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ARTICLE

*xArx*2: An Aristaless Homolog That Regulates Brain Regionalization During Development in *Xenopus laevis*

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Summary: The aristaless-related gene, *Arx*, plays a fundamental role in patterning the brain in humans and mice. *Arx* mutants exhibit lissencephaly among other anomalies. We have cloned a *Xenopus aristaless* homolog that appears to define specific regions of the developing forebrain. *xArx2* is transcribed in blastula through neurula stages, and comes to be restricted to the ventral and lateral telencephalon, lateral diencephalon, neural floor plate of the anterior spinal cord, and somites. In this respect, *Arx*2 expresses in regions similar to *Arx* with the exception of the somites. Overexpression enlarges the telencephalon, and interference by means of antisense morpholino-mediated translation knockdown reduces growth of this area. Overexpression and inhibition studies demonstrate that misregulation of *xArx*2 imposes dire consequences upon patterns of differentiation not only in the forebrain where the gene normally expresses, but also in more caudal brain territories and derivatives as well. This suggests that evolutionary changes that expanded *Arx*-expression from ventral to dorsal prosencephalon might be one of the determinants that marked development and expansion of the telencephalon. genesis 00:000–000, 2008. © 2008 Wiley-Liss, Inc.

Key words: *Xenopus laevis*; Arx2; homeobox; telencephalon; embryogenesis; neural development; morpholino; XSLAG; lissencephaly; evolution

INTRODUCTION

The vertebrate brain undergoes a complex evolution of patterning, and elucidation of the cellular and molecular mechanisms that underlie the differentiation of this organ is the focus of intense research. It is estimated that fully two thirds of mouse all genes are expressed at some point in the brain (Abbott, 2003): embryological and genetic studies are only just beginning to define some of the many genes that are involved. Several organizing regions appear to be critical to normal elaboration of the brain: the isthmus at the mid-hindbrain junction (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Echevarria *et al.*, 2003; Larsen *et al.*, 2001); and the anterior neural ridge which demarcates the junction between neural plate and ectoderm. This latter structure is necessary for the maintenance of forebrain identity (Shimamura and Rubenstein, 1997). For example, *FoxG*1/*Bf*1 encodes a winged-helix transcription factor that is required for regionalization and growth of the telencephalic and optic vesicles. Mice mutant for *FoxG*1/*Bf*1 have a small telencephalon and lack expression of a basal telencephalic marker, *Dlx*2 (Xuan *et al.*, 1995). Excision of the anterior neural ridge has been shown to eliminate expression of *FoxG*1/*Bf*1 in neural plate explants (Shimamura and Rubenstein, 1997). Moreover, transplantation of anterior neural ridge cells from zebrafish into more caudal regions of the neural plate induces the expression of *Nkx*2.1 and *Emx*, genes typically expressed in the telencephalon (Houart *et al.*, 1998).

The aristaless family of transcription factors is characterized by the structure of its homeodomain and by the presence of a C-terminal motif termed the OAR or aristaless domain. The family is comprised of three broad groups, the second of which includes genes such as *Arx* and *Rx* that express in the anterior neural ridge (Beverdam and Meijlink, 2001). *Arx* is one of the more recent to have been linked to a diverse array of congenital defects in human. In mammals, there are several related *Arx* genes, however, mutation of the gene that is most closely related to the *Drosophila* prototype, namely *ARX*, can lead to autism, epilepsy, abnormal cortical development, spasticity, and distonia (Sherr, 2003). Other anomalies in this X-linked disorder include the development of brain cysts and ambiguous genital development

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(Kitamura *et al*., 2002; Uyanik *et al*., 2003). Impaired development results from various types of mutation including point mutation, mis-sense, and deletion mutations, as well as expansion of a region that encodes a poly-alanine tract (Bienvenu *et al*., 2002; Kitamura *et al*., 2002; Stromme *et al*., 2002). In mice and in zebrafish, *Arx* is expressed in the developing cerebral cortex as well as in the neural floorplate (Miura *et al*., 1997), and disruption of *Arx* in mice leads to abnormal cortical and genital development, though this latter phenotype may result from incomplete inactivation of the gene (Collombat *et al*., 2003; Kitamura *et al*., 2002). In mutant mice, neural anomalies are linked to a failure of normal neural migration and differentiation of the absal ganglia (Colombo *et al*., 2007).

A related gene, x*Arx* (hereafter called *xArx*), has also been cloned in *Xenopus* (El-Hodiri *et al*., 2003), however, the expression pattern of this locus diverges slightly from that of mouse and zebrafish in that there does not appear to be expression in the somites. More recently, through the application of ectopic expression and loss-of-function studies, *Xenopus Arx* has been shown to play a role in brain regionalization (Seufert *et al*., 2005). Misexpression of this gene results in morphological deficits in forebrain derivatives as well as in the development of ectopic otic vesicles. We report the cloning of a second *Xenopus* locus, *xArx*2 (accession number AY519474), that shares 90% amino acid identity with *xArx*, expresses in similar domains, but which in addition expresses in anterior neural ridge and developing somites. Like its *Xenopus* homolog, it first expresses ventrally in the telencephalon. This is in marked distinction to murine *Arx* which expresses dorsally and the difference may serve to indicate why frogs essentially lack a neocortex. Through ectopic expression and antisense morpholino-mediated translation knockdown studies, we sought to define the importance of *xArx*2 to development of the *Xenopus* forebrain. By either assay, *Arx*2 appears to modulate forebrain development, and indirectly, to affect the differentiation of more caudal neural structures.

RESULTS

*XArx*2 is a Conserved Member of the Vertebrate *Arx Family*

x*Arx*2 encodes a conceptual open reading frame encoding a protein of 528 amino acids. It contains a glutamine at Position 50 of its homeodomain. The sequence also encodes a conserved octapeptide sequence, a nuclear localization domain, and a C-terminal aristaless domain (Fig. 1a). Alignment of the predicted Arx amino
F1 acid sequences among vertebrates revealed a high degree of homology between Arx2 and homologs from human, mouse, and zebrafish. There was 100% identity among all Arx sequences analyzed in the octapeptide, nuclear localization, homeodomain, and the C-terminal aristaless domain (Fig. 1b). *xArx*2 (Genbank accession number AY519474) and *xArx* (El-Hodiri *et al*., 2003; GenBank accession number AY130460) share 90% similarity at both the nucleotide and the amino acid levels. Both *Xenopus* Arx homologues share 67% identity with mouse and zebrafish. However, *xArx*2 was found to be marginally more similar at the amino acid level to human ARX than was *xArx* (68% versus 66%) (Fig. 1c).

Temporal Expression of *xArx*2 by RT-PCR Analysis

The temporal expression profile of *xArx*2 during early *Xenopus* development was analyzed by RT-PCR (see Fig. 2). *xArx*2 is detectable as a maternal transcript and is F2
present at moderate levels up until the end of gastrulation. Just following the onset of neurulation, the expression level at Stage 14 markedly increases. *xArx*2 expression continues to increase throughout neurulation into tailbud stages.

xArx2-Directed Morpholino Inhibits Translation of xArx2

To confirm the specificity of the morpholino oligonucleotides (MO) used in our studies, we assayed levels of in vitro translated ^{35}S-labeled products using constructs that contained the open reading frame of *xArx*2 fused to morpholino target or positionally equivalent sites (see Fig. 3). Translation was inhibited in a construct that con- F3
tained the *Arx*2-MO equivalent site when *Arx*2-MO was introduced. Moreover, translational levels were unaffected by *xArx*-MO with construct that contained the *Arx*2-MO site. The standard control morpholino had no effect on *xArx*2 translation and neither morpholino impaired translational levels of the control GFP protein.

