The EMBO Journal Vol.17 No.1 pp.191–203, 1998

Xiro3 encodes a *Xenopus* homolog of the *Drosophila Iroquois* genes and functions in neural specification

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We have identified in *Xenopus* and in the mouse two highly related genes, *Xiro3* and *Irx3* respectively, that encode a *Drosophila Iroquois*-related homeobox transcription factor. *Xiro3* in *Xenopus* and *Irx3* in the mouse are expressed early in the prospective neural plate in a subset of neural precursor cells. In *Xenopus*, injection of *Xiro3* mRNA expands the neural tube and induces ectopic neural tissue in the epidermis, based on the ectopic expression of early neural markers such as *Xsox3*. In contrast, the differentiation of the early forming primary neurons, as revealed by the expression of the neuronal marker N-tubulin, is prevented by *Xiro3* expression. Activation of *Xiro3* expression itself requires the combination of a neural inducing (noggin) and a posteriorizing signal (basic fibroblast growth factor). These results suggest that *Xiro3* activation constitutes one of the earliest steps in the development of the neural plate and that it functions in the specification of a neural precursor state.

Keywords: homeobox/*Iroquois*/mouse/neural specification/prepattern gene/*Xenopus*

Introduction

During embryonic development, the precursor cells of the vertebrate central nervous system are induced in the dorsal ectoderm by vertical and/or planar signals from a region of the dorsal mesoderm called the organizer (Spemann and Mangold, 1924). A number of secreted molecules (e.g. noggin, follistatin and chordin) have been identified that are expressed in the organizer and that have neuralizing activity. These factors are thought to act by antagonizing BMP4, which serves as an epidermalizing signal (Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1995; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; for review, see Bier, 1997).

By the neural plate stage, the neural ectoderm is patterned in remarkable detail. This is shown by the highly regionalized expression pattern of several genes along the A–P and M–L (prospective D–V axis) of the neural plate (e.g. Papalopulu and Kintner, 1993, 1996a,b; Bang *et al.*, 1997). In addition, in lower vertebrates, a number of neuroectodermal cells exit the cell cycle as early as the neural plate stage and start differentiating (Hartenstein, 1989). In *Xenopus*, this is shown by the expression of a neuronal marker, N-tubulin, which is observed in three longitudinal domains on either side of the dorsal midline (Chitnis *et al.*, 1995). Cells that differentiate in these longitudinal domains of the posterior neural plate correspond to the three classes of primary neurons, namely motor neurons, interneurons and sensory neurons, in a medial-to-lateral order. Within these domains of neuronal differentiation, N-tubulin expression is detected in scattered cells among a non-expressing population.

The sequence and molecular nature of the events that follow the initial neuralization of the ectoderm by organizer molecules and which lead to the differentiation of neurons of distinct identities in a precise pattern and temporal order are still poorly understood. As far as A–P patterning is concerned, all neural inducers that are known to date induce neural tissue of anterior character (Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1995). Therefore, it has been suggested that additional signals from the posterior dorsal axial and non-axial mesoderm are involved in the posteriorization of the induced neural tissue. Recently, it has been reported that anterior neural tissue can be posteriorized by signals such as fibroblast growth factor (FGF), retinoic acid (RA) and Wnt-3A (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; McGrew *et al.*, 1995; Crossley *et al.*, 1996; Papalopulu and Kintner, 1996a; Pownall *et al.*, 1996; Bang *et al.*, 1997; Blumberg *et al.*, 1997).

As far as the differentiation of primary neurons within the neural ectoderm is concerned, it is thought to be under the control of proneural and neurogenic genes. In *Drosophila*, proneural genes, which are typically transcription factors of the basic helix–loop–helix (bHLH) class, confer neural potential on groups of ectodermal cells, called proneural clusters (for review, see Campuzano and Modelell, 1992; Ghysen and Dambly-Chaudière, 1993; Skeath and Carroll, 1994; Lewis, 1996). Subsequently, neurogenic genes, in particular the membrane-bound ligand Delta and the Notch receptor, restrict proneural gene expression to a single cell within each proneural cluster. This cell then becomes a neuroblast (for review, see Campos-Ortega, 1993; Muskavitch, 1994; Artanavis-Tsakonas *et al.*, 1995).

Several transcription factors of the bHLH family have been identified in vertebrates and some appear to be homologues of the *Drosophila* proneural *achaete–scute* complex (AS-C) and *atonal* genes (Guillemot *et al.*, 1993; Zimmerman *et al.*, 1993; Ferreiro *et al.*, 1994; Turner and Weintraub, 1994; Lee *et al.*, 1995; Sommer *et al.*, 1995;

Ma *et al.*, 1996; Takebayashi *et al.*, 1997). As mentioned above, in *Xenopus*, primary neurons form in a scattered pattern within longitudinal domains in the posterior neural plate. Based on expression studies and functional analysis, *Xenopus* bHLH genes are thought to confer neuronal potential in cells within each longitudinal domain. Thus, these domains have been viewed as analogous to the proneural clusters in *Drosophila*. In addition, vertebrate homologues of the neurogenic genes Notch and Delta have also been identified (Coffman *et al.*, 1993; Chitnis *et al.*, 1995; Henrique *et al.*, 1995; Simpson, 1995). Functional studies in *Xenopus* have shown that, similarly to the *Drosophila* neurogenic genes, the vertebrate homologues of Notch and Delta have a role in limiting the number of cells that differentiate to neurons within each proneural domain (Coffman *et al.*, 1993; Chitnis *et al.*, 1995). The expression of neurogenic genes is activated by bHLH transcription factors but, in turn, the expression and/or the activity of certain bHLH transcription factors is restricted by the Notch–Delta inhibitory interactions. This feedback loop between proneural and neurogenic genes is thought to result in the selection of a few cells that differentiate into neurons within each longitudinal proneural domain.

From studies in *Xenopus* embryos, it has become clear that although all bHLH proneural genes isolated to date can promote neuronal differentiation, they differ in their time and place of expression; in general, genes that are expressed earlier are expressed in a wider domain that encompasses the domain of expression of genes that are expressed later. Furthermore, all the proneural bHLH genes that have been tested have the ability to activate the neurogenic gene Delta, but they differ in their sensitivity to Notch-mediated inhibition. In general, earlier expressed proneural genes (e.g. *XASH3* and *X-ngnr-1*) tend to be more sensitive to inhibition by activated Notch than later expressed genes (e.g. *NeuroD*) or certain combinations of genes (*X-ngnr-1/X-MyT1*) (Bellefroid *et al.*, 1996; Chitnis and Kintner, 1996). Thus, while both early and late bHLH factors convey neuronal potential to cells in which they are expressed, later acting genes or combinations of genes may act to stabilize the process of neuronal differentiation by allowing cells to escape lateral inhibition. In order to emphasize the distinct roles of early and late acting bHLH proneural genes, they have been termed 'neuronal determination' and 'neuronal differentiation' genes, respectively. Additional support for this model is provided by the observation that neuronal determination genes such as *X-ngnr-1* have been shown to activate the expression of neuronal differentiation genes such as *NeuroD* in a unidirectional cascade (Ma *et al.*, 1996). These results support the idea that in the developing central nervous system (CNS), cells go through successive stages of specification/commitment, defined by the sequential activation of different sets of transcription factors.

In contrast, very little is known about the events which follow neural induction and precede the selection of neuronal precursor cells. Most vertebrate proneural genes isolated to date are expressed in a punctate manner within the longitudinal domains of the posterior neural plate. One exception is the bHLH gene *XASH3*, which is uniformly expressed. However, *XASH3* expression is restricted to only a small part of the neural plate. Thus, one would predict the existence of additional bHLH proneural genes which are uniformly expressed within the open neural plate and which are responsible for conveying neuronal potential to a large population of neural precursor cells. In addition, one would predict the existence of regulatory genes which are responsible for the site-specific activation of these proneural genes. These regulators of proneural genes would lie downstream of neural induction and they would act before the neuronal potential becomes limited to a few cells by lateral inhibitory interactions. Such genes could be the homologues of the *Drosophila* homeobox-containing *Iroquois* genes which have been shown to be involved in the site-specific activation of the proneural AS-C genes (Dambly-Chaudière and Leyns, 1992; Gomez-Skarmeta *et al.*, 1996) and which have therefore been termed prepattern genes.

We now report on the identification of two vertebrate homeobox genes, *Xenopus Xiro3* and mouse *Irx3*, which are homologous to each other and to the *Drosophila Iroquois* genes. Our analysis of the function of *Xiro3* in *Xenopus* embryos suggests that it functions in the earliest step of neural development to specify a neural precursor state, characterized by the expression of early neural and the absence of neuronal differentiation markers.

