**XBF-1**, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in *Xenopus* competent ectoderm

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**SUMMARY**

Neuronal differentiation in the vertebrate nervous system is temporally and spatially controlled by mechanisms which are largely unknown. Here we investigate the role of XBF-1, an anterior neural plate-specific winged helix transcription factor, in controlling the pattern of neurogenesis in *Xenopus* ectoderm. We show that, in the anterior neural plate of normal embryos, prospective neurogenesis is positioned at the anterior boundary of the XBF-1 expression domain. By misexpressing XBF-1 in the posterior neural plate we show that a high dose of XBF-1 has a dual effect; it suppresses endogenous neuronal differentiation in high expressing cells and induces ectopic neuronal differentiation in adjacent cells. In contrast, a low dose of XBF-1 does not suppress but instead, expands the domain of neuronal differentiation in the lateral and ventral sides of the embryo. XBF-1 regulates the expression of *XSox3*, *X-ngnr-1*, *X-Myt-1* and *X-Delta-1* suggesting that it acts early in the cascade leading to neuronal differentiation. A fusion of XBF-1 to a strong repressor domain (EnR) mimics most of the XBF-1 effects suggesting that the wild type XBF-1 is a transcriptional repressor. However, fusion of XBF-1 to a strong activation domain (E1A) specifically suppresses neuronal differentiation suggesting that XBF-1 may also work as a transcriptional activator. Based on these findings, we propose that XBF-1 is involved in positioning neuronal differentiation by virtue of its concentration dependent, dual activity, as a suppressor and an activator of neurogenesis.

Key words: XBF-1, qin, Neurogenesis, Forebrain, Neuronal patterning, *Xenopus laevis*

**INTRODUCTION**

During embryonic development, the ectoderm on the dorsal side of the gastrula embryo is induced to form the neuroectoderm which contains the precursor cells of the vertebrate nervous system. The neuroectoderm is induced by signals from the organiser (reviewed in Saxen, 1989), which antagonise BMP-4, an epidermalising signal (reviewed in Tanabe and Jessell, 1996; Bier, 1997). In fish and amphibians, a number of neuroectodermal cells exit the cell cycle as early as the end of gastrulation and start differentiating (Hartenstein, 1989). These early differentiating neurons form a primary nervous system and many are later replaced by secondary neurons (Forehand and Farel, 1982). Because of its simple organisation and accessibility, the primary nervous system is an excellent model system in which to study the interactions that lead to neuronal differentiation.

Neuronal differentiation (neurogenesis) within the neural ectoderm is thought to be under the control of proneural and neurogenic genes. Proneural genes, which are typically members of the bHLH family of transcription factors are thought to confer neuronal potential in ectodermal cells (Guillemot et al., 1993; Zimmerman et al., 1993; Ferreiro et al., 1994; Turner and Weintaub, 1994; Lee et al., 1995; Ma et al., 1996; Bellefroid et al., 1996; Takebayashi et al., 1997; Kim et al., 1997; Ravassard et al., 1997; Bellefroid et al., 1998; Dubois et al., 1998). It has been suggested that neuroectodermal cells go through successive stages of specification/commitment, defined by the sequential activation of different sets of proneural transcription factors. These proneural genes have been termed 'neuronal determination' or 'neuronal differentiation' genes, depending on whether they act early or late, respectively, in the regulatory cascade that leads to neuronal differentiation (Lee et al., 1995; Chitnis and Kintner, 1996; Ma et al., 1996; Bellefroid et al., 1998). While proneural genes have a role in promoting neuronal differentiation, neurogenic genes, such as the transmembrane receptor *X-Notch-1*, its ligand, *X-Delta-1*, and the intracellular mediator of *X-Notch-1* signalling, *X-Su(H)*, have a role in limiting the number of cells that undergo neuronal differentiation (Coffman et al., 1990; Chitnis et al., 1995; Wettstein et al., 1997; reviewed in Chitnis, 1995; Lewis, 1996). The expression of neurogenic genes is activated by the proneural genes and in turn, the expression and/or the activity
of certain proneural genes is restricted by the activated Notch receptor (e.g. Chitnis and Kintner, 1996; Ma et al., 1996). This feedback loop between proneural and neurogenic genes is thought to result in the selection of a few cells that differentiate into neurons within the neural plate (reviewed in Tanabe and Jessell, 1996).

In Xenopus, the sites of prospective neuronal differentiation are marked by the expression of a number of proneural and neurogenic genes and finally by the expression of N-tubulin, a marker of differentiated neurons (e.g. Chitnis et al., 1995; Bellefroid et al., 1996; Ma et al., 1996; Dubois et al., 1998). The expression pattern of these genes shows that not all neuroepithelial cells differentiate at the same time and that neuronal differentiation is not random; rather, it follows a stereotyped temporal and spatial order. The temporal order dictates that neuronal differentiation takes place posteriorly at the neural plate stage while anteriorly is delayed until after neural tube closure. The spatial order dictates that in the posterior neural plate, neuronal differentiation takes place in three longitudinal domains on either side of the dorsal midline (Chitnis et al., 1995). Cells that differentiate in these longitudinal domains correspond to the three classes of primary neurons, namely motor neurons, interneurons and sensory neurons, in a medial-to-lateral order. Anteriorly, in the Xenopus forebrain, the spatial order dictates that neuronal differentiation first appears in four clusters, located in the olfactory placodes, the telencephalon, the ventral diencephalon and the epiphysis (Papalopulu and Kintner, 1996). These initial sites become gradually enlarged and differentiation spreads to the rest of the brain (see also Hartenstein, 1993; Ross et al., 1992).