Spatio-Temporal Expression Analysis of *xArx*2 by Whole Mount Riboprobe In Situ Hybridization

The spatiotemporal expression of *xArx*2 was analyzed by whole-mount in situ hybridization (see Fig. 4). The F4
transcript is initially visualized at blastula stage (Fig. 4a) on the prospective dorsal side of the embryo and is not again detectable until Stage 14 (Fig. 4b), where it expresses as a pair of bands straddling the anterior neural plate, and where it remains throughout neurulation, as well as a low level of transcription in the neural apical ridge (Fig. 4c). During early tailbud stages (Fig. 4d,e) *xArx*2 is detected in the prosencephalon, or presumptive forebrain area, and in the somites. At the late tailbud stage (Fig. 4f) *xArx*2 expresses strongly in the ventral and lateral telencephalon, the lateral diencephalon, and in the anterior neural tube (Fig. 4g,h). Analysis of sectioned tadpoles subjected to in situ hybridization revealed that in anterior sections, *xArx*2 is expressed in all but the dorsal-most region of the telencephalon (Fig. 4i) and in the medio-lateral diencephalon (Fig. 4j). There is no staining observed with sense probe at any of the stages. Moreover, the stringency of hybridization employed was such that there was no apparent cross-reactivity between *Arx* and *Arx*2 probes (Fig. 4k). Finally, embryos that were unilaterally injected with *Arx*2 morpholino at doses sufficient to generate a pro-

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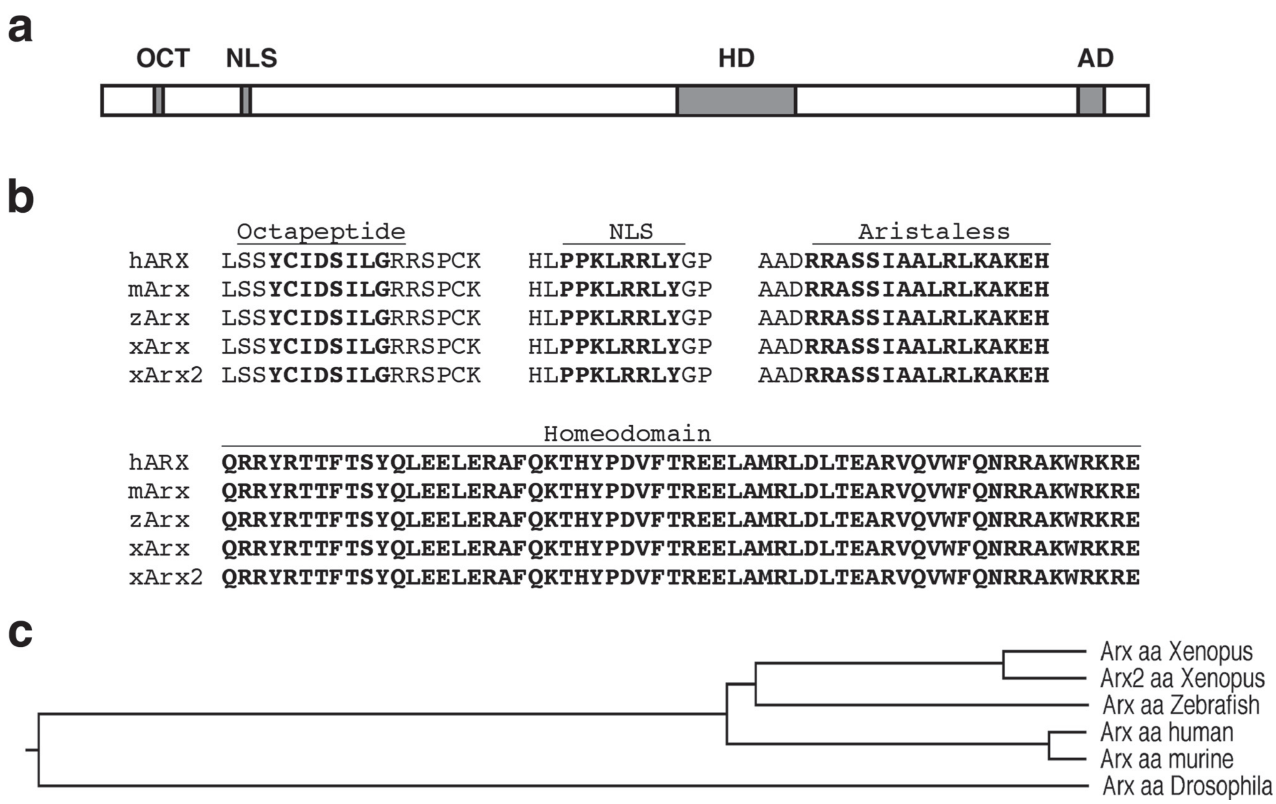

FIG. 1. *xArx*2 encodes a protein with a structure that is highly conserved among vertebrate Aristaless-related products. **a:** The N-terminal octapeptide (OCT) and nuclear localization signal (NLS) are followed by a central homeodomain (HD) and C-terminal aristaless domain (AD). **b:** *xArx*2 shares 100% identity with zebrafish, mouse, human and *xArx*2 in the octapeptide, nuclear localization signal (NLS), homeo-, and aristaless domains. **c:** The pattern of dendrogram clustering suggests that the *Xenopus* loci are homologous to previously characterized *Arx* homologs.

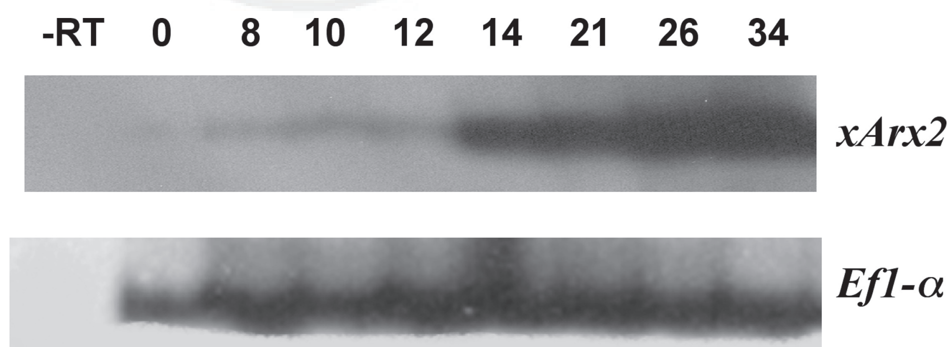

FIG. 2. RT-PCR of *xArx*2 expression over the course of early *Xenopus* development. Blastula stages include Stage 8, gastrulation commences Stage 10, and neurulation, late stage 12.

found phenotype had the effect of inhibiting *Arx*2 expression of the injected side in presumptive forebrain at early stages, and in somites at later ones. (Fig. 4l). Either the morpholino/transcript hybrids expose transcript to premature degradation or *Arx*2 is normally autoregulatory.

In summary, although expression in the diencephalon is conserved, expression patterns for the two genes diverge in that *xArx* expression is uniquely apparent in the ectoderm immediately below the cement gland (El-Hodiri *et al*., 2003), whereas Arx2 enjoys unique expression in the ridge that demarcates the anterior neural plate, and briefly, in the somites.

*xArx*2 expression can be more precisely localized to the posterior telencephalon and anterior diencephalon by comparison to other markers. For example, in early neurulae, *xArx*2 expresses just caudal to telencephalon marker *FoxG*1/*Xbf*1 (Fig. 5a), and slightly overlapping F5
and caudal to *Rx* (Fig. 5b). *xArx*2 is rostral and slightly overlapping with diencephalon/mesencephalon marker

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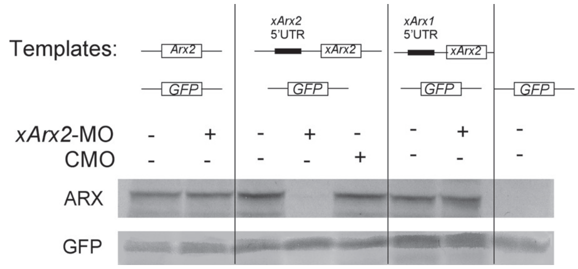

FIG. 3. Radio-labeled Western blot of in vitro translated reactions shows that *xArx*2 antisense morpholino specifically blocks translation from *xArx*2 template. Transcripts that lacked the *xArx*2 5′-untranslated 25mer sequence were unaffected by treatment with either *xArx*2 antisense morpholino (*xArx*2-MO), or control morpholino (CMO).

*Otx*1 (Fig. 5c), and is completely rostral to isthmus (midbrain/hindbrain junction) marker engrailed (Fig. 5d).

Misexpression of *xArx*2 Results in Anterior Defects

Ectopic *xArx*2 expression results in distinct and reproducible phenotypes in the anterior region of the embryo. The majority of morphological effects of *xArx*2 appear to be dose-dependent until the RNA injected reaches 400 pg. Beyond this dose (at 600 and 800 pg) survival rate to swimming tadpole stage dramatically declines, and head structures are barely recognizable. Several different phenotypes can emerge, and when they are compounded, they are hard to interpret (Fig. 6a–d). Misexpression of *xArx*2 results in several distinct
F6 anterior abnormalities, which include microcephaly (11% at 200 pg *xArx2* mRNA, $n = 119$), midline defects such leading to cone shaped or fused eyes (under 5%), or diminished or absent eyes (6% 200 pg) (Fig. 6c). Occasionally, the olfactory organs were displaced dorsally (Fig. 6a,b). In this respect, the phenotypes in ectopic expression mutants are similar to those reported for *xArx* (Seufert *et al*., 2005). A more common and dose-dependent consequence of *xArx*2 overexpression is enlargement of the forebrain that occurs in a morphologically obvious manner 8%–22% of the time in doses ranging from 200–800 pg respectively ($n = 119$ and 53, respectively). This percentage underestimates the effect of *Arx*2 overexpression insofar as only those embryos with no midline defects could be faithfully analyzed with respect to unilateral forebrain size changes. Midline defects were also caused by antisense morpholino-mediated loss-of-function, and with comparable frequency (Fig. 6d). In contrast to ectopic expression phenotypes, morpholino-mediated loss-of-function tended to diminish forebrain size (compare 7A, C with 7B, D) (18 ng 16% $n = 72$). Other forebrain abnormalities seen following either gain- or loss-of-function treatments included a fusion of forebrain lobes often associated with a reduction in the craniofacial development. Embryos injected with GFP or with control morpholino at comparable concentrations displayed no abnormalities. Injections at the four- or eight-cell staged produced identical effects. Finally, the morphological anomalies produced by *Arx*2 misexpression appear very similar in character to those produced in *Arx*-disrupted embryos (Seufert *et al*., 2005). In contrast to *xArx, xArx*2 does not induce the formation of ectopic otic vesicles as assessed by morphology or by expression of a specific marker, *xDlx*5 (data not shown).

