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Xiro, a *Xenopus* homolog of the *Drosophila Iroquois* complex genes, controls development at the neural plate

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The *Drosophila* homeoproteins Ara and Caup are members of a combination of factors (prepattern) that control the highly localized expression of the proneural genes *achaete* and *scute*. We have identified two *Xenopus* homologs of *ara* and *caup*, *Xiro1* and *Xiro2*. Similarly to their *Drosophila* counterparts, they control the expression of proneural genes and, probably as a consequence, the size of the neural plate. Moreover, *Xiro1* and *Xiro2* are themselves controlled by noggin and retinoic acid and, similarly to *ara* and *caup*, they are overexpressed by expression in *Xenopus* embryos of the *Drosophila cubitus interruptus* gene. These and other findings suggest the conservation of at least part of the genetic cascade that regulates proneural genes, and the existence in vertebrates of a prepattern of factors important to control the differentiation of the neural plate.

Keywords: neural crest/neural plate/proneural genes/*Xenopus*/*Xiro*

Introduction

In amphibia, the formation of the embryonic nervous system results from inductive interactions between the dorsal mesoderm and the ectoderm. As a consequence of these interactions, the dorsal ectoderm thickens and becomes a flat sheet of cells called the neural plate. At the lateral margins of the neural plate, changes in cell shape and interactions with surrounding tissue combine to generate the neural folds. The neural folds rise and fuse at the dorsal midline to form a cylindrical tube that subsequently differentiates into the central nervous system (CNS). Neural induction starts during gastrulation, when the dorsal mesoderm sends to the ectoderm vertical and/or planar inductive signals that commit it to a neural fate (Spemann, 1938; Kintner and Melton, 1987; Dixon and Kintner, 1989; Doniach, 1993; Ruiz i Altaba, 1993; for reviews, see Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). In the last few years, several molecules involved in neural induction have been identified. Thus, in the amphibian *Xenopus*, the secreted molecules noggin, chordin and follistatin, synthesized by the dorsal mesoderm, induce neuralization in the ectoderm (Smith and Harland, 1992; Lamb *et al.*, 1993; Smith *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1994).

In the absence of induction, dorsal ectodermal cells become epidermis. However, recent experiments suggest that the default fate for ectoderm is neural, and this fate is inhibited by signals present in the normal ectoderm (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). This antineural activity is probably mediated by BMP-4 (Graff *et al.*, 1994; Schmidt *et al.*, 1995; Suzuki *et al.*, 1995), a member of the transforming growth factor-β (TGF-β) family of secreted molecules. During neural induction, the inhibitory signal would be removed by the inducers emanating from the dorsal mesoderm; therefore, the inhibitory signal would be restricted to the ventral ectoderm, which is fated to be epidermis (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). As the neural inducers appear to direct cells to an anterior fate (Lamb *et al.*, 1993; Hemmati-Brivanlou and Melton, 1994; Hemmati-Brivanlou *et al.*, 1994; Hawley *et al.*, 1995; Sasai *et al.*, 1995), later additional signals emanating from the posterior neuroectoderm, such as retinoic acid (RA) and members of the fibroblast growth factor (FGF) and Wnt families, are thought to confer to cells a more posterior fate (Blumberg *et al.*, 1997; for reviews, see Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). The limit between dorsal and ventral ectoderm, where positive and negative signals also interact, would define the position of the neural folds and the limits and size of the neural plate (Moury and Jacobson, 1990; Dickinson *et al.*, 1995; Liem *et al.*, 1995; Mayor *et al.*, 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996).

The *Xenopus* genes *XASH-3*, neurogenin (*X-ngnr-1*) or *ATH-3*, which encode basic helix–loop–helix (bHLH) factors homologous to the proneural proteins of the *Drosophila achaete–scute* complex (AS–C), appear to participate in the development of the CNS. Thus, overexpression of *XASH-3* causes expansion of the neural plate, that of *X-ngnr-1* produces ectopic neurons, and that of *ATH-3* causes both effects (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994; Ma *et al.*, 1996; Takebayashi *et al.*, 1997). In the wild-type embryos, *XASH-3* mRNA starts to accumulate at stage 11–11.5. It does so within the neural plate, in two symmetric patches lateral to the midline that correspond to the prospective spinal cord, and within the future hindbrain, in two anterior transverse stripes (Zimmerman *et al.*, 1993; Turner and Weintraub, 1994). *X-ngnr-1* mRNA is detected initially at stage 10.5–11 in the territories that will give rise to the three rows of primary neurons, which are located at each side of the midline, and in the trigeminal placode (Ma *et al.*, 1996). *ATH-3* mRNA begins to be expressed at stage 12 in primary neuron precursors (Takebayashi *et al.*, 1997).

Among the *Drosophila* AS-C genes, *achaete* (*ac*) and *scute* (*sc*) are most important for the generation of the peripheral nervous system (PNS), which includes the epidermal sensory organs (SOs) of the adult fly. Loss- or gain-of-function alleles of these genes eliminate SOs or induce ectopic SOs, respectively. In the imaginal discs, the precursors of most of the adult epidermis, *ac* and *sc* are co-expressed in clusters of cells, the proneural clusters, from which SO mother cells arise. Thus, the pattern of expression of *ac–sc* pre-figures the spatial distribution of SOs of the fly (reviewed in Campuzano and Modolell, 1992). The expression of *ac–sc* in each cluster is due to position-specific enhancers present in the AS-C DNA, which are presumed to respond to local combinations of factors (Gómez-Skarmeta *et al.*, 1995). The combination of these factors, each of them distributed in domains larger than the proneural clusters, constitutes in fact a prepattern (Stern, 1954) that subdivides the tissue and thus creates positional information. Recently, two components of this prepattern, the products of the *araucan* (*ara*) and *caupolican* (*caup*) genes, members of the *Iroquois* complex (IRO-C), have been characterized (Gómez-Skarmeta *et al.*, 1996; Leyns *et al.*, 1996). They are related homeoproteins that accumulate in coincident broad domains of the wing discs and which overlap many proneural clusters. Ara and Caup bind to AS-C enhancers and are required for *ac–sc* activation (Gómez-Skarmeta *et al.*, 1996).

