Development 125, 4889-4900 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV1333

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

Catherine Bourguignon[1], Jian Li[2] and Nancy Papalopulu[1,*]

[1]Wellcome/CRC Institute, Tennis Court Road, Cambridge CB2 1QR, UK and Department of Anatomy, Downing Street, Cambridge CB2 3DY, UK
[2]The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA
*Author for correspondence (e-mail: np209@mole.bio.cam.ac.uk)

Accepted 2 October; published on WWW 12 November 1998

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 stereotypical 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 *Not*I, 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-1*myc 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 *Not*I, 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 *Bam*HI-*Eco*RI 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 coinjected 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-1*^{stu} RNA 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-MytT1* (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-phopshate (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-phopshate (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,

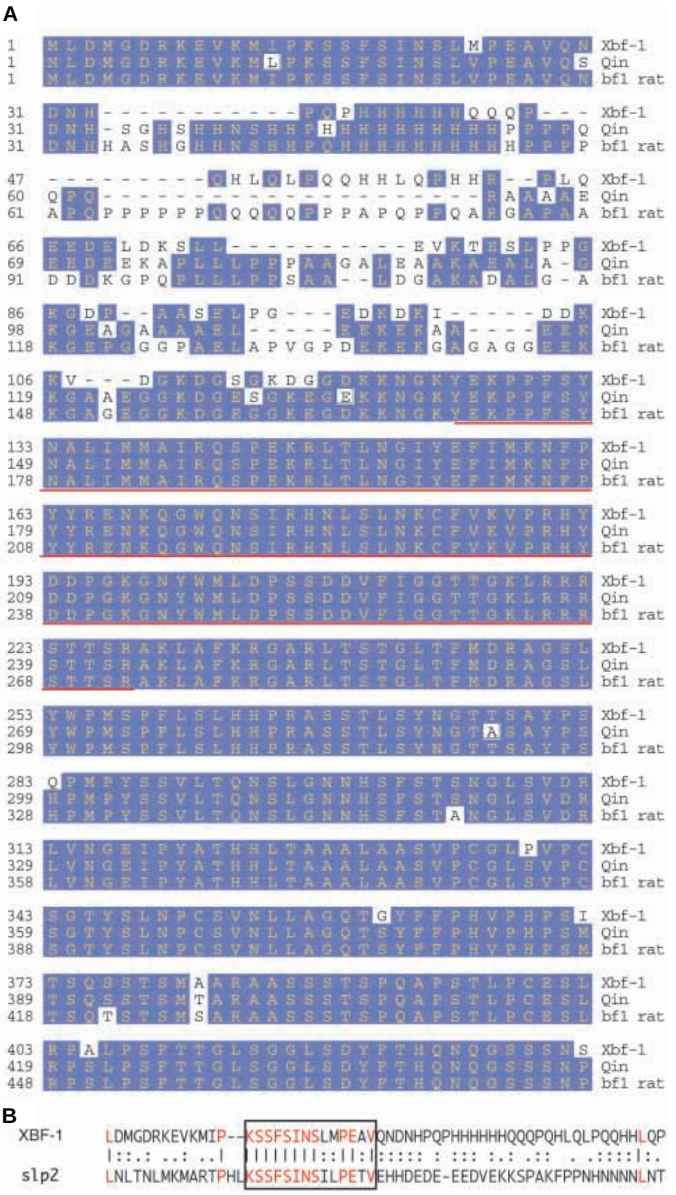

Fig. 1. Sequence of *XBF-1* and sequence comparisons. (A) Predicted amino acid sequence of a full length *XBF-1* cDNA clone, compared to sequence derived from the chicken *qin* and the rat *BF-1* genes. Sequence conservation is shown in blue. The DNA binding domain is underlined in red. (B) Aminoacid sequence alignment showing (in red) a region that is highly conserved between the *Xenopus* XBF-1 and the *Drosophila* slp2 proteins. In both proteins this region is located in the N terminus. The GenBank accession no. for the XBF-1 cDNA clone is AF101387.