As outlined above, a number of genes that allow the cells within the longitudinal domains of the posterior neural plate to switch from an epidermal to a neural and subsequently neuronal fate have been characterised. By contrast, little is known about the mechanisms whereby the temporal and spatial pattern of neuronal differentiation is achieved. In particular, the mechanisms by which the sites of neuronal differentiation are positioned on the neural plate is largely unknown, although Gli/Zic genes appear to have a role (Brewster et al., 1998). As far as the temporal pattern of neuronal differentiation is concerned, we have previously suggested that it may be controlled by the process of anteroposterior patterning. In support of this hypothesis, we have shown that down regulation of anterior genes and up regulation of posterior genes by retinoic acid (RA) treatment accelerates the timing of neuronal differentiation in anterior neuroectoderm in vivo and in vitro (Papalopulu and Kintner, 1996). One of the anterior genes that is down regulated by RA is the winged helix transcription factor XBF-1. This has led us to speculate that XBF-1 may be involved in preventing anterior neural plate cells from undergoing early neuronal differentiation. This interpretation is consistent with the observation that when the mouse homologue, BF-1, is knocked-out, there is premature neuronal differentiation in the forebrain (Xuan et al., 1995).

Here we examine directly the role of XBF-1 in primary neurogenesis by misexpressing it in the posterior neural plate of Xenopus embryos. Our findings support our initial hypothesis since neuronal differentiation is specifically suppressed in cells in which XBF-1 is expressed at high levels. Surprisingly, we have uncovered an opposing biological activity since neuronal differentiation is ectopically induced in cells adjacent to high XBF-1-expressing cells. This dual activity results in ectopic neuronal differentiation in the lateral or ventral side of the embryo, along the border of high XBF-1-expressing ectoderm. We propose that in vivo, a similar boundary effect is created around the XBF-1 expression domain and positions prospective neurogenesis in the anterior neural plate. We show that the activity of XBF-1 is concentration dependent and evolutionarily conserved. Finally, we provide evidence for the molecular mechanism of action and potential targets of XBF-1.

MATERIALS AND METHODS

Isolation of XBF-1 cDNA and plasmid constructions

A 210 bp cDNA fragment was previously isolated (Papalopulu and Kintner, 1996) and it was used as probe for a high stringency screen of a stage 17 Xenopus embryo cDNA library in λgt10. Several positive clones were isolated and the longest was cloned in the expression vector pCS2+ (Rupp et al., 1994; Turner and Weintaub, 1994) and fully sequenced. The full length XBF-1 coding region was amplified by PCR from the cloned cDNA and subcloned into the pCS2+ vector. After linearisation with NotI, the vector was transcribed in vitro with SP6 polymerase in the presence of GpppG to produce capped XBF-1 transcripts that lack the 5’ and 3’ non coding sequences. XBF-1myc was produced by PCR cloning the XBF-1 coding region into the pCS2+MT vector (Turner and Weintaub, 1994), thus fusing 6 myc epitope tags in the N terminus of XBF-1. XBF-1-E1A and XBF-1-EnR were produced by PCR cloning the XBF-1 coding region into the pCS2+NLS-MT-EnR and pCS2+NLS-MT-E1A vectors respectively (a generous gift from Dr E. Bellefroid; see also Bellefroid et al., 1996; Marine et al., 1997). These vectors carry the SV40 large T antigen nuclear localisation signal (NLS) in front of 6 myc tag repeats and the repressor domain, EnR, of the Drosophila Engrailed gene (Han and Manley, 1993) or the activation domain of the E1A gene (Lillie and Green, 1989), respectively. The resulting fusions carry the NLS and myc tag in frame at the 5’ end of XBF-1 and either the E1A or the EnR fragment in frame at the 3’ end. The same strategy has been employed before in creating a dominant activator of the Retinoic acid receptor a (Blumberg et al., 1997). All constructs were linearised with NotI, and transcribed in vitro with SP6 polymerase in the presence of GpppG to produce capped transcripts using the Ambion mMessage mMachine kit. The qin construct was made by cloning the cDNA fragments into the BamHI-EcoRI sites of pCS2+ vector.

Embryo culture and RNA injections

Embryos were obtained from Xenopus laevis adult frogs by hormone-induced egg laying and in vitro fertilisation using standard methods. Embryos were staged according to Nieuwkoop and Faber (1967). One blastomere of two-cell stage embryos was injected with capped, synthetic RNAs. 0.5 ng or 90 pg of XBF-1 RNA were injected in a volume of 10 nl. Experimental RNAs were co-injected with lacZ RNA which serves as a lineage label for cells that have inherited the injected RNA mixture. As a negative control, embryos were similarly injected with lacZ RNA alone. At neural plate stage, the injected embryos were fixed, stained with X-gal to reveal the distribution of the lacZ tracer, and then analysed by whole-mount in situ hybridization. The lacZ RNA used in this study carries a nuclear localisation signal and therefore the blue staining is localised in the nucleus. In contrast, the in situ hybridisation signal is predominantly cytoplasmic. RNA was prepared in vitro using SP6 RNA polymerase. X-Delta-1RNA was transcribed from clones previously described (Chitnis et al., 1995).
In situ hybridisation

In situ hybridisation was performed essentially as described by Harland (1991). Antisense RNA probes from N-tubulin (Chitnis et al., 1995), X-MylT1 (Bellefroid et al., 1996), X-Delta-1 (Chitnis et al., 1995), XSox3 (kindly provided by Dr R. M. Grainger), Xotx2 (Lamb et al., 1993), XBF-1 (entire cDNA) were prepared by in vitro transcription of the linearised DNA templates in the presence of digoxigenin-11-UTP or fluorescein-12-UTP (Boehringer Mannheim) as described by Harland (1991). The substrate for the chromogenic reaction was Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP; purple colour).