To learn about the control of *Xenopus* proneural genes and to examine whether the mechanisms that regulate proneural gene expression are evolutionarily conserved, we have searched for *Xenopus* homologs of *ara* and *caup*. We have found two genes, *Xiro1* and *Xiro2*, which are similarly expressed in the neural plate earlier than *XASH-3*, *X-ngnr-1* and *ATH-3* and in regions that partially overlap with and are larger than the expression domains of these three genes. Overexpression of *Xiro* induces ectopic expression of *XASH-3*, *X-ngnr-1* and, weakly, of *ATH-3* and expansion of the neural plate. In addition, we find that the *Xiro* genes are regulated by noggin, RA and, similarly to *ara* and *caup* (Gómez-Skarmeta and Modolell, 1996), can be activated by the *Drosophila* Gli protein Cubitus interruptus (Ci). Thus, the regulation of *ac–sc* and the *Xenopus* proneural genes by the Iro homeoproteins and probably the regulation of *Xiro* by Gli proteins appear to be conserved during evolution. The results support the presence in vertebrates of a prepattern of factors that help control patterning in the neural plate.

Results

Molecular cloning of the Xiro genes

We screened a *Xenopus* gastrula (stages 10.5–11.5) cDNA library at low stringency with a DNA probe encoding the homeodomain of the *Drosophila caup* gene (Gómez-Skarmeta *et al.*, 1996). Twelve cDNAs, that apparently correspond to two different genes, were recovered. The predicted proteins encoded by these cDNAs indicate that they belong to the Iro family of homeoproteins. Xiro1 and Xiro2 have 313 and 455 amino acids, respectively. The similarity between Xiro1, Xiro2, Ara, Caup and the products encoded in five human partial cDNAs is restricted mainly to the homeodomains and to a short region (15 amino acids) at the carboxy-terminus (Figure 1). Within the homeodomain, the similarity is very high, which suggests that Xiro1 and Xiro2 are homologs of Ara, Caup and the novel human proteins. The carboxy-terminal region of homology may be an exclusive motif of the Iro proteins, since similar sequences are not found in protein databases. The human HIRX-5 protein also presents high identity with Xiro1 within the region flanking the amino end of the homeodomain (Figure 1C). This suggests that HIRX-5 is the human ortolog of Xiro1. Similarly to the *Drosophila* Iro proteins, Xiro1 and Xiro2 have acidic regions close to and at the carboxy side of the homeodomains (not shown). These regions may be implicated in transcriptional activation.

Xiro genes are expressed in the ectoderm

The expression of *Xiro1* and *Xiro2* in developing embryos was examined by whole-mount *in situ* hybridization using probes specific for each gene. As the patterns of expression were very similar, only that of *Xiro2* is described. The earliest detectable expression, at gastrula stage 10 (Niewkoop and Faber, 1967), occurs in a large region of the dorso-lateral ectoderm, which is presumably larger that the prospective neural plate (Figure 2A). No expression is detected in the mesoderm, the endoderm or the ventral ectoderm. Subsequently, *Xiro2* expression is split into two dorso-lateral patches (Figure 2B and C). At later stages (15–20), each patch is divided into two domains of expression (Figure 2D–F). Two of them, at each side of the midline, are confined within the neural folds. The other two, in the lateral ectoderm, extend from and beyond the neural crests. This has been verified by double *in situ* hybridizations using *Xiro2* and *Xslug* probes (the latter is a specific marker of the neural folds, Mayor *et al.*, 1995). Clearly, the lateral domains of *Xiro2* expression are outside the neural plate (Figure 2G).

To define more precisely the expression of *Xiro2* within the neural plate, we resorted to double and triple *in situ* hybridizations using probes for *engrailed-2*, *Otx-2* and *Krox-20*, which are expressed at the midbrain–hindbrain junction, in the midbrain, and in the third and fifth rhombomeres of the hindbrain, respectively (Hemmati-Brivanlou *et al.*, 1991; Bradley *et al.*, 1993; Blitz and Cho, 1995; Pannese *et al.*, 1995). The anterior border of *Xiro2* expression is located in the midbrain–hindbrain junction (Figure 2H and I). The spacing between maxima of expression within the anterior neural plate appears to be in register with the division of the hindbrain in rhombomeres, as detected by *Krox-20* expression (Figure 2F and I).

Ectopic expression of Xiro enlarges the CNS and reduces the neural crests

As the pattern of expression of *Xiro* overlaps with the presumptive neural plate, we analyzed whether these genes participate in the development of neural tissue. Two-cell stage embryos were injected in one blastomere with *Xiro2* mRNA, and the expression of the neural plate marker *Xsox-2* (R.Grainger, personal communication) was examined. The injected side showed a significantly enlarged neural plate (Figure 3A and B). *Xiro2* mRNA injected at the one-cell stage caused expansion of both sides of this plate, an effect also observed with *caup* mRNA (not shown), a *Drosophila* homolog of *Xiro2*. In

Fig. 1. Conserved sequences among the Xiro proteins and other members of the IRO family. (**A**) Comparison of the homeodomains of Ara, Caup, Mirror (another member of the IRO-C, McNeil *et al.*, 1997), Xiro1 and Xiro2, and conceptual translations of five human cDNAs obtained from DDBJ/EMBL/GenBank (accession Nos U90308, U90304, U90305, U90306 and U90307). Identical amino acids are boxed with a black background, and conservative substitutions are stippled. The extent of the four helices is indicated by brackets. Arrows point to amino acids that appear to confer specificity for DNA-binding sites. These are largely conserved among these members of the family. (**B**) Conservation of a carboxy-terminal motif of Ara, Caup, Mirror, Xiro1, Xiro2 and HIRX-2a. (**C**) Comparison of the regions flanking the amino side of the homeodomain of Xiro1 and HIRX-5. Xiro1 and Xiro2 accession numbers are AJ001834 and AJ001835, respectively.

contrast, no expansion was detected by injecting a truncated *Xiro* mRNA that lacked the homeodomain and C-terminal encoding region (not shown).