Inhibition of xArx2 affects the developing forebrain. We analyzed the effect of loss-of-function of *xArx*2 by means of antisense morpholino oligonucleotide (MO)-mediated translational knockdown. This commonly results in a reduction of the telencephalon, both mediolaterally and rostrally, (Fig. 6d) as well as asymmetrical, or underdeveloped craniofacial modeling (Fig. 6c,d). Embryos injected with the control morpholino display an infrequent (2% of embryos) incidence of hypomorphic eyes, but otherwise develop normally.

Histological examination of tadpoles reveals forebrain size abnormalities. To better examine the forebrain region in tadpoles misexpressing *xArx*2,

FIG. 4. Expression of *xArx*2 during *Xenopus* development. At blastula stage (**a**), *xArx*2 is observed in dorsal blastomeres. Expression later restricts to a pair of distinct stripes in the anterior neural plate, and more diffusely in the apical neural ridge (**b** arrow, which later refines to a light crescent, arrow in **c**). Expression is later observed in somites (**d**,**e**), and presumptive forebrain (**f**,**g**), which consequently refines to diencephalon (di) and telencephalon (tel) (g). In cleared embryos, expression is detected in the anterior neural tube (**h**). Cross sections of *Xenopus* embryos demonstrate expression of *xArx*2 in the ventral and lateral telencephalon (**i**), lateral diencephalon (**j**). In situ hybridization using *Arx* and *Arx*2-specific probes show distinct expression patterns (**k**). Unilaterally injected *Arx*2 morpholino (arrows) perturbs expression of *Arx*2 (**l**).

FIG. 5. Double stained in situ hybridizations show that *Arx*2 is expressed caudal to telencephalon markers *FoxG*1/*Xbf*1 (**a**) and *Rx* (**b**), but rostral to diencephalon/mesencephalon marker *Otx*1 (**c**) and isthmus marker engrailed (**d**).

FIG. 6. Misexpression of *xArx*2 causes anterior developmental anomalies. Dorsal views of tadpoles injected with *xArx*2 (**a–c**) or antisense morpholino (**d**). Anomalies following ectopic expression included: hypomorphic craniofacial modeling (a); mispositioned forebrain (arrow in b); micropthalmia on the side of RNA injection (right side of c); extension of the retinal pigmented epithelium - RPE (d). (Frequency of phenotype is indicated in brackets).

FIG. 7. *Arx*2 misexpression perturbs forebrain development. Unilaterally injected embryos develop enlarged forebrains if injected at the 2-cell stage with *Arx2* transcript (**a**), diminished forebrains if injected with antisense morpholinos (**b**). Asymmetrical growth is particularly evident in longitudinal sections: ectopic expression of *xArx*2 expands the forebrain growth relative to contralateral tissues in Hoechst-stained sections (**c**), and inhibition by antisense morpholino inhibits growth (**d**).

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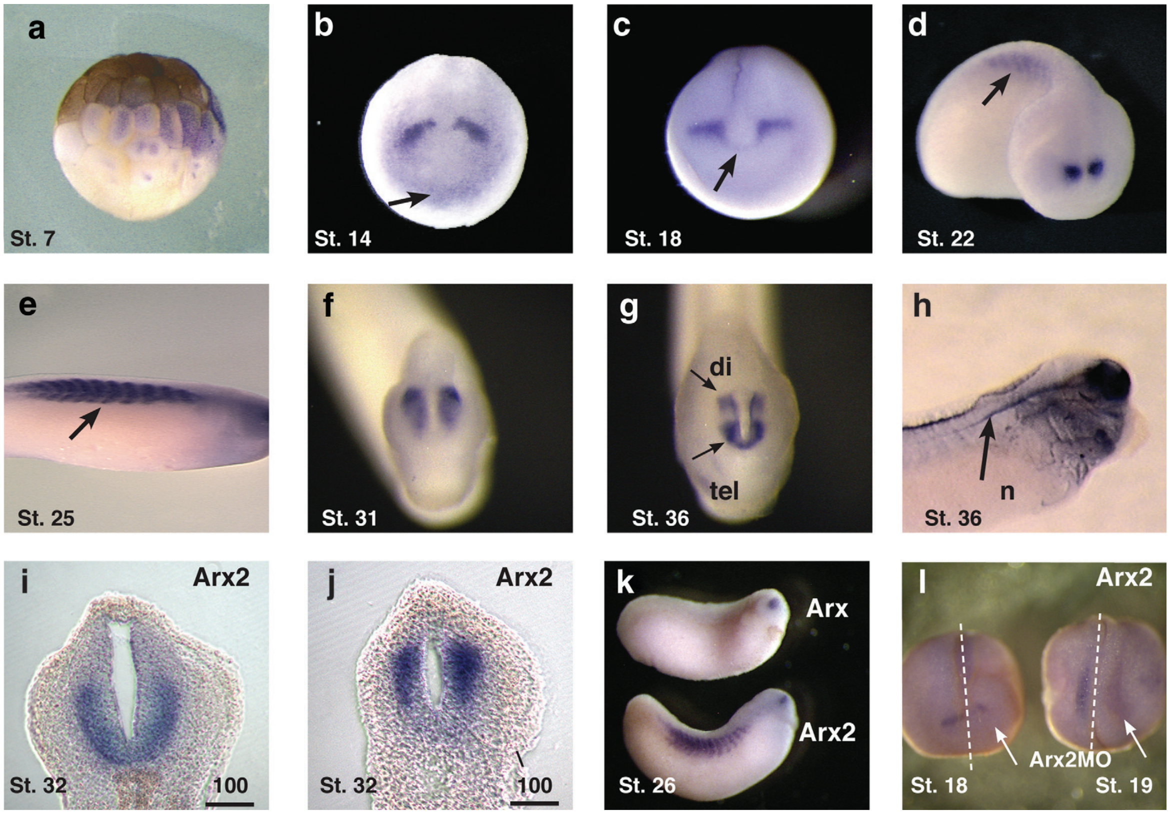

FIG. 4.

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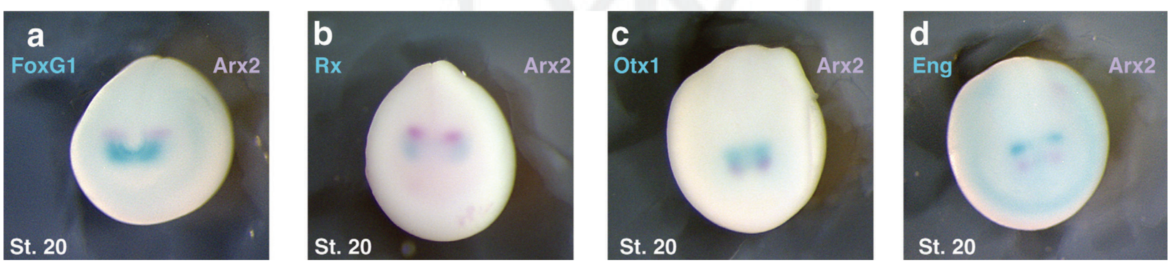

FIG. 5.

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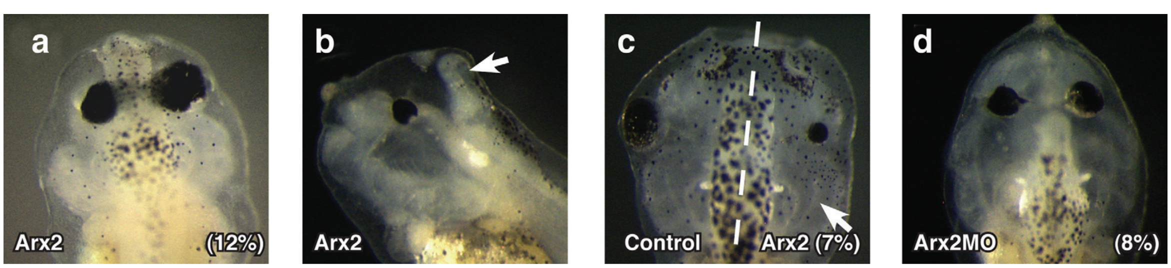

FIG. 6.