Results

Identification of the Xiro3/Irx3 genes

Degenerate primers corresponding to part of the homeodomain of the *Drosophila Iroquois* genes were used in a PCR to amplify related sequences from a stage 12 *Xenopus* cDNA library (see Materials and methods). By screening a stage 17 *Xenopus* cDNA library with the amplified fragment, we have obtained a 1.9 kb cDNA clone, termed *Xiro3*, which encodes a polypeptide of 448 amino acids containing a homeodomain showing 92% identity to the *Drosophila Iroquois* homeodomain (Figure 1A). In the mouse, three distinct cDNA clones encoding *Drosophila Iroquois*-related sequences have been isolated (Bosse *et al.*, 1997). Here, we present one of these clones, named *Irx3*, that showed 81% identity to *Xiro3* in the region upstream of the homeobox and 100% identity within the homeodomain itself (Figure 1A). In human, a partial cDNA sequence encoding an identical homeodomain and showing 97% identity in the portion of sequence that precedes the homeobox region has also been reported (HS90308) (Figure 1B). Less homology was detected between *Xiro3* and the two other murine *Irx* clones, as well as with other members of the family isolated from *Xenopus* (*Xiro1* and *2*) (Gómez-Skarmeta *et al.*, 1998) and from human (Figure 1B), arguing that *Xiro3* might indeed be the homologue of *Irx3*. In the region 3′ to the homeodomain, *Irx3* and *Xiro3*, like all members of the family, have stretches rich in acidic residues that could be involved in transactivation. In addition, two potential phosphorylation sites for mitogen-activated protein kinase (MAPK) (Clark-Lewis *et al.*, 1991; Gonzales *et al.*, 1991) and a short region close to the carboxy-terminus are also conserved (Mc Neill *et al.*, 1997).

Xiro3 expression pattern in Xenopus embryos

We have analysed *Xiro3* expression in *Xenopus* embryos by *in situ* hybridization using the entire *Xiro3* cDNA clone

A

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Xenopus  MSFPQLGYQYIRPLYPSDR---------QSVGVTRSG-----TELSPAGTLSNVLSSVYG   46
Mouse    MSFPQLGYQYIRPLYPPERPGAAGGGGGGSSAGGRSGPGAGASELAASGSLSNVLSSVYG   60

         APY----AAAAAAQAYGAFLPYSAELPIFPQLGSXYDMKDSPGVQH----AAFSHPHPAF   98
         APYAAAAAAAAAAQGYGAFLPYATELPIFPQLGAQYELKDSPGVQHPATAAAFPHPHPAF  120

         YPYGQYQFGDPSRPKNATRESTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVSTW  158
         YPYGQYQFGDPSRPKNATRESTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVSTW  180

         FANARRRLKKENKMTWAPRSRTDEEGNAYGSDH-EEDKHEDEEEI--DLENIDTEDIESK  215
         FANARRRLKKENKMTWAPRSRTDEEGNAYGSEREEEDEEEDEEESKRELE-MEEEELAXR  239

         -EDLDDPDTD-IHSDSKTDTRSDSEVSDGFE-DLNVPEDR---L-LKSV----VGQRQLL  264
         GGGHGGRGAGRRRRDEEIDLENLDSAAAGSELTLAGAAHRNGDFGLGPISDCKTSDSDDS  299

         NEEPQDK--CALS---SDAKVPQ-PACEQIKLNRLPPSPPQENNMPVAHKPKIWSLAETA  318
         SEGLEDRPLSVLSLAPPPPVARAPASPPSPPSSLDPCAPAPAPSSALQKPKIWSLAETA  359

         TTPDNP-RNSPNTGGS-----VN--TQNL--------IAQHRLIASPGSRFQGWTGRAF-  361
         TSPDNPRRSPPGAGGSPPGAAVAPPTLQLSPAAAAAAAAAHRLVSAPLGKFPAWTNRPFP  419

         ------SAQQLSLLNSA--HFLQGLGVSHTAPCSGNASFPKAAESKCSTNSLTDRPSTVD  413
         GPPAGPRPHPLSMLGSAPQHLL-GLPGAAGHPAAAAAAYARPAE----PESGTDRCSALE  473

         IEKTIPVLNTAFQPVQRRSQDHLDAAMILSALSSS                           448
         VEKKL--LKTAFQPVPRRPQNRLDAALVLSALSSS                           507
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B

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                  HELIX I          HELIX II     HELIX III+IV

Xiro3       FGDPSRPKNATRESTSTLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKENKMTWAPRSR
Irx3        ------------------------------------------------------------------------------
U90308      ------------------------------------------------
U90304      Y---AYR-----DA-AI-------------------------------------------------------T--N-
U90305      LSGAG-R------T-----------------------------------------
U90306      MDSGT-R------T--------Q-------------------T----------
U90307      Y---G--------N-----------------------------------------
U90309      Y---AYR-----DA-A-------------------------------------------------------T--N-
Araucan     YDLAA-R--------A---------K-----------------------------------------E-KN-
Caupolican  YDLAA-R--------A------S--K-----------------------------------------E-KNK
Mirror      DLNGA-R------T-----------K-----------------------------------------E--N-
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Fig. 1. Sequence of *Xenopus Xiro3*, mouse *Irx3* and related homeobox factors. (**A**) Alignment of the entire predicted amino acid sequences of *Xenopus Xiro3* and mouse *Irx3* products. The homeodomain is boxed. The strongly acidic regions, the MAPK consensus phosphorylation sites and a conserved region present in all members of the *Iroquois* family are indicated by the first, second and third brackets, respectively. (**B**) Alignment of the *Xiro3* and *Irx3* homeodomains with the homeodomain of other members of the *Iroquois* subfamily. Conserved residues in comparison with *Xiro3* are indicated by dashes. References for the sequences are as follows: araucan and caupolican (Gomez-Skarmeta *et al.*, 1996); Mirror (McNeill *et al.*, 1997). U90304–U90309 are conceptual translations of human partial cDNA sequences.

as probe. Expression of *Xiro3* was found primarily in the developing CNS. The first neuroectodermal expression was detected at gastrula stages in two wide bilaterally symmetrical patches within the presumptive neural plate (Figure 2B and C). During early neurula stages, *Xiro3* was expressed in two transverse anterior bands and two short longitudinal stripes, one on either side of the dorsal midline of the embryo (Figure 2D). As neurulation proceeds, these longitudinal stripes of expression spread posteriorly in the prospective hindbrain and spinal cord areas (Figure 2E and F). Co-hybridization of late neurula embryos with a mixture of *Xiro3* and *en-2* probes showed that the intense transverse stripes of *Xiro3* expression overlapped those of *en-2* (data not shown) and were thus located at the midbrain–hindbrain junction. *Xiro3* was not expressed in the anterior neural plate, from which the forebrain is derived. Double labelling with *Xiro3* and the neuron-specific N-tubulin gene at stage 14 showed that *Xiro3* was expressed in both the superficial and deep layers of the neuroectoderm, in regions corresponding to the medial and intermediate stripes of primary neurons (Figure 2J–L). Within the deep layer, *Xiro3* expression