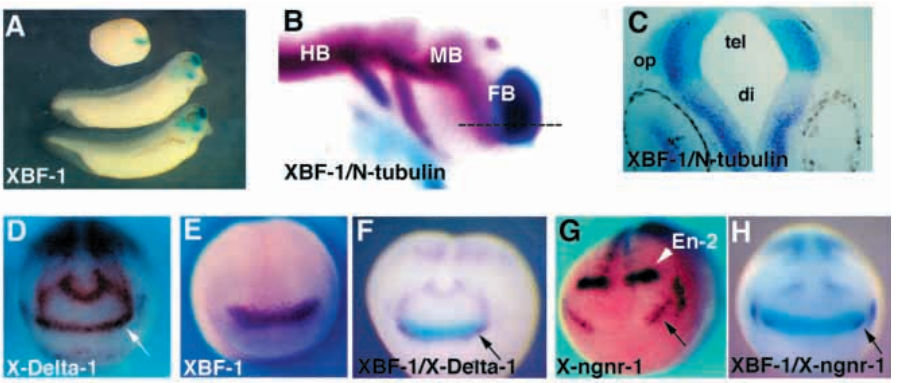

Fig. 2. Expression pattern of *XBF-1*, in relation to the expression of *N-tubulin*, *X-Delta-1* and *X-ngnr-1*. (A) Expression of *XBF-1* in stage 15, 32 and 35 embryos, lateral views, dorsal up. (B) Lateral view and (C) horizontal section, (plane of section indicated by a broken line in B), of a stage 35 embryo hybridised with *N-tubulin* (magenta) and *XBF-1* (light blue). High *XBF-1* expression is restricted to the telencephalon and olfactory placodes. *N-tubulin* expression is localised towards the mantle while *XBF-1* is localised towards the ventricular area of the neural tube. (D-H) Expression on the anterior neural plate of (D) *X-Delta-1*, (E) *XBF-1*, (F) *XBF-1* (light blue) and *X-Delta-1* (magenta, arrow), (G) *X-ngnr-1* (arrow) and *En-2* (arrowhead) (H) *XBF-1*(light blue) and *X-ngnr-1* (magenta, arrow). Expression of *X-Delta-1* in the anterior neural plate occurs in an anterior and a posterior curved stripe and that of *X-ngnr-1* in two bilateral patches. The anterior stripe of *X-Delta-1* (arrow) and the patches of *Xngnr-1* expression (arrow) are positioned around the anterior edge of the *XBF-1* expression domain. di, diencephalon; FB, forebrain; HB, hindbrain; MB, midbrain; op, olfactory placode; tel, telencephalon.

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,

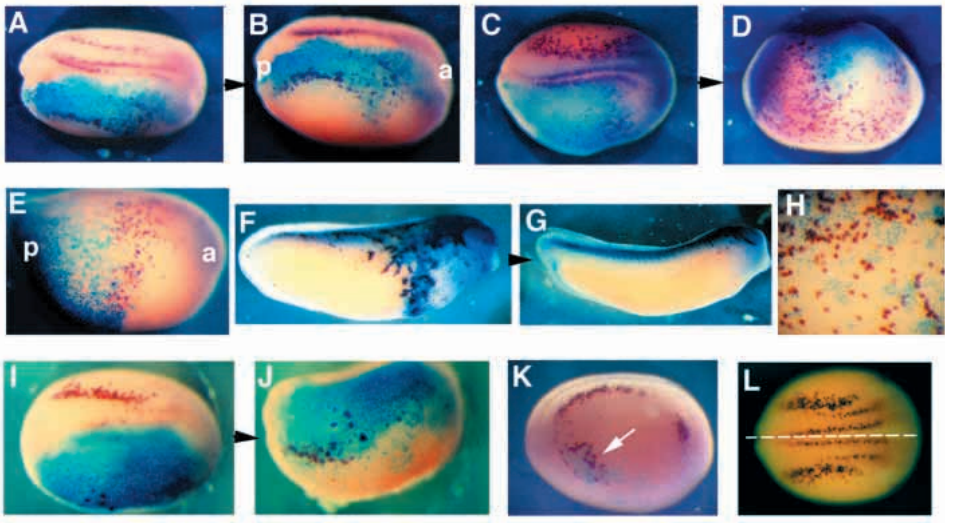

Fig. 3. Misexpression of *XBF-1* and *qin* suppresses endogenous and induces ectopic primary neurogenesis. Embryos were injected with *XBF-1/lacZ* (A-G,K), *qin/lacZ* (H-J) RNA, or were uninjected (L) and were processed for X-gal staining (light blue) and whole-mount in situ hybridisation for *N-tubulin* (purple). Dorsal (A,C,I,L) and side (B,D,E,J,K) views are shown, anterior to the right. Black arrowheads connect dorsal and lateral views of the same embryos. The lateral views show that the ectopic *N-tubulin* forms far from the dorsal midline at the lateral and even ventral side of the embryo, outside the *XBF-1/lacZ*-expressing ectoderm but at the boundary of the high expressing ectoderm. Similarly, *XBF-1/lacZ* injection of one blastomere of a 32 cell stage embryo (K), produces ectopic *N-tubulin* (arrow in K) at the boundary of the high *XBF-1/lacZ*-expressing patch. (F) The *XBF-1*-injected side and (G) the uninjected control side of a tadpole stage embryo. In E and F, note that the ectopic *N-tubulin* stripe follows the boundary of X-gal staining and has formed perpendicular, rather than the normal parallel, to the antero-posterior axis. (H) A high magnification view of the lateral side of an embryo similar to the one shown in (D); it shows that there is no overlap between the high XBF-*1/lacZ*-expressing cells (blue) and the *N-tubulin*-expressing cells (brown/purple). The X-gal staining is nuclear while the in situ signal is cytoplasmic. In L a white broken line indicates the dorsal midline of the neural plate and separates the three bilaterally symmetrical stripes of *N-tubulin* expression. a, anterior; p, posterior.

