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xArx2: An Aristaless Homolog That Regulates Brain Regionalization During Development in Xenopus laevis

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**Introduciton**

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 (Martinez, 2001); and the total structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001); the zona limitans intrathalamica, a pivotal structure separating the dorsal and ventral thalami (Martinez, 2001).}

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**article**

xArx2: An Aristaless Homolog That Regulates Brain Regionalization During Development in Xenopus laevis

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**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, FoxG1/Bf1 encodes a winged-helix transcription factor that is required for regionalization and growth of the telencephalic and optic vesicles. Mice mutant for FoxG1/Bf1 have a small telencephalon and lack expression of a basal telencephalic marker, Dlx2 (Xuan et al., 1995). Excision of the anterior neural ridge has been shown to eliminate expression of FoxG1/Bf1 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 Nkx2.1 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 (Bevendam 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 dystonia (Sherr, 2003). Other anomalies in this X-linked disorder include the development of brain cysts and ambiguous genital development.

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Impaired development results from various types of mutation including point mutation, missense, 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 abso ganglia (Colombo et al., 2007).

A related gene, xArx (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, xArx2 (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 xArx2 to development of the Xenopus forebrain. By either assay, Arx2 appears to modulate forebrain development, and indirectly, to affect the differentiation of more caudal neural structures.

RESULTS

xArx2 is a Conserved Member of the Vertebrate Arx Family

xArx2 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 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). xArx2 (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, xArx2 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 xArx2 by RT-PCR Analysis

The temporal expression profile of xArx2 during early Xenopus development was analyzed by RT-PCR (see Fig. 2). xArx2 is detectable as a maternal transcript and is present at moderate levels up until the end of gastrulation. Just following the onset of neurulation, the expression level at Stage 14 markedly increases. xArx2 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 35S-labeled products using constructs that contained the open reading frame of xArx2 fused to morpholino target or positionally equivalent sites (see Fig. 3). Translation was inhibited in a construct that contained the xArx2-MO equivalent site when Arx2-MO was introduced. Moreover, translational levels were unaffected by xArx-MO with construct that contained the Arx2-MO site. The standard control morpholino had no effect on xArx2 translation and neither morpholino impaired translational levels of the control GFP protein.

Spatio-Temporal Expression Analysis of xArx2 by Whole Mount Riboprobe In Situ Hybridization

The spatiotemporal expression of xArx2 was analyzed by whole-mount in situ hybridization (see Fig. 4). The 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) xArx2 is detected in the prosencephalon, or presumptive forebrain area, and in the somites. At the late tailbud stage (Fig. 4f) xArx2 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, xArx2 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 Arx2 probes (Fig. 4k).

Finally, embryos that were unilaterally injected with Arx2 morpholino at doses sufficient to generate a pro-
found phenotype had the effect of inhibiting Arx 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 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 Arx enjoys unique expression in the ridge that demarcates the anterior neural plate, and briefly, in the somites.

xArx expression can be more precisely localized to the posterior telencephalon and anterior diencephalon by comparison to other markers. For example, in early neurulae, xArx expresses just caudal to telencephalon marker FoxG1/Xbf1 (Fig. 5a), and slightly overlapping and caudal to Rx (Fig. 5b). xArx is rostral and slightly overlapping with diencephalon/mesencephalon marker

FIG. 1. xArx 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 shares 100% identity with zebrafish, mouse, human and xArx 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 expression over the course of early Xenopus development. Blastula stages include Stage 8, gastrulation commences Stage 10, and neurulation, late stage 12.
FIG. 3. Radio-labeled Western blot of in vitro translated reactions shows that xArx2 antisense morpholino specifically blocks translation from xArx2 template. Transcripts that lacked the xArx2 5’-untranslated 25mer sequence were unaffected by treatment with either xArx2 antisense morpholino (xArx2-MO), or control morpholino (CMO).

FIG. 4. Expression of xArx2 during Xenopus development. At blastula stage (a), xArx2 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 (d) and telencephalon (tel) (g). In cleared embryos, expression is detected in the anterior neural tube (h). Cross sections of Xenopus embryos demonstrate expression of xArx2 in the ventral and lateral telencephalon (i), lateral diencephalon (j). In situ hybridization using Arx and Arx2-specific probes show distinct expression patterns (k). Unilaterally injected Arx2 morpholino (arrows) perturbs expression of Arx2 (l).

FIG. 5. Double stained in situ hybridizations show that Arx2 is expressed caudal to telencephalon markers FoxG1/Xbf1 (a) and Rx (b), but rostral to diencephalon/mesencephalon marker Otx1 (c) and isthmus marker engrailed (d).