Double in situ hybridisation was performed according to a protocol developed by Dr T. Doniach and described by Knecht et al. (1995). The substrate of the first chromogenic reaction was 5-bromo-4-chloro-3-indolyl phosphate (BCIP; light blue colour) and of the second, 5-bromo-6-chloro-3-indolyl phosphate (magenta phosp.; magenta colour). Some specimens were sectioned after staining, and these were post-fixed O/N in MEMFA, and then embedded in gelatin/albumin mixture, solidified with glutaraldehyde. Sections (10 μm or 30 μm, as appropriate) were cut on a Leica VT1000M vibratome, mounted in 90% glycerol, and photographed with Nomarski optics.

X-gal staining

Embryos were grown to the desired stage, de-vitellinised and fixed in MEMFA for 1 hour. Following a brief wash in phosphate buffer (pH 6.3) embryos were transferred into the X-gal staining solution (Coffman et al., 1990) until staining was apparent, typically 1-2 hours. The reaction was terminated by rinsing in phosphate buffer and the embryos were dehydrated and stored in ethanol at −20 °C.

Antibody staining

To combine in situ hybridisation with immunohistochemistry, embryos were taken through the whole-mount in situ hybridisation procedure first (omitting the proteinase K digestion), post-fixed overnight in MEMFA at 4°C and then labelled with an anti-myc antibody (9E10, Santa Cruz Biotechnology). Antibody binding was revealed with a horseradish peroxidase coupled secondary antibody (Stratech Scientific) and diaminobenzidine staining.

RESULTS

Isolation of full length XBF-1

We previously reported the isolation by PCR of a partial cDNA, XBF-1, that showed homology to the DNA binding domain of the mammalian gene, BF-1 (Papalopulu and Kintner, 1996). By screening a Xenopus cDNA library with this PCR fragment we isolated the full length XBF-1 cDNA (Fig. 1A).

XBF-1 is a member of the winged helix family of transcription factors, which has been subdivided into several groups based on sequence similarity within the DNA binding domain. The founding group of the winged helix family is represented by the Drosophila forkhead gene (fkh) and a number of vertebrate forkhead-related genes (FKH) (Clevidence et al., 1993; Kaufmann and Knochel, 1996). XBF-1 is more closely related to genes of the group defined by the Drosophila slp1 and slp2 genes (Clevidence et al., 1993; Kaufmann and Knochel, 1996). XBF-1 is approximately 80% identical at the amino acid level to the chicken qin (Chang et al., 1995) and approximately 70% to the rat BF-1 (Tao and Lai, 1992) genes (Fig. 1A). The vertebrate BF-1 genes are very highly homologous in the DNA binding domain and the C terminus of the protein (Fig. 1A). Upstream of the DNA binding domain, homology is high in the N terminus but is followed by a divergent region of variable length and rich on homopolymeric amino acid runs, such as histidine and proline. The similarity between the Drosophila and vertebrate genes is mainly restricted to the DNA binding domain. However, an N-terminal region shows significant sequence similarity to the Drosophila slp2 protein (Fig. 1B) suggesting that it may be important for the function of the protein.

In the anterior neural plate, X-Delta-1 and X-ngnr-1 are expressed around the anterior boundary of XBF-1

In situ hybridisation with the full length XBF-1 clone gave the same results as the shorter PCR clone (Papalopulu and Kintner, 1996).
1996). At the neural plate stage, XBF-1 was expressed in a band across the anterior neural plate and at the tadpole stage it was highly expressed in the olfactory placodes and the telencephalon (Fig. 2A-C,E). An additional site of expression was found in the neural crest that migrates in the first branchial arch (Fig. 2A and data not shown).

Double in situ hybridisation with N-tubulin showed that in the tadpole forebrain XBF-1 is highly expressed in proliferating undifferentiated neuroectodermal cells (Fig. 2C). A direct comparison of XBF-1 expression with that of N-tubulin at an earlier stage was not possible since N-tubulin is not expressed in the anterior ectoderm at the neural plate stage (Papalopulu and Kintner, 1996; see also Fig. 9 and 10, control). Therefore, in order to map the expression of XBF-1 in relation to the sites of neuronal differentiation in the anterior neural plate we used gene markers whose expression precedes that of N-tubulin, such as the neurogenic gene X-Delta-1 and the neuronal determination gene X-ngnr-1. In the posterior neural plate the sites of prospective neuronal differentiation are marked by the expression of such genes and some (e.g., X-Delta-1, X-ngnr-1, X-Myt-1,) are also expressed in the anterior neural plate even though neuronal differentiation is delayed (Chitnis et al., 1995; Ma et al., 1996; Bellefroid et al., 1996).

Specifically, in the anterior neural plate, the expression pattern of X-Delta-1, occurs in two curved stripes, the anterior of which (arrow in Fig. 2D) coincides with the edge of the anterior neural plate. X-ngnr-1 is also expressed in the edge of the anterior neural plate, in two lateral patches (Fig. 2G). According to the fate map, the edge of the anterior neural plate contains laterally the anlage of the epiphysis and medially the anlage of the prospective olfactory placodes and part of the telencephalon (Eagleson and Harris, 1989; Eagleson et al., 1995; Couly and LeDouarin,
Fig. 4. A high concentration of *XBF-1* suppresses endogenous and induces ectopic *N-tubulin* while a low concentration only induces additional *N-tubulin*. Embryos were injected with *XBF-1* RNA, *lacZ* RNA (control), a mixture of *XBF-1* and *lacZ* RNA or *XBF-1-myc* RNA at a high (0.5 ng) or low (90 pg) concentration and processed by in situ hybridisation for *N-tubulin* (purple), either alone (*XBF-1*), or combined with X-gal staining, (light blue; *XBF-1/mcZ* and *lacZ*) or with myc antibody staining, (orange; *XBF-1-myc*). In all panels, anterior is to the right and injected side towards the lower end. The left and right panels show dorsal views while the middle panel shows lateral views of the embryos shown on the left. At high concentrations ectopic tubulin forms at the boundary of the *XBF-1*-expressing ectoderm (light blue in embryos co-injected with *lacZ* and orange in embryos injected with *XBF-1-myc*) while at low concentrations ectopic (i.e. supernumerary) *N-tubulin* forms within the *XBF-1*-expressing ectoderm.