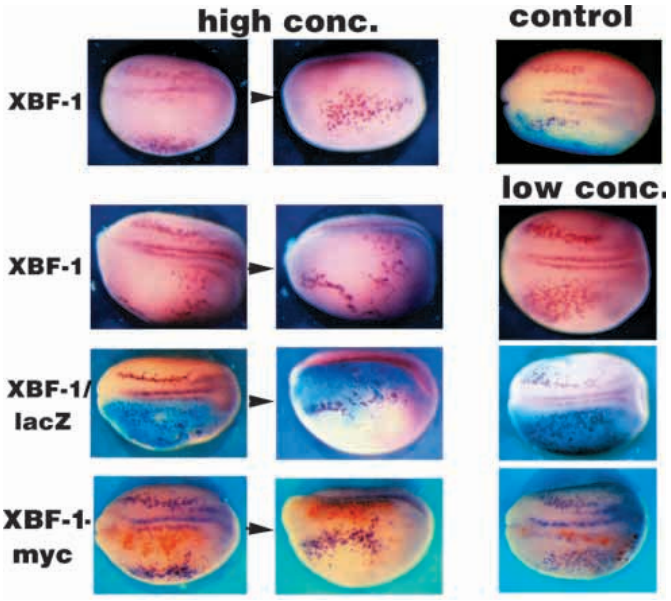

Fig. 4. A high concentration of *XBF-1* suppresses endogenous and induces ectopic *N-tubulin* while a low concentration only induces additional *N-tubulin*. Embryos were injected with *XBF-1* RNA, *lacZ* RNA (control), a mixture of *XBF-1* and *lacZ* RNA or *XBF-1-myc* RNA at a high (0.5 ng) or low (90 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/lacZ* 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.

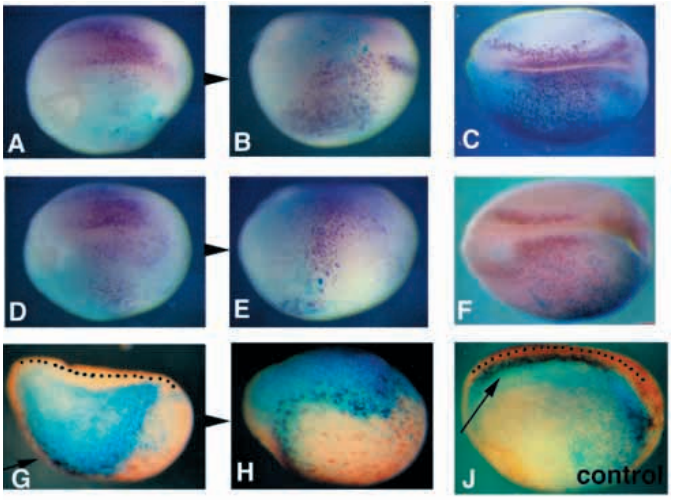

Fig. 5. *XBF-1* regulates the expression of *X-Myt-1* and *X-ngnr-1*. Embryos injected with *XBF-1/lacZ* (A-F) and *qin/lacZ* (G,H) or *lacZ* (J) RNA and analysed for *X-Myt-1* (A-E and G-J) and *X-ngnr-1* expression (F). Black arrowheads connect dorsal and lateral or ventral views of the same embryos. (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 lateroventral 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 Graziadei, 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). At the low concentration, the remaining embryos were normal (no effect=33% *n*=21; 50% *n*=18); in none of these embryos was endogenous *N-tubulin* suppressed within the *XBF-1*-expressing ectoderm.

