fig. 4C). XBF-2-injected explants contain dense clumps of neurons whose processes extend within the explant to form a tangled basket of axons much like that seen in X-NeuroD-expressing explants (Fig. 4D). Therefore, we show that not only can XBF-2 neuralize ectodermal tissue but is sufficient to cause neuronal differentiation.

Explants expressing XBF-2 were also analyzed for the expression of regional neural markers by RT-PCR (see Fig. 4E). The neural inducer noggin, as well as other BMP antagonists, induces neural tissue that is anterior rather than

posterior in character (Lamb et al., 1993). Like noggin-neuralized explants, XBF-2-expressing explants strongly express the anterior gene *Otx-2*, which marks the midbrain, forebrain and eyes (Lamb et al., 1993). To our surprise, unlike neural tissue induced by noggin, neural tissue formed by XBF-2 also exhibits strong expression of more posterior CNS markers including *Engrailed 2* (Hemmati-Brivanlou and Harland, 1989), *Krox-20* (Bradley et al., 1993) and the spinal cord marker *HoxB9* (Sharpe et al., 1987).

In order to examine the effect of XBF-2 in the context of embryonic signaling, we injected mRNA into whole embryos. Embryos were injected at the 2-cell stage in the animal pole of one of the two blastomeres. This allowed us to examine the effect of mRNA injection in approximately half of the embryo while keeping the other half as a control. Embryos were cultured until late neurula stages when they were analyzed for the expression of neural markers. When embryos were examined for the expression of the pan-neural markers N-CAM, *nrl1* or the 6F11 antigen, we saw a remarkable expansion of neural tissue on the injected side (Fig. 5B-D,G-I). Co-injections with a lineage tracer demonstrated that the expansion of neural tissue was coincident with the side of injection; XBF-2-injected cells contributed to the neural ectoderm on the injected side (Fig. 5S). In fact, increasing amounts of injected XBF-2 RNA correlated with increasing expansion of the region that was neuralized. At high doses the neuralized field extended all the way around to the ventral side of the embryo. At all doses, the ectopic neural tissue contacted the endogenous neural plate and was composed of a uniform field of neural cells. This contrasts with the phenotype generated by neurogenin overexpression, which results in patches of neural cells on the injected side, but not in a continuous expansion of the neural plate (Fig. 5E,J; Ma et al., 1996).

Embryos were examined at the earlier open neural plate



**Fig. 4.** XBF-2 upregulates differentiated neural markers. (A-D) Explants are stained with mAb 2G9 and Tor 25.4. Insets at low power show that explants neuralized by XBF-2 or X-NeuroD stain strongly for these markers. High-power view of explants shows the extending neurites that make up the tangled mesh of neurons in the XBF-2 and X-NeuroD-expressing explants (scale bar, 30  $\mu$ m). (A) In uninjected explants, these mAbs often mark nonneural cells that are clearly distinguishable from mature neurons. (B) Noggin-expressing (50 pg) explants do not have mature neurons at this stage. (C) XBF-2 (500 pg) injected. (D) NeuroD injected (1 ng). (E) RT-PCR for Otx-2, En2, Krox-20 and HoxB9 showing that XBF-2 upregulates the expression a variety of regional markers in explants at stage 28. No explants express the mesodermal marker, muscle actin (MA).

stage for the expression of neural-specific  $\beta$ -tubulin (N-tubulin), a marker of the primary neurons (Chitnis et al., 1995; Good et al., 1989). Instead of the characteristic three stripes of primary neurons, the injected side lost all of the N-tubulin-expressing neurons or was left with scattered N-tubulin-expressing cells (Fig. 5R). Thus, the increase in the field expressing general neural markers and the loss of differentiating cells suggests that XBF-2-expressing cells contribute to a proliferating neural plate and are delayed from differentiating.

Neural tissue in embryos injected with XBF-2 is formed at the expense of other ectodermally derived tissues. Embryos expressing XBF-2 unilaterally typically lack anterior features such as eyes and cement glands (data not shown). On the injected side, there is a loss of slug and twist expression and therefore a loss in cranial crest derivatives (Fig. 5N,P; Mayor et al., 1995; Hopwood et al., 1989). Embryos that are stained for an epidermal marker, epidermal Keratin, show a striking loss of expression on the injected side (Fig. 5L), illustrating that, in XBF-2-injected embryos, epidermis is lost in compensation for the expansion of neural tissue.

#### BMP antagonists induce XBF-2

XBF-2 could neuralize by upregulating the expression of BMP antagonists which would subsequently induce neural tissue. Alternatively, XBF-2 may lie downstream of these molecules. In order to test these possibilities, explants injected with XBF-2 were assayed for the expression of *noggin*, *cerberus* or

*chordin*. None of these genes was upregulated by XBF-2 (Fig. 6E). However, explants expressing *noggin*, *cerberus* or *gremlin* (another BMP antagonist that is expressed during the tailbud stage; Hsu et al., 1998), exhibited strong induction of XBF-2 (Fig. 6B-D). Thus, in the explant assay, blocking BMP signaling with a BMP antagonist leads to the upregulation of XBF-2. Consistent with its time of expression, XBF-2 lies downstream of the organizer-specific BMP antagonists in the neural induction pathway.