![Image](image.png)

**Fig. 5.** *XBF-1* regulates the expression of *X-Myt-1* and *X-ngnr-1*. Embryos injected with *XBF-1/lacZ* (A-F) and *qin/lacZ* (G,H) or *lacZ* (J) RNA and analysed for *X-Myt-1* (A-E and G-J) and *X-ngnr-1* expression (F). Black arrowheads connect dorsal and lateral or ventral views of the same embryo. (A,B,D,E,G,H) represent the ‘high dose’ phenotype, where *X-Myt-1* is suppressed over the X-gal stained ectoderm but ectopically induced in adjacent cells. In G and J a black dotted line indicates the dorsal midline. The lateral stripe of *X-Myt-1* expression (arrow in G and J) appears at a great distance from the dorsal midline in experimental embryos (G) compared to controls (J). A lateral ventral view (H) of the embryo shown in (G) shows that *X-Myt-1* expression formed along the boundary of the ectoderm that stained highly and uniformly with X-gal and in a punctate pattern outside it. C and F represent the low dose phenotype, where the normal expression domain of *X-Myt-1* and *X-ngnr-1* is greatly expanded on the injected side. The expression of *X-ngnr-1* at high concentrations of *XBF-1* or *qin* was not determined.

1988; Knouff, 1935; Klein and Graziaedi, 1983). When the neural plate closes into a tube, these are the first sites to undergo neuronal differentiation in the anterior brain (Papalopulu and Kintner, 1996; see also Wilson et al., 1990; Ross et al., 1992; Hartenstein, 1993). Thus, *X-Delta-1* and *X-ngnr-1* expression is likely to be a marker of the area of prospective neuronal differentiation in the anterior neural plate. Within this area, *X-Delta-1* may have a role in limiting the number of cells that differentiate, as it does in the posterior neural plate (Chitnis et al., 1995). By performing double in situ hybridisation with *X-Delta-1* or *X-ngnr-1* and *XBF-1*, we found that the outer stripe of *X-Delta-1* and the two patches of *X-ngnr-1* expression occur around the anterior edge of the *XBF-1* expression domain (Fig. 2F and H).

**In XBF-1- and qin-injected embryos ectopic N-tubulin forms at the boundaries of highly expressing ectoderm**

Previously, we have observed an inverse correlation between *XBF-1* expression and neuronal differentiation, in vitro and in vivo, and therefore we suggested that *XBF-1* may act as a negative regulator of neuronal differentiation (Papalopulu and Kintner, 1996). To test this hypothesis more directly, we injected a mixture of in vitro transcribed *XBF-1* (0.5 ng) and

lacZ (0.2 ng) RNA in one blastomere of the two-cell stage *Xenopus* embryo. Embryos were injected in the animal pole in order to target the ectoderm, and the distribution of the RNA was followed with X-gal staining. To test whether the function of *BF-1*-related genes is evolutionarily conserved, we also misexpressed the chicken homologue, *qin*, in the same manner. Embryos that received *XBF-1* or *qin* RNA, showed suppression of endogenous *N-tubulin* expression (Fig. 3). As shown by the X-gal staining, *N-tubulin* was suppressed in the areas that received a high and uniform dose of experimental RNA (Fig. 3A,C,I). However, while endogenous *N-tubulin* was suppressed in the blue area, abundant ectopic *N-tubulin* formed outside it, on the lateral and ventral side of the injected embryos (Fig. 3B,D,E,J). Ectopic *N-tubulin* expression was observed in two patterns. In the first pattern, ectopic *N-tubulin* followed the border of the X-gal stained area, either as a tight stripe or a wide band (Fig. 3B,F,E). The ectopic stripe/band was placed on the lateral (Fig. 3B) or even as far as the ventral side of embryo (Fig. 3J); in others, it formed perpendicular, rather than parallel, to the A-P axis of the embryo (Fig. 3F and E). In the second pattern, ectopic *N-tubulin* formed extensively in a punctate pattern outside the area of heavy X-gal staining and extended well into the ventral side of the embryo (Fig. 3D). High magnification showed that in these embryos the punctate *N-tubulin*-positive cells were interspersed with blue cells (Fig. 3H). Injection of *XBF-1/lacZ* into one animal-pole blastomere
of the 32-cell stage embryo also resulted in formation of ectopic N-tubulin (45%; n=11) surrounding a patch of X-gal stained cells (Fig. 3K). Ectopic neuronal differentiation was stable as it was maintained in the tadpole (Fig. 3F).

In addition to the effect on N-tubulin, 30% of the neural plate stage embryos showed an externally visible thickening and buckling of the ectoderm. This may be related to the oncogenic properties of XBF-1 and qin (Li et al., 1997) and will be described elsewhere.

**Misexpression of different concentrations of XBF-1 leads to opposite phenotypes**

Because in the injections of 0.5 ng of XBF-1 RNA, a small but variable number of embryos displayed expansion instead of suppression of endogenous N-tubulin, we decided to test whether the phenotype was dose dependent. Embryos were injected with either high dose (0.5 ng/embryo) or a low dose (90 pg/embryo) of XBF-1 RNA either alone or mixed with a constant concentration of lacZ RNA (0.2 ng/embryo) (Fig. 4).

At the high dose of XBF-1, the majority of the embryos showed suppression of endogenous N-tubulin (55%, n=18; 78%, n=19; no effect=0% in both cases). The remaining embryos showed expansion of N-tubulin expression. X-gal staining revealed that, as described above, suppression of N-tubulin was accompanied by ectopic N-tubulin either along the boundary of the expressing, blue, ectoderm (Fig. 4) or in widespread punctate pattern outside it, in what would normally be prospective epidermal ectoderm (see Fig. 3C,D). In contrast, at the low dose of XBF-1 we found increase of the endogenous N-tubulin, such that the width of the lateral N-tubulin stripe was greatly expanded (66%, n=21; 50% n=18), with no or little ectopic N-tubulin along the boundaries of XBF-1-expressing tissue or in the adjacent ectoderm (Fig. 4).