FIG. 6. Misexpression of xArx2 causes anterior developmental anomalies. Dorsal views of tadpoles injected with xArx2 (a-c) or antisense morpholino (d). Anomalies following ectopic expression included: hypomorphic craniofacial modeling (a); mispositioned forebrain (arrow in b); microphthalmia 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. Arx2 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 xArx2 expands the forebrain growth relative to contralateral tissues in Hoechst-stained sections (c), and inhibition by antisense morpholino inhibits growth (d).

Otx1 (Fig. 5c), and is completely rostral to isthmus (midbrain/hindbrain junction) marker engrailed (Fig. 5d).

Misexpression of xArx2 Results in Anterior Defects

Ectopic xArx2 expression results in distinct and reproducible phenotypes in the anterior region of the embryo. The majority of morphological effects of xArx2 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 xArx2 results in several distinct 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 xArx2 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 Arx2 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 Arx2 misexpression appear very similar in character to those produced in Arx2-disrupted embryos (Seufert et al., 2005). In contrast to xArx, xArx2 does not induce the formation of ectoptotic vesicles as assessed by morphology or by expression of a specific marker, xDlx5 (data not shown).

Inhibition of xArx2 affects the developing forebrain. We analyzed the effect of loss-of-function of xArx2 by means of antisense morpholino oligonucleotide (MO)-mediated translational knockdown. This common 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 xArx2,
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 xArx2 injected side of the embryo is observed (Fig. 7a,c). Conversely, the forebrain on the injected side of the Arx2-MO-injected embryos appears substantially reduced (Fig. 7b,d).

**Interfering with Proper xArx2 Function Results in Midline Defects**

Misexpressing xArx2 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)(Arx2 RNA 200 pg 16% n = 119; Arx2 morpholino 18 ng 28% n = 72). This directed us to see if xArx2 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 fused brain lobes that are reminiscent of those observed in midline defects in humans with a disruption of "Shh" signaling (Roessler and Muenke, 2001). Misexpressing xArx2 caused only slightly observable changes in xPax6 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 Pax6 expression by the stages at which eye differentiation begins in earnest.

**Midbrain.** Analysis of xOtx2, a prosencephalon and mesencephalon expressing gene (Pannese et al., 1995), showed that ectopic xArx2 caused a reduction in the size of the xOtx2 expression domain in early embryos (Fig. 9a). Later xArx2 diminished xOtx2 expression in the eye, whereas the expression in the brain was reduced at the posterior boundary and expanded laterally (Fig. 9a'). Conversely, inhibition of xArx2 translation resulted in an expansion of the xOtx2 expression domain early (Fig. 9b) and up-regulated xOtx2 levels later in the eye (Fig. 9b'). Pax6 marks the presumptive isthmus (midbrain-hindbrain) region (Rowitch and McMahon, 1995). In both xArx2 and xArx2-MO-injected embryos, a slight reduction in xPax2 expression levels was observed in this domain (Fig. 9c,c',d,d'). There was no effect on xPax2 expression in its other domains (Fig. 9c').

**Hindbrain.** XGbx2a has an anterior expression border in the region of the first rhombomere (von Bubnoff et al., 1996). Ectopic xArx2 reduced the early expres-

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**FIG. 8.** The effect of misexpression of xArx2 on forebrain markers FoxG1/XBF1, Rx1, and Pax6. Following unilateral injection at the 2-cell stage with xArx2 mRNA (400 pg) or xArx2 antisense morpholino oligonucleotides (18 ng xArx2-MO), embryos were stained for marker gene expression at late neurula (a-f) or later during organogenesis (a'-f'). FoxG1/XBF1 following transcript or morpholino injection; a, a' and b, b' respectively; Rx1 expression following transcript or morpholino injection c, c', d, d' respectively; Pax6 expression following transcript or morpholino injection e, e', f, f respectively.

**FIG. 9.** The effect of misexpression of xArx2 on midbrain markers xOtx2 and xPax2. As before, embryos were unilaterally injected on the left side (right side of picture). Marker gene perturbation is induced by xArx2 mRNA (400 pg) or xArx2 antisense morpholino oligonucleotides (18 ng xArx2-MO) at early (a-d) and later (a'-d') developmental stages and are shown from an anterior perspective. xArx2 transcript or antisense morpholino injected embryos were stained for xOtx2 (a, a', and b, b' respectively), or for xPax2 (c, c', and d, d' respectively).