We also investigated the effect that XBF-2 may have on the expression of BMP-4. Embryos were injected with XBF-2 RNA at the 1-cell stage, and animal cap explants were taken at blastula stages and cultured until stage 10.5, when they were fixed for analysis by in situ hybridization. BMP-4 is expressed in non-neural ectoderm at this stage (Fig. 6F; Fainsod et al., 1994) and has been shown to have epidermis-inducing activity (Wilson and Hemmati-Brivanlou, 1995). As expected, uninjected explants stained strongly for BMP-4 expression (Fig. 6G). Surprisingly, however, XBF-2-injected explants exhibited a dramatic decrease in BMP-4 expression (Fig. 6H). Thus, XBF-2 upregulates the expression of neural-specific markers while at the same time downregulating the expression of an epidermal inducer, BMP-4.

#### XBF-2 has transcriptional repressor activity

Since XBF-2 is a winged helix transcription factor, we next wanted to determine whether it is responsible for activating or repressing transcription. We fused the DNA-binding domain of



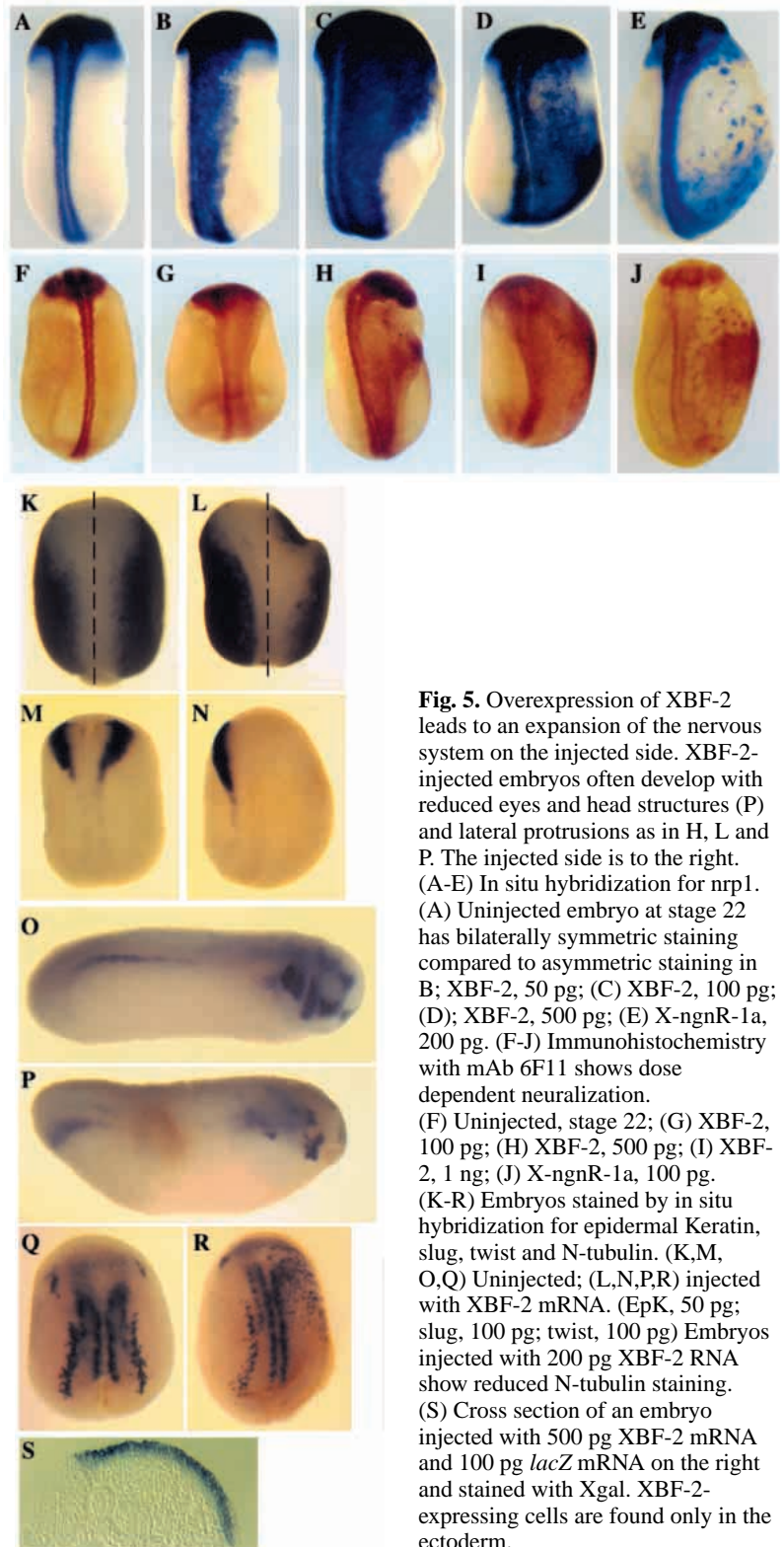
XBF-2 to previously characterized activating or repressing domains, including the repressor domain from *Drosophila* engrailed (EnR; Jaynes and O'Farrell, 1991), the repressor domain of hairy (Barolo and Levine, 1997) and the activator domain of VP16 (Sadowski et al., 1988; Fig. 7A). Fusion constructs were tested in the ectodermal explant assay and within the context of the whole embryo – the same assays used to characterize the activity of XBF-2. The DNA-binding domain alone was also tested for activity.