To verify these findings at the protein level, we tagged the XBF-1 protein with a myc epitope and injected XBF-1-myc RNA at high and low concentrations (Fig. 4, lower panels). The results of these experiments were consistent with those obtained with XBF-1/lacZ RNA injections.

**XBF-1 regulates the expression of X-Myt-1 and X-ngnr-1**

To find out whether XBF-1 affects the expression of genes which are involved in neuronal determination, we looked at the expression of X-Myt-1 and X-ngnr-1 (Fig. 5). Similarly to N-tubulin, at high concentration of XBF-1 and qin, X-Myt-1 showed a dual phenotype of endogenous suppression and simultaneous ectopic induction outside the high XBF-1 or qin/lacZ-expressing ectoderm (Fig. 5A-E,G,H). Also similarly to N-tubulin, at low concentration of XBF-1 there was no endogenous suppression but instead the normal expression domain of X-Myt1 (Fig. 5C) and X-ngnr-1 (Fig. 5F) was greatly expanded.

**High XBF-1-expressing cells do not overlap with N-tubulin positive cells**

High magnification pictures of embryos injected with 0.5 ng of XBF-1 RNA suggested that the population of XBF-1/lacZ-and N-tubulin-expressing cells do not overlap (Fig. 3H). In order to look in more detail at the distribution of the N-tubulin-positive cells relative to the cells that express XBF-1, we sectioned embryos injected either with a high or low dose of XBF-1 and co-injected with a constant dose of lacZ RNA (Fig. 6A-D). Similarly, we sectioned embryos injected with XBF-1-myc (Fig. 6E-H). Consistent with the results from the whole-mount analysis, we found that over the region that expresses XBF-1/lacZ or XBF-1-myc, a high dose of XBF-1 suppressed endogenous N-tubulin expression. In contrast, a low dose increased endogenous N-tubulin (compare Fig. 6A to 6C and Fig. 6E to 6G). Thus, the neuronal differentiation promoting activity is maintained at low concentrations while the suppressing activity is lost. In order to verify that there was a genuine increase in numbers rather than a spreading out of the normal number of differentiated cells, we counted N-tubulin-positive

![Fig. 6](image)
positive cells on serial sections of the embryo shown in Fig. 6C and we found a 4-fold increase of \textit{N-tubulin}-positive cells on the injected side, over the whole embryo.

Since the \textit{X-gal} and myc antibody staining are nuclear while the in situ signal (\textit{N-tubulin}) is cytoplasmic, we were able to determine whether the \textit{N-tubulin}-positive cells express \textit{lacZ} or \textit{myc}, in serial sections under high magnification. In embryos injected with a high \textit{XBF-1} concentration, suppression of \textit{N-tubulin} was observed in cells that expressed \textit{XBF-1/lacZ} while ectopic \textit{N-tubulin} was expressed at high frequency in cells that did not express detectable levels of \textit{lacZ} or \textit{XBF-1-myc} (Fig. 6B and F). Thus, 90\% of the \textit{N-tubulin}-positive cells were apparently \textit{lacZ} negative, while only 10\% co-expressed \textit{N-tubulin} and detectable \textit{lacZ} \((n=259\text{ cells})\). However, in
embryos injected with a low dose of XBF-1, the percentage of the N-tubulin-positive cells that co-express lacZ increased to 38% (n=249 cells). Interestingly, additional N-tubulin within the normal N-tubulin stripe or ectopic N-tubulin in the epidermal ectoderm, induced by low or high doses of XBF-1 respectively, was observed only in cells of the deep layer of the posterior ectoderm, where N-tubulin is normally expressed (Fig. 6).

**XBF-1-expressing cells that do not express N-tubulin, express XSox3**

We have shown that XBF-1 and qin inhibit neuronal differentiation in cells where they are highly expressed. To rule out a non-specific toxic effect, we examined the expression pattern of a neural marker, the HMG box containing gene XSox3 (Zygar et al., 1988). In the embryo, XSox3 is expressed in undifferentiated neuroectodermal cells, covering the area between the medial (motorneuron) and intermediate (interneuron) stripes of N-tubulin expression (Bellefroid et al., 1998; see Fig. 7G). We found that both XBF-1 and qin induced ectopic XSox3 (Fig. 7A,C) and that in contrast to N-tubulin, ectopic XSox3 expression co-localised largely with the X-gal staining (Fig. 7D-F). This shows that XBF-1/ lacZ-expressing cells adopt a neural fate but are specifically inhibited from undergoing neuronal differentiation. Double in situ hybridisation with XSox3 and N-tubulin in XBF-1-injected embryos showed that the expression of N-tubulin and XSox3 was mutually exclusive. In embryos exhibiting the high dose phenotype, XSox3 was laterally expanded and ectopic N-tubulin formed around the XSox3-expressing ectoderm (Fig. 7H and K). In embryos exhibiting the low dose phenotype, the outer stripe of N-tubulin expression was expanded but XSox3 was not detectably affected (Fig. 7L).

**X-Delta-1 mediated interactions limit the number of cells that turn on N-tubulin in response to XBF-1 or qin**

We have shown that misexpression of 0.5 ng XBF-1 in the posterior neural plate induces ectopic N-tubulin expression, in the ectoderm outside the region that expresses uniformly high levels of XBF-1. The ectopic N-tubulin occurred in a highly scattered pattern suggesting that cell interactions may limit the number of cells that respond to the inducing signal by the XBF-1-expressing cells. We hypothesised further, that this interaction may be mediated by the transmembrane receptor Notch and its ligand X-Delta-1, that are involved in limiting the number of cells that differentiate in the neural plate. Indeed, we found that X-Delta-1 was ectopically induced on the injected side of XBF-1 or qin-injected embryos (Fig. 8A). Furthermore, co-injection of XBF-1 or qin with a dominant negative form of X-Delta-1, X-Delta-1<sup>dn</sup>, resulted in a less scattered pattern of ectopic N-tubulin compared to injections...