To examine whether the expansion of the neural plate modified the neural crest, we analyzed the expression of the neural crest marker *Xslug* (Mayor *et al.*, 1995). At the injected side, *Xslug* expression was reduced and localized to a more ventral position (Figure 3C). This change was most likely due to enlargement of the neural plate. Indeed, injected embryos doubly hybridized with *Xsox-2* and *Xslug* probes showed that the reduction and shifting of the neural crest were accompanied by an expansion of the neural plate (Figure 3D). This suggests that at least some of the *Xiro2*-induced expansion of the neural plate occurs by recruitment of cells of the neural crest. Similar results were obtained by injecting *Xiro1* mRNA.

Xiro controls the expression of Xenopus proneural genes

In *Drosophila*, Ara and Caup bind to AS-C enhancers and are necessary to activate the *ac–sc* genes (Gómez-Skarmeta *et al.*, 1996). In *Xenopus*, we have shown that ectopic expression of *Xiro* expands the neural plate, similarly to the effect of overexpressing *XASH-3* or *ATH-3* (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994; Takebayashi *et al.*, 1997). In addition, *Xiro* expression precedes and partially includes (Figure 4) the domains of expression of *XASH-3* and *ATH-3*, and that of *X-ngnr-1*, another proneural gene which, when overexpressed, causes the differentiation of ectopic neurons (Ma *et al.*, 1996). Thus, we tested whether *Xiro* overexpression affects the patterns of expression of these genes. Injected *Xiro2* mRNA in one blastomere of a two-cell embryo enlarged the domain of expression of *XASH-3* and *X-ngnr-1* in ~50 and 40% of the embryos, respectively (Figure 5A and B). *ATH-3* overexpression was observed in only 17% of injected embryos (Figure 5C).

We also examined whether *Xiro2* promotes proneural gene expression in the absence of neural inducers. Animal caps dissected (stage 10) from *Xiro2* mRNA-injected embryos were cultured *in vitro* until stage 17, and *XASH-3*, *X-ngnr-1* and *ATH-3* expression were determined. *XASH-3* was expressed at variable levels, but not *X-ngnr-1* or *ATH-3* (Figure 5D and not shown). Taken together, these