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*-

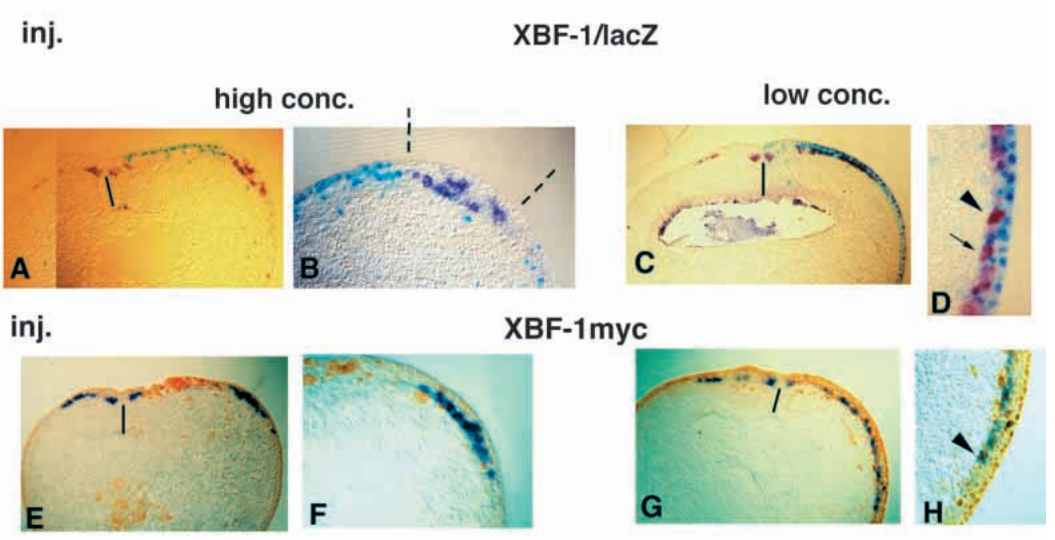

Fig. 6. High *XBF-1*-expressing cells do not overlap with *N-tubulin*-positive cells. (A-H) Transverse sections through embryos injected with high and low concentrations of *XBF-1*/*lacZ* of *XBF-1-myc* as indicated. Staining for X-gal (light blue) and myc (orange) is nuclear while the in situ signal for *N-tubulin* (purple) is cytoplasmic. In all panels a solid line passes through the notochord and indicates the dorsal midline. (B,D,F,H) High magnification views of the lateroventral side of the embryo, therefore *N-tubulin* shown in these frames is ectopic. At the high *XBF-1* concentration (B), broken lines delimit a cluster of ectopic *N-tubulin* cells formed adjacent to the ectoderm that shows detectable X-gal staining. At high *XBF-1* concentration (A,B,E,F) *N-tubulin* cells do not express detectable *lacZ* or myc. By contrast at low *XBF-1* concentration (C,D,G,H) some cells that express ectopic *N-tubulin* have detectable levels of nuclear X-gal or myc staining (arrows) while others do not (arrowhead). See text for details. Note that ectopic *N-tubulin* forms in the deep layer of the ectoderm, where the endogenous *N-tubulin* is also located.

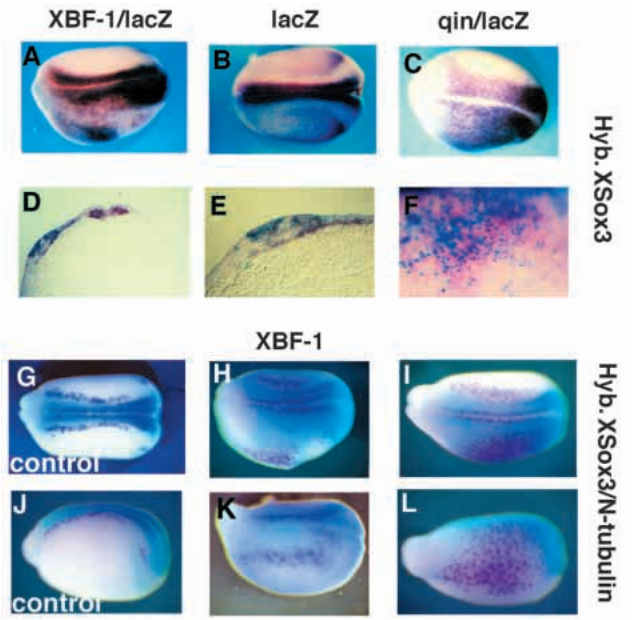

Fig. 7. *XBF-1* and *qin* induce ectopic *XSox3*. Upper panel (A-F) shows embryos injected with a high dose of *XBF-1/lacZ* (A,D,E) or *qin/lacZ* (C,F) and with *lacZ* alone (B) and were processed for X-gal staining and in situ hybridisation for *XSox*3. (D,E) Transverse sections at low (D) and high (E) magnification through an embryo similar to the one shown in A). The lateral ectoderm is thickened and expresses ectopic *XSox3*, over the area that also misexpresses *XBF-1/lacZ*. (F) A high magnification view of ectopic *XSox3*-expressing ectoderm located laterally on an embryo similar to the one shown in C. It shows that ectopic *XSox3* expression is largely coincident with X-gal staining, in contrast to the expression of *N-tubulin*, shown in Fig. 2H. Lower panels (G-L) show embryos injected with *XBF-1* (H,I,K,L), or were uninjected (G,J) and processed with double in situ hybridisation for *N-tubulin* (magenta) and *XSox3* (light blue). All embryos are shown with anterior to the right and black arrowheads connect dorsal and side views of the same embryo. (H,K) The high dose phenotype, (I,L) the low dose phenotype. The control embryo (G), shows that there is no overlap between *XSox3* and the lateral most stripe of *N-tubulin* expression; the experimental embryos (H,I,K,L) show that ectopic *XSox3* and ectopic *N-tubulin* are mutually exclusive (injected side towards the lower end of the panel). The embryo shown in H and K formed ectopic *N-tubulin* on the lateral side, outside an expanded *XSox3* expression domain. The embryo shown in I and L formed a greatly expanded lateral *N-tubulin* stripe that did not express ectopic *XSox3*.

positive cells on serial sections of the embryo shown in Fig. 6C and we found a 4-fold increase of *N-tubulin*-positive cells on the injected side, over the whole embryo.

Since the *X-gal* and myc antibody staining are nuclear while the in situ signal (*N-tubulin*) is cytoplasmic, we were able to determine whether the *N-tubulin*-positive cells express *lacZ* or myc, in serial sections under high magnification. In embryos injected with a high *XBF-1* concentration, suppression of *N-tubulin* was observed in cells that expressed *XBF-1/lacZ* while ectopic *N-tubulin* was expressed at high frequency in cells that did not express detectable levels of *lacZ* or XBF-1-myc (Fig. 6B and F). Thus, 90% of the *N-tubulin*-positive cells were apparently *lacZ* negative, while only 10% co-expressed *N-tubulin* and detectable *lacZ* (*n*=259 cells). However, in