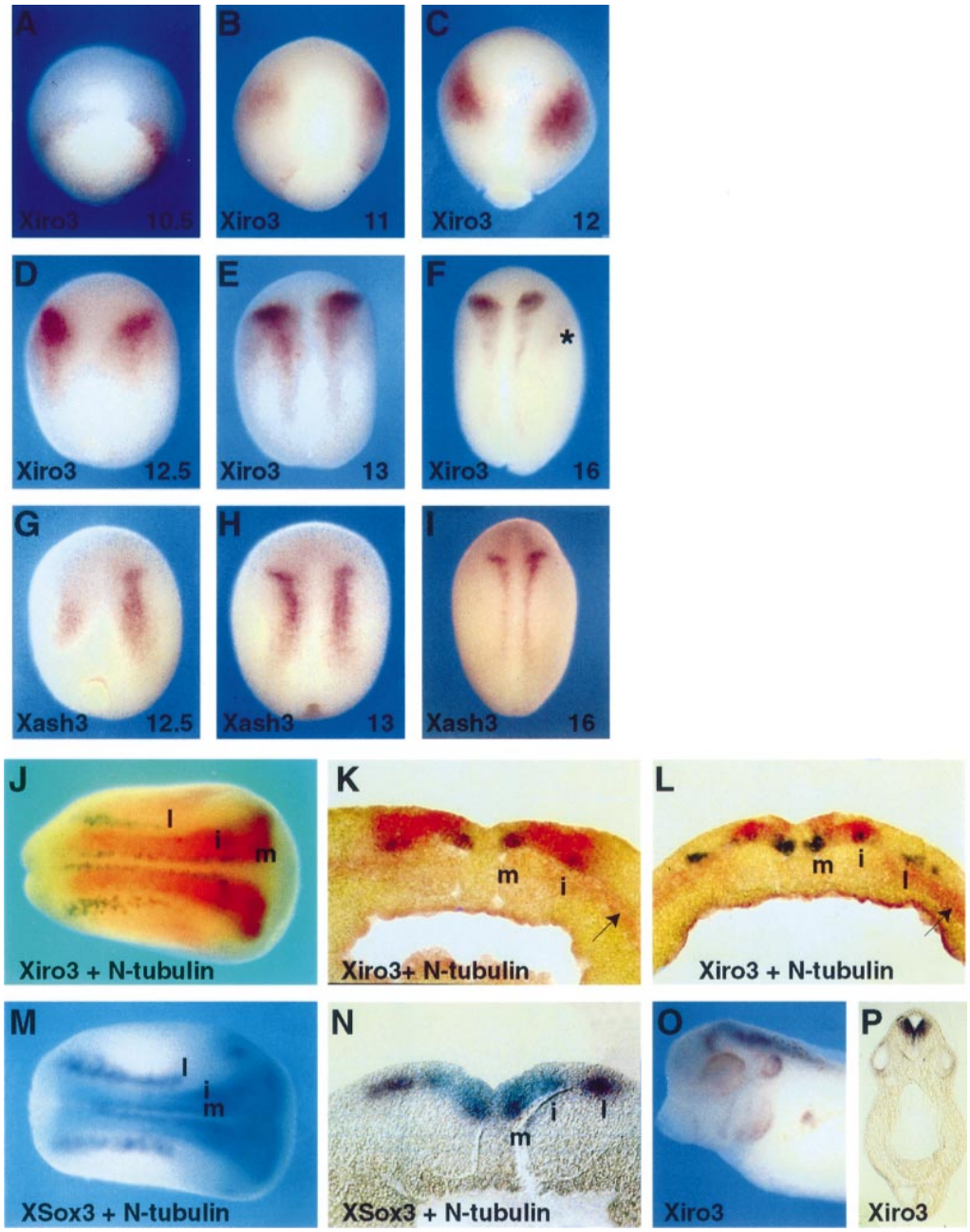

Fig. 2. *Xiro3* expression during early embryogenesis. (A–I) Embryos at the indicated stages are analysed by *in situ* hybridization with probes for *Xiro3* (**A–F**) and *XASH3* (**G–I**). Note that *Xiro3* expression within the prospective neural plate is first detected at stage 11 in the form of two wide symmetrical patches (B) and that later *Xiro3* and *XASH3* are similarly expressed in the neural plate in the form of two symmetrical longitudinal stripes and two transverse anterior bands. In addition, expression of *Xiro3* also occurs in the ectoderm lateral to the anterior neural plate (asterisk). (**J**) Double *in situ* hybridization of *Xiro3* (red) and N-tubulin (purple) in a stage 15 embryo (m, medial; i, intermediate; l, lateral). (**K**) Cross-sections of a stage 15 *Xiro3*/N-tubulin-stained embryo showing that *Xiro3* is expressed in regions corresponding to the medial and intermediate stripes of N-tubulin and that it is detected in cells located more superficially than those expressing N-tubulin. (**L**) Cross-section of a stage 15 *Xiro3*/N-tubulin-stained embryo at a more posterior position. Note that *Xiro3* expression appears in both the superficial and deep layer of the neurectoderm. *Xiro3* is also expressed in anterior lateral mesoderm (arrowheads). (**M**) Double *in situ* hybridization of *XSox3* (blue) and N-tubulin (purple). (**N**) Cross-section of the *XSox3*/N-tubulin-stained embryo shown in (M). Note that *XSox3* is expressed like *Xiro3* in the posterior neural plate in superficial layers of the neurectoderm in between the intermediate and medial N-tubulin stripes. (**O**) Lateral view of the head of a tadpole embryo. (**P**) Transverse section at the level of the otic vesicle. All embryos are shown in a dorsal view, with anterior to the top, except in (J), (M) and (O).

was detected in cells located more superficially than cells expressing the N-tubulin gene (Figure 2K). In tadpole stage embryos, *Xiro3* expression was detected in the brain, extending well anterior to the midbrain–hindbrain junction but excluding the forebrain, and decreasing in the spinal cord (Figure 2O). In cross-sections in the hindbrain region, *Xiro3* expression was restricted to an intermediate position along the D–V axis of the neural tube and was localized mainly to the ventricular zone (Figure 2P). By contrast, N-tubulin expression at that stage was only detected in the outermost cell layer of the neural tube containing differentiated neurons that do not express *Xiro3* (Bellefroid

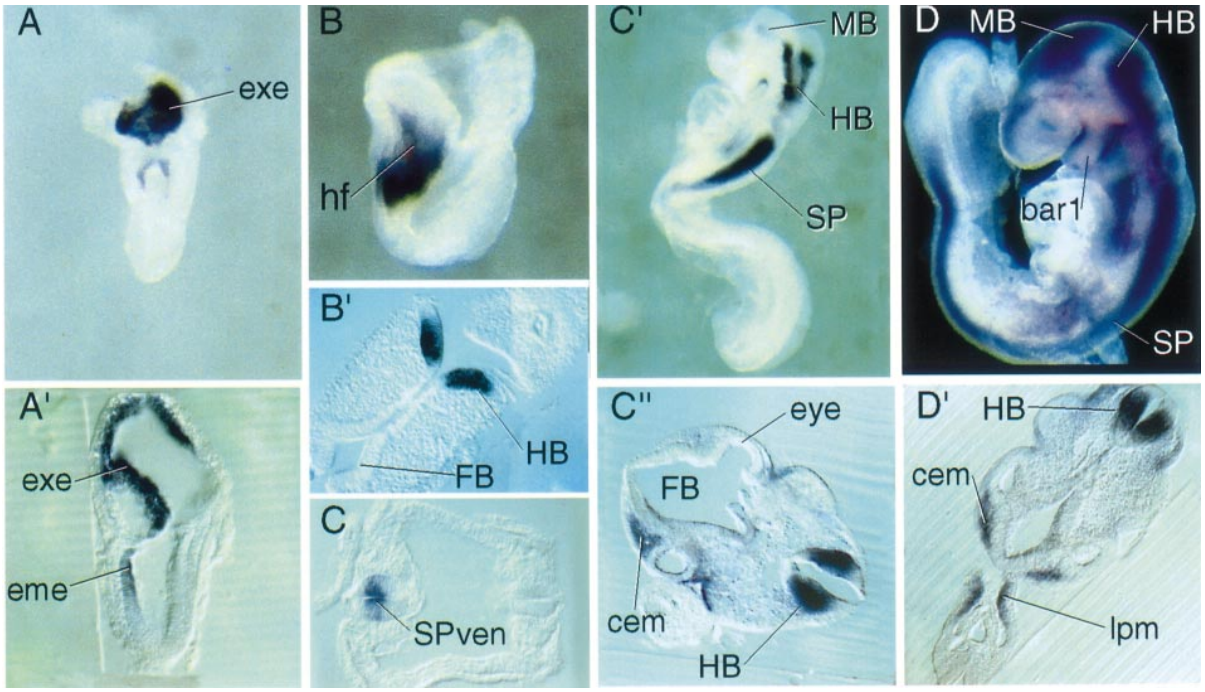

Fig. 3. *In situ* hybridization of *Irx3* during early mouse embryogenesis. (**A** and **A′**) Ventral view of an E7.5 embryo and transverse section respectively, with *Irx3* expression in the embryonic ectoderm (eme) and extra-embryonic ectoderm (exe). (**B**) The ventral view of an E8.0 embryo. Strong *Irx3* expression is seen in the posterior head fold (hf) portion giving rise to the hindbrain. (**B′**) Cross-section of an E8.0 embryo at the prospective hindbrain level exhibiting restricted expression into the hindbrain (HB, hinbrain; FB, forebrain). (**C**) Cross-section of an E8.5 embryo showing *Irx3* transcripts concentrated to the ventricular zone of the developing spinal cord (Spven). (**C′**) Dorsolateral view on an E8.5 embyo. *Irx3* expression is confined to the midbrain (MB), hindbrain (HB) and the rostral part of the spinal cord (SP). (**C″**) *Irx3* expression in a frontal section at the hindbrain level is found in the hindbrain (HB) and in cephalic mesodermal cells (cem). (**D**) *Irx3* expression in an embryo after neurulation. Strong expression is detected in the midbrain (MB), hindbrain (HB), spinal cord (SP) and in the first branchial arch (bar1). (**D′**) A corresponding frontal section at the hindbrain level showing *Irx3* expression in the lateral plate mesoderm (lpm).

et al., 1996). Thus, both at neurula and tadpole stages, high levels of *Xiro3* expression were observed in undifferentiated neurectodermal cells while lower levels were observed in cells that express N-tubulin.

Compared with the other neural-specific genes, *Xiro3* expression appears to be similar to that of the proneural HLH gene *XASH3* (Zimmerman *et al.*, 1993) and of the HMG box-encoding gene *XSox3* (Koyano *et al.*, 1997; Grainger *et al.*, personal communication). Like *Xiro3*, *XASH3* is also expressed within the neural plate in the form of two bilaterally symmetrical longitudinal stripes that end in anterior transverse bands (Figure 2G–I) and it is also expressed in superficial layers of the neurectoderm (data not shown). However, on a side-by-side comparison, we have found that *XASH3* was expressed later than *Xiro3*. For instance, *XASH3* expression was not detectable until advanced gastrula stages (stage 11.5–12) while *Xiro3* was already detected in the corresponding regions by early gastrulation (stage 11, Figure 2B). We also found that the anterior transverse bands of *Xiro3* expression extended slightly more anterior than those of *XASH3* (data not shown). The posterior longitudinal stripes of *Xiro3* expression overlap with but are broader than those of *XASH3* (compare Figure 2F with Figure 2I). *XSox3*, like *Xiro3*, is also expressed within the posterior neurectoderm in regions excluding the lateral-most area that gives rise to the Rohon–Beard cells, and the *XSox3* staining appears to be more superficial than that of N-tubulin (Figure 2M and N). Thus, both *XASH3* and *XSox3* appear to be expressed in undifferentiated cells of the neural plate, where *Xiro3* is also highly expressed.