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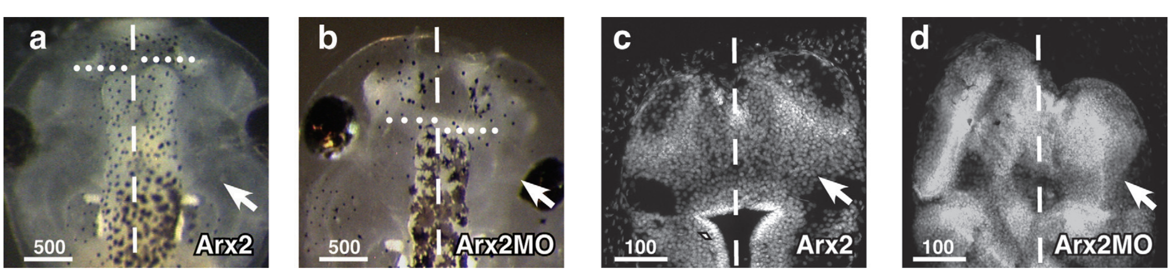

FIG. 7.

embryos were stained with either Hoechst or with Hematoxylin and eosin (H&E). At this level, a striking expansion of the forebrain territory on the *xArx*2 injected side of the embryo is observed (Fig. 7a,c). Conversely, the forebrain on the injected side of the *Arx*2-MO-injected embryos appears substantially reduced in (Fig. 7b,d).

F7

Interfering with Proper *xArx*2 Function Results in Midline Defects

Misexpressing *xArx*2 induces anterior deformities, such as abnormal optic stalk patterning, cyclopia, and fused brain lobes that are reminiscent of those observed with a disruption of "Shh" signaling (Roessler and Muenke, 2001)(*Arx*2 RNA 200 pg 16% $n = 119$; *Arx*2 morpholino 18 ng 28% $n = 72$). This directed us to see if *xArx*2 features in the elaboration of left-right asymmetry. We examined the developed heart and gut in Stage 46 tadpoles. We observed a low (1%–6%) frequency of reversed looping in both of these organs among *xArx*2-injected and *xArx*2-MO-injected embryos, but not in GFP-injected control embryos. Since laterality was not the focus of this project, further studies of gut and heart development were not pursued.

*xArx*2 Misexpression Alters the Expression of Genes Expressed in the Brain

To gain a better appreciation of the effects of *xArx*2 misexpression on the developing *Xenopus* brain, we examined its effects upon marker genes of the brain and eye territories. Embryos were injected unilaterally at the two-cell stage with either *xArx*2 mRNA (400 pg) or *xArx*2-morpholino oligonucleotides (MO) (18 ng). The uninjected side of the embryo was used as a contra-lateral control and represented the normal expression of each marker. Ectopic and inhibited *xArx*2 expression results in anomalous expression of various marker genes.

Forebrain. *FoxG*1/*XBf*1is a winged helix gene and marks the telencephalic territory of the embryo (Papalopulu and Kintner, 1996). As a result of ectopic *xArx*2 expression, the *FoxG*1/*XBf*1 domain of both early and late embryos (Fig. 8a,a′, respectively) slightly expanded laterally. Although not much change was observed in early embryos injected with *xArx*2-MO (Fig. 8b), a reduction was apparent in the late tailbud stage (Fig. 8b′). *Rx*1 is also expressed in the anterior prosencephalon and subsequently in the developing eye and forebrain (Casarosa *et al*., 1997; Mathers *et al*., 1997). Ectopic *xArx*2 expression resulted in a reduction in the early and late expression levels of *Rx*1 (Fig. 8c,c′), while *xArx*2-MO induced the opposite effects, as the *Rx*1 expression levels were marginally up-regulated (Fig. 8d,d′). *Pax*6 demarcates the telencephlon/diencephalon border before it later expresses during eye development (Li *et al*., 1997). *xArx*2-injected embryos showed a marked expansion of *Pax*6 expression in late neurula stage embryos (Fig. 8e,e′) and a dissipated expression in embryos injected with *xArx*2-MO-injected embryos at a similar stage (Fig. 8f,f′). Injection caused only slightly observable changes in *xPax*6 expression in tailbud stage embryos; whatever the consequence of perturbation early in development, the system is apparently robust enough to reconstitute the elements necessary to restore normal *Pax*6 expression by the stages at which eye differentiation begins in earnest.

F8

Midbrain. Analysis of *xOtx*2, a prosencephalon and mesencephalon expressing gene (Pannese *et al*., 1995), showed that ectopic *xArx*2 caused a reduction in the size of the *xOtx*2 expression domain in early embryos (Fig. 9a). Later *xArx*2 diminished *xOtx*2 expression in the eye, whereas the expression in the brain was reduced at the posterior boundary and expanded laterally (Fig. 9a′). Conversely, inhibition of *xArx*2 translation resulted in an expansion of the *xOtx*2 expression domain early (Fig. 9b) and up-regulated *xOtx*2 levels late in the eye (Fig. 9b′). *Pax*2 marks the presumptive isthmus (midbrain-hindbrain) region (Rowitch and McMahon, 1995). In both *xArx*2 and *xArx*2-MO-injected embryos, a slight reduction in *xPax*2 expression levels was observed in this domain (Fig. 9c,c′,d,d′). There was no effect on *xPax*2 expression in its other domains (Fig. 9c′).

F9

Hindbrain. *XGbx*2*a* has an anterior expression border in the region of the first rhombomere (von Bubnoff *et al*., 1996). Ectopic *xArx*2 reduced the early expres-

FIG. 8. The effect of misexpression of *xArx*2 on forebrain markers *FoxG*1/*XBF*1, *Rx*1, and *Pax*6. Following unilateral injection at the 2-cell stage with *xArx*2 mRNA (400 pg) or *xArx*2 antisense morpholino oligonucleotides (18 ng *xArx*2-MO), embryos were stained for marker gene expression at late neurula (**a–f**) or later during organogenesis (**a′–f′**). *FoxG*1/*XBF*1 following transcript or morpholino injection- a, a′ and b, b′ respectively; *Rx*1 expression following transcript or morpholino injection c, c′ d, d′ respectively; *Pax*6 expression following transcript or morpholino injection e, e′, f, f′ respectively.

FIG. 9. The effect of misexpression of *xArx*2 on midbrain markers *xOtx*2 and *xPax*2. As before, embryos were unilaterally injected on the left side (right side of picture). Marker gene perturbation is induced by *xArx*2 mRNA (400 pg) or *xArx*2 antisense morpholino oligonucleotides (18 ng *xArx*2-MO) at early (**a–d**) and later (**a′–d′**) developmental stages and are shown from an anterior perspective. *xArx*2 transcript or antisense morpholino injected embryos were stained for *xOtx*2 (a, a′, and b, b′ respectively), or for *xPax*2 (c, c′, and d, d′ respectively).

FIG. 10. The effect of misexpression of *xArx*2 on hindbrain markers *Gbx*2 and *Krox*-20. As before, embryos were unilaterally injected on the left side (right side of picture). Marker gene perturbation is induced by *xArx*2 mRNA (400 pg) or *xArx*2 antisense morpholino oligonucleotides (18 ng *xArx*2-MO) at early (**a–d**) and later (**a′–d′**) developmental stages and are shown from an anterior perspective. *xArx*2 transcript or antisense morpholino injected embryos were stained for *Gbx2* (a, a′, and b, b′ respectively), or for *Krox*-20 (c, c′, and d, d′ respectively).

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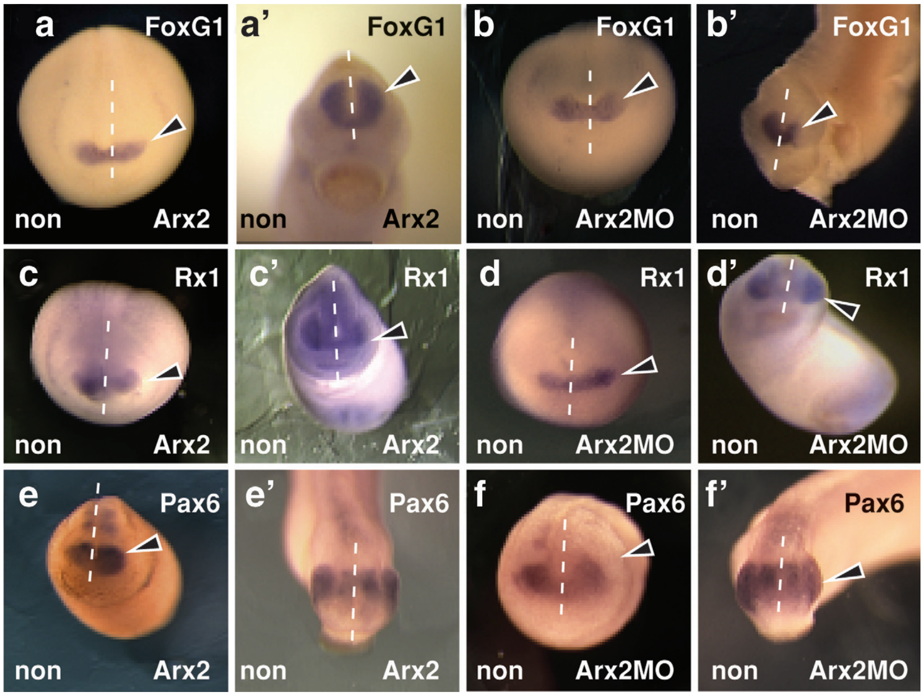

FIG. 8.