When mRNA from the repressor constructs EnR::XBF and XBF::hairy was injected, neural tissue formed at the expense of epidermal tissue in whole embryos (Fig. 7C,E,G) and in ectodermal explants (Fig. 7H) just as with wild-type XBF-2. The DNA-binding domain alone, although properly localized to the nucleus (data not shown), was incapable of inducing neural tissue or neural plate expansion (Fig. 7I). Thus, the neuralizing activity of EnR::XBF is mediated by the repressor function of the construct (from En or hairy) rather than by the XBF-2 DNA-binding domain. Since the activity of these repressor fusions phenocopies the effect of overexpression of XBF-2 in both the explant assay and in the context of the whole embryo, XBF-2 likely acts as a transcriptional repressor.

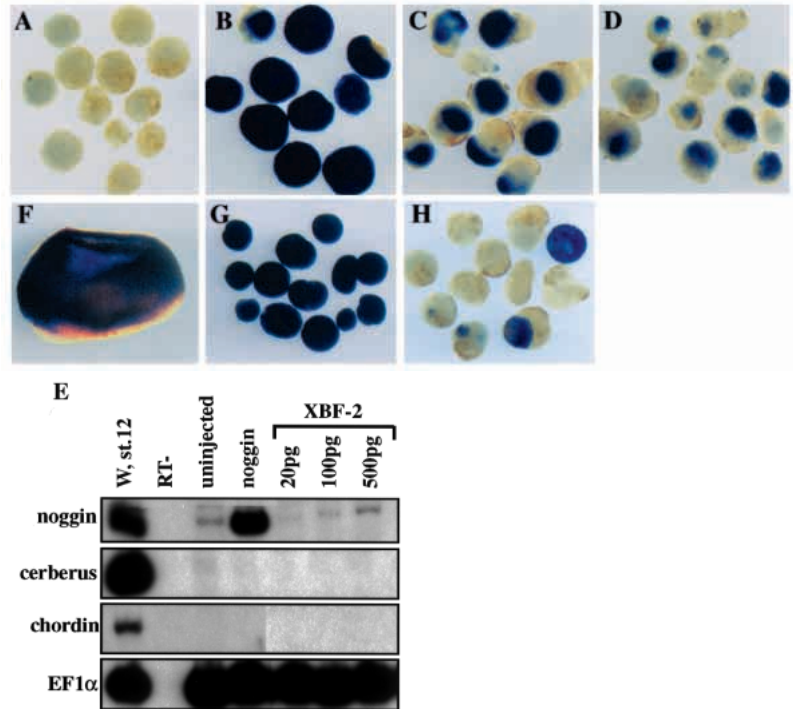
In further support of this idea, the XBF DNA-binding domain fused to the VP16 activator, XBF::VP16 is capable of reversing the neuralizing effects of XBF-2. As increasing amounts of XBF::VP16 were coinjected with XBF-2 RNA, N-CAM expression decreases in the explant (Fig. 7I). XBF::VP16 presumably interferes with XBF-2 activity by binding to XBF-2-binding sites and upregulating genes normally repressed by XBF-2. The EnR::XBF and XBF::VP16 results suggest that the conversion of ectoderm into neural tissue by XBF-2 is mediated specifically through transcriptional repression.

In order to identify the region or regions of XBF-2 with repressor activity and to demonstrate repressor activity with a heterologous DNA-binding domain, the amino-terminal or the carboxy-terminal portion of XBF-2 was fused in frame to the GAL4 DNA-binding domain. Each of the fusion proteins was transfected into COS cells along with a reporter plasmid containing two GAL4 DNA-binding sites upstream of the thymidine kinase promoter driving Firefly luciferase (Fig. 7A). COS cells were also transfected with a plasmid expressing Renilla luciferase to monitor transfection efficiency and cell survival. Renilla and Firefly luciferase use different substrates, so their activities are strictly separable. In the COS cell assay, both the N-terminal and C-terminal fusions have repressor activity (Fig. 7J); the C-terminal fusion is an especially potent repressor. Repression is apparent when as little as 0.1 ng of the fusion is transfected with 2 µg of reporter plasmid and 0.1 µg fusion decreases luciferase activity 80-fold. The N-terminal fusion is less potent; 0.1 µg transfected reduces luciferase activity only 8-fold.