In addition to the neural progenitor cells of the posterior nervous system, *Xiro3* was also expressed outside of the CNS. At early gastrula stages, *Xiro3* was expressed around the closing blastopore in two symmetrical dorsolateral domains (Figure 2A). During early to late gastrula stages, *Xiro3* was no longer expressed in the circumblastoporal mesoderm but was expressed in the anterior involuted lateral plate mesoderm (Figure 2K and L, arrows). During late neurula stages, in addition to the staining in the neural plate, weak staining was also detected lateral to the anterior neural plate (Figure 2F). In tadpoles (Figure 2O), *Xiro3* expression was detected in the ectodermal layer of the branchial arches, in the otic vesicle and the kidney.

Expression pattern of Irx3 in the early mouse embryo

In the mouse, the earliest embryonic *Irx3* expression was detected at the end of gastrulation, at E7.5 (Figure 3A). Saggital sections of embryos at this stage revealed *Irx3* transcripts to be restricted to the ectodermal layer, which gives rise to the nervous sytem. In addition, intense staining was detected in the extra-embryonic tissue of the chorionic ectoderm (Figure 3A′). At E8.0, *Irx3* expression is confined to the thickening neural ectoderm corresponding to regions of the future mesencephalon and rhombencephalon (Figure 3B and B′). From E8.5 onwards, *Irx3* expression was detected in the prospective midbrain and

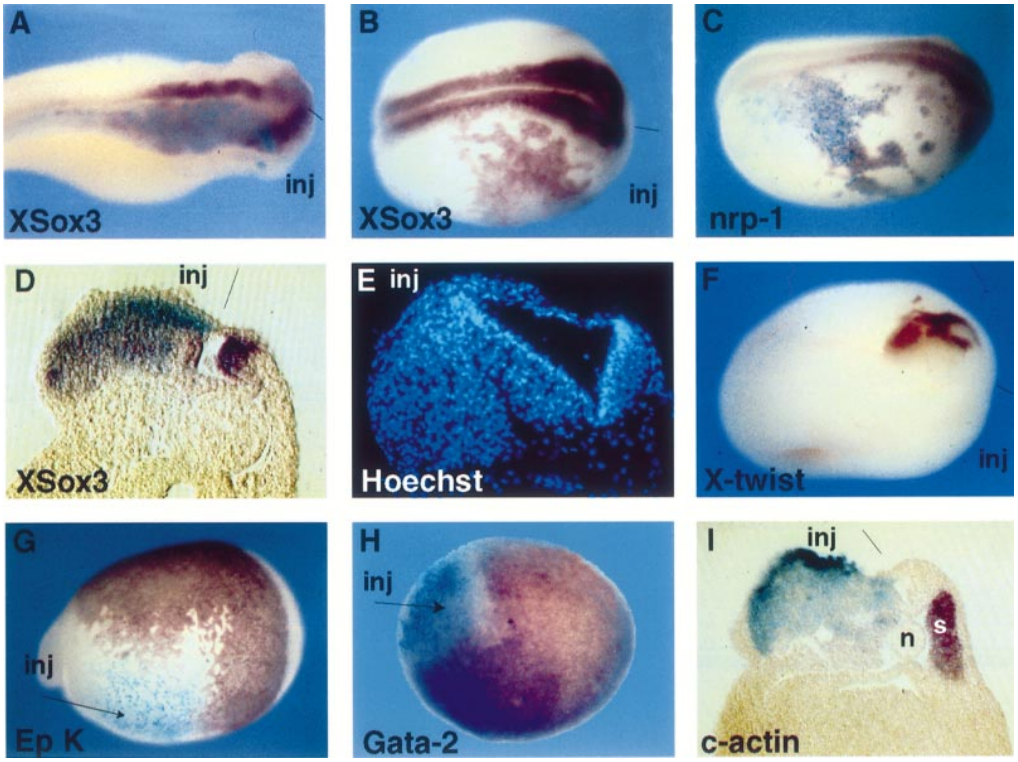

Fig. 4. Neural expansion in *Xiro3*-injected embryos. The injected side of the embryos is indicated and can be visualized by nuclear LacZ staining. Anterior is toward the right. Embryos are viewed from the dorsal or dorso-lateral side in (A), (B), (C) and (F) and from the the ventral side in (G) and (H). (**A**) Stage 30 embryo with increased *XSox3* expression in the expanded neural tube. (**B**) Stage 15 embryo showing ectopic *XSox3* expression in the ectoderm. (**C**) Ectopic expression of *nrp-1* in the lateral ectoderm. (**D**) A section through the hindbrain region of a stage 30 embryo labelled for *XSox3* expression. (**E**) A section through the hindbrain region of a Hoechst-stained stage 30 embryo showing that the number of cells in the expanded neural tube is increased. (**F**) *X-twist* is expressed in cranial neural crest on the control side but is absent on the injected side of the embryos. (**G**) Epidermal keratin is expressed outside the neural plate and is blocked in the *Xiro3*-injected area. (**H**) Like epidermal keratin, *GATA-2* is expressed in the non-neural ectoderm and is absent after *Xiro3* injection. (**I**) A section through the hindbrain of a stage 30 embryo showing that the formation of the somites as revealed by cardiac actin expression is affected on the injected side (n, notochord; s, somite).

hindbrain and in the rostral part of the closing neural tube (Figures 3C′). Cross-sections at the level of the spinal cord revealed *Irx3* transcripts confined to the ventricular zone of the neural tube, which contains uncommited progenitor cells (Figure 3C). In cross-sections at the level of the hindbrain, *Irx3* expression was detected in the ventricular zone of the neural tube, excluding the floor plate and roof plate, and in the cephalic mesenchyme (Figure 3C″). After closure of the neural tube at E9.5 (Figure 3D and D′), predominant *Irx3* expression persisted in the nervous system in the midbrain, hindbrain and spinal cord. Outside of the CNS, *Irx3* expression was detectable in the lateral plate mesoderm and the ectodermal layer of the branchial arches. Thus, both in the mouse and in *Xenopus*, *Irx3*/*Xiro3* is activated at the earliest steps of the development of the CNS and is expressed in neural progenitor cells.

Ectopic expression of Xiro3 in the dorsal ectoderm of the Xenopus embryo causes an increase in neural tissue at the expense of adjacent non-neural tissue

To determine the role of *Xiro3* in neural development, we examined the phenotype of embryos that were injected with *Xiro3* mRNA. Injection of 0.5–1 ng of *Xiro3* mRNA into one cell of the two-cell stage *Xenopus* embryos results in a failure to gastrulate normally. To circumvent this problem, we directed the expression of *Xiro3* into neural tissue and dorsal epidermis by injecting a single dorsal animal pole blastomere of 4 to 32-cell stage embryos with 50–250 pg of *Xiro3* RNA. Co-injection of LacZ mRNA served as a lineage marker in order to identify the injected side. In these experiments, overexpression of *Xiro3* consistently produced a bulge of tissue on the dorsal side of the embryo (51 out of 57 embryos). When the *Xiro3* mRNA was directed to the head region, eye and otic vesicle were reduced or absent. To test for the neural character of this extra tissue, we used a probe for the *Xenopus* HMG box transcription factor-encoding gene *XSox3*, a neural marker that is expressed similarly to *Xiro3* in the posterior neural plate (Figure 2M and N). *In situ* hybridization analysis of *Xiro3*-injected embryos with *XSox3* showed that the tissue bulge was neural (Figure 4A). Ectopic expression of *XSox3* could be detected as early as the neural plate stage, not only in the dorsal ectoderm, but also in lateral or ventral regions of the ectoderm that are not contiguous with the CNS (100% of the embryos examined; 25 embryos) (Figure 4B). Similar results were obtained using the general neural marker *nrp-1* (100% of the embryos examined; 20 embryos) (Figure 4C) (Knecht *et al.*, 1995). In those experiments, the ectopic neural tissue was coincident with the co-injected LacZ RNA in 21 out of 23 embryos examined. Sectioning of these embryos verified the dramatic expansion of the neural tube (Figure 4D). Hoechst labelling of nuclei in injected embryos further demonstrated that the cell number on the injected side of the expanded neural tube is strongly increased (Figure 4E).

A similar lateral expansion of the neural tube has been observed upon ectopic expression of *XASH3* (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994) and of a constitutively active form of *X-Notch-1* (Coffman *et al.*, 1993); these effects have been attributed mainly to conversion of non-neural cells to a neural fate. To test if this was also true for *Xiro3*-injected embryos, we looked at the expression of non-neural markers in the injected embryos. The expression of the *X-twist* gene, a marker of cephalic neural crest cells (Hopwood *et al.*, 1989a) was reduced or absent on the injected side (10 out of 10 embryos tested (Figure 4F). Expression of the epidermal keratin gene (Jonas *et al.*, 1985) and the *GATA-2* gene (Bertwistle *et al.*, 1996), which are both expressed in the non-neural ectoderm, was also severely blocked in the injected area of all embryos tested ($n = 20$ for epidermal keratin and 12 for *GATA-2*) (Figure 4G and H). In addition, in those cases where *Xiro3* RNA was directed to mesodermal regions, somite formation was severely impaired, as revealed by the suppression of cardiac actin gene expression (Figure 4I). To test if increased cell proliferation also contributes to the expansion of neural tissue, we blocked cell division in *Xiro3*-injected embryos at mid-gastrula stage with a mixture of hydroxyurea and aphidicolin (HUA) (Harris and Hartenstein, 1991). Embryos injected with *Xiro3* and treated with HUA developed an expanded neural tube just like *Xiro3*-injected untreated control embryos. Taken together, these results suggest that overexpression of *Xiro3* induces extra neural tissue, at least in part by converting adjacent non-neural cells to a neural fate.