![Fig. 9. Misexpression of XBF-1-E1A versus XBF-1-EnR has opposite effects on primary neurogenesis. (A,B) Injection of XBF-1-E1A completely suppresses N-tubulin (purple) on the injected side, identified by X-gal staining (A) or anti-myc staining (B). C shows that XBF-1-E1A suppresses N-tubulin (magenta) but does not affect XSox3 expression (light blue). None of the embryos in A-C showed any N-tubulin on the lateral or ventral side. Injection of XBF-1-EnR results in dispersed and ectopic N-tubulin expression (purple in E-G). In some XBF-1-EnR embryos, ectopic N-tubulin is also found anteriorly (I), while XBF-1-E1A-injected (J) and control (H) embryos show no N-tubulin in the anterior neural plate. Injected areas are identified by X-gal staining (light blue) in E and anti-myc staining (light brown) in F,G,I and J.](image)

![Fig. 10. Misexpression of XBF-1-E1A versus XBF-1-EnR and XBF-1 has opposite effects on anterior development. Embryos were injected with various RNAs as indicated on the side of each set of panels and were analysed by double in situ hybridisation for N-tubulin (magenta) and XOtx2 (light blue), or by in situ hybridisation for XOtx2 (purple) and X-gal staining (light blue), as indicated. Dorsal (left panels) and anterior (middle and right panels; injected side to the left) views are shown. XBF-1-EnR reduces XOtx2 expression while XBF-1-E1A expands XOtx2 expression locally. XBF-1 also reduces XOtx2 expression (bottom right). The XBF-1-injected embryo shown in the middle panel has normal XOtx2 expression, presumably because it did not receive RNA anteriorly. Some XBF-1-EnR embryos show ectopic N-tubulin anteriorly, in the area where XOtx2 is suppressed (second row from top).](image)
of XBF-1 or qin alone (Fig. 8B). In XBF-1- or qin/X-Delta-1

**DISCUSSION**

In this paper, we examine the role of the Xenopus gene XBF-

**XBF-1 can act either as a repressor or an activator of a transcription**

XBF-1 is a putative transcription factor and as such, it could be acting as a transcriptional repressor, a transcriptional activator or both. To distinguish between these possibilities we fused XBF-1 to either a strong activation domain from the adenoviral E1A protein (Lillie and Green, 1989), or to the strong repressor domain, EnR, derived from the Drosophila Engrailed protein (Han and Manley, 1993).

In embryos injected with XBF-1-EnR RNA, ectopic N-tubulin formed (42%, n=24) but endogenous N-tubulin was not suppressed as effectively as with the wild type RNA (i.e. 21%, n=24 versus 55%, n=18; Fig. 9E-G). Embryos injected with XBF-1-E1A RNA showed suppression of endogenous N-tubulin (100%, n=8) but unlike the result of injections of wild-type RNA, none of these embryos showed any ectopic N-tubulin (Fig. 9A-C). This was also true in cases where half of the embryos showed no effect (i.e. N-tubulin suppression =50%, no effect =50%, n=10), suggesting that unlike the wild type XBF-1, the XBF-1-E1A construct does not induce neuronal differentiation at low concentrations. XBF-1-E1A-injected embryos that showed N-tubulin suppression, had normal Xsox3 expression suggesting that the suppressing effect is specific for N-tubulin (Fig. 9C). In contrast, ectopic Xsox3 was induced by XBF-1-EnR (data not shown).

Embryos injected with XBF-1-EnR showed a reduction of head development, as marked by Xotx2 expression (61%, n=13). Injections of the wild-type XBF-1 also reduced Xotx2 expression (42%, n=7). Interestingly, in XBF-1-EnR-injected embryos we found an expansion of N-tubulin into the anterior neural plate (17%, n=24; Fig. 9I), in a subset of embryos that showed suppression of Xotx2 (Fig. 10). Embryos injected with XBF-1-E1A showed the opposite phenotype i.e. a local enhancement of Xotx2 expression (53%, n=15; Fig. 10). None of the XBF-1-E1A-injected embryos showed any N-tubulin in the anterior neural plate (Fig. 9J). When we used X-gal staining to selectively analyse embryos that had received RNA anteriorily we found that the percentage of Xotx2 reduction by XBF-1-EnR (n=5), XBF-1 (n=4) and of local Xotx2 expansion by XBF-1-E1A (n=7) rose to 100% (Fig. 10).

**XBF-1 can act both as an activator and a repressor of neuronal differentiation**

Our experiments showed that misexpression of XBF-1 leads to two opposite phenotypes that co-exist at high concentrations but are separable at low concentrations. Thus, embryos injected with high concentration of XBF-1 show suppression of neuronal differentiation in the injected area and at the same time ectopic and extensive neuronal differentiation outside the main injected area of the ectoderm. Embryos injected with low concentrations do not show the suppressing effect but show only supernumerary neuronal differentiation inside the injected area. The pattern of ectopic N-tubulin expression is mirrored by the pattern of the neuronal determination genes X-Myt-1 and X-ngnr-1, suggesting that XBF-1 acts early in the cascade that leads to neuronal differentiation.

A detailed examination of the positioning of N-tubulin cells in relation to XBF-1-expressing cells in whole-mount and sectioned material showed that, cells that express high XBF-1 have a low chance of expressing N-tubulin. Thus, the neuronal inhibitory effect is mainly cell-autonomous (although we cannot exclude the possibility that it also has a very short range non-cell autonomous effect). By contrast, ectodermal cells which are adjacent to high XBF-1-expressing cells are induced to differentiate. In several cases this creates a ‘border’ or a ‘zone’ of N-tubulin-positive cells surrounding a high XBF-1-expressing area that is itself N-tubulin negative.