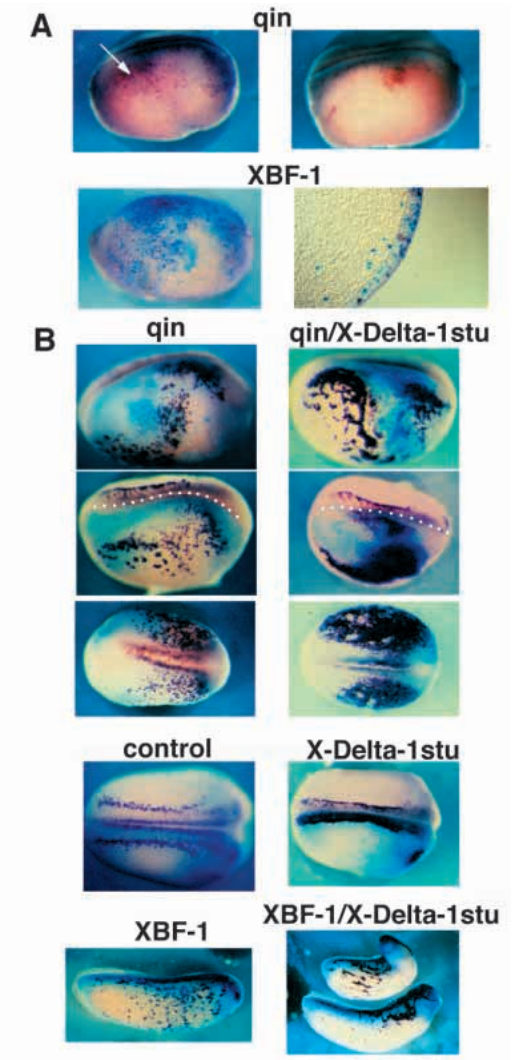

Fig. 8. Lateral inhibition is activated by *XBF-1* and *qin* and contributes to the dispersed pattern of ectopic *N-tubulin* expression. (A) Embryos were injected with 0.5 ng *qin* or *XBF-1* RNA, co-injected with *lacZ* RNA and assayed for X-gal staining (light blue) and *X-Delta-1* expression (purple). In the *qin*-injected embryo, the injected side (shown on the left) expresses ectopic *X-Delta-1* (arrow) while the control side (shown on the right) does not. In the *XBF-1*-injected embryo, the left panel represents a ventral view and the right panel a section through the ventral side of the embryo under high magnification, both showing ectopic *X-Delta-1* expression in purple. (B) Embryos were injected with *qin*, *XBF-1*, *qin/X-Delta-1* ^{stu} (a dominant negative form of *X-Delta-1*), *XBF-1/X-Delta-1* ^{stu} or *X-Delta-1* ^{stu} RNA, co-injected with *lacZ* RNA and assayed for X-gal staining (light blue) and *N-tubulin* expression (purple). In the *qin*- and *XBF-1*-injected embryos, the pattern of ectopic neuronal differentiation is less dispersed in the presence of *X-Delta-1* ^{stu} suggesting that lateral inhibition limits the number of cells that adopt a neuronal fate in response to *qin* or *XBF-1*. However, neuronal differentiation is not observed in areas that express high levels of *qin* (identified by strong uniform X-gal staining, middle panels), even in the presence of *X-Delta-1* ^{stu}. A white dotted line indicates the dorsal midline, for comparison between the injected and uninjected side. Note that both the high dose 'suppression of endogenous-ectopic induction' phenotype (top two frames, middle panel) and the low dose ' expansion of endogenous' *N-tubulin* phenotype (lower frame, middle panel) are affected by co-expression of *X-Delta-1*^{stu}.

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. 7I and L).

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*^{stu}, resulted in a less scattered pattern of ectopic *N-tubulin* compared to injections

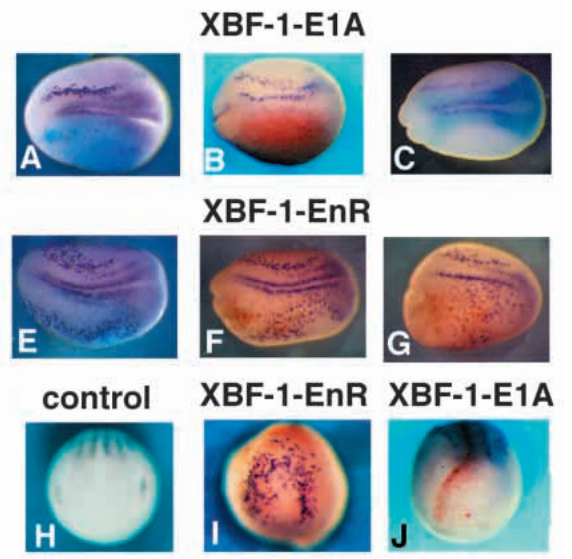

Fig. 9. Misexpression of *XBF-1-E1A* versus *XBF-1-EnR* has opposite effects on primary neurogenesis. (A,B) Injection of *XBF-1-E1A* completely suppresses *N-tubulin* (purple) on the injected side, identified by X-gal staining (A) or anti-myc staining (B). C shows that *XBF-1-E1A* suppresses *N-tubulin* (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.