Surprisingly, this repression is seen even in control experiments where the reporter lacks GAL4-binding sites (Fig. 7J; see Discussion). However, despite the observation that the specific GAL4-binding sites are not essential for the repression of luciferase expression, the COS cells transfections further demonstrate that XBF-2 has repressor function and, in



**Fig. 5.** Overexpression of XBF-2 leads to an expansion of the nervous system on the injected side. XBF-2-injected embryos often develop with reduced eyes and head structures (P) and lateral protrusions as in H, L and P. The injected side is to the right. (A-E) In situ hybridization for *nrp1*. (A) Uninjected embryo at stage 22 has bilaterally symmetric staining compared to asymmetric staining in B; XBF-2, 50 pg; (C) XBF-2, 100 pg; (D); XBF-2, 500 pg; (E) X-*ngnR-1a*, 200 pg. (F-J) Immunohistochemistry with mAb 6F11 shows dose dependent neuralization. (F) Uninjected, stage 22; (G) XBF-2, 100 pg; (H) XBF-2, 500 pg; (I) XBF-2, 1 ng; (J) X-*ngnR-1a*, 100 pg. (K-R) Embryos stained by in situ hybridization for epidermal Keratin, slug, twist and N-tubulin. (K,M, O,Q) Uninjected; (L,N,P,R) injected with XBF-2 mRNA. (EpK, 50 pg; slug, 100 pg; twist, 100 pg) Embryos injected with 200 pg XBF-2 RNA show reduced N-tubulin staining. (S) Cross section of an embryo injected with 500 pg XBF-2 mRNA and 100 pg *lacZ* mRNA on the right and stained with Xgal. XBF-2-expressing cells are found only in the ectoderm.



**Fig. 6.** (A-D) XBF-2 is upregulated by BMP antagonists. RNA in situ hybridization of explants at stage 22 for XBF-2 expression showing that blocking BMP signaling with secreted BMP antagonists induces the expression of XBF-2. Explants that were injected with noggin, cerberus and gremlin RNA at doses known to induce neural tissue (this was verified by analyzing sibling explants for N-CAM expression) induce XBF-2 expression. (A) Water injected; (B) Noggin injected, 500 pg; (C) Cerberus injected, 1 ng; (D) Gremlin injected, 500 pg. Explants expressing XBF-2 (E) do not upregulate the expression of noggin, cerberus or chordin, nor do explants expressing noggin RNA (100 pg). PCR amplified band in the noggin lane detects the injected message. (F-H) XBF-2 downregulates the expression of BMP-4. In situ hybridization with BMP-4 probe showing that explants expressing XBF-2 downregulate the expression of an epidermal inducer. (F) Embryo at stage 10.5 showing the intensity of BMP-4 staining in this experiment. (G) Uninjected explants express BMP-4 strongly. (H) By stage 10.5, XBF-2 (200 pg)-injected explants do not express BMP-4.

addition, suggests that there may be multiple domains within XBF-2 responsible for this activity.

### Expression of the XBF::VP16 fusion disrupts neural tissue development

The demonstration that the XBF::VP16 fusion blocks the neuralizing effects of XBF-2 and that XBF-2 can act as a transcriptional repressor, prompted us to examine the effect of XBF::VP16 fusion on normal development. We therefore injected embryos with XBF::VP16 mRNA into one blastomere at the 2-cell stage. Injected embryos cleaved, gastrulated and neurulated normally; thus, the XBF::VP16 fusion has no gross effect on these developmental processes. However, when embryos are analyzed at stage 21 by in situ hybridization, expression of the general neural marker *nrp1* decreased dramatically on the injected side along the entire extent of the embryo (20 out of 27 embryos affected; see Fig. 8B). Other neural markers, *Otx2* and *HoxB9*, also exhibit reduced expression (data not shown). This reduction in neural tissue is reversible since embryos injected with both XBF::VP16 fusion as well as XBF-2 mRNA have expanded *nrp1* staining on the injected side (22 of 22 embryos rescued; see Fig. 8C). Epidermal Keratin expression is also reduced on the injected side (Fig. 8E) suggesting that the fusion protein affects cell fate outside the neural plate.

### The mouse homologue is also active in *Xenopus*

In order to test the specificity of XBF-2, other winged-helix transcription factors were tested in the explant assay. Pintallavis is a winged-helix protein that can induce ectopic floor plate in *Xenopus* embryos but is incapable of inducing the expression of a neural-specific antigen marker recognized by the antibody Xen1 in ectodermal explants (Ruiz i Altaba and Jessell, 1992). As expected, in our assays, pintallavis does not induce neural tissue (Fig. 9). Thus, simply overexpressing a

winged-helix transcription factor does not convert ectoderm into neural tissue, implying that the effect of XBF-2 is specific. However, the product of the mouse gene, *BF-2* (Hatini et al., 1996), the probable homologue of *XBF-2*, was able to neuralize explants of *Xenopus* ectoderm, despite extensive divergence of sequence. Further studies will be needed to determine whether any of the regions shared among BF-2 homologues are the domains responsible for repressor function (Fig. 1).