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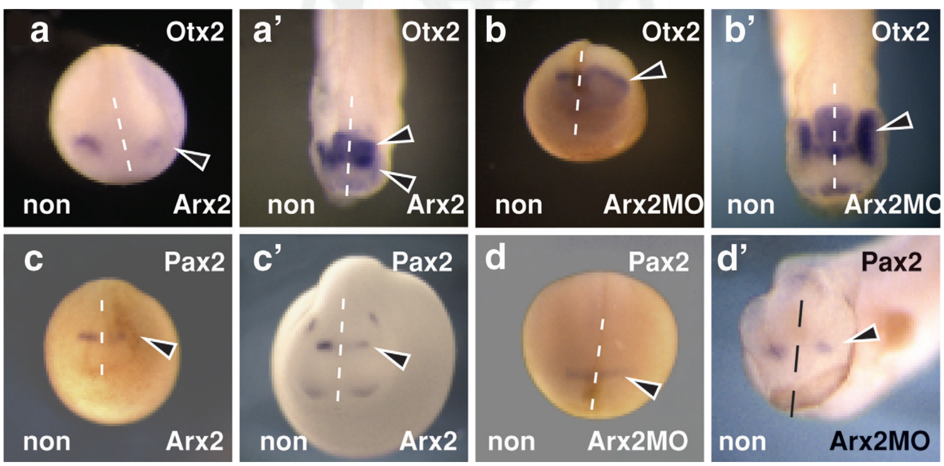

FIG. 9.

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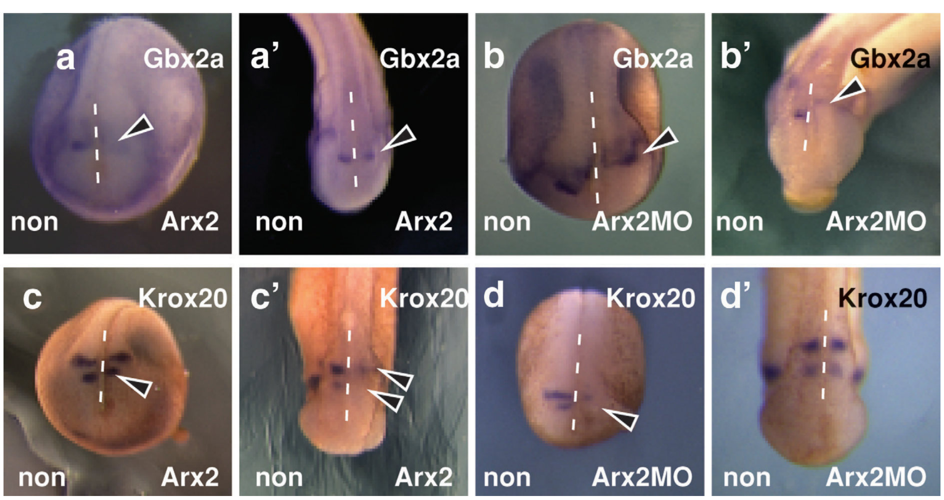

FIG. 10.

F10 sion of *xGbx2a* (Fig. 10a) and posteriorized its later expression (Fig. 10a′). This posteriorization of the *xGbx2a* expression domain was also observed in *xArx*2-MO-injected embryos (Fig. 10b,b′), and late embryos also showed a reduction in the expression level of *xGbx2a*. Analysis of the hindbrain marker *Krox*20 (Bradley *et al*., 1992), revealed that ectopic *xArx*2 expression posteriorized the expression of this hindbrain marker in early stage embryos (Fig. 10c), while in late embryos, *xKrox*20 expression was barely detectable (Fig. 10c′). This substantial reduction in expression was also observed in early embryos following translational inhibition of *xArx*2 (Fig. 10d), but appeared to have normalized by tailbud stage (Fig. 10d′).

We were also interested in the effects that misregulation of *xArx*2 had on the homologous transcript *xArx* (El-Hodiri *et al*., 2003), as it represents an alternate forebrain marker, and analysis could provide insight into the different roles of the two genes. We noticed that ectopic *xArx*2 up-regulated the level of *xArx* expression both early (Fig. 4k) and to a more subtle degree, late (not shown). Conversely, its inhibition downregulated *xArx* expression early and late (not shown).

Generally speaking, for any given marker, *xArx*2 gain-of-function has the opposite effect of loss-of-function. *xArx*2 overexpression subtly increases early expression of forebrain ventralizing factors such as *FoxG1/Xbf*1 and *Pax*6, while decreasing expression of the ventral forebrain marker *Rx*1. The Forebrain/midbrain marker *Otx*2 is repressed by *Arx*2 activity, and this in turn presumably has effects upon the definition of the midbrain/hindbrain boundary that consequently depresses expression of *Pax*2 and *Gbx*2. Subsequently, differentiation of the rhombomeres is perturbed as reflected by altered patterning of *Krox*-20.

DISCUSSION

Recently, another *Xenopus Arx* has been cloned (El-Hodiri *et al*., 2003; Seufert *et al*., 2005) which differs in several respects from the *xArx*2 characterized in this study. Sequence comparisons between the two reveal that the peptide sequences differ by 10%. It is likely that *xArx*2 and *xArx* represent genes that have diverged slightly since the ancestral *Xenopus laevis* genome underwent duplication to become pseudotetraploid. Analysis by ClustalW indicates that the two *Xenopus* loci diverge more than the human differs from the mouse gene. *xArx*2 shows a slightly higher degree of similarity to human ARX than does *xArx* (68 vs. 66%). The absolute conservation of the octapeptide, nuclear localization signal, homeo- and aristaless domains from frogs to humans suggests that they are essential to an evolutionarily shared function, and more specifically that Arx proteins bind to highly conserved regulatory sequences within their target genes (El-Hodiri *et al*., 2003; Miura *et al*., 1997; Stromme *et al*., 2002).

*xArx*2 Expression Suggests it May Perform a Role in Forebrain Development

Our findings augment those of El-Hodiri *et al*. (2003) since the anterior expression of both *xArx* transcripts were identical in many of the stages analyzed. However, we observed that *xArx*2 is also expressed visibly in the blastula, then faintly in the anterior neural ridge, and later, strongly in the somites. Unlike *xArx*, *xArx*2 does not express below the cement gland at any stage of development. Consistent with the expression patterns that we observed, it has been shown that *Arx* expresses in similar structures and at similar times throughout development in other vertebrates. In mouse, *Arx* was first expresses at E9 in the dorsal telencephalon, anterior diencephalon, and the floor plate. Unlike frog, murine *Arx* also expresses in the isthmus. Expression remains persistent in the dorsal telencephalon (presumptive cerebral cortex), ganglionic eminence and ventral thalamus. Expression in the somites was also detected (Miura *et al*., 1997). Zebrafish *Arx* was initially detected in the presumptive diencephalon, and is soon after temporarily expressed in the caudal telencephalon. By 40 h the expression of *zArx* is restricted to telencephalic and diencephalic bands, along the telencephalon/diencephalon boundary, and the hypothalamus. Zebrafish *Arx* expression in the floor plate and the somites was also observed (Miura *et al*., 1997). Human *ARX* has been reported to express in neuronal precursors in the germinal matrix of the ganglionic eminence and in the ventricular zone of the telencephalon in fetal tissue (Ohira *et al*., 2002). Moreover, since somites give rise to vertebrae and other tissues including skeletal muscle it is worth noting that *ARX* has been reported to express strongly in human skeletal muscle (Ohira *et al*., 2002). *xArx*2 may play a role during somitogenesis or muscle differentiation, however, disorders of this nature have not yet been reported to associate with mutations in *ARX*. Whether or not the presence of transcripts indicates the eventual activity functional protein remains to be elucidated.

Since *xArx*2 expresses in the anterior neural plate during neurulation, and then in derivatives of this territory, it is possible that it plays a crucial role in the establishment of the forebrain in *Xenopus*. Moreover, its early detection by both RT-PCR and in situ hybridization suggests that *xArx*2 may help to establish this territory very early in development. The absence of *xArx*2 signal during gastrulation by in situ hybridization likely reflects the differential sensitivity of this method of analysis compared to RT-PCR.