Suppression of differentiation of the early forming primary neurons in Xiro3-injected embryos

The data presented so far demonstrate that overexpression of *Xiro3* in *Xenopus* embryos induced ectopic neural tissue, as revealed by the ectopic expression of the neural markers *XSox3* and *nrp-1*. In *Xenopus*, a number of neuroectodermal cells exit the cell cycle at the neural plate stage to differentiate as primary neurons (Hartenstein, 1989). To determine the effect of *Xiro3* overexpression on the process of primary neuronal differentiation, we examined the expression of the neuronal-specific N-tubulin gene (Chitnis *et al.*, 1995). In *Xiro3*-injected embryos, N-tubulin was suppressed within the neural plate (25 out of 25 embryos tested) (Figure 5A). In 40% of these embryos, we observed a displacement of one or more of the stripes of N-tubulin expression at the border of the *Xiro3/lacZ*-injected area (Figure 5B and C). None of the embryos that were injected outside the neural plate had ectopic N-tubulin expression (19 embryos tested). Similar results were obtained with the *X-MyT1* zinc finger gene which is expressed earlier in differentiating primary neurons (Bellefroid *et al.*, 1996) (Figure 5D).

In contrast, at tadpole stages, when the injected *Xiro3* mRNA was most probably degraded, we found that the expanded neural tube was positive for the N-tubulin marker (Figure 5E). However, the level of N-tubulin expression was higher in the portion of the neural tube that was weakly stained for X-gal. In addition, when the injected *Xiro3* mRNA was directed into the lateral ectoderm, 68 out of 84 embryos examined at the tadpole stage showed bulges in the non-neural ectoderm, 70% of

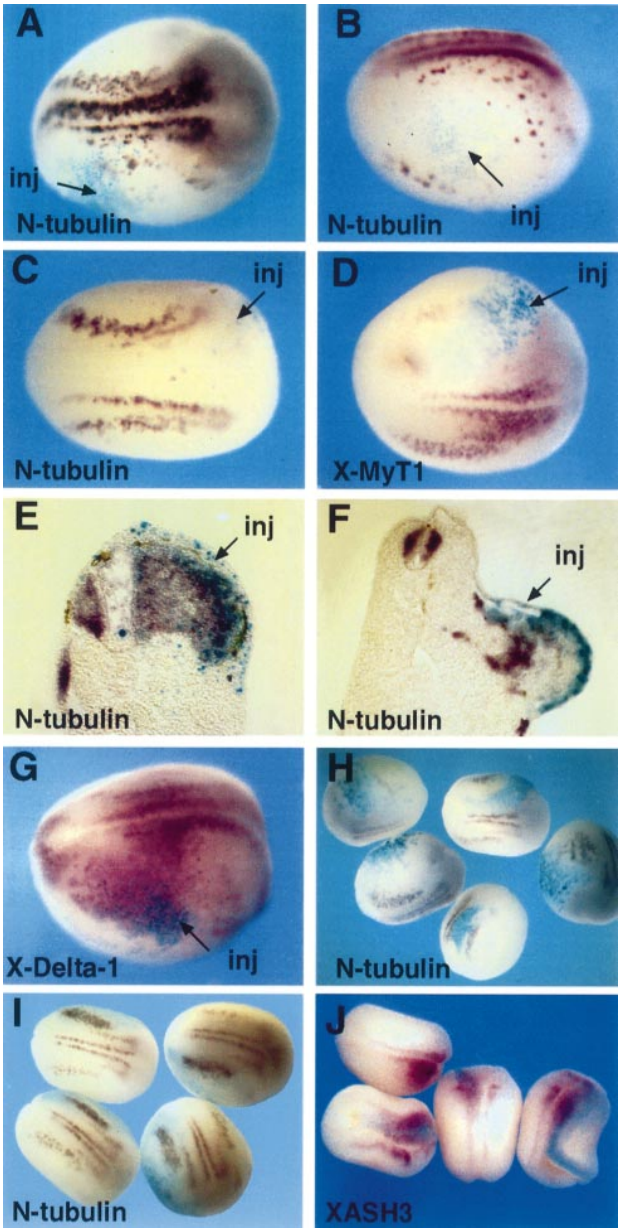

Fig. 5. *Xiro3* blocks the differentiation of the primary neurons and expands the expression of *XASH3*. All embryos are viewed from the dorsal side except in (B) (lateral view). In (A–D and G), embryos are viewed with the anterior end toward the right. The injected side of the embryos is marked by blue staining for nuclear LacZ expression. (**A**) Stage 15 embryo injected with *Xiro3* mRNA. Note that the lateral stripe of N-tubulin expression is suppressed on the injected side. (**B**) Stage 15 embryo injected with *Xiro3* mRNA showing disorganized N-tubulin expression at the border of the injected area. (**C**) Stage 15 embryo injected with *Xiro3* mRNA showing that the lateral and intermediate stripes of N-tubulin expression are moved to a more ventral position. (**D**) Stage 14 embryo injected with *Xiro3* mRNA. Expression of *X-MyT1* is blocked on the injected side of the embryo. (**E**) Section through the hindbrain region of a stage 30 embryo labelled for N-tubulin expression. (**F**) Cross-section through a stage 30 embryo injected with *Xiro3* in the lateral ectoderm. Note that *Xiro3* induces extra tissue in the lateral epidermis of the embryo that is partially N-tubulin positive. (**G**) Stage 14 embryo injected with *Xiro3* showing ectopic expression of *X-Delta-1* over the injected area. (**H**) Stage 14 embryos injected with a combination of *Xiro3* and a dominant-negative form of *X-Delta-1*, *X-Delta-1*^{stu} that block lateral inhibition. No rescue of the blocking of N-tubulin expression is observed. (**I**) Control embryos injected with *X-Delta-1*^{stu} alone showing an increase of density of N-tubulin-positive cells within the stripes. (**J**) Stage 16 embryos injected with *Xiro3*. The expression of *XASH3* is slightly expanded on the injected side.

them being N-tubulin positive. As in the expanded neural tube, the N-tubulin staining was localized mainly within the area that stained less intensely with X-gal and therefore most probably contains less of the injected *Xiro3* mRNA (Figure 5F).

The inhibition of neuronal differentiation we observed in *Xiro3*-injected embryos might be due to increased lateral inhibition (Chitnis and Kintner, 1996). Therefore, we looked at the effect of *Xiro3* on the expression of *X-Delta-1*. We found that 63% of the *Xiro3*-injected embryos show ectopic *X-Delta-1* expression ($n = 19$) (Figure 5G). However, co-injection of an antimorphic version of *X-Delta-1*, called *X-Delta-1*^{stu}, which blocks lateral inhibition, did not result in the rescue of the inhibition of N-tubulin expression ($n = 68$) (Figure 5H). In this experiment, control embryos injected with *X-Delta-1*^{stu} alone showed an increased in the density of N-tubulin-expressing cells as previously reported (Chitnis *et al.*, 1995) (Figure 5I). These results suggest that *Xiro3* prevents the formation of primary neurons and that this inhibition is not the consequence of *X-Delta-1* activation of lateral inhibition.

The fact that the effects of *Xiro3* on N-tubulin expression were different at early neurula and tadpole stages suggest that the neural tissue induced by *Xiro3* was competent to undergo neuronal differentiation but that high levels of *Xiro3* expression might be incompatible with neuronal differentiation. To test this idea, we looked at the effect of increased neuronal differentiation on *Xiro3* expression. We injected embryos with the *X-ngnr-1* neuronal determination gene and asked if it alters the normal expression pattern of *Xiro3*. As shown in Figure 7A and B, embryos that were injected with *X-ngnr-1* showed strong and widespread ectopic N-tubulin expression, as previously described (Ma *et al.*, 1996). In contrast, *Xiro3* expression was blocked on the injected side of those injected embryos (24/24 embryos examined). Together, these results suggest that a high level of *Xiro3* expression promotes an early neural state but that this induced neural tissue is competent to undergo further neuronal diffentiation only when a lower *Xiro3* concentration is achieved.