## DISCUSSION

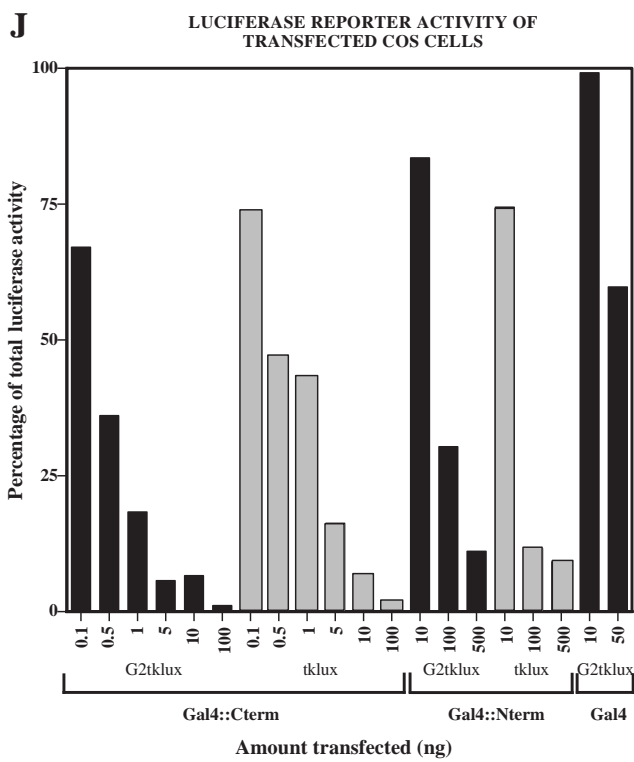
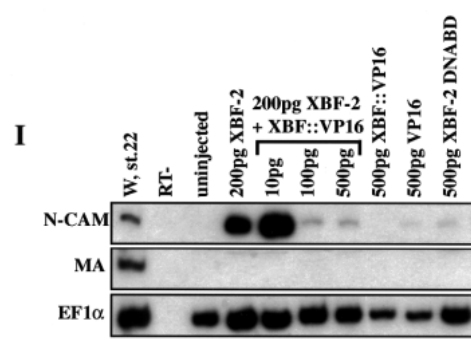
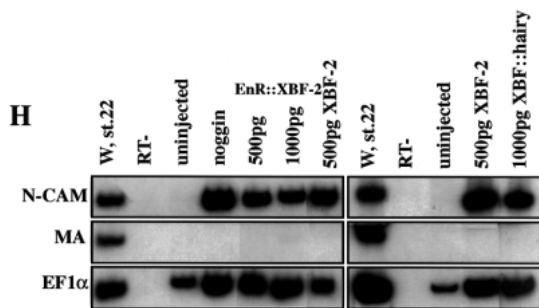
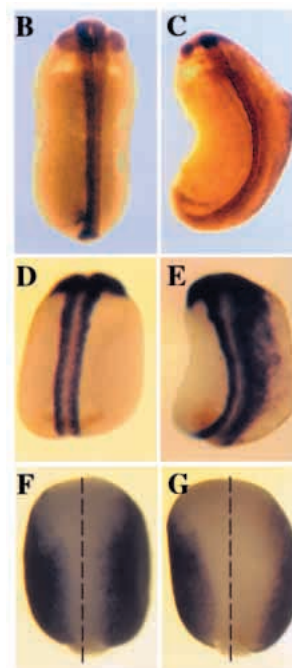
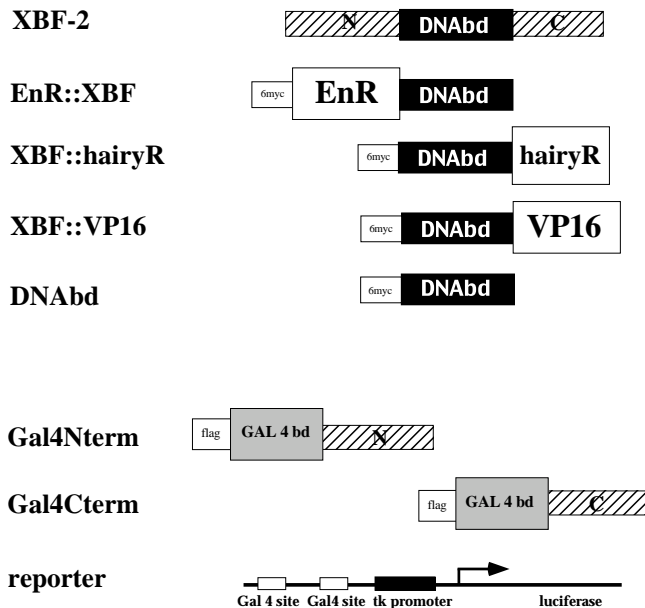
### XBF-2 converts ectoderm into neural tissue