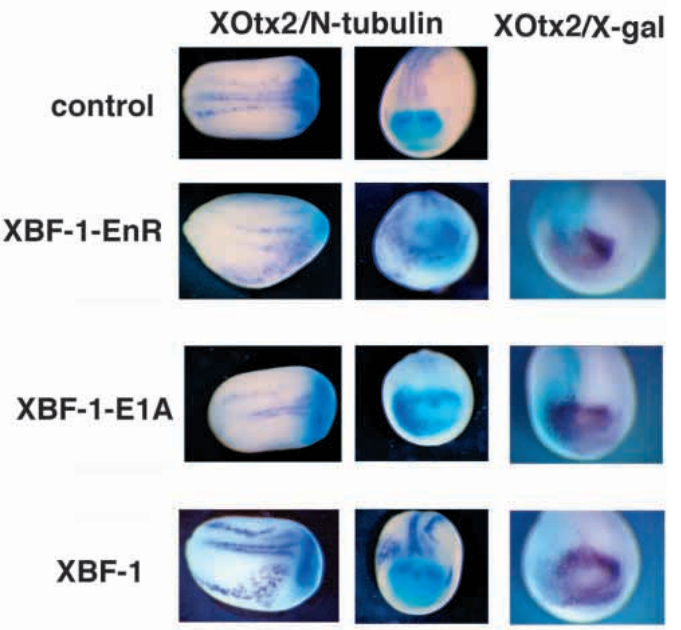

Fig. 10. Misexpression of *XBF-1-E1A* versus *XBF-1-EnR* and *XBF-1* has opposite effects on anterior development. Embryos were injected with various RNAs as indicated on the side of each set of panels and were analysed by double in situ hybridisation for *N-tubulin* (magenta) and *XOtx2* (light blue), 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).

of *XBF-1* or *qin* alone (Fig. 8B). In *XBF-1*- or *qin/X-Delta-1^{stu}/lacZ*-injected embryos, *N-tubulin*-positive cells were connected in 'islands' of positive cells around *XBF-1*- or *qin*-expressing cells. However, areas that stained heavily with X-gal and that presumably expressed high and uniform levels of *XBF-1* or *qin* did not express *N-tubulin* even in the presence of *X-Delta-1^{stu}*. This finding suggested that, while lateral inhibition contributes to generating a punctate neuronal differentiation pattern in response to *XBF-1*, the inhibition of neuronal differentiation in high *XBF-1*-expressing areas is not due to increased lateral inhibition.

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 =0%, *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 anteriorly 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).

DISCUSSION

In this paper, we examine the role of the *Xenopus* gene *XBF-1* in primary neurogenesis. *XBF-1* is a member of the winged helix family of transcription factors, and is homologous to a number of vertebrate genes, such as the rat *BF-1* and chicken *qin* genes (Tao and Lai, 1992; Chang et al., 1995). The *BF-1* subgroup of vertebrate winged helix genes is related to the *Drosophila slp* genes, which are important for head development and for maintaining the polarity of parasegments (Grossniklaus et al., 1994; Cadigan et al., 1994). *XBF-1* is expressed in the anterior neural plate which gives rise to the telencephalon and olfactory placodes. The rat and chicken homologues are also expressed in the telencephalon (Chang et al., 1995; Tao and Lai, 1992). Previous experiments suggested that *XBF-1* and the mouse homologue *BF-1* may act to suppress early neuronal differentiation in the anterior neural plate (Papalopulu and Kintner, 1996) and in the forebrain (Xuan et al., 1995). In this paper, we ectopically express *XBF-1* and its chicken homologue *qin* in the posterior neural plate of *Xenopus* embryos and assay the pattern of neuronal differentiation. We find that *XBF-1* and *qin* have identical activities in this assay, suggesting that their function is evolutionarily conserved.

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 can not 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 *XOtx2* 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 *XOtx2* 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*

(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).

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 *Hairy*-related genes (reviewed in Fisher and Caudy, 1998). In support of this idea, we note that the knock-out of a *Hairy*-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-ngnr-1* expression occur around the anterior border of the *XBF-1* expression domain. Thus, *XBF-1* may a have 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.

We thank Enrique Amaya and Eric Bellefroid for critical comments on the manuscript, Chuck Kimmel for a critical discussion and Sarah Bray for mentioning the case of Kruppel. We also thank Eric Bellefroid for his generosity with various reagents, Rob Grainger for making available a *XSox3* probe prior to publication and Alex Sossick and Desmond Schmidt for their expert help with computing. N. P. and C. B. were supported by the Wellcome Trust, J. L. was supported by the Council for Tobacco Research, USA (Grant 4053) and the American Cancer Society (Grant RPG-97-069-01-VM). This work was supported by a Career Development Award from the Wellcome Trust to N. P.

REFERENCES

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish *nk2.2* influenced by *sonic hedgehog/vertebrate hedgehog-1* and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121,** 1755-1768.

Bellefroid, E.J., Bourguignon, C., Holleman, T., Ma, Q., Anderson, D. J., Kintner, C. and Pieler, T. (1996). X-Myt1, a *Xenopus* C2HC-type zinc finger protein with a regulatory function in neuronal differentiation. *Cell* **87,** 1191-1202.