How is this border effect created? When RNA is injected into the two-cell stage embryo it diffuses from the site of injection as the embryo divides. Therefore, the simplest interpretation is that at the neural plate stage, areas of the ectoderm that express high levels of XBF-1/lacZ or XBF-1 myc are surrounded by cells that express lower levels. It is possible that these levels of lacZ or XBF-1 myc RNA are below our limit of detection by X-gal or myc staining, yet are sufficient to induce neuronal differentiation. An alternative interpretation is that XBF-1-expressing cells produce an autonomous repressor and a non-cell autonomous activator of neurogenesis that has an effect only on non-XBF-1-expressing cells. At present we can not distinguish between these two possibilities, but experiments where XBF-1-expressing cells are unambiguously distinguished from non-expressing cells are currently under way.
Only the deep layer of the posterior ectoderm responds to XBF-1 by forming ectopic N-tubulin

It is interesting to note that, in our misexpression experiments, XBF-1 induces neuronal differentiation only in the deep layer of the posterior ectoderm. In the neuroectoderm, the deep and superficial layers of the posterior neural plate contain the precursors of primary and secondary neurons, respectively (Hartenstein, 1989). The secondary precursors divide longer and differentiate later in larval life while the primary precursors differentiate early. Thus, at the neural plate stage the expression of N-tubulin and of several proneural and neurogenic genes is restricted to the deep layer of the posterior neuroectoderm (N.P. unpublished data). The implication of the finding that XBF-1 can not induce neuronal differentiation in superficial ectoderm is two-fold. First, it shows that there is a difference in neuronal competence between the deep and superficial layers, throughout the ectoderm. Second, it suggests that the inducing activity of XBF-1 could be influenced by negative and/or positive co-factors present in the superficial and deep layer of the posterior ectoderm respectively.

High levels of XBF-1 specify a neural precursor fate

In the embryo, XSox3 is highly expressed in undifferentiated, N-tubulin negative, neuroectodermal cells that are located between the medial and intermediate stripe of N-tubulin expression (Bellefroid et al., 1998 and Fig. 7). In our misexpression experiments, areas of the ectoderm that express high levels of XBF-1 do not express N-tubulin but express XSox3, resulting in an expansion of the endogenous XSox3 expression domain. Thus, XBF-1 shows similarity to the recently described homeobox gene, Xiro3, in that Xiro3 also upregulates XSox3 and suppresses neuronal differentiation, suggesting that it specifies a neural precursor fate (Bellefroid et al., 1998). Since XBF-1 and Xiro3 are expressed only in the anterior and posterior neural plate respectively, while XSox3 is expressed in both, it is possible that in normal development Xiro3 regulates XSox3 posteriorly while XBF-1 regulates XSox3 anteriorly. This is consistent with the observation that Xiro3 is not ectopically induced by XBF-1 (N. P. unpublished observations) suggesting that XSox3 can be induced either by XBF-1 or Xiro3, through parallel pathways.

Lateral inhibition and cell mixing contribute to the punctate pattern of ectopic N-tubulin

One characteristic of the XBF-1/qin phenotype is that the pattern of ectopic N-tubulin is punctate, both at high and low doses. Misexpression of XBF-1/qin results in ectopic X-Delta-1 expression, and blocking X-Delta-1 signalling results in uniform ectopic N-tubulin expression. Interestingly however, blocking X-Delta-1 signalling does not lift the inhibition of neuronal differentiation within the high XBF-1/qin-expressing areas. These results suggest that lateral inhibition, mediated by X-Delta-1, contributes to the ‘salt-and-pepper’ pattern of ectopic N-tubulin but that high XBF-1/qin-expressing cells produce an additional neuronal inhibitory signal, distinct from X-Delta-1.

In addition to lateral inhibition, cell mixing between injected and non injected cells is likely to contribute to the punctate pattern of ectopic neuronal differentiation. Indeed, we observed a high degree of interspersion of lacZ-positive with lacZ-negative cells along the boundary of expressing and non-expressing ectoderm, especially along the lateral and ventral sides of the embryos (see also Bradley et al., 1998). In the future, it would be interesting to examine the effect of XBF-1 in embryos where cell mixing in the ectoderm is inhibited.

XBF-1 can act either as a repressor or an activator of a transcription

Our results suggest that XBF-1 represses and activates neuronal differentiation in a dose dependent manner. At the molecular level, XBF-1 could be acting either as a transcription activator or as a transcriptional repressor or as both.

To distinguish between these possibilities we fused XBF-1 either to a strong activation domain, E1A, derived from adenovirus, or to a strong repressor domain, EnR, derived from Engrailed. The rationale behind this experiment is that if XBF-1 is always a repressor or always an activator, one of the two constructs would have the same effect as the wild type and the other would have the opposite effect. If on the other hand, XBF-1 can act either as a repressor or an activator neither construct will fully reproduce the wild-type XBF-1 phenotype but both would show a phenotype that is a subset of the wild type. Injections of XBF-1-E1A and XBF-1 EnR showed that the latter possibility is more likely. Injection of XBF-1 fused to the strong repressor domain EnR phenocopied most aspects of the wild-type XBF-1 injections. Specifically, both the wild-type XBF-1 and the XBF-1 EnR fusion, expanded XSox3, suppressed XOt2 and resulted in ectopic N-tubulin expression, suggesting that the wild type XBF-1 is a strong transcriptional repressor. However, endogenous N-tubulin was not suppressed as effectively with XBF-1-EnR as with the wild type XBF-1. By contrast, XBF-1-E1A-injected embryos were similar to wild type XBF-1 ones, in that they showed strong suppression of endogenous N-tubulin. In all other aspects tested, XBF-1-E1A differed from the wild type and from XBF-1-EnR; specifically it did not cause any ectopic or supernumerary N-tubulin, did not affect XSox3 and enhanced XOt2 expression.