We have shown that XBF-2 has neuralizing activity in ectodermal explants as well as within the context of the embryo. Explants expressing XBF-2 RNA not only consist of neural tissue but also contain mature neurons with extended neurites (Figs 3A-E, 4A-D). These explants do not contain mesoderm that could have induced neural tissue secondarily. XBF-2 is normally expressed in the brain and developing tailbud (Fig. 2). Consistent with this, XBF-2 induces the expression of both anterior and posterior markers in animal cap explants. These markers include the anterior CNS marker, *Otx-2*, the midbrain and hindbrain markers, *En2* and *Krox-20*, and even the spinal cord marker, *HoxB9* (Fig. 4E). If XBF-2 only specifies the most anterior and posterior fates (consistent with its expression) then the intermediate fates must be induced indirectly (see Lamb and Harland, 1995).

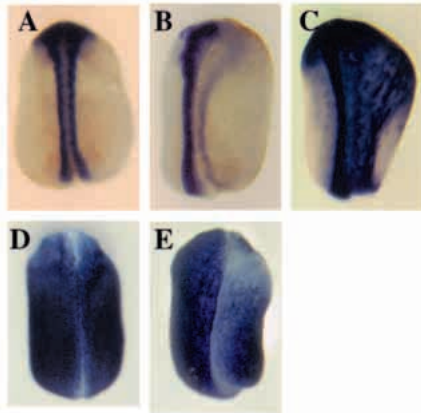
Within the context of the embryo, cells that would not ordinarily express XBF-2 and would not ordinarily become neurons are converted to neurons by XBF-2 mRNA overexpression. In injected embryos, ectoderm extending to the ventral midline and along the entire axis can be converted to the neural fate (Fig. 5B-D,G-I). As with injection of noggin mRNA, this neural tissue forms at the expense of nonneural



**A.**



**Fig. 7.** XBF-2 fusion protein activity. (A) Schematic diagram of fusion constructs used for COS cell transfections and for expression in *Xenopus* tissue. (B-E) Injection of the EnR::XBF fusion protein RNA into embryos phenocopies the overexpression of XBF-2. Immunohistochemistry with mAb 6F11 and in situ hybridization for N-CAM shows an expanded neural plate in EnR::XBF-injected embryos; embryos injected with the EnR alone do not have expanded neural tissue. EnR::XBF-injected embryos also exhibit a compensatory loss of epidermal Keratin expression. (B) Uninjected, mAb 6F11; (C) EnR::XBF, 500 pg, mAb 6F11; (D) EnR alone, N-CAM probe; (E) EnR::XBF, 500 pg, N-CAM probe; (F) Uninjected, epidermal Keratin probe; (G) EnR::XBF, 500 pg, epidermal Keratin probe. (H) Repressor fusions also have neuralizing activity in the ectodermal explant assay. Both the EnR::XBF fusion and the XBF::hairy fusion upregulate N-CAM expression. (I) The XBF::VP16 fusion can act as a dominant negative. Increasing amounts of XBF::VP16 titrate out the neuralizing effect of XBF-2. Neither the fusion alone, VP16 alone or the DNA-binding domain of XBF-2 can induce N-CAM expression. (J) COS cells transfected with both Gal4::Cterm or Gal4::Nterm along with a luciferase reporter. The data is expressed as a percentage of the luciferase activity of each reporter plasmid when transfected alone. Filled bars represent transfections with pG2tklux (contains two Gal4-binding sites), stippled bars represent transfections with ptklux (no Gal4-binding sites).

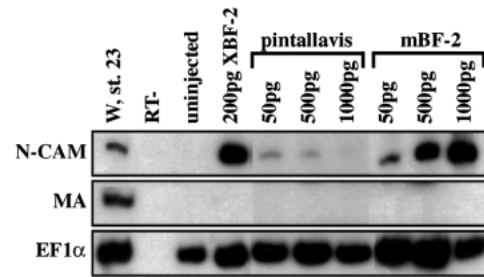


**Fig. 8.** Expression of the XBF::VP16 fusion disrupts neural tissue development. Embryos were injected into one blastomere at the 2-cell stage and then processed for RNA in situ hybridization with the general neural marker, *nrp1*. Embryos injected with 400 pg of the XBF::VP16 fusion, (B), exhibit a striking loss of *nrp1* staining on the injected side. This could be rescued with a coinjection of 50pg XBF-2 mRNA (C). Embryos were also examined for the expression of epidermal Keratin (D, uninjected). Those injected with 400 pg of the XBF::VP16 fusion loose epidermal Keratin expression on the injected side (E).

tissue; there is a decrease in the amount of epidermis, as measured by epidermal Keratin (Fig. 5L), and neural crest, as measured by *slug* and *twist* expression (Fig. 5N,P); there is also a suppression and loss of anterior structures. Thus, as shown by several assays, XBF-2 converts ectoderm into neural tissue at the expense of nonneural tissue.