Bellefroid, E.J., Kobbe, A., Gruss, P., Pieler, T., Gurdon, J. B. and Papalopulu, N. (1998). *Xiro3* encodes a *Xenopus* homolog of the *Drosophila Iroquois* genes and functions in neural specification. *EMBO J.* **17,** 191-203.

Bier, E. (1997). Anti-neural inhibition: a conserved mechanism for neural induction. *Cell* **89,** 681-684.

Blumberg, B., Bolado, J. Jr., Moreno, T. A., Kintner, C., Evans, R. and Papalopulu, N. (1997). An essential role for retinoid signalling in anteroposterior neural patterning. *Development* **124,** 373-379.

Bradley, R. S., Espeseth, A. and Kintner, C. (1998). NF-protocadherin, a novel member of the cadherin superfamily, is required for *Xenopus* ectodermal differentiation. *Curr. Biol.* **18,** 325-334.

Brewster, R., Lee, J. and Ruiz i Altaba, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* **393**, 579-583.

Cadigan, K. M., Grossniklaus, U. and Gehring, W. J. (1994). Localised expression of *sloppy-paired* protein maintains the polarity of *Drosophila* parasegments. *Genes Dev.* **8,** 899-913.

Chalepakis, G., Jones, F. S., Edelman, G. M. and Gruss, P. (1994). Pax-3

contains domains for transcription activation and transcription inhibition. *Proc. Natl. Acad. Sci. USA* **91,** 12745-12749.

Chang, H. W., Li, J., Kretzschmar, D. and Vogt, P. K. (1995). Avian cellular homolog of the *qin* oncogene. *Proc. Natl. Acad. Sci. USA* **92,** 447-451.

Chitnis, A., Hernique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* **375,** 761-766.

Chitnis, A. and Kintner, C. (1996). Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in *Xenopus* embryos. *Development* **122,** 2295-2301.

Clevidence, D. E., Overdier, D. G.,Tao, W., Qian, X., Pani, L., Lai, E. and Costa, R. H. (1993). Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding-domain family. *Proc. Natl. Acad. Sci. USA* **90,** 3948-3952.

Coffman, C., Harris, W. and Kintner, C. (1990). *Xotch,* the *Xenopus* homolog of *Drosophila Notch*. *Science* **249,** 1438-1441.

Couly, G. and LeDouarin, N. M. (1988). The fate map of the cephalic neural primordium at the presomitic to the 3-somite stage in the avian embryo. *Development* **103 Supplement,** 101-113.

Dubois, L. L., Bally-Cuif, M., Crozatier, J., Moreau, L., Paquereau, ? and Vincent, A. (1998) XCoe2, a trascription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in *Xenopus. Curr. Biol.* **8,** 199-209.

Eagleson, G. W. and Harris, W. A. (1989). Mapping of the presumptive brain regions in the neural plate of *Xenopus laevis*. *J. Neurobiol.* **21,** 427-440.

Eagleson, G., Ferreiro, B. and Harris, W. (1995). The fate of the anterior neural ridge and the morphogenesis of the *Xenopus* forebrain. *J. Neurobiol.* **28,** 146-158.

Ferreiro, B., Kintner, C., Zimmerman, K., Anderson, D. and Harris, W. (1994). XASH genes promote neurogenesis in *Xenopus* embryos. *Development* **120,** 3649-3655.

Fisher, A. and Caudy, M. (1998). The function of hairy-related bHLH repressor proteins in cell fate decisions. *BioEssays* **20,** 298-306.

Forehand, C. J. and Farel, P. B. (1982). Spinal cord development in anuran larvae: I. Primary and secondary neurons. *J. Comp. Neurol.* **209,** 386-394.

Zygar, C. A., Cook,T.L., and Grainger, R.M. (1998). Gene activation during early stages of lens induction in Xenopus. *Development* **125,** 3509-3519.

Grossniklaus, U., Cadigan, K. M. and Gehring, W. J. (1994). Three maternal coordinate systems cooperate in the patterning of the *Drosophila* head. *Development* **120,** 3155-3171.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian *achaete-scute homolog 1* is required for the early development of olfactory and autonomic neurons. *Cell* **75,** 463-476.

Han, K. and Manley, J. L. (1993). Functional domains of the *Drosophila* engrailed protein. *EMBO J.* **12,** 2723-2733.

Harland, R. M. (1991). In situ hybridization:an improved whole-mount method for *Xenopus* embryos. In *Methods in Cell Biology* 36 (ed. B. K. Kay and H. B. Peng), pp. 685-695. San Diego, CA: Academic Press.

Hartenstein, V. (1989). Early neurogenesis in *Xenopus*: The spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. *Neuron* **3,** 399-411.

Hartenstein, V. (1993). Early pattern of neuronal differentiation in the *Xenopus* embryonic brainstem and spinal cord. *J. Comp. Neurol.* **328,** 213-231.

Ishibashi, M., Ang, S.-L., Shiota, K., Nakahishi, R.and Guillemot, F.(1995). Targeted disruption of mammalian hairy and *Enhancer of split* homolog-1 (*HES-1*) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Gen. Dev.* **9,** 3136-3148.

Kaufmann, E. and Knochel, W. (1996). Five years on the wings of *forkhead. Mech. Dev.* **57,** 3-20.