Although the perturbation of molecular markers for early brain development altered predictably with *xArx*2 gain- and loss-of-function, the phenotypes identified at later stages were quite variable and complex. *XArx*2 misexpression appears to disrupt both rostral-caudal brain as well as midline patterning. The extent to which the different phenotypes dominated could be a product of the local concentration, stability, and distribution of injected products, as well as of the variable stochastic dynamics that commonly contribute to phenotypic variability in many mammalian mutant phenotypes. In its simplest manifestation, *xArx*2 overexpression results in an expansion of the forebrain territory as well as in disruption of normal craniofacial and eye development. In contrast, impaired translation of *xArx*2 inhibits forebrain development on the side of injection, and perturbs development of the craniofacial structures and of the eyes. Often these latter phenotypes were not as severe as seen in the ectopic expression assays. We speculate that over expression of *xArx*2 yields circumstances in which both *Arx* and *xArx*2 target genes are precociously affected. So, it is clear that the genes differentiate targets at some level as *xArx*2 did not induce ectopic otic vesicles like *xArx* (El-Hodiri, personal communication). By contrast, the morpholino-induced knockdown would be predicted to affect only those genes normally targeted by *Arx*2. Given their overlapping expression domains, the two *xArx* genes likely possess some overlapping functions and perhaps endogenous *xArx* can partially compensate for the loss of *xArx*2.

The phenotypic effects appear, for the most part, to be dose dependent as frequencies increase with increasing amounts of *xArx*2 mRNA or *xArx*2-MO, however, some variability is observed. For instance, at high doses of *xArx*2 the frequency of microcephaly and "fused brain" is less than that observed at lower doses (7.5% and 1.5% at 800 pg compared to 12% and 6%, respectively, at 400 pg). This is probably a result of the high mortality rate of highly dosed tadpoles, where phenotypes compounded, and embryos with severe defects did not survive long enough to be analyzed. Craniofacial anomalies are unlikely to be a direct consequence of *xArx*2 action under normal circumstances: *xArx*2 does not express in the facies. However, induced brain territory anomalies could alter developmental programming of other cell types, such as the neural crest, which contribute to craniofacial modeling.

Brain Regionalization is Altered in Embryos Misexpressing *xArx*2

We used a panel of eight genes (*FoxG*1/*XBf*1, *xGbx2a, xKrox*20, *xArx, xOtx*2, *Xrx*1, *xPax*2, and, *xPax*6), representative of a broad range of markers of positional identity in the developing brain and eye fields to obtain a more thorough assessment of the role of *xArx*2. Ectopic *xArx*2 expression has a similar effect on two forebrain markers, *FoxG*1/*XBf*1, a winged helix gene which is expressed in the anterior neural plate, the region fated to become forebrain (Papalopulu and Kintner, 1996), and the *xArx*2 homologous gene, *xArx* (El-Hodiri *et al*., 2003). The *FoxG*1/*XBf*1 and *xArx* expression domains expanded. Conversely, inhibition of *xArx*2 translation via antisense *xArx*2-MO reduced the level of expression of both *FoxG*1/*XBf*1 and *xArx. FoxG*1/*XBf*1 is thought to play a role in preventing anterior neural plate cells from undergoing early neuronal differentiation (Bourguignon *et al*., 1998). High levels of *FoxG*1/*XBf*1 suppress neural differentiation and permit proliferation, and low concentrations result in the precocious induction of differentiation in competent ectoderm (Bourguignon *et al*., 1998). Mouse embryos lacking *FoxG*1/*XBf*1 die at birth with hypoplasia of the cerebral hemispheres due to premature neuronal differentiation in the forebrain (Xuan *et al*., 1995). Moreover, *FoxG*1/*XBf*1 null mutant mice exhibit profound deficits in ventral forebrain patterning, and appear to lose both the *fgf*8 expression that is critical to proliferation, and the expression of sonic hedgehog that is so important for midline patterning (Martynoga *et al*., 2005). Expansion of the *FoxG*1/*XBf*1 domain in *xArx*2-injected embryos may cause expansion of cell populations that are competent to undergo neurogenesis in the anterior neural plate, while simultaneously impeding ventral differentiation and midline patterning. This interpretation is lent some credence by the depression of ventral telencephalon marker *Rx*1, and the concomitant increase in dorsal neural marker *Pax*6 (Li *et al*., 1997). It also resonates well with the fused brain and eye phenotypes that arise in *Arx*2-perturbed embryos: *Rx*1 is required for normal eye development (Mathers *et al*., 1997). Moreover, since *Rx*1 has been shown to regulate anterior neurogenesis by maintaining neuronal precursors in a proliferative state (Andreazzoli *et al*., 1999, 2003), expansion or retraction of the forebrain by *xArx*2 overexpression or MO-mediated knockdown respectively, can also be explained by the effects of *xArx*2 upon *Rx*1 expression. Finally, *FoxG*1/*Xbf*1 and *Rx*1 are inversely and reciprocally regulated, and downregulation of *Rx*1 is also known to result in a commensurate increase in *Pax*6 expression (Chuang and Raymond, 2001)—relationships that are internally consistent with the results.

The effect that misexpression of *xArx*2 had upon *xArx* may indicate that *xArx*2 normally impinges upon *xArx* to activate transcription or that the *xArx* genes auto-regulate and *xArx*2 is mimicking an x*Arx* effect ectopically. Since specific translation knockdown of *xArx*2 exerts effects upon brain development we conclude that the two *Arx* genes are not completely redundant and that depletion of one either prohibits activation of specific targets, or results in a gene dosage effect. Differences in the ability of the two Arx proteins to induce supernumerary otic vesicles tend to support the former proposition.

Ectopic *xArx*2 expression reduces the expression levels of genes that play a role in midbrain and eye development. *xOtx*2 is a homeobox gene involved in patterning the body axis and head (Pannese *et al*., 1995). Late in development it is restricted to the fore- and midbrain, as well as the eye. Mice deficient in *Otx*2 lack eyes and *Otx*2-/- mice lack forebrain, midbrain, and rostral hindbrain (Acampora, 1995; Matsuo *et al*., 1995). It has been recently suggested that *Otx*2 potentiates the functional interaction among eye field transcription factors (Zuber *et al*., 2003). *Otx*2 expression is decreased by fgf8 (Joyner *et al*., 2000), and since *xArx*2 increased ventral telencephalon markers such as *FoxG*1/*Xbf*1, we entertain the possibility that fgf8-mediated proliferative expansion in the telencephalon has consequences upon midbrain patterning via depression of *Otx*2. Both early and late expressions of *xOtx*2 and *xPax*2 were decreased in *xArx*2-injected embryos. Conversely, expression levels of *xOtx*2 were increased in *xArx*2-MO-injected embryos, while the level of *xPax*2 was again reduced. A mutually restrictive relationship between *XGbx*2 and *Otx*2 positions the midbrain-hindbrain boundary, thereby also establishing the *Pax*2 expression domain (Rowitch and McMahon, 1995; Tour *et al*., 2002a,b).

Ectopic *xArx*2 reduced or posteriorized expression of posterior markers such as *xGbx2a* (von Bubnoff *et al*., 1996), and *xKrox*20 (Seitanidou *et al*., 1997), which mark the rhombomeres 1, 3, and 5 respectively. The expression domains of these two genes do not overlap with either *Arx* gene, so direct regulation is unlikely. Since such embryos display an expanded forebrain later in development, more posterior regions of the brain may be pushed back as a result of overproliferation of cells in more anterior regions. Alternatively, cells determined to a forebrain fate could be increased at the expense of those in more caudal territories. However, morpholino-mediated knockdown of *xArx*2 also resulted in posteriorized and reduced *xGbx2a* expression and decreased levels of early *xKrox*20 expression. Possibly, proper specification of the anterior region of the brain may be required to maintain positional identities of more posterior domains. Since temporal and spatial attributes of brain specification are linked but poorly understood, it remains unclear whether the observed pattern of mid/hindbrain differentiation is a consequence of orthographic posteriorization or temporally delayed inhibition. Alternatively, the *Xenopus Arx* genes may express in the isthmus like their murine relative, and play a role in patterning there, but if so they would have to express at levels below the sensitivity of in situ hybridization to detect.

Arx Function May be Conserved Among Vertebrates

Mutations in human *ARX* generate a wide range of phenotypes including X-linked infantile spasms, Partington syndrome, characterized by mental retardation, ataxia, and dystonia, and various forms of mental retardation (Kitamura *et al*., 2002; Stromme *et al*., 2002). Because of these effects, it is thought to regulate genes involved in cellular processes and functions required for cognitive development and to play a role in neuronal migration (Bienvenu *et al*., 2002; Ohira *et al*., 2002). The first functional studies on *Arx* were conducted using mouse knockouts, which resulted in developmental abnormalities of the brain and testis similar to with human XLAG (Kitamura *et al*., 2002). These researchers suggested that proliferation was affected, and that neuronal migration is regulated by *Arx*. We speculate that *Arx* may is playing a similar role in *Xenopus* and that it plays a crucial role in forebrain patterning. Whether it does so by regulating mechanisms pertaining to cell differentiation, neuronal migration, or cellular proliferation remains to be elucidated. We conducted experiments in unilaterally injected embryos that were designed to detect differences in apoptosis or in proliferation: these differences, whatever they may be, were too slight or spread over too long a developmental period to be discernable in the "snapshot" afforded by fixed tissues. We speculate that the repositioning of territorial boundaries is more likely to cause the anomalous differentiation recorded. Since this gene has been found to play a significant role in human cognitive function, determining its precise function in forebrain specification is of great importance. Moreover, it is tempting to speculate that the development of a neocortex was enhanced by an evolutionary expansion of *Arx* expression to the dorsal telencephalon where it would have promoted growth of the telencephalon. What is clear, however, is that anterior neural ridge plays a potent role in organizing the brain, and the *xArx*2 activity is necessary for this function.