**FIG. 10.** The effect of misexpression of xArx2 on hindbrain markers Gbx2 and Krox-20. As before, embryos were unilaterally injected on the left side (right side of picture). Marker gene perturbation is induced by xArx2 mRNA (400 pg) or xArx2 antisense morpholino oligonucleotides (18 ng xArx2-MO) at early (a-d) and later (a'-d') developmental stages and are shown from an anterior perspective. xArx2 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).
Discussion

Recently, another Xenopus Arx has been cloned (El-Hodiri et al., 2003; Seufert et al., 2005) which differs in several respects from the xArx2 characterized in this study. Sequence comparisons between the two reveal that the peptide sequences differ by 10%. It is likely that xArx2 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. xArx2 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 aristless 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).

xArx2 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 xArx2 is also expressed visibly in the blastula, then faintly in the anterior neural ridge, and later, strongly in the somites. Unlike xArx, xArx2 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 expressed 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 xArx 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 vertebral 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). xArx2 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 xArx2 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 xArx2 may help to establish this territory very early in development. The absence of xArx2 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 xArx2 gain- and loss-of-function, the phenotypes identified at later stages were quite variable and complex. xArx2 mis-expression 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, xArx2 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 xArx2 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 xArx2 yields circumstances in which both Arx and xArx2 target genes are precociously affected. So, it is clear that the genes differentiate targets at some level as xArx2 did not induce ectopic otic vesicles like Arx (El-Hodiri, personal communication). By contrast, the morpholino-induced knockdown would be predicted to affect only those genes normally targeted by Arx. 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 xArx2.

The phenotypic effects appear, for the most part, to be dose dependent as frequencies increase with increasing amounts of xArx2 mRNA or xArx2-MO, however, some variability is observed. For instance, at high doses of xArx2 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 did not survive long enough to be analyzed. Craniofacial abnormalities are unlikely to be a direct consequence of xArx2 action under normal circumstances: xArx2 does not express in the facies. However, induced brain territory abnormalities 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 xArx2**

We used a panel of eight genes (FoxG1/XBf1, xGbx2a, xKrox20, xArx, xOtx2, Xr1, xPax2, and, xPax6), 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 xArx2. Ectopic xArx2 expression has a similar effect on two forebrain markers, FoxG1/XBf1, a winged helix gene which is expressed in the anterior neural plate, the region fated to become forebrain (Papalopulu and Kintner, 1996), and the xArx2 homologous gene, xArx (El-Hodiri et al., 2003). The FoxG1/XBf1 and xArx expression domains expanded. Conversely, inhibition of xArx2 translation via antisense xArx2-MO reduced the level of expression of both FoxG1/XBf1 and xArx. FoxG1/XBf1 is thought to play a role in preventing anterior neural plate cells from undergoing early neuronal differentiation (Bourguignon et al., 1998). High levels of FoxG1/XBf1 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 FoxG1/XBf1 die at birth with hypoplasia of the cerebral hemispheres due to premature neuronal differentiation in the forebrain (Xuan et al., 1995). Moreover, FoxG1/XBf1 null mutant mice exhibit profound deficits in ventral forebrain patterning, and appear to lose both the fgf8 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 FoxG1/XBf1 domain in xArx2-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 Rx1, and the concomitant increase in dorsal neural marker Pax6 (Li et al., 1997). It also resonates well with the fused brain and eye phenotypes that arise in Arx2-perturbed embryos: Rx1 is required for normal eye development (Mathers et al., 1997). Moreover, since Rx1 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 xArx2 overexpression or MO-mediated knockdown respectively, can also be explained by the effects of xArx2 upon Rx1 expression. Finally, FoxG1/XBf1 and Rx1 are inversely and reciprocally regulated, and down-regulation of Rx1 is also known to result in a commensurate increase in Pax6 expression (Chuang and Raymond, 2001)—relationships that are internally consistent with the results.

The effect that misexpression of xArx2 had upon Arx may indicate that xArx2 normally impinges upon Arx to activate transcription or that the xArx genes auto-regulate and xArx2 is mimicking an Arx effect ectopically. Since specific translation knockdown of xArx2 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 xArx2 expression reduces the expression levels of genes that play a role in midbrain and eye development. xOtx2 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 Otx2 lack eyes and Otx2/- mice lack forebrain, midbrain, and rostral hindbrain (Acampora, 1995; Matsuo et al., 1995). It has been recently suggested that Otx2 potentiates the functional interaction among eye field transcription factors (Zuber et al., 2003). Otx2 expression is decreased by fgf8 (Joyner et al., 2000), and since xArx2 increased ventral telencephalon markers such as FoxG1/XBf1, we enter-
tain the possibility that fgf8-mediated proliferative expansion in the telencephalon has consequences upon midbrain patterning via depression of Otx2. Both early and late expressions of xOtx2 and xPax2 were decreased in xArx2-injected embryos. Conversely, expression levels of xOtx2 were increased in xArx2-MO-injected embryos, while the level of xPax2 was again reduced. A mutually restrictive relationship between XGbx2 and Otx2 positions the midbrain-hindbrain boundary, thereby also establishing the Pax2 expression domain (Rowitch and McMahon, 1995; Tour et al., 2002a,b).