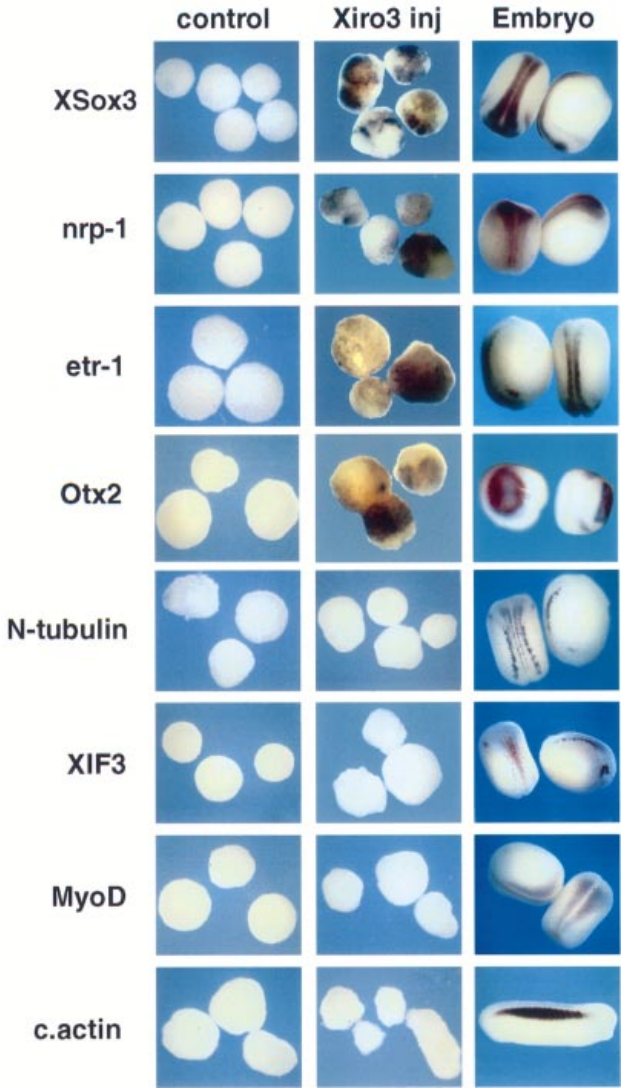

Fig. 6. *Xiro3* induces early neural genes in animal caps. *Xiro3* mRNA was injected in both cells of two-cell stage embryos and animal caps were dissected at stages 9–10. Explants were cultured to stage 16 and then analysed by whole-mount *in situ* hybridization. For cardiac actin expression, explants were cultured to tadpole stage. Note that *Xiro3* injection induces the expression of all neural, but not neuronal markers tested.

Xiro3 participates in the control of XASH3 expression

As neuronal differentiation is under the control of proneural genes and since in *Drosophila*, *Iroquois* genes participate in the positive regulation of AS-C genes, we asked if overexpression of *Xiro3* affects proneural gene expression. *XASH3* is a putative proneural gene whose expression overlaps with, but appears later than that of *Xiro3* (see above). Thus, we tested whether *Xiro3* increases expression of *XASH3*. We found that overexpression of *Xiro3* outside the areas where *XASH3* is normally expressed did not result in ectopic expression of *XASH3* ($n = 78$). However, when *Xiro3* was directed into the neural plate, where *XASH3* is normally expressed, we observed that in 21 out of 35 embryos the domain of *XASH3* expression was slightly expanded (Figure 5J). These results suggest that *Xiro3* is involved in the positive regulation of at least some of the proneural genes. The fact that we observed no ectopic expression of *XASH3* and in some embryos, only a moderate expansion of *XASH3* expression in the neural plate might indicate that additional factors cooperate with *Xiro3* to induce proneural gene expression.

Direct activation of early neural gene expression by Xiro3 in animal caps

We have shown that *Xiro3* overexpression causes an expansion of the neural tube which is at least partly due to conversion of epidermal tissue into a neural fate (see above). To test this further, we asked if *Xiro3* can induce neural tissue in isolated animal caps which, in the absence of additional inducing signals, adopt an epidermal fate. Animal caps were isolated from blastula stage embryos that had been injected bilaterally with *Xiro3*-encoding RNA, cultured to the equivalent of stage 16, and then assayed for the expression of different marker genes by whole-mount *in situ* hybridization (Figure 6). As expected, no neural markers were expressed in uninjected animal caps. Injection of *Xiro3* mRNA was sufficient to induce

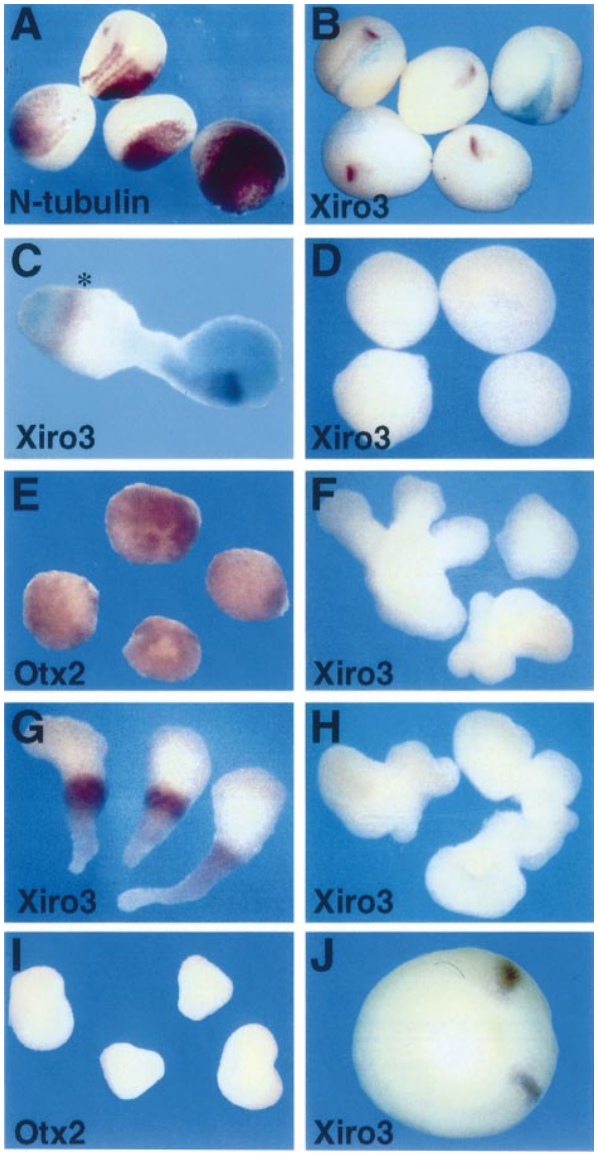

Fig. 7. Regulation of *Xiro3* expression. (**A**) N-tubulin expression is strongly induced in embryos injected unilaterally with *X-ngnr-1* mRNA. (**B**) *Xiro3* expression, in contrast to N-tubulin, is blocked by overexpression of *X-ngnr-1*. (**C**) *Xiro3* expression (purple) in a Keller explant is detected in the form of a stripe overlapping with that of engrailed (blue). A second band of expression (asterisk) is also detected in the cephalic mesendoderm. (**D**) *Xiro3* is not expressed in noggin-injected animal caps. (**E**) *Otx2* expression is induced in noggin-injected animal caps. (**F**) *Xiro3* expression in animal caps treated with bFGF. (**G**) *Xiro3* is induced in the form of a transverse band in elongated animal caps injected with noggin and treated with bFGF. (**H**) *Xiro3* is not induced in noggin-injected animal caps treated with RA. (**I**) *Otx2* expression is down-regulated in noggin-injected animal caps treated with RA. (**J**) *Xiro3* expression in a stage 15 embryo injected at the two-cell stage in each blastomere with the dominant-negative form of the FGF receptor.

the expression of general neural markers such as *XSox3*, *nrp-1* and *etr-1*. *Xiro3* also induced expression of *Otx2* which is co-expressed with *Xiro3* in the area outside the lateral-anterior neural plate (see Figure 6). Induction of the above neural markers was also observed when development of the injected caps was stopped at the equivalent of early neurula stage (stage 13) and was found to persist, although at a lower level, after 2 days in culture. These results support the idea that *Xiro3* can induce neural tissue in the absence of neural-inducing signals, as opposed to simply enhancing the proliferation of neural tissue.

To test if *Xiro3* induced the same type of neural tissue in animal caps as it does in the embryo, we examined the expression of N-tubulin. Consistent with the results in the embryo, N-tubulin was not induced by *Xiro3*. Another neuronal marker, *XIF3* (Sharpe *et al.*, 1989), was also not induced. To determine if the induction of neural tissue by *Xiro3* was a secondary consequence of induction by mesodermal tissue, *Xiro3* mRNA-injected animal caps were also analysed for the expression of mesodermal markers. No MyoD and cardiac actin expression was detected. These data indicate that ectopic *Xiro3* expression is sufficient to convert animal cap explants directly to an early neural state, characterized by the presence of early neural markers and the absence of neuronal differentiation markers.

Xiro3 expression is induced by a combination of noggin and bFGF

To characterize the inductive signals that initiate *Xiro3* expression, we first examined *Xiro3* expression in Keller sandwich explants, i.e. in the absence of vertical mesoderm–ectoderm interactions (Keller and Danilchick, 1988). In these explants (5/5), *Xiro3* was expressed in an intense stripe that overlaps with that of *en-2* but extends anteriorly, suggesting that it corresponds to the anterior transverse band of *Xiro3* expression in whole embryos (Figure 7C). Weaker expression was also observed in more posterior neuroectodermal regions that may correspond to expression in the hindbrain and spinal cord. As in whole embryos, expression of *Xiro3* was also detected in the cephalic mesendoderm that has migrated away from the neuroectoderm (Doniach *et al.*, 1992). These results suggested that signals originating from the dorsal mesoderm induce *Xiro3* expression and that planar signalling is sufficient to direct correct A–P patterning of its expression.