In conclusion, these results suggest that XBF-1 may work as a bimodal transcription factor; the induction of ectopic N-tubulin by wild type XBF-1 is due to transcriptional repression, while for the repression of N-tubulin a transcriptional activation function is also important, presumably by activating an inhibitor of neuronal differentiation.

Sequence data support the idea that XBF-1 may be a bimodal transcription factor. On the one hand, the N terminus of XBF-1 contains a sequence motif conserved between the Drosophila and Xenopus genes (see Fig. 1B). A very similar motif is found in transactivation domain II located in the C terminus of HNF3β (Pani et al., 1992) suggesting that it may be part of an N-terminal transactivation domain. On the other hand, data from the chick suggest that the C terminus of qin is important for transcriptional repression in transient transfection assays (Li et al., 1995; Li et al., 1997). Since the entire C terminus of the protein is highly conserved between XBF-1 and qin (see Fig. 1A) it is likely that the repression function is also conserved.

It is interesting to note that other developmentally important transcription factors have also been reported to act as concentration-dependent positive and negative regulators of transcription, as for example the products of the Drosophila Kruppel (Sauer and Jackle, 1991) and the vertebrate Pax3.
A model for the function of XBF-1 in vivo

What is the role of XBF-1 in normal development? XBF-1 is expressed in the anterior neuroectoderm as early as the neural plate stage. We have shown previously that compared to the posterior neural plate, the anterior neural plate undergoes neurogenesis with a marked delay. This delay is also observed in higher vertebrates and may reflect a mechanism to ensure that sufficient number of progenitors are maintained for the subsequent development of the forebrain (Xuan et al., 1995; Ishibashi et al., 1995). We have hypothesised that anterior-specific genes, such as XBF-1, may be involved in preventing early neuronal differentiation (Papalopulu and Kintner, 1996). Such a role would be consistent with two observations presented here. First, in normal development, XBF-1 is highly expressed on the ventricular side of the neuroepithelium where proliferating undifferentiated cells are located. Second, misexpression experiments show that XBF-1 inhibits neurogenesis in cells in which it is highly expressed.

How does XBF-1 inhibit neuronal differentiation in the anterior neural plate? In embryos injected with a XBF-1-EnR fusion, N-tubulin expression expands into the anterior neural plate which itself is reduced, a phenotype similar to the mouse BF-1 knock-out (Xuan et al., 1995). This finding suggests that in normal development, suppression of N-tubulin expression in the anterior neural plate is due to the XBF-1 acting as an activator of transcription, presumably activating the transcription of an inhibitor of neuronal differentiation. We note that this inhibitor is distinct from the inhibitory ligand X-Delta-1 (see above and also Papalopulu and Kintner, 1996). Instead, XBF-1 may be mediating its inhibitory effects via activation of neuronal inhibitors of the bHLH class such as Hairless-related genes (reviewed in Fisher and Caudy, 1998). In support of this idea, we note that the knock-out of a Hairless-related gene in the mouse, HES-1, leads to a phenotype similar to that of the BF-1 knock-out (Ishibashi et al., 1995; Xuan et al., 1995).

While our experiments suggested that XBF-1 has a role in suppressing neuronal differentiation, they have unexpectedly uncovered a second role for XBF-1 in inducing neuronal differentiation in competent ectoderm, when expressed at lower concentrations. In experimental embryos, this property coupled with the high concentration inhibition, results in a border of neuronal differentiation around a patch of undifferentiated, high XBF-1-expressing, neural ectoderm. The same phenomenon may take place in normal development, where the anterior stripe of X-Delta-1 and the anterior patches of X-ngrl-1 expression occur around the anterior border of the XBF-1 expression domain. Thus, XBF-1 may have a role in defining the sites of prospective neuronal differentiation in the anterior neural plate. Perhaps, if during development the concentration and/or activity of XBF-1 becomes lower, neurogenesis would spread from those initial sites at the border of XBF-1 expression further into the XBF-1 expression domain. Indeed, studies in the zebrafish showed that neuronal differentiation starts from well defined but small clusters in the forebrain; these initial clusters become gradually enlarged as neighbouring cells are recruited to a neuronal fate (Ross et al., 1992).

Clearly however, not all sites of neurogenesis can be defined by XBF-1. In particular, because of its restricted expression, XBF-1 may only be responsible for the neuronal differentiation clusters in the olfactory placodes and the telencephalon, that are derived from the anterior neural edge. Genes related to XBF-1 may be performing a similar function in positioning the other sites of neuronal differentiation in the anterior neural plate (such as the posterior stripe of X-Delta-1) and perhaps also in the posterior neural plate.

It has been previously suggested that boundaries of gene expression in the zebrafish brain define the sites of neuronal differentiation and axonal tracts (MacDonald et al., 1994; Barth and Wilson, 1995). Here, we have provided functional evidence that a forebrain specific transcription factor, XBF-1, sets up a boundary along which neuronal differentiation takes place, when the ectoderm becomes competent to differentiate. We have shown that this boundary is formed as early as the neural plate stage. Finally, we have proposed that the mechanism by which prospective neuronal differentiation is organised around the XBF-1 boundary of expression, consists of a combination of autonomous, or short range, inhibition in high expressing cells coupled with activation of neuronal differentiation in adjacent low expressing cells.

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(Chalepakis et al., 1994) genes. The molecular mechanism by which XBF-1 would convert from a transcriptional activator to a repressor is presently unclear; it could depend either on an interaction of XBF-1 with different co-factors or on concentration-dependent dimer formation, as it is the case for Kruppel (Sauer and Jackle, 1993).
contains domains for transcription activation and transcription repression. 