Ectopic expression of neurogenin and neuroD promote the formation of ectopic mature neurons in *Xenopus* embryos (Lee et al., 1995; Ma et al., 1996). At early neural plate stages, neurogenin induces a wide swath of primary neurons on the injected side. At later stages, however, the neural tube is not expanded but many isolated patches of primary neurons persist lateral to the neural tube (Fig. 5E,J). In contrast, already at the neural plate stage, ectopic XBF-2 mRNA reduces primary neuron number or induces only scattered patches of primary neurons. This is similar to the activities of Xiro3 (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998) or XASH-3 (Chitnis and Kintner, 1996). Chitnis and Kintner (1996) suggest that neurogenic transcription factors differ in their sensitivity to lateral inhibitory mechanisms mediated by X-Notch-1 and X-Delta-1; they also induce expression of the inhibitory delta ligand. Thus, small amounts of XASH-3 induce neurogenesis in a scattered pattern while greater amounts induce delta expression and suppress primary neuron formation. Although XBF-2 differs from XASH-3 in its ability to induce neural fates far from the dorsal midline, XBF-2 may be similar to XASH-3 in its effects on lateral inhibition. Whether or not the disruption in N-tubulin expression is due to a sensitivity to lateral inhibition or some other mechanism is yet to be determined. Clearly this early block to neural differentiation is not permanent as XBF-2 animal cap tissue, when analyzed at later stages, contains fully differentiated neurons (see Fig. 4C).

Although few or scattered primary neurons form on the XBF-2-injected side, XBF-2 embryos have an expanded neural plate consisting of a contiguous field of neural cells. This



**Fig. 9.** The mouse homologue, BF-2 also neuralizes ectodermal explants and induces N-CAM expression in the absence of muscle. Pintallavis-expressing explants do not upregulate N-CAM.

neural plate expands along the rostral-caudal axis of the embryo and borders the neural tube. Again, this is in contrast to the effects of ectopic NeuroD or Neurogenin where the neural tube is not expanded and is more similar to the effects of ectopic XASH-3, which can also lead to a modest expansion of the neural plate. Only some of the cells that make up the expanded neural field on the XBF-2-injected side initially differentiate into primary neurons, leaving the remaining cells predominantly in an undifferentiated state. The idea that XBF-2 can specify neural precursors correlates with the expression of XBF-2 in the forebrain and tailbud where neural precursors are born and remain in an undifferentiated stem cell state after their siblings in the spinal cord have begun to differentiate (Hartenstein, 1993; Knecht et al., 1995). The correlation of XBF-2 expression and the undifferentiated state extends to the mouse *BF-2* gene which is expressed at its highest levels in the ventricular zone of the developing diencephalon (Hatini et al., 1994).

### XBF-2 lies downstream of the BMP antagonists and downregulates BMP-4 expression

Endogenous XBF-2 expression begins at the early neural plate stage after the time expression of the known secreted neural inducers has initiated. This suggested that XBF-2 might lie downstream of BMP antagonism in the genetic pathway to neural tissue. Indeed, we have demonstrated that XBF-2 is strongly induced by noggin and other BMP antagonists whereas XBF-2 is incapable of upregulating noggin, chordin or cerberus expression (Fig. 6A-E).

Explants expressing XBF-2 exhibit a dramatic decrease in the expression of the epidermal inducer BMP-4 (Fig. 6H). Thus, neural tissue may form because XBF-2 blocks BMP-4 signaling at the transcriptional level. Whether the lack of BMP transcription is sufficient to determine neural fates or whether XBF-2 provides additional information to determine neural fates has not been determined.

We have shown that ectopic XBF-2 inhibits the expression of the neural crest markers *slug* and *twist* (Fig. 5N,P). Simply inducing an expansion of the neural plate does not have this effect. Ectopic *Zic3* or *Zicr-1*, for example, results in an expanded neural tube as well as a marked increase in the number of *slug*- and *twist*-expressing cells on the injected side (Nakata et al., 1997; Mizuseki et al., 1998a). Liem et al. (1995, 1997) have shown that neural crest cells can be induced by BMP-4. Since XBF-2 represses BMP-4 expression, a loss of BMP-4 due to ectopic XBF-2 expression could lead to decrease

in slug and twist expression. It is important to note that XBF-2 cannot be functioning solely to inhibit BMP signaling, since its effects on anterior-posterior identity and neuronal differentiation in animal cap explants are very different from those of extracellular or intracellular BMP antagonists.