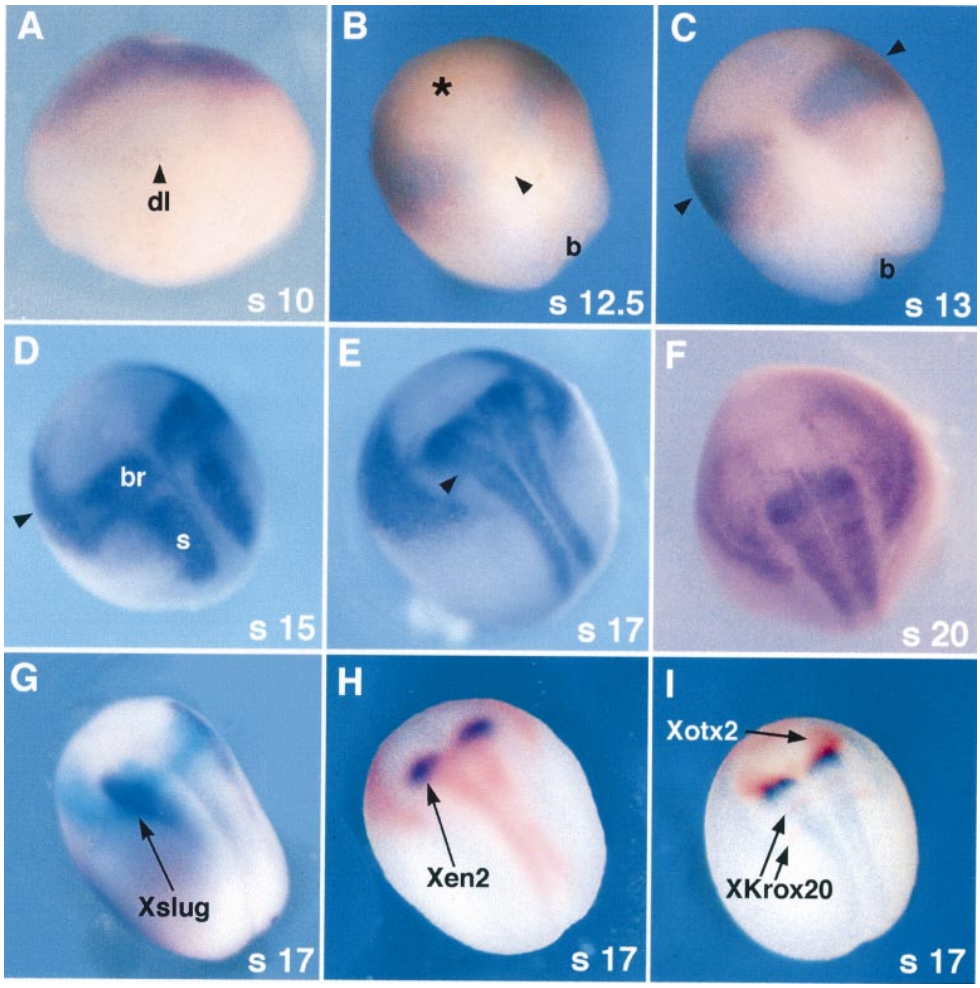

Fig. 2. Expression patterns of *Xiro* mRNA in *Xenopus* embryos at different developmental stages. Views are from the dorsal side, anterior is up. (**A**) Stage 10; *Xiro2* is expressed on the anterior dorsal ectoderm; dl, dorsal lip. (**B**) Stage 12.5; *Xiro2* mRNA disappears from the anterior-most ectoderm (asterisk) and from the dorsal midline (arrowhead); b, blastopore. (**C**) Stage 13; *Xiro2* mRNA accumulates in two dorso-lateral regions of the ectoderm (arrowheads). The anterior border of expression becomes stronger than the posterior one. (**D**) Stage 15; expression occurs in the CNS (br, brain; s, spinal chord) and in two arches that spread out of the neural plate and into the anterior ectoderm (arrowhead). (**E**) Stage 17; expression continues in the CNS and lateral anterior ectoderm. Notice the change in the intensity of expression in the hindbrain region (arrowhead). (**F**) Stage 20; expression in the CNS and in the non-neural ectoderm becomes segmented into bands that apparently correspond to the rhombomeric divisions of the hindbrain. (**G**) Stage 17; double *in situ* hybridization of *Xiro2* (green) and *Xslug* (blue). *Xiro2* is expressed both inside and outside of the neural plate border as marked by *Xslug*. (**H**) Stage 17; double *in situ* hybridization of *Xiro2* (pink) and *en-2* (purple). The anterior border of *Xiro2* expression overlaps with that of *en-2* in the hindbrain. (**I**). Stage 17; triple *in situ* hybridization of *Xiro2* (blue) and*Otx2* and *Krox20* (pink). The anterior border of *Xiro2* expression overlaps with the posterior expression of *Otx2*.

results suggest that *Xiro* positively regulates the expression of at least some of the *Xenopus* proneural genes, and that it requires additional factors, present in the neural plate but absent in animal caps, to activate *X-ngnr-1* and *ATH-3* ectopically.

Control of Xiro expression

Our results suggest that *Xiro2* expression is an early event in neural differentiation. Accordingly, we examined whether the *Xiro* genes are regulated by a neural inducer like noggin. This was apparently the case since animal caps taken from embryos injected with noggin mRNA, but not control caps from uninjected embryos, expressed *Xiro* (Figure 6A and B), albeit at relatively low levels. Note, however, that noggin, similarly to chordin and follistatin, induces the expression of anterior neural markers (Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997) and that the domain of *Xiro* expression seems located at the posterior limit of this activation (Lamb and Harland, 1995). Thus, noggin may participate in the activation of *Xiro* at the placodes region. *Xiro* expression within the neural plate may require posteriorizing signals, such as RA, FGF or Wnts, which transform the anterior neural plate into posterior plate. Accordingly, we analyzed *Xiro* expression in RA-treated embryos, which are known to be posteriorized. In these embryos, the domain of *Xiro* expression within the neural plate was apparently expanded anteriorly and that outside of the plate was reduced (compare Figure 2E with Figure 6C). These data thus suggest that *Xiro* is controlled by both anterior neural inducers and posteriorizing agents.

The *Drosophila ara* and *caup* genes are positively controlled, in some domains of expression, by the putative transcription factor Cubitus interruptus (Ci) (Gómez-

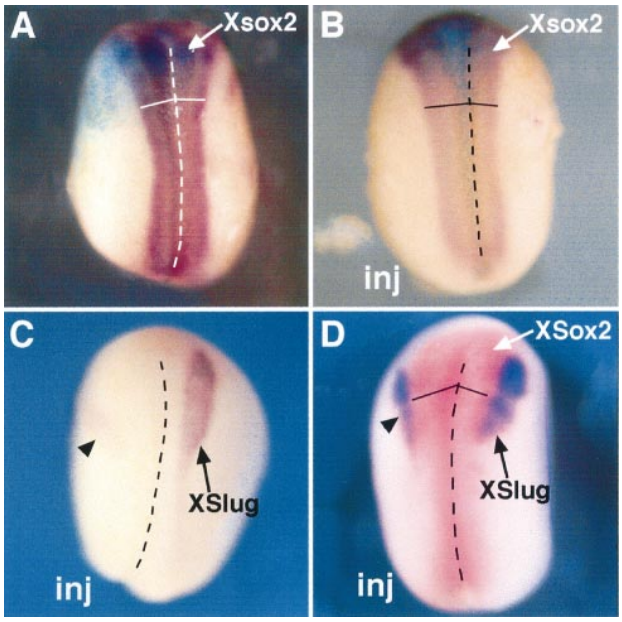

Fig. 3. Effect of *Xiro* overexpression on neural plate and neural crest differentiation. Embryos were fixed at stage 17 and *Xsox2* (A, B and D) and *Xslug* (C and D) mRNA distribution was determined. Embryos are oriented as in Figure 2. (**A**) Control embryo injected in one blastomere of the two-cell stage with 0.3 ng of *lacZ* mRNA showing the expression of the neural plate marker *Xsox2* (purple) and X-Gal staining (green). No effect on the size of the neural plate is observed in the injected side. (**B**) Embryo injected in one blastomere of the two-cell stage with 4 ng of *Xiro2* mRNA and 0.3 ng of *lacZ* mRNA. Notice the expansion of *Xsox2* expression in the injected side of the embryo, which is marked by the X-Gal staining in green (62% of embryos, $n = 170$). (**C**) Embryo injected in one blastomere of the two-cell stage with 4 ng of *Xiro2* mRNA. In the injected side, *Xslug* expression is moved to a more ventral position and its level is strongly decreased (arrowhead, 56% of embryos, $n = 134$). (**D**) Embryo injected as in (C) and double *in situ* hybridized with *Xsox2* (pink) and *Xslug* (purple). At the injected side, the neural plate is enlarged and the neural fold is reduced (arrowhead). The sizes of each side of the neural plate in (A), (B) and (D) are indicated by bars. 'inj' in (B–D) indicates the side injected with *Xiro* mRNA.