To examine further the idea that *Xiro3* is induced by signals from the organizer, we first tested if *Xiro3* is induced by known neural inducers. We observed that noggin was not able to induce *Xiro3* expression in animal caps aged to late neurula or tailbud stages (Figure 7D). Since *Xiro3* is only expressed in the posterior neural plate and noggin only induces anterior neural markers, we reasoned that *Xiro3* activation may require a posteriorizing factor. We therefore tested if FGF or RA, two factors which are thought to lead to posteriorization, can induce *Xiro3* expression in noggin-treated caps. In these experiments, *Xiro3* expression was induced by bFGF, in a stripe near the middle of the elongated tissue (Figure 7G). Weak staining was also observed at one end of the elongated structure. No *Xiro3* expression was detected in animal caps treated with bFGF alone (Figure 7F). In contrast to FGF, RA was not efficient in inducing *Xiro3* expression in noggin animal caps (Figure 7H) although it was very efficient in suppressing *Otx2* expression (Figure 7E and I). In elongated caps seen after combined noggin + bFGF treatment, no muscle actin expression was detected, suggesting that *Xiro3* induction was not a secondary induction due to mesoderm induction by bFGF (data not shown). To determine if *Xiro3* inductive signals require FGF in the whole embryo, a dominant-negative FGF receptor

(XFD) was overexpressed. These embryos failed to complete gastrulation due to disruption of mesoderm formation as previously described (Amaya *et al.*, 1991). Whole-mount *in situ* analysis of the injected embryos shows that XFD did not block the transverse stripe of *Xiro3* expression in the midbrain–hindbrain area. This is consistent with previous reports that used other markers for this area of the CNS in XFD-injected embryos (Kroll and Amaya, 1996; Godsave and Durston, 1997). XFD injection also did not block the more posterior *Xiro3* expression, although some embryos showed reduced posterior *Xiro3* expression on one side (Figure 7J). These observations suggest that the *Xiro3*-inducing signals can be mimicked by a combination of noggin and FGF, although endogenous FGF may not be directly involved in *Xiro3* activation.

Discussion

Xiro3 is expressed uniformly by neural precursors of the posterior CNS

We have shown that *Xiro3* is expressed uniformly in a restricted medio-lateral portion of the neural plate that is coincident with *XSox3* expression, and that *Xiro3* expression overlaps but is wider than that of *XASH3*. Unlike *XSox3*, *Xiro3* is not expressed in the anterior neural plate and later on in the forebrain, suggesting that the neural programme in the forebrain is distinct from that in the posterior CNS (see Papalopulu and Kintner, 1996a). The expression of *Xiro3* differs from the other known proneural genes in that it is uniform rather than punctate. This punctate pattern of gene expression which precedes the punctate pattern of neuronal differentiation is thought to be generated by the Notch–Delta-mediated inhibitory cell interactions that limit proneural gene expression to a few cells within each longitudinal domain of primary neurons (Chitnis *et al.*, 1995; Chitnis and Kintner, 1996; Ma *et al.*, 1996). We speculate, therefore, that lateral inhibition may not play a central role in regulating *Xiro3* expression.

We have found that *Xiro3* is expressed in both the deep and superficial cell layers of the neural plate which give rise to the primary and secondary neuronal precursors, respectively (Hartenstein, 1989). Within the deep layer, cells that express N-tubulin do not appear to express *Xiro3* or express it at low levels. Similarly, later on, *Xiro3* is expressed mainly in progenitor cells of the ventricular zone whereas N-tubulin is expressed in differentiated cells located in the external-most layer of the neural tube. This early uniform expression of *Xiro3* in pluripotent precursors of both primary and secondary neurons and glia suggests that it is an earlier acting gene than all proneural genes identified in vertebrates, including *Mash1*, neurogenin and *NeuroD*. These are expressed later in descendant cells and regulate subsequent steps of neurogenesis (Guillemot *et al.*, 1993; Lee *et al.*, 1995; Ma *et al.*, 1996).

Xiro3 expression is induced by noggin plus bFGF but not RA

Xiro3 expression starts at gastrula stage in the prospective posterior CNS at around the midbrain–hindbrain region and then spreads posteriorly. This restricted expression suggests that *Xiro3* is activated by organizer signals associated with the formation of the posterior CNS. We have found that *Xiro3* is induced by noggin and bFGF, a putative posterioring signal. The *Xiro3* expression observed in such explants is reminiscent of that in whole embryos and is consistent with the observation that noggin and bFGF generate the anterior–posterior neural pattern in animal caps (Lamb *et al.*, 1995). Cox and Hemmati-Brivanlou (1995) have shown that an interaction between anterior and posterior neural tissue generates tissue of intermediate positional values. Therefore, it is possible that the *Xiro3* stripe in the middle of the explant is induced by a secondary interaction of anterior with posterior neural tissue.

Molecules other than bFGF, namely RA and Wnt-3A, have also been reported to have posteriorizing activity. It is not clear how the activities of these molecules differ from each other and how signalling from these diverse pathways is integrated to produce the posterior nervous system. Here we report one difference between two putative posteriorizing signals by showing that *Xiro3* is induced by FGF in noggin-neuralized animal caps but is not induced by RA. We have shown previously that RA co-operates with *XASH3* to induce neurogenesis in noggin-neuralized animal caps (Papalopulu and Kintner, 1996a). In these experiments, *XASH3* was introduced in the animal caps by mRNA injection; therefore, we assume that RA was not able to induce *XASH3*. Since RA was also unable to induce *Xiro3* in noggin-neuralized animal caps (this work), we propose that posterior neuronal differentiation requires RA as well as additional signals that promote the expression of proneural genes. Our findings suggest that one of these signals could be bFGF or a related molecule.

Endogenous FGF is expressed in the posterior of the embryo and is therefore in the right place and time to be inducing *Xiro3*. However, the role of endogenous FGF in the induction of *Xiro3* is not clear since blocking FGF signalling in the whole embryo with a dominant-negative FGF receptor (XFD) does not abolish *Xiro3* expression. We note, however, that some XFD-injected embryos express *Xiro3* at a reduced level in posterior regions. One possibility is that endogenous FGF may be involved in *Xiro3* activation but there are inputs by other signalling pathways that allow some *Xiro3* expression in the absence of FGF signalling. In any case, it seems likely that *Xiro3* is regulated at multiple levels. We note, for example, that the *Xiro3* protein contains MAPK phosphorylation sites, suggesting that not only the expression but also the activity of the *Xiro3* protein could be regulated by bFGF or a related tyrosine kinase.

Finally, although the nature of the endogenous *Xiro3*-inducing signals remains unclear, we have shown that they can act within the plane of the ectoderm. Thus, planar signals that act over an extended range, either by diffusion or a relay mechanism, are sufficient to induce a range of genes that mark position along the A–P axis (Doniach *et al.*, 1992; Papalopulu and Kintner, 1993; Blitz and Cho, 1995), neural specification (this work) and neuronal differentiation (Sater *et al.*, 1993).

Xiro3 induces neural tissue de novo

When ectopically expressed in embryos, *Xiro3* leads to a dramatic enlargement of the neural tube. This enlargement appears to be due at least in part to conversion of surrounding non-neural tissue to a neural fate. Several findings support the idea that *Xiro3* can induce neural

tissue *de novo*. First, blocking cell division in mid-gastrulation does not eliminate the enlarged neural tube phenotype. Second, when the RNA is directed to lateral and ventral regions of the embryo, neural tissue forms non-contiguously with the host neural plate. Third, neural crest and epidermal markers are suppressed over that affected area and, finally, *Xiro3* can induce neural tissue in isolated animal caps.

Xiro3 expression prevents the differentiation of the early forming primary neurons

In contrast to the ectopic expression of *XSox3* and other early neural markers, neuronal differentiation markers such as N-tubulin are not induced within the epidermis of neurula embryos in response to *Xiro3* overexpression. Furthermore, we have shown that the expression of N-tubulin is down-regulated when *Xiro3* mRNA is injected into cells located inside the neural plate. These results differ from findings obtained with the bHLH genes that have proneural activities. Overexpression of *NeuroD* or *XATH3* induces the ectopic expression of both early neural specification markers and neuronal differentiation markers (Lee *et al*., 1995; Ma *et al.*, 1996). Another bHLH gene, *XATH1*, can only induce ectopic expression of neuronal differentiation markers (Kim *et al.*, 1997). Thus, *Xiro3* appears to be unique compared with the known *Xenopus* proneural genes, in that it induces only some early neural fate.

Why do high levels of *Xiro3* induce an early neural state and prevent the completion of neuronal differentiation? We have shown that a high level of *Xiro3* expression blocks the expression of neuronal regulatory factors such as *X-MyT1*. We have also observed that overexpression of *Xiro3* induces ectopic *X-Delta-1* expression, suggesting the lack and/or suppression of neuronal differentiation might be due to X-*Delta-1* activation of lateral inhibition (Chitnis *et al.*, 1995). However, co-injection of *X-Delta-1*^{stu}, an antimorphic form of *X-Delta-1*, showed that this is not the case.