Ectopic xArx2 reduced or posteriorized expression of posterior markers such as xGbx2a (von Bubnoff et al., 1996), and xKrox20 (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 xArx2 also resulted in posteriorized and reduced xGbx2a expression and decreased levels of early xKrox20 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, Paragangion 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 be 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 xArx2 activity is necessary for this function.

Finally, activity of Arx2 during somitogenesis does not appear to be critical for the segmentation of somitic mesoderm, and in Arx2 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 Arx2 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 Arx2 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**

Arx2 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 Nieuwoop and Faber (Nieuwoop and Faber, 1967). For injections, two-cell stage embryos were transferred to 1.5% Ficoll-400 (Sigma) in 0.3 MBS for injections.
**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 xArx2 rather than the previously published sequence (El-Hodiri et al., 2003). PCR conditions were determined empirically to establish the linear range of amplification for xArx2 and reactions were conducted using a therm-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 [32P]dCTP, and 1 µM of each primer (xArx2-5'-CCGACTGGAGCTCTGCT-3' and 5'-ACACTTCTTTGCCGGTGC-3'; Efl-α - 5'-CAGATTTGCTGAGATG-3' and 5'-ACTGCGCTGAT-GACTCTCA-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 xArx2 expression construct was derived using Vent polymerase (New England Biolabs) and primers (forward 5'-GAAGGCTTGGCCAGCAGTTGA-3' and reverse 5'-GCTCTAGACTGATAAAAAGTTACACTC-3') which bracketed the open reading frame and possessed restriction sites for StuI and XbaI, respectively, to facilitate directional insertion into the plasmid. Synthetic capped mRNA of xArx2 and Green Fluorescent Protein (GFP) was made from linearized template using Message Machine (Ambion). Capped mRNAs were resuspended in nuclease free water and coinjected into the animal pole of embryos at the two-cell stage with a Drummond nanoinjector. Concentrations of the xArx2 capped mRNA ranged from 100 pg to 800 pg. 400 pg of GFP capped mRNA was used for coinjection 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 stage, which was determined by GFP fluorescence under UV light, and to the required stages. The uninjected side served as a control.

**In Situ Hybridization**

To examine the putative effects of xArx2 misexpression on various brain and eye marker genes, embryos, injected with either synthetic capped xArx2 (600 pg) and GFP (400 pg) mRNA or with xArx2 morpholino oligonucleotides (18 ng), were subjected to whole mount in situ hybridization, performed according to Harland (Harland, 1991) using digoxigenin 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: xBf1 (N. Papalopulu), xArx20 (D. Wilkinson), xArx (H. El-Hodiri), xOtx2 (I. Blitz), xRx1 (G. Barsacchi), xPax2 (N. Heller), xDlx5 (el-Hodiri), and xPax6 (W. Harris). XGbx2a was amplified from a whole embryo cDNA library using primers (forward 5'-GGGAATTCTAGGCTT-CATTGACTCTCAG-3' and reverse 5'-AAAGGCCCTA-CATTCAAGGCTG-3') that contained Stud and XbaI restriction sites, respectively, to facilitate directional insertion into pCS2.

**Histology**

Stage 46 tadpoles that had been injected with 400 pg of xArx2 mRNA or 18 ng of xArx-MO, and which showed slight forebrain defects, were fixed in MEMFA 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 µg/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**

xArx2-MO specificity was assessed by means of an in vitro translation approach previously described (Winckbauer et al., 2001). A construct containing a xArx2 morpholino-equivalent site was created in the pCS2-Myc vector. The oligonucleotides (5'-GATTCACGAGACAGTC TGACGCCAGCA-3' and 5'-GCTGCGCTGGCTGAGGA CTGTGCGAGGAGGAG-3'), which contained restriction sites for BamHI and Clal, respectively, and which complemented the xArx2 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'-GATCTTGAAGACGTCCGGAGAGTC-3' and 5'-GCTGCGCTGGCTGAGGA CTGTGCGAGGAGGAG-3').
GCATTG-3' and 5'-CGCAATGCTGAGCTCCGGACTCTGCT-CAA-3'). Subsequently, xArx2 was directionally cloned into these constructs using primers (forward 5'-GAATGCCTCTAGAGCCGCCTACCAA-3' and reverse 5'-GGCTCTAGACTGCAAAATGTTACCTGCT-3'), which contained restriction sites for Stul and XbaI, respectively. xArx2-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 35S, 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 synthesis.

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LITERATURE CITED


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