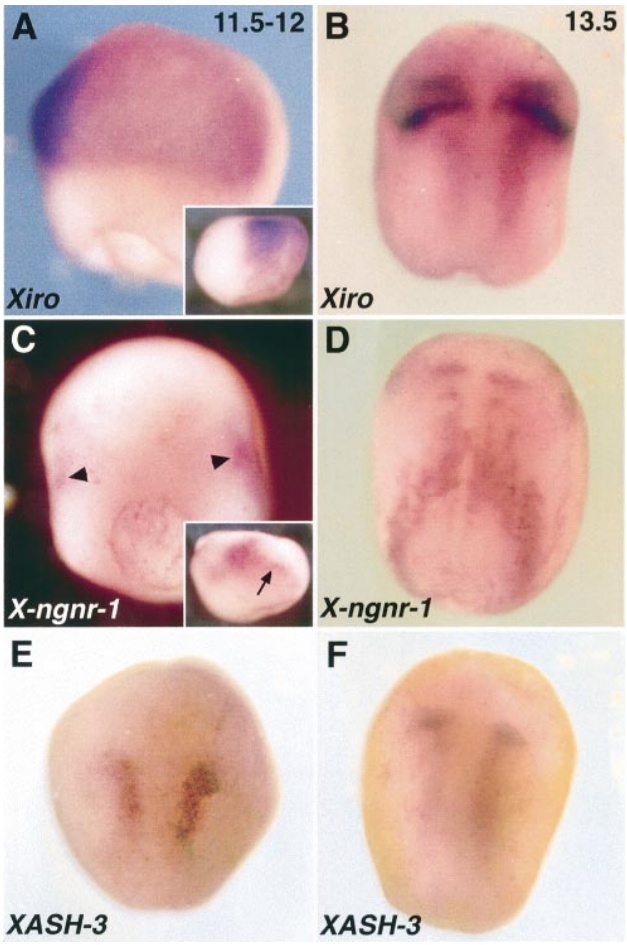

Fig. 4. Sequential expression of *Xiro* and proneural genes. Stage 11.5–12 (A, C and E) and 13.5 (B, D and F) embryos hybridized with *Xiro2* (**A** and **B**), *X-ngnr-1* (**C** and **D**) and *XASH-3* (**E** and **F**) probes. All panels are dorsal views (anterior up), while insets in (A) and (C) are lateral views (anterior to the right). *Xiro2* expression occurs before that of proneural genes (stage 10, Figure 2A; Zimmerman *et al.*, 1993; Ma *et al.*, 1996; Takebayashi *et al.*, 1997). At stage 11.5–12, the expression domains of *X-ngnr-1* (C, arrowheads) are partially within the *Xiro* territory (A). This is clearer from a lateral view (compare insets in A and C). The arrow in (C) inset points to the anterior domain of *X-ngnr-1* included in the *Xiro* territory. At stage 13.5, only the anterior part of the *X-ngnr-1* domain is within the *Xiro* territory (compare B and D). At stages 11.5–12 and 13.5 (E and F respectively), *XASH-3* expression was totally comprised within the *Xiro* domains of expression (compare with A and B). The embryo in (E) is slightly tilted with respect to that in (A), so that more of the anterior region is shown. *ATH-3* expression was not examined since this gene is expressed at later stages and within the domain of *X-ngnr-1* expression (Takebayashi *et al.*, 1997).

Skarmeta and Modolell, 1996). Ci is up-regulated by the Hedgehog (Hh) signaling molecule (Johnson *et al.*, 1995; Motzny and Holmgren, 1995; Slusarski *et al.*, 1995; Alexandre *et al.*, 1996; Domínguez *et al.*, 1996; Sánchez-Herrero *et al.*, 1996; Hepker *et al.*, 1997). In *Xenopus*, several *hh* homologs are expressed in different domains which surround the *Xiro* domains of expression (Ekker *et al.*, 1995). Moreover, the mouse homologs of Ci, the Gli proteins, are expressed in territories adjacent to these Hh domains, as expected if they mediate Hh signaling (Hui *et al.*, 1994; Mo *et al.*, 1997). We thus examined whether, similarly to *ara–caup*, *Xiro2* is controlled by Gli proteins. Since *Xenopus* Gli proteins were not available, we used *Drosophila* Ci. Injected *ci* mRNA enhanced the expression of *Xiro2*, most significantly in the anterior region of the embryo (Figure 6D). These data thus suggest that Gli proteins control *Xiro*, and that at least several members of the genetic cascade that regulates proneural gene expression are conserved in *Drosophila* and vertebrates.

Discussion

In *Drosophila*, the IRO-C genes *ara* and *caup* encode homeoproteins that act as prepattern factors necessary for the expression of the proneural genes *ac* and *sc* (Gómez-Skarmeta *et al.*, 1996; Leyns *et al.*, 1996). We have now isolated cDNAs corresponding to two *Xenopus* genes, *Xiro1* and *Xiro2*, which putatively encode proteins with homeodomains very similar to those of Ara and Caup. This similarity and, as discussed below, the conservation of some of their functions, suggest that the Xiro proteins are *Xenopus* homologs of Ara and Caup.