Kim, P., Helms, A. W., Johnson, J. E.and Zimmerman, K. (1997). XATH-1, a vertebrate homolog of *Drosophila* atonal, induces neuronal differentiation within ectodermal progenitors. *Dev. Biol.* **187,** 1-12.

Klein, S. L. and Graziadei, P. P. C. (1983). The differentiation of the olfactory placode in *Xenopus laevis*: a light and electron microscope study. *J. Comp. Neurol.* **217,** 17-30.

Knecht, A. K., Good, P. J., Dawid, I. B and Harland, R. M. (1995). Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. *Development* **121,** 1927-1936.

Knouff, R. A. (1935). The developmental pattern of ectodermal placodes in *Rana Pipiens*. *J. Comp. Neurol.* **62,** 17-71.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel,S. E., Economides, A., Stahl, N., Yancopoulos, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262,** 713-718.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of X*enopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268,** 836-844.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr. Opin. Neurobiol.* **6,** 3-10.

Li, J., Chang, H. W., Lai, E., Parker, E. J. and Vogt, P. (1995). The oncogene qin codes for a transcriptional repressor. *Cancer Research* **55,** 5540-5544.

Li, J., Thurm, H., Chang, H. W., Ianocovi, J. S. and Vogt, P. (1997). Oncogenic transformation induced by the Qin protein is correlated with transcriptional repression. *Proc. Natl. Acad. Sci.* **94,** 10885-10888.

Lillie, J. W. and Green, M. R. (1989). Transcription activation by the adenovirus E1A protein. *Nature* **338,** 39-44.

Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal differentiation gene. *Cell* **87,** 43-52.

MacDonald, R., Xu, Q. Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S. and Wilson, S. (1994). Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic Zebrafish forebrain. *Neuron* **13,** 1039-1053.

Marine, J.-C., Bellefroid, E. J., Pendeville, H. Martial, J. A. and Pieler, T. (1997). A role for *Xenopus* Gli-type zinc finger proteins in the early embryonic patterning of mesoderm and neuroectoderm. *Mech. Dev.* **63,** 211-225.

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

Pani, L., Overdier, D. G., Porcella, A., Qian, X., Lai, E. and Costa, R. H. (1992). Hepatocyte nuclear factor 3β contains two transcriptional activation domains one of which is novel and conserved with the *Drosophila* Fork Head protein. *Mol. Cell. Biol.* **12,** 3723-3732.

Papalopulu, N. and Kintner, C. (1996). A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in *Xenopus* neuroectoderm. *Development* **122,** 3409-3418.

Penzel, R., Oschwald, R., Chen, Y., Tacke, L., Grunz, H. (1997). Characterization and early embryonic expression of a neural specific transcription factor *xSOX3* in *Xenopus laevis*. Int J Dev Biol **41**:667-677.

Ravassard, P., Vallin, J., Mallet, J. and Icard-Liepkalns, C. (1997). Relax promotes ectopic neuronal differentiation in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **94,** 8602-8605.

Ross, L. S., Parrett, T. and Easter, S. S. J. (1992). Axonogenesis and morphogenesis in the embryonic zebrafish brain. *J. Neurosc.* **12,** 467-482.

Rupp, R. A. W., Snider, L. and Weintraub, H. (1994). *Xenopus* embryos regulate the nuclear localisation of XMyoD. *Genes Dev.* **8,** 1311-1323.

Sauer, F. and Jackle, H. (1991). Concentration-dependent transcriptional activation or repression by *Kruppel* from a single binding site. *Nature* **353,** 563-566.

Sauer, F. and Jackle, H. (1993). Dimerization and the control of transcription by Kruppel. **364,** 454-457.

Saxen, L. (1989). Neural Induction. *Int. J. Dev. Biol.* **33,** 21-48.

Takebayashi, K., Takahashi, S., Yokota, C., Tsuda, H., Nakanishi, S., Asashima, M.and Kageyama, R. (1997). Conversion of ectoderm into a neural fate by *ATH-3*, a vetrebrate helix-loop-helix gene homologous to the *Drosophila* proneural gene *atonal*. *EMBO J.* **16,** 384-395.

Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274,** 1115-1122.

Tao, W. and Lai, E. (1992). Telencephalon-restricted expression of BF-1, a new member of the *HNF-3/fork head* gene family in the developing rat brain. *Neuron* **8,** 957-966.

Turner, D. L. and Weintaub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8,** 1434-1447.

Wettstein, D. A., Turner, D. L.and Kintner, C. (1997). The *Xenopus* homolog of *Drosophila* Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124,** 693-702.

Wilson, S. W., Ross, L. S., Parrett, T. and Easter, S. S. Jr. (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. *Development* **108,** 121-145.

Xuan, S., Baptista, C. A, Balas, G., Tao, W., Soares,V. C and Lai, E. (1995). Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron* **14,** 1141-1152.

Zimmerman, K., Shih, J., Bars, J., Collazo, A. and Anderson, D. J. (1993). *XASH-3*, a novel *Xenopus achaete-scute* homolog, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate. *Development* **119,** 221-232.

Zygar, C. A., Cook,T. L. and Grainger, R. M. (1998). Gene activation during early stages of lens induction in Xenopus. *Development* **125,** 3509-3519.