One interpretation of the lack of neuronal differentiation in *Xiro3*-expressing tissue is that additional factors are needed. These missing factors may be indepedent of *Xiro3* and act in parrallel with it. Alternatively, they may be linearly downstream of *Xiro3* but their expression and/or activity may be prevented by the elevated expression of *Xiro3*. A number of observations favour this second possibility. First, the effects of *Xiro3* on N-tubulin expression were stage dependent such that N-tubulin expression was detected in later stage embryos. Since the levels of the injected RNA and resulting protein decline during development, we speculate that the differentiation programme is initiated by *Xiro3* but is not completed until the levels of *Xiro3* decline. Second, there is an inverse correlation between *Xiro3* expression on one hand and N-tubulin on the other. Embryos with increased *Xiro3* expression do not express N-tubulin, and inversely, embryos with increased, ectopic and premature N-tubulin expression (caused by *X-ngnr-1* overexpression) do not express *Xiro3*. Finally, we have shown that in the embryo, neural precursors express high levels of *Xiro3* while differentiated neurons do not express *Xiro3* or express it at lower levels. Thus, cells that express high levels of *Xiro3* appear not to differentiate and, inversely, cells that differentiate do not express high levels of *Xiro3*. According to this model, high levels of *Xiro3* expression are incompatible with neuronal differentiation; the neural programme is initiated by *Xiro3* but *Xiro3* expression and/or activity must be down-regulated in order for the neural programme to be completed. It is not clear how cells turn off *Xiro3* expression in order to differentiate. Our results suggest that cells that express high levels of *X-ngnr1* turn off *Xiro3*, so *X-ngnr1* could be involved directly in down-regulating *Xiro3* expression.

Does Xiro3 induce proneural gene expression?

One of the functions of the *Drosophila* genes of the *Iroquois* complex is to activate proneural gene expression. Does the *Xenopus Xiro3* gene perform a similar function? We have shown that *Xiro3* expression precedes and overlaps extensively with that of *XASH3* and that *Xiro3* injections into the embryo result in moderate up-regulation of *XASH3*. Additionally, *Xiro3* overexpression gives embryos with an expanded neural tube, a phenotype also observed after *XASH3* overexpression (Fereirro *et al.*, 1994; Turner *et al.*, 1994). These results suggest that *Xiro3* may act upstream of *XASH3*. However, the observations that *XASH3* expression is only moderately expanded within the neural plate of *Xiro3*-injected embryos and that it is not induced in *Xiro3*-injected animal caps (unpublished observations) suggest that factors additional to *Xiro3* might be required to fully activate *XASH3* expression, perhaps on a co-operative basis.

Additional but indirect evidence that *Xiro3* regulates positively the expression and/or function of other proneural bHLH genes is provided by several observations. First, overexpression of *CASH4* and *XATH3* in *Xenopus* embryos produces a neural tube enlargement similar to that produced by *Xiro3* (Henrique *et al.*, 1997; Takebayashi *et al.*, 1997). The same phenotype has been also observed after overexpression of an activated from of the *X-Notch-1* receptor (Coffman *et al.*, 1993) which may be due to a proneural function of X-*Notch-1* early in development, as has been described recently in *Drosophila* (Baker and Yu, 1997). Second, proneural genes have been shown to induce the expression of *X-Delta-1* (Ma *et al.*, 1996). Therefore, the ectopic expression of *X-Delta-1* in *Xiro3*-injected embryos is compatible with the idea that *Xiro3* induces proneural gene expression which subsequently induces the expression of *X-Delta-1*. It remains to be seen more directly whether *Xiro3* activates the expression of proneural genes such as *X-ngnr-1* (Ma *et al.*, 1996), *XATH3* (Takebayashi *et al.*, 1997) or the homologue of the chicken gene *CASH4* (Henrique *et al.*, 1997), which has not been isolated from *Xenopus* yet. The *Xenopus* homologue of the HMG transcription factor *XSox3* is also one of the putative targets of *Xiro3* since it is expressed in a very similar pattern in the posterior neural plate and it is induced by *Xiro3* overexpression. Although *XSox3* has been hypothesized to have a role in neural competence (Rex *et al.*, 1997), functional studies in *Xenopus* or mice are needed to establish its role in neural development. We assume that additional proneural genes exist that could be targets of *Xiro3* and that these will be found in the future.

In conclusion, we have shown that *Xiro3* is expressed in undifferentiated neural precursor cells in the posterior CNS before neural differentiation begins and that it can

induce an early neural fate. Furthermore, its expression is induced by a combination of noggin, a neural-inducing signal, and bFGF, a posteriorizing signal. We therefore suggest that *Xiro3* is one of the earliest genes acting in the genetic cascade that leads to neurogenesis; we suggest that *Xiro3* is downstream of neural induction but upstream of neuronal differentiation and that it functions in specifying neural precursors.

Materials and methods

Isolation of Xiro3 cDNA

Degenerate oligonucleotide primers were designed based on two amino acid sequences within the homeodomain of the *Drosophila Iroquois* proteins: WLNEHKKNP [TGG CT(ACTG) AA(CT) GA(AG) CA(CT) AA(AG) AA(AG) AA(CT) CC] and KKENKMTWEP [GG (TC)TC CCA (AGCT)GT CAT (TC)TT (GA)TT (TC)TC (TC)TT (TC)TT]. These primers were used for PCR (94°C for 1 min, 56°C for 1 min, 72°C for 1 min, 35 cycles) using as template cDNA from a stage 12 library in pcDNA1 (provided by Dr N.Garret). A 150 bp fragment was obtained, cloned in pGEM-T and sequenced. The PCR product was then used as probe to screen 10^6 phages from a stage 17 *Xenopus* embryo cDNA library in λgt10 (Kintner and Melton, 1987). Six positive clones were isolated, out of which five were identical to each other as judged from their restriction pattern. The longest of these clones was sequenced and termed *Xiro3*. The *Xiro3* sequence has been deposited in DDBJ/EMBL/GenBank (accession No. AF027175)

In situ hybridization of mouse embryos

Whole-mount *in situ* hybridizations were performed as described (Wilkinson, 1992) using digoxigenin-labelled antisense RNAs corresponding to the 0.6 kb *Eco*RV–*Sma*I fragment of mouse *Irx3* cDNA.

RNA injection and in situ hybridization of Xenopus embryos

The entire *Xiro3* coding region was subcloned into the pCS2+ vector (Turner and Weintraub, 1994). After linearization with *Not*I, the vector was transcribed *in vitro* with SP6 polymerase in the presence of GpppG to produce capped *Xiro3* transcripts. *Xiro3* RNA (100–250 pg) along with 20 pg of LacZ RNA (Chitnis *et al.*, 1995) was injected in a volume of 10 nl into a single blastomere of 2- to 32-cell stage *Xenopus* embryos. As a negative control, embryos were similarly injected with LacZ RNA alone. *X-ngnr-1* (0.5 ng; Ma *et al.*, 1996) RNAs were prepared as originally described and injected in the animal region at the two-cell stage in only one blastomere. XFD (1.0 ng; Amaya *et al.*, 1991) and noggin (0.5 ng; Lamb *et al.*, 1993) mRNAs were injected into each of the blastomeres at the two-cell stage embryo. At various stages, 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 as described (Bellefroid *et al.*, 1996). Antisense RNA probes from N-tubulin (Chitnis *et al.*, 1995), *X-MytT1* (Bellefroid *et al.*, 1996), *X-Delta-1* (Chitnis *et al.*, 1995), *X-twist* (Hopwood *et al.*, 1989a), *MyoD* (Hopwood *et al.*, 1989b), *Otx2* (Pannese *et al.*, 1995), *etr-1* (Knecht *et al.*, 1995), *XASH3* (Turner and Weintraub, 1994), *engrailed-2* (Hemmati-Brivanlou *et al.*, 1991), *XSox3* (kindly provided by Dr R.M.Grainger), cardiac actin (Mohun *et al.*, 1984), *XIF3* (Sharpe *et al.*, 1989), *nrp-1* (Knecht *et al.*, 1995), *GATA-2* (Bertwistle *et al.*, 1996) and epidermal keratin (Jonas *et al.*, 1985) were transcribed with digoxigenin or fluorescein as previously described. For double *in situ* hybridization, probes were visualized sequentially with the corresponding specific alkaline phosphatase conjugates using first Fast Red (Boehringer) or BCIP alone for the fluorescein-labelled probe and then NBT/BCIP or Magenta phos (Molecular Probes Europe) for the second digoxigenin-labelled probe.

Embryos, animal cap explants, HUA, FGF and RA treatment

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg laying and *in vitro* fertilization using standard methods. Animal caps were dissected from stage 9–10 embryos and cultured in 1× MBSH + 0.1% bovine serum albumin. For RA treatment, caps were treated with 2×10^{-6} M RA in dimethylsulfoxide. For FGF treatment, caps were incubated in the presence of 100 ng/ml bFGF. Animal caps were cultured until neurula or tailbud stages and fixed for *in situ* hybridization. To block cell division, embryos at stage 11 were maintained in 20 mM hydroxyurea and 150 μM aphidicolin as previously described (Harris and Hartenstein, 1991).

Acknowledgements

We thank Dr Rob Grainger for providing a *XSox3* plasmid prior to publication and Dr Thomas Sargent for providing the epidermal keratin clone. We are also grateful to E.Tweed and K.Butler for technical assistance. This work was supported by a grant from the Wellcome Trust to N.P., by a grant from the Cancer Research Campaign to J.B.G. and by funds from the Deutsche Forchungsgemeinschaft to P.G. and T.P. (SFB 271). E.J.B. is a recipient of an EEC fellowship.

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Received August 21, 1997; revised October 10, 1997; accepted October 10, 1997