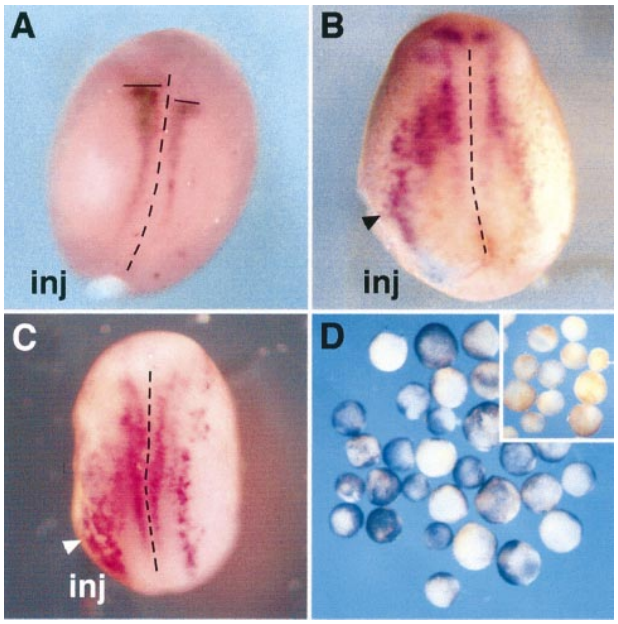

Fig. 5. *Xiro* controls the expression of proneural genes. (**A**) Stage 17 embryo injected in one blastomere of the two-cell stage with 2 ng of *Xiro2* mRNA. Notice the expansion of *XASH-3* expression in the injected side (47% of embryos, $n = 78$). (**B**) Embryo injected in one blastomere of the two-cell stage with 2 ng of *Xiro2* mRNA and 0.3 ng of *lacZ* mRNA. At stage 15, *X-ngnr-1* expression is expanded at the side with X-Gal staining (43% of embryos, $n = 51$). (**C**) Stage 15 embryo injected in one blastomere of the two-cell stage with 2 ng of *Xiro2* mRNA and 0.3 ng of *lacZ* mRNA. *ATH-3* expression is expanded in the injected side, although X-Gal staining is hardly visible at this focus. This effect was observed in only 17% of embryos ($n = 36$). (**D**) Animal caps from embryos injected with 0.2 ng of *Xiro2* mRNA taken at stage 10 and cultured until the equivalent of stage 17. *XASH-3* is strongly expressed (42% of caps, $n = 96$). Control caps (inset, $n = 110$) do not express *XASH-3*.

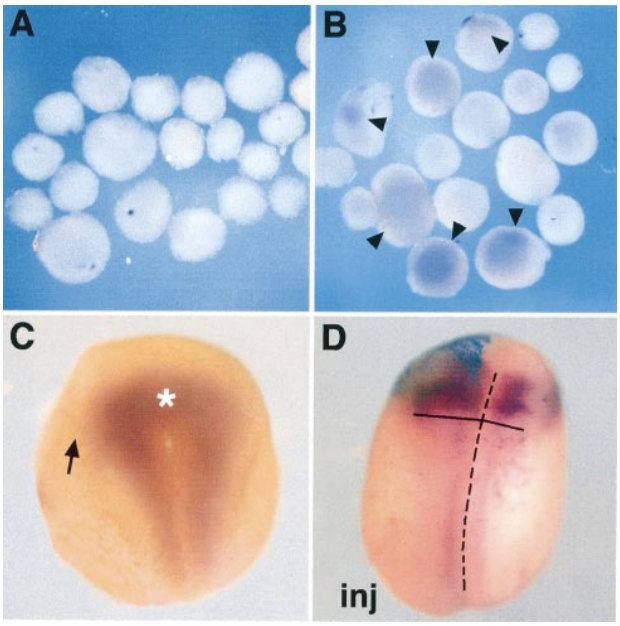

Fig. 6. Regulation of *Xiro* expression. Noggin mRNA (0.1 ng) was injected at the one-cell stage and animal caps were dissected at stage 10.5, cultured until the equivalent of stage 17, and *Xiro2* expression was analyzed. (**A**) Uninjected animal caps: no expression is detectable ($n = 45$). (**B**) Animal caps from noggin-injected embryos: *Xiro2* is expressed (arrowheads; 39% of caps, $n = 41$). (**C**) *Xiro2* expression in a stage 17 embryo treated with retinoic acid. *Xiro2* is expressed in the most anterior neural plate which may be transformed to posterior neuroectoderm (asterisk). Notice that *Xiro* is suppressed from the lateral ectoderm (arrow, compare with Figure 2E). (**D**) Embryo injected with 0.3 ng of *ci* mRNA and 0.3 ng of *lacZ* mRNA in one blastomere of the two-cell stage. At stage 17, *Xiro2* expression is expanded in the injected side (50% of embryos, $n = 64$), which suggests a morphological enlargement of the neural tissue at the injected side. The size of each side of the neural plate is indicated by bars.

Expression of Xiro

During *Xenopus* early development, *Xiro1* and *Xiro2* are expressed in very similar patterns. Expression starts early (stage 10) in a domain that includes most of the prospective neural plate. Slightly later, expression occurs in two domains, one within the anterior ectoderm (outside the neural plate) and the other within the neural plate. At the end of neurulation, the first domain is subdivided into rings that correspond to the placodal cells. At the same stage, the second domain consists of two longitudinally oriented broad bands separated by the midline. They extend from the midbrain–hindbrain junction to, and including, the spinal chord. *Xiro* expression precedes and overlaps with the domains of expression of *XASH-3*, *X-ngnr-1* and *ATH-3* (Zimmerman *et al.*, 1993; Turner and Weintraub, 1994; Ma *et al.*, 1996; Takebayashi *et al.*, 1997), genes encoding proneural proteins related to the *Drosophila* Ac and Sc proteins. This co-localization and structural similarities suggested that the *Xiro* genes control *XASH-3*, *X-ngnr-1* and *ATH-3*, as their putative *Drosophila* homologs *ara* and *caup* control *ac* and *sc*.

Xiro controls proneural genes and neural plate development

The overexpression of *Xiro* mRNAs indicates that, in the neural plate, *Xiro* positively regulates *XASH-3*, *X-ngnr-1* and possibly *ATH-3*. Thus, while *XASH-3* and *X-ngnr-1* are ectopically activated in almost half of *Xiro*-injected embryos, *ATH-3* is overexpressed in ~17% of these embryos. It has been shown that both *XASH-3* and *X-ngnr-1* can activate the expression of *Delta* which, in turn and by means of the *Notch* pathway, suppresses neural differentiation (Chitnis and Kintner, 1996; Ma *et al.*, 1996). Thus, the *Xiro*-dependent overexpression of *XASH-3* and *X-ngnr-1* may overactivate this pathway and prevent a large up-regulation by these early proneural genes of the later expressing *ATH-3* in neural precursors. Alternatively, *Xiro* may directly overactivate *Delta* or other genes of the lateral inhibition pathway, such as those of the *Enhancer of split* complex, and block any substantial activation of *ATH-3* within the enlarged domains of *XASH-3* and *X-ngnr-1*.