Finally, activity of *Arx*2 during somitogenesis does not appear to be critical for the segmentation of somitic mesoderm, and in *Arx*2 misexpressing embryos, somite derivatives apparently elaborate in a normal fashion. Murine *Arx* also expresses in somites during development (Colombo *et al*., 2004). The midline phenotypes and the rare laterality defects obtained in frog may reflect a role for *Arx*2 in sustaining cues necessary both to dorsal midline integrity as well as to the provision of a barrier to laterality cues. If so, this role is unique to frogs as neither human nor murine mutants appear to express similar deficits. Possibly, somitic expression of *Arx*2 exerts an indirect effect upon differentiation reproductive organs to produce the abnormal genitalia in mammals. We did not foster disrupted embryos long enough to assess urogenital differentiation in *Xenopus*.

METHODS

Cloning and Sequence Analysis

*Arx*2 was isolated from a *Xenopus* head and heart cDNA library (Stage 28–35) that was constructed using a commercially prepared vector (Stratagene). The clone was bidirectionally sequenced, and ClustalW was used to generate an alignment of the conceptual *Xenopus* Arx2 protein with known homologues from other organisms (human, mouse, and zebrafish) and with the previously published *Xenopus xArx* sequence (El-Hodiri *et al*., 2003).

Embryo Preparation

Xenopus eggs were obtained, fertilized, dejellied, and cultured as previously described (Drysdale and Elinson, 1991). Developmental staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For injections, two-cell stage embryos were transferred to 1.5% Ficoll-400 (Sigma) in 0.3× MBS for injections.

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RT-PCR

Embryos were reared in 0.1× MBS at 12°C, 17°C, or room temperature until all of the desired developmental stages had been achieved. Poly (A)$^+$ RNA purifications from 10 pooled embryos of each developmental stage were performed in parallel using oligo dT-polystyrene beads (Sigma DMN-10). mRNA equivalent to one embryo was utilized for first strand cDNA synthesis in the presence of RNasin (Promega) using reverse transcriptase according to the manufacturer's instructions (Omniscript, Qiagen). One fifth of the reactions were employed as templates for amplifications. Primers were designed to specifically amplify *xArx*2 rather than the previously published sequence (El-Hodiri *et al*., 2003). PCR conditions were determined empirically to establish the linear range of amplification for *xArx*2 and reactions were conducted using a thermo-stable polymerase in 10 mM Tris (pH 9.0), 50 mM KCL, 0.1% Triton X-100, 3 mM MgCl2, 0.2 mM dNTPs, 0.1 mM [3232P]dCTP, and 1 μM of each primer (*xArx*2-5′-CCGCACTGGACTCTGCT-3′ and 5′-ACACTTCTTTGCCGGTGC-3′; *Ef*1-α - 5′-CAGATTGGTGCTGGATATG-3′ and 5′-ACTGCCTTGATGACTCCTA-3′). All amplifications were preceded by a 4-min denaturation step at 94°C, then immediately cycled 29 times at 94°C for 45 s, 57°C for 1 min, and 72°C for 45 s. One tenth of each reaction was electrophoresed on 4% polyacrylamide in 0.5× TBE and visualized by autoradiography.

Microinjection

An *xArx*2 expression construct was derived using Vent polymerase (New England Biolabs) and primers (forward 5′-GAAGGCCTGCAGCCCAGCATTGA-3′; reverse 5′-GCTCTAGACTGCATAAAAGTTACACTC-3′) which bracketed the open reading frame and possessed restriction sites for *StuI* and *XbaI*, respectively, to facilitate insertion into pCS2. Synthetic capped mRNA of *xArx*2 and Green Fluorescent Protein (GFP) was made from linearized template using mMessage Machine (Ambion). Capped mRNAs were resuspended in nuclease free water and coinjected into the animal pole of embryos at the 2-cell stage with a Drummond nanoinjector. Concentrations of the *xArx*2 capped mRNA ranged from 100 pg to 800 pg. 400 pg of GFP capped mRNA was used for coinjections and 800 pg for control injections. Injection volumes never exceeded 4.6 nl. Injected embryos were cultured in 2% Ficoll-400 in 0.3× MBS at 12°C overnight. The solution was subsequently changed to 0.1× MBS and embryos were reared at 17°C until they reached early tailbud stage. Embryos were separated on the basis of side of injection, which was determined by GFP fluorescence under UV light, and to the required stages. The uninjected side served as a contra-lateral control.

Loss-of-function assays were conducted similarly using fluoresceinated *xArx*2 antisense morpholino oligonucleotides (*xArx*2-MO) (Gene Tools) (5′-TGCTGGGCTG CAGGACTGTGTCGGT-3′). Concentrations of 6, 9, and 18 ng were injected into one blastomere at the two-cell stage. Fluoresceinated control morpholino oligonucleotides which represented a random sequence were injected at a concentration of 20 ng (5′-CCTCCTACCT CATTACAATTTATA-3′).

In Situ Hybridization

To examine the putative effects of *xArx*2 misexpression on various brain and eye marker genes, embryos, injected with either synthetic capped *xArx*2 (600 pg) and GFP (400 pg) mRNA or with *xArx*2 morpholino oligonucleotides (18 ng), were subjected to whole mount in situ hybridization, performed according to Harland (Harland, 1991) using digoxygenin labeled probe (Roche). The side of injection was predetermined prior to fixation on the basis of fluorescence of the injected side under UV light, and the uninjected side was assessed as a contra-lateral control. All of the constructs used, with the exception of *xGbx2a*, were obtained as gifts: *xBf*1 (N. Papalopulu), *xKrox*20 (D. Wilkinson), *xArx* (H. El-Hodiri), *xOtx*2 (I. Blitz), *xRx*1 (G. Barsacchi), *xPax*2 (N. Heller), xDlx5 (el-Hodiri), and *xPax*6 (W. Harris). *XGbx2a* was amplified from a whole embryo cDNA library using primers (forward 5′-CGGAATTCAGGCTTCATTTGACTCTCAG-3′ and reverse 5′-AAGGCCTGAACATTTCAAGGTCTTGC-3′) that contained *StuI* and *XbaI* restriction sites, respectively, to facilitate directional insertion into pCS2. AQ2

Histology

Stage 46 tadpoles that had been injected with 400 pg of *xArx*2 mRNA or 18 ng of *xArx*-MO, and which showed slight forebrain defects, were fixed in MEMPFA and then stained with Hoechst or hematoxylin and eosin (H&E). For Hoechst staining, tadpoles were subsequently gradually dehydrated to 100% methanol, removed to 5 ug/ml Hoechst 33258 for 1 h, gradually rehydrated to water, and then embedded in 5% agarose. They were then sectioned vertically, 30-um thick on a vibratome (Leica VT 1000S) and visualized under filtered UV light.

For H&E staining, fixed tadpoles were embedded in paraplast, and 20 um horizontal sections were cut using a microtome (Spencer 820).

In Vitro Protein Synthesis

*xArx*2-MO specificity was assessed by means of an in vitro translation approach previously described (Winklbauer *et al*., 2001). A construct containing a *xArx*2 morpholino-equivalent site was created in the pCS2-Myc vector. The oligonucleotides (5′-GATCCACCGAGACAGTCC TGCAGCCCAGCA-3′ and 5′-CGTGCTGGGCTGCAGGA CTGTGTCGGAG-3′), which contained restriction sites for *BamHI* and *ClaI*, respectively, and which complemented the *xArx*2 morpholino oligonucleotide used in our loss-of-function studies, were used to create the site. A second construct, containing a *xArx* morpholino-equivalent site was created in a similar manner using the oligonucleotides (5′-GATCTTGAGACAGTCCGGAGCTCA

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GCATTG-3′ and 5′-CGCAATGCTGAGCTCCGGACTGTCT-CAA-3′). Subsequently, *xArx2* was directionally cloned into these constructs using primers (forward 5′-GAA GGCCTATGAGCGGCCACTACCAA-3′ and reverse 5′-GCTCTAGACTGCATAAAAGTTACACTC-3′), which contained restriction sites for *StuI* and *XbaI*, respectively. *xArx*-containing constructs included *pCS2-myc-Arx*, *pCS2-myc-Arx* with the *xArx*-MO-equivalent site, and *pCS2-myc-Arx* with the *xArx2*-MO-equivalent site. In vitro protein translations, using ^{35}S, were performed according to the manufacturer's protocol (Retic Lysate, Ambion), in the presence and absence of 18 ng of *xArx2* MO using 1 ug of each mRNA template. Additionally, 20 ng of control morpholino was added to one of the reactions, and 1 μg of GFP mRNA was utilized as an internal control in each reaction in order to equate levels of protein syntheses.