### **XBF-2 acts as a transcriptional repressor**

We have demonstrated that XBF-2 has repressor activity by several criteria. Within the context of the embryo, we tested fusions with protein domains already known to have repressive or activating transcriptional activities: the repressor domains from the *Drosophila* engrailed or hairy proteins (Jaynes and O'Farrell, 1991) and the activator domain from HSV VP16 (Sadowski et al., 1988). This kind of approach has been used to characterize the activity of several transcriptional activators involved in *Xenopus* development (Conlon et al., 1996; Ryan et al., 1996; Fan and Sokol, 1997; Kessler, 1997; Horb and Thomsen, 1997). By fusing these domains to the XBF-2 DNA-binding domain, we were able to confirm the repressive activity of XBF-2. For example, when the En repressor domain (or the hairy repressor domain) is fused to the XBF-2 DNA-binding domain, this fusion has the same biological activity as the wild-type XBF-2 molecule. The EnR::XBF fusion neuralizes explants and causes an expansion of the neural plate in whole embryos at the expense of epidermis (Fig. 7C,E,G). Since only the fusion of a repressor domain to the XBF-2 DNA-binding domain has neuralizing activity (the DNA-binding domain alone and the EnR do not neuralize (Fig. 7D,H)), we have demonstrated that the full-length XBF-2 converts ectoderm into neural tissue by acting as a repressor. Conversely, when the VP16 fusion protein is coexpressed with the full-length XBF-2 RNA, it acts as an antagonistic molecule and prevents the neuralizing activity of XBF-2. The XBF::VP16 fusion protein likely upregulates genes that would ordinarily be repressed by XBF-2.

In a transfection assay, we tested the N- or C-terminal half of XBF-2 fused to the Gal4 DNA-binding domain. The COS cell assays suggest that both the N terminus and in particular the C terminus are capable of mediating repression. These experiments have not ruled out the possibility that the XBF DNA-binding domain is also involved in repression (as with the *msx-1* DNA-binding domain (Zhang et al., 1996)), but do demonstrate that the N and C termini are likely sufficient to mediate repression in the absence of the XBF-2 DNA-binding domain. Surprisingly, the fusion proteins can still mediate repressor activity in the absence of GAL4 DNA-binding sites. This suggests several possibilities: (1) that DNA binding is not required for their repressive activity, (2) that the fusion proteins are acting by interacting directly with other transcription factors or even the basal transcriptional machinery, or (3) that the GAL4 fusions can bind to some cryptic site in the thymidine kinase promoter of the reporter construct. In embryos, the neuralizing activity of the molecule does require the XBF-2 DNA-binding domain (not shown), confirming that embryonic assays are more stringent than the COS cell assays.

### **Is XBF::VP16 a dominant negative molecule?**

If the XBF::VP16 fusion only interfered with wild-type XBF-2 function, then it would be expected to suppress neural specification only where XBF-2 is normally expressed.

However, the XBF::VP16 fusion inhibits neural development wherever it is expressed (Fig. 8B). Furthermore, instead of prospective neural cells being diverted towards epidermal fates, the injected cells do not express epidermal cytokeratins, and high levels of XBF::VP16 even suppress cytokeratin expression in the prospective epidermis (Fig. 8E). These and other results with such fusions must be interpreted in light of their likely mechanism of action. The strong transcriptional activation or repressor domain will act wherever there is an accessible XBF-2-binding site and not just in cells that express XBF-2. This will inevitably lead to pleiotropic effects on differentiation by altering transcription levels in the vicinity of XBF-2-binding sites. An extreme view would be that such fusions are simply toxic and suppress any kind of differentiation. A strong argument against non-specific toxicity comes from rescue experiments, where modest amounts of XBF-2 can neuralize in the presence of the XBF::VP16 fusion (see Fig. 8C). These experiments demonstrate that neural specification requires that transcription must be inhibited in the region of XBF-2-binding sites; however, it would be premature to conclude that XBF-2 itself is essential for neural differentiation. From these and results with the mouse knockout (Hatini et al., 1996), it seems more reasonable to suggest that normal neural differentiation employs many factors, some of which are redundant.

### **Conservation of XBF-2 function**

Since the mouse homologue, BF-2, is also active in the neural induction assay, it is possible that it also acts as a transcriptional repressor. Sequence comparison identifies three regions of similarity outside the DNA-binding domain that are conserved among the mouse, human, rat and chicken homologues; these do not share obvious similarity to known repressor motifs. A more detailed analysis will be required to identify which, if any, of these amino acid sequences are responsible for transcriptional repression.

In the mouse, BF-2 is expressed in the forebrain, specifically in the temporal retina, optic stalk, thalamus and hypothalamus, and in the kidneys. Mice lacking BF-2 function have major anomalies in the kidneys and only subtle abnormalities in the retina, forebrain and adrenal gland (Hatini et al., 1996). It is possible that the BF-2 mutant mouse has no obvious loss of neural tissue because of the presence of other neuralizing genes with overlapping expression patterns in the forebrain. The lack of reciprocity between the mouse knockout and *Xenopus* overexpression studies emphasizes that, in order to find genes that contribute to neural identity, it will be necessary to identify genes by gain of function as well as by loss of function.

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