The regulation of proneural gene expression by Xiro seems spatially restricted, since injected *Xiro* mRNA does not activate these genes outside the neural plate. Xiro probably requires additional factor(s), exclusive of the neural plate, to activate proneural genes or its action is blocked by inhibitor(s) outside the neural plate. We have also found, in animal caps and therefore in the absence of neural inductive signals emanating from the mesoderm, that *Xiro* can activate *XASH-3*, but not *X-ngnr-1* and *ATH-3*, and that this activation is stronger than that detected in whole embryos. This suggests that Xiro is

sufficient to activate *XASH-3* and that negative factors, controlled by inductive signals, block its activation in the whole embryo. In contrast, Xiro most likely requires other factor(s), present in at least part of the neuroectoderm and absent in animal caps, to activate *X-ngnr-1* and *ATH-3*. Note that the territories that express *XASH-3* are contained within the domains of Xiro accumulation while part of those expressing *X-ngnr-1* and *ATH-3* are outside of them. This also suggests that other regulators activate *X-ngnr-1* and *ATH-3*.

In *Drosophila*, *ara* and *caup* directly activate *ac* and *sc*. Thus, in *Xenopus*, *Xiro* may also directly activate proneural genes. Moreover, similarly to the effect of the overexpression of *Xiro*, overexpression of *ara* in *Drosophila* does not lead to generalized overexpression of *ac* or *sc*, which suggests that additional factor(s) participate in the control of these proneural genes (Gómez-Skarmeta *et al.*, 1996).

Overexpression of *Xiro* expands the neural plate and reduces the neural crest. A similar effect has been found by overexpressing *XASH-3* (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). Moreover, in both cases, the expansion of the neural plate is apparently due to a change in fate of the cells laying at the border of the neural plate, which are specified as cells of this rudiment rather than of the neural crest or epidermis (Turner and Weintraub, 1994; this study). This indicates that *Xiro* overexpression, probably by ectopic activation of proneural genes, commits cells that are normally outside of the neural plate to adopt a neural fate, thus altering initial choices within the ectoderm.

Cascade of neural development in Xenopus

In vertebrates, the initial steps of neural induction are mediated by several molecules, chordin, noggin and probably follistatin, that antagonize the BMP-4 antineuralizing function (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). However, these molecules seem to induce only anterior genes. So, after this initial anterior neural induction, several factors, such as RA and members of the FGF and Wnt families, posteriorize part of the induced neural tissue to generate the full complement of anterior–posterior neural identities (Sasai and de Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). We find that *Xiro* expression is induced optimally in the presence of both an anterior neural inducer, such as noggin, and a posteriorizing agent, like RA. This fits with the localization of *Xiro* expression at the posterior limit of the region inducible by noggin. RA receptors recently have been implicated in primary neurogenesis in *Xenopus* (Papalopulu and Kintner, 1996; Blumberg *et al.*, 1997; Sharpe and Goldstone, 1997). It is possible that at least part of this function is mediated by *Xiro*.

In *Drosophila*, growth and patterning of the wing depend on a genetic cascade which is initiated by Hh signaling between the cells of the posterior and anterior compartments (Lecuit *et al.*, 1996, and references therein; Nellen *et al.*, 1996). This signal induces in anterior cells the up-regulation of the Ci transcription factor (Johnson *et al.*, 1995; Motzny and Holmgren, 1995; Slusarski *et al.*, 1995; Alexandre *et al.*, 1996; Domínguez *et al.*, 1996; Hepker *et al.*, 1996; Sánchez-Herrero *et al.*, 1997), which in turn promotes the expression of *ara–caup* (Gómez-Skarmeta and Modolell, 1996). In vertebrates, members of the Hh family organize the pattern in many different tissues. However, the genes that transduce Hh signals are almost unknown (reviewed by Hammerschmidt *et al.*, 1997). In *Xenopus*, three Hh homologs are known: *Xenopus sonic* (*X-shh*), *banded* (*X-bhh*) and *cephalic hedgehog* (*X-chh*) (Ekker *et al.*, 1995). During neurulation, *X-shh* and *X-bhh* are expressed in the midline and in the peripheral region of the neural plate, respectively, and *X-chh* in anterior structures encompassing both neural and endodermal cells (Ekker *et al.*, 1995). Three targets of Hh signaling, the genes *Gli*, *Gli2* and *Gli3*, homologs to *Drosophila ci*, have been found in the mouse (*Xenopus* homologs have not yet been described). They are expressed in different domains that comprise most, if not all, the neural plate (Hui *et al.*, 1994). This pattern of expression is consistent with the proposal that different Hh molecules induce the expression of different *Gli* genes (Mo *et al.*, 1997). We have now found that *Drosophila ci* mRNA ectopically activates *Xiro* genes, which suggests that they are positively controlled by Gli proteins. Assuming that mouse and fly data can be extrapolated to *Xenopus*, *Xiro* should be expressed within the domains of accumulation of Gli proteins. Moreover, we have provided evidence that *Xiro* in turn regulates *XASH-3*, *X-ngnr-1* and possibly *ATH-3*. Hence, these data suggest that, after neural induction, the genetic cascade of proneural gene activation, namely *hh:X-shh/X-bhh/X-chh, ci:Gli, ara–caup:Xiro* and *ac–sc:XASH-3/X-ngnr-1/ATH-3*, is largely conserved in *Drosophila* and vertebrates.