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LITERATURE CITED

AQ3 Abbott A. 2003. Neuroscience: genomics on the brain. Nature 426:757.

Acampora D, Mazan S, Lallemand Y, Avantaggiato V, Maury M, Simeone A, Brûlet P. 1995. Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. Development 121:3279–3290.

Andreazzoli M, Gestri G, Angeloni D, Menna E, Barsacchi G. 1999. Role of Xrx1 in Xenopus eye and anterior brain development. Development 126:2451–2460.

Andreazzoli M, Gestri G, Cremisi F, Casarosa S, Dawid IB, Barsacchi G. 2003. Xrx1 controls proliferation and neurogenesis in Xenopus anterior neural plate. Development 130:5143–5154.

Beverdam AMeijlink F. 2001. Expression patterns of group-I aristaless-related genes during craniofacial and limb development. Mech Dev 107:163–167.

Bienvenu T, Poirier K, Friocourt G, Bahi N, Beaumont D, Fauchereau F, Ben Jeema L, Zemni R, Vinet MC, Francis F, Couvert P, Gomot M, Moraine C, van Bokhoven H, Kalscheuer V, Frints S, Gecz J, Ohzaki K, Chaabouni H, Fryns JP, Desportes V, Beldjord C, Chelly J. 2002. ARX, a novel Prd-class-homeobox gene highly expressed in the telencephalon, is mutated in X-linked mental retardation. Hum Mol Genet 11:981–991.

Bourguignon C, Li J, Papalopulu N. 1998. XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in Xenopus competent ectoderm. Development 125:4889–4900.

Bradley LC, Snape A, Bhatt S, Wilkinson DG. 1992. The structure and expression of the *Xenopus Krox*-20 gene: Conserved and divergent patterns of expression in rhombomeres and neural crest. Mech Dev 40:73–84.

Casarosa S, Andreazzoli M, Simeone A, Barsacchi G. 1997. Xrx1, a novel Xenopus homeobox gene expressed during eye and pineal gland development. Mech Dev 61:187–198.

Chuang JC, Raymond PA. 2001. Zebrafish genes rx1 and rx2 help define the region of forebrain that gives rise to retina. Dev Biol 231:13–30.

Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P. 2003. Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes Dev 17:2591–2603.

Colombo E, Collombat P, Colasante G, Bianchi M, Long J, Mansouri A, Rubenstein JL, Broccoli V. 2007. Inactivation of Arx, the murine ortholog of the X-linked lissencephaly with ambiguous genitalia gene, leads to severe disorganization of the ventral telencephalon with impaired neuronal migration and differentiation. J Neurosci 27:4786–4798.

Colombo E, Galli R, Cossu G, Gecz J, Broccoli V. 2004. Mouse orthologue of ARX, a gene mutated in several X-linked forms of mental retardation and epilepsy, is a marker of adult neural stem cells and forebrain GABAergic neurons. Dev Dyn 231:631–639.

Drysdale TA, Elinson RP. 1991. Development of the *Xenopus laevis* hatching gland and its relationship to surface ectoderm patterning. Development 111:469–478.

Echevarria D, Vieira C, Gimeno L, Martinez S. 2003. Neuroepithelial secondary organizers and cell fate specification in the developing brain. Brain Res Brain Res Rev 43:179–191.

El-Hodiri HM, Qi XL, Seufert DW. 2003. The Xenopus arx gene is expressed in the developing rostral forebrain. Dev Genes Evol 212:608–612.

Houart C, Westerfield M, Wilson SW. 1998. A small population of anterior cells patterns the forebrain during zebrafish gastrulation. Nature 391:788–792.

Joyner AL, Liu A, Millet S. 2000. Otx2. Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. Curr Opin Cell Biol 12:736–741.

Kitamura K, Yanazawa M, Sugiyama N, Miura H, Iizuka-Kogo A, Kusaka M, Omichi K, Suzuki R, Kato-Fukui Y, Kamiirisa K, Matsuo M, Kamijo S, Kasahara M, Yoshioka H, Ogata T, Fukuda T, Kondo I, Kato M, Dobyns WB, Yokoyama M, Morohashi K. 2002. Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. Nat Genet 32:359–369.

Larsen CW, Zeltser LM, Lumsden A. 2001. Boundary formation and compartition in the avian diencephalon. J Neurosci 21:4699–4711.

Li H, Tierney C, Wen L, Wu JY, Rao Y. 1997. A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. Development 124:603–615.

Martinez S. 2001. The isthmic organizer and brain regionalization. Int J Dev Biol 45:367–371.

Martynoga B, Morrison H, Price DJ, Mason JO. 2005. Foxg1 is required for specification of ventral telencephalon and region-specific regulation of dorsal telencephalic precursor proliferation and apoptosis. Dev Biol 283:113–127.

Mathers PH, Grinberg A, Mahon KA, Jamrich M. 1997. The Rx homeobox gene is essential for vertebrate eye development. Nature 387:603–607.

Matsuo I, Kuratani S, Kimura C, Takeda N, Aizawa S. 1995. Mouse Otx2 functions in the formation and patterning of rostral head. Genes Dev 9:2646–2658.

Miura H, Yanazawa M, Kato K, Kitamura K. 1997. Expression of a novel aristaless related homeobox gene '*Arx*' in the vertebrate telencephalon, diencephalon and floor plate. Mech Dev 65:99–109.

Nieuwkoop PD, Faber J. 1967. Normal table of *Xenopus laevis* (Daudin). Amsterdam: North Holland Press.

Ohira R, Zhang YH, Guo W, Dipple K, Shih SL, Doerr J, Huang BL, Fu LJ, Abu-Khalil A, Geschwind D, McCabe ER. 2002. Human ARX gene: genomic characterization and expression. Mol Genet Metab 77:179–188.

Pannese M, Polo C, Andreazzoli M, Vignali R, Kablar B, Barsacchi G, Boncinelli E. 1995. The *Xenopus* homologue of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. Development 121:707–720.

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

Roessler E, Muenke M. 2001. Midline and laterality defects: Left and right meet in the middle. Bioessays 23:888–900.

Rowitch DH, McMahon AP. 1995. Pax-2 expression in the murine neural plate precedes and encompasses the expression domains of Wnt-1 and En-1. Mech Dev 52:3–8.

Seitanidou T, Schneider-Maunoury S, Desmarquet C, Wilkinson DG, Charnay P. 1997. Krox-20 is a key regulator of rhombomere-specific gene expression in the developing hindbrain. Mech Dev 65:31–42.

Seufert DW, Prescott NL, El-Hodiri HM. 2005. Xenopus aristaless-related homeobox (*xARX*) gene product functions as both a transcriptional activator and repressor in forebrain development. Dev Dyn 232:313–324.

Sherr EH. 2003. The ARX story (epilepsy, mental retardation, autism, and cerebral malformations): One gene leads to many phenotypes. Curr Opin Pediatr 15:567–571.

Shimamura K, Rubenstein JL. 1997. Inductive interactions direct early regionalization of the mouse forebrain. Development 124:2709–2718.

Stromme P, Mangelsdorf ME, Shaw MA, Lower KM, Lewis SM, Bruyere H, Lutcherath V, Gedeon AK, Wallace RH, Scheffer IE, Turner G, Partington M, Frints SG, Fryns JP, Sutherland GR, Mulley JC, Gecz J. 2002. Mutations in the human ortholog of Aristaless cause X-linked mental retardation and epilepsy. Nat Genet 30:441–445.

Tour E, Pillemer G, Gruenbaum Y, Fainsod A. 2002a. *Gbx2* interacts with *Otx2* and patterns the anterior-posterior axis during gastrulation in Xenopus. Mech Dev 112:141–151.

Tour E, Pillemer G, Gruenbaum Y, Fainsod A. 2002b. Otx2 can activate the isthmic organizer genetic network in the Xenopus embryo. Mech Dev 110:3–13.

Uyanik G, Aigner L, Martin P, Gross C, Neumann D, Marschner-Schafer H, Hehr U, Winkler J. 2003. ARX mutations in X-linked lissencephaly with abnormal genitalia. Neurology 61:232–235.

von Bubnoff A, Schmidt JE, Kimelman D. 1996. The Xenopus laevis homeobox gene *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm. Mech Dev 54:149–160.

Winklbauer R, Medina A, Swain RK, Steinbeisser H. 2001. Frizzled-7 signalling controls tissue separation during Xenopus gastrulation. Nature 413:856–860.

Xuan S, Baptista CA, Balas G, Tao W, Soares VC, Lai E. 1995. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron 14:1141–1152.

Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development 130:5155–167.

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