Neural induction and prepattern genes

In *Drosophila*, the spatial distribution of the transcription factors of that genetic cascade, namely, Ci, Ara–Caup and Ac–Sc, occurs in overlapping but progressively more restricted domains. In the case of Ara and Caup, their expression is restricted, within the territory of Ci accumulation, by repression mediated by the homeoprotein Engrailed, the Wnt protein Wingless and other as yet unknown factors (Gómez-Skarmeta and Modolell, 1996). Similarly, Ara–Caup, in combination with the inputs of other factors, direct the even more restricted expression of *ac–sc* (Gómez-Skarmeta *et al.*, 1996). Thus, at each step of the genetic cascade and by the participation of combinations of factors distributed in different landscapes, positional information is gradually gained. These combinations of factors, both activators and repressors, have been addressed as a 'prepattern' (Stern, 1954; Ghysen and Dambly-Chaudière, 1988, 1989). The conservation of the postulated cascade in the vertebrate neural plate, together with the expression of *XASH-3*, *X-ngnr-1* and *ATH-3* within the more extensive expression domains of *Xiro*, suggest that a similar prepattern is generated in the neural ectoderm. Accordingly, Xiro would be one of the prepattern factors that define, at least in part, the restricted expression of *XASH-3*, *X-ngnr-1* and *ATH-3*. This view is supported by the fact that *Xiro* generalized overexpression only expands the expression of these genes in a moderate way, which indicates that other factors co-operate in the regulation of *Xenopus* proneural gene expression. Another putative *Xenopus* prepattern gene is *Pax-3*, which is expressed very early (stage 11) in a broad mediolateral domain of the presumptive neural plate. The medial

expression is rapidly turned off (stage 11.5), leaving expression in only two lateral posterior domains (Bang *et al.*, 1997), which seem to overlap partially with those of *Xiro* (compare Figure 4A with Figure 2A and B in Bang *et al.*, 1997). Thus the combination of *Xiro*, *Pax-3* and other prepattern genes may subdivide the neuroectoderm into specific domains. Evidently, the postulated prepattern may also control the expression of other genes.

In summary, the inductive signals that operate during neural induction, namely noggin, chordin, follistatin, RA, Wnt proteins, FGF and members of the Hh family, appear to activate broad domains of expression of prepattern genes, among them the *Xiro* and *Pax-3* genes. The combinatorial activity of the prepattern genes should activate a second set of genes, such as *XASH-3*, *X-ngnr-1* and *ATH-3*, that are expressed in more restricted patterns and reflect the progressive subdivision of the CNS as it develops.

Materials and methods

Molecular cloning of the Xiro genes

Approximately 10^6 phages from a *Xenopus* gastrula (stages 10.5–11.5) UniZap-XR cDNA library (Cho *et al.*, 1991) were screened with a 195 bp probe (nucleotides 1355–1550 of the *caup* cDNA sequence) encoding the *caup* homeodomain, in hybridization buffer (40% formamide, 4× SSC, 5× Denhartd's, 0.1% SDS, 20 mM phosphate buffer pH 6.8, 0.1 mg/ml salmon sperm DNA and 0.1% sodium pyrophosphate) at 42°C. Filters were washed with 2× SSC, 0.1% SDS at 42°C. Twelve positives were obtained, were purified and the corresponding plasmids were excised. As judged from their sequences, these cDNAs correspond to two different *Xiro* genes. In addition, the partial sequences of three of these cDNAs indicate that they were extremely similar, but not identical, to *Xiro1*. According to the sequence divergence of *Xiro1* and these cDNAs, they may correspond to a *Xiro1* pseudo allele (Graff and Kobel, 1991).

DNA sequencing

The longest *Xiro1* (3.3 kb) and *Xiro2* (2.5 kb) cDNAs were sequenced in both strands with ABI chemistry in an automatic DNA sequencer, using T3, T7 and custom synthesized oligonucleotides (ISOGEN Bioscience BV, Maarsesen, The Netherlands) as primers. Consensus sequences were assembled and analyzed with the University of Wisconsin GCG software packages (Devereux *et al.*, 1984).

Whole-mount in situ hybridization and X-Gal staining

Antisense RNA probes were prepared from *Xiro*, *Xslug* (Mayor *et al.*, 1995), *XASH-3* (Ferreiro *et al.*, 1994), *X-ngnr-1* (Ma *et al.*, 1996), *ATH-3* (Takebayashi *et al.*, 1997), *Xsox-2* (kindly provided by Dr R.M.Grainger), *engrailed-2* (Hemmati-Brivanlou *et al.*, 1991), *Otx-2* (Pannese *et al.*, 1995; Blitz and Cho, 1995) and *Krox-20* (Bradley *et al.*, 1993) cDNAs using digoxigenin or fluorescein as labels. Specimens were prepared, hybridized and stained by the method of Harland (1991) with modifications (Mancilla and Mayor, 1996). Double and triple *in situ* hybridizations were made by mixing different probes and using different alkaline phosphatase substrates for developing color. The substrates used were BM purple and BCIP (both from Boehringer Mannheim) and Magenta Phos (Molecular Probes). X-Gal staining was performed according to Coffman *et al.* (1993).

In vitro RNA synthesis and plasmid constructs

Xiro cDNAs were cloned in Bluescript or in SP35T vectors (no qualitative difference was observed in the effects of the mRNAs produced using these different vectors). All the vectors were linearized and transcribed as described by Harland and Weintraub (1985) with GTP cap analog (New England Biolabs). SP6, T3 or T7 RNA polymerase were used. After DNase treatment, RNA was extracted with phenol–chloroform and precipitated with ethanol. Injected mRNA was resuspended in water.

Embryos, explants, microinjection of mRNA and retinoic acid treatment

Xenopus embryos were obtained as described previously (Mayor *et al.*, 1993) and staged according to Niewkoop and Faber (1967). Animal caps, dissected at stage 10, were cultured in 3/8 NAM (Slack, 1984) until the equivalent of stage 17. Synthetic mRNA was injected at the one- or two-cell stage embryo in 8–12 nl volume as described (Mayor *et al.*, 1993). For RA treatment, embryos were incubated at stage 10 for 3 h in a 10 mM solution. After removing this solution, embryos were incubated until stage 17 and fixed for *in situ* hybridization.

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Note added in proof

Xenopus genes encoding Gli proteins have recently been isolated [Lee *et al*. (1997) *Development*, **124**, 2537–2552]. Their expression patterns appear to be controlled by Hedgehog signaling and are consistent with their activating *Xiro* expression.

Another member of the *Xenopus* iro family (*Xiro3*) has been characterized [Bellefroid *et al*. (1998) *Xiro3* encodes a *Xenopus* homolog of the *Drosophila Iroquois* genes and functions in neural specification. *EMBO J.*, **17**, 191–203].