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

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Recommended Citation
Wolanski, Marian; Khosrowshahian, Farhad; Kelly, LE; and Crawford, Michael J., "xArx2: An Aristaless Homolog That Regulates Brain Regionalization During Development in Xenopus laevis" (2009). Genesis, 47, 1, 19-31.
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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. *xArx*2 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 *xArx2* 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, *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 (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 dystonia (Sherr, 2003). Other anomalies in this X-linked disorder include the development of brain cysts and ambiguous genital development.
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 aristless 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 aristless domain (Fig. 1b). XArx2 (GenBank accession number AY519474) and xArx (EH 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 Arx2-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-
The found phenotype had the effect of inhibiting Arx expression in the injected side in presumptive forebrain at early stages, and in somites at later ones. (Fig. 4). 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.

xArx2 expression can be more precisely localized to the posterior telencephalon and anterior diencephalon by comparison to other markers. For example, in early neurulae, xArx2 expresses just caudal to telencephalon marker FoxG1/Xbf1 (Fig. 5a), and slightly overlapping and caudal to Rx (Fig. 5b). xArx2 is rostral and slightly overlapping with diencephalon/mesencephalon marker.
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 xArx2 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 ectopic 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,
FIG. 4.

FIG. 5.

FIG. 6.

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 $x_{Arx2}$ 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 in (Fig. 7b,d).

**Interfering with Proper $x_{Arx2}$ Function Results in Midline Defects**

Misexpressing $x_{Arx2}$ 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). $Arx2$ mRNA (400 pg), $x_{Pax6}$ expression, the pulu and Kintner, 1996). As a result of ectopic expression, the uninjected side of the embryo was used as a contra-lateral control and represented the normal expression of uninjected side of the embryo (Fig. 8e,e, respectively; Fig. 9a,a). Laterally, although not much change was observed in the late tailbud stage (Fig. 8b), $Rx1$ is also expressed in the anterior prosencephalon and subsequently in the developing eye and forebrain (Casarosa et al., 1997; Mathers et al., 1997). Ectopic $x_{Arx2}$ expression resulted in an expansion in the early and late expression levels of $Rx1$ (Fig. 8c,c'), while $x_{Arx2}$-MO induced the opposite effects, as the $Rx1$ expression levels were marginally up-regulated (Fig. 8d,d'). $Pax6$ demarcates the telencephalon/diencephalon border before it later expresses during eye development (Li et al., 1997). $Arx2$-injected embryos showed a marked expansion of $Pax6$ expression in late neurula stage embryos (Fig. 8e,e') and a dissipated expression in embryos injected with $x_{Arx2}$-MO-injected embryos at a similar stage (Fig. 8f,f'). Injection caused only slightly observable changes in $x_Pax6$ 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 $x_{Otx2}$, a prosencephalon and mesencephalon expressing gene (Pannese et al., 1995), showed that ectopic $x_{Arx2}$ caused a reduction in the size of the $x_{Otx2}$ expression domain in early embryos (Fig. 9a). Later $Arx2$ diminished $x_{Otx2}$ expression in the eye, whereas the expression in the brain was reduced at the posterior boundary and expanded laterally (Fig. 9a'). Conversely, inhibition of $x_{Arx2}$ translation resulted in an expansion of the $x_{Otx2}$ expression domain early (Fig. 9b) and up-regulated $x_{Otx2}$ levels late in the eye (Fig. 9b'). $Pax6$ marks the presumptive isthmus (midbrain-hindbrain) region (Rowitch and McMahon, 1995). In both $Arx2$ and $x_{Arx2}$-MO-injected embryos, a slight reduction in $x_Pax6$ expression levels was observed in this domain (Fig. 9c,c',d,d'). There was no effect on $x_{Pax6}$ 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 $x_{Arx2}$ reduced the early expres-

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**FIG. 8.** The effect of misexpression of $x_{Arx2}$ on forebrain markers $FoxG1/XBF1$, $Rx1$, and $Pax6$. Following unilateral injection at the 2-cell stage with $x_{Arx2}$ mRNA (400 pg) or $x_{Arx2}$ antisense morpholino oligonucleotides (18 ng $x_{Arx2}$-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 $x_{Arx2}$ on midbrain markers $x_{Otx2}$ and $x_{Pax2}$. As before, embryos were unilaterally injected on the left side (right side of picture). Marker gene perturbation is induced by $x_{Arx2}$ mRNA (400 pg) or $x_{Arx2}$ antisense morpholino oligonucleotides (18 ng $x_{Arx2}$-MO) at early (a-d) and later (a'-d') developmental stages and are shown from an anterior perspective. $x_{Arx2}$ transcript or antisense morpholino injected embryos were stained for $x_{Otx2}$ (a, a', and b, b' respectively), or for $x_{Pax2}$ (c, c', and d, d' respectively).

**FIG. 10.** The effect of misexpression of $x_{Arx2}$ 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 $x_{Arx2}$ mRNA (400 pg) or $x_{Arx2}$ antisense morpholino oligonucleotides (18 ng $x_{Arx2}$-MO) at early (a-d) and later (a'-d') developmental stages and are shown from an anterior perspective. $x_{Arx2}$ 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).
Expression of XGbx2a (Fig. 10a) and posteriorized its later expression (Fig. 10a'). This posteriorization of the XGbx2a expression domain was also observed in XArx2-MO-injected embryos (Fig. 10b,b'), and late embryos also showed a reduction in the expression level of XGbx2a. Analysis of the hindbrain marker Krox20 (Bradley et al., 1992), revealed that ectopic XArx2 expression posteriorized the expression of this hindbrain marker in early stage embryos (Fig. 10c), while in late embryos, XKrox20 expression was barely detectable (Fig. 10c'). This substantial reduction in expression was also observed in early embryos following translational inhibition of XArx2 (Fig. 10d), but appeared to have normalized by tailbud stage (Fig. 10d').

We were also interested in the effects that misregulation of XArx2 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 XArx2 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, XArx2 gain-of-function has the opposite effect of loss-of-function. XArx2 overexpression subtly increases early expression of forebrain ventralizing factors such as FoxG1/Xbf1 and Pax6, while decreasing expression of the ventral forebrain marker Rx1. The Forebrain/midbrain marker Otx2 is repressed by Arx2 activity, and this in turn presumably has effects upon the definition of the midbrain/hindbrain boundary that consequently depresses expression of Pax2 and Gbx2. Subsequently, differentiation of the rhombomeres is perturbed as reflected by altered patterning of Krox20.

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 pseudotraploid. 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 emience and ventral thalamus. Expression in the somites was also detected (Miura et al., 1997). Zebrfish 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. Zebrfish 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 emience 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). 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 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
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 xArx may indicate that xArx normally impinges upon xArx to activate transcription or that the xArx genes auto-regulate and xArx2 is mimicking an xArx 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 hindbrain, 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 posteriorization 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, 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 XLAS (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 Arx2 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 Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For injections, two-cell stage embryos were transferred to 1.5% Ficoll-400 (Sigma) in 0.5× 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) T RNA purifications from 10 pooled embryos of each developmental stage were performed in parallel using oligo d(Poly)strene 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 (Omni-

Microinjection

An xArx2 expression construct was derived using Vent polymerase (New England Biolabs) and primers (forward 5'-GAAGGCCTGCAGCCAGTTGA-3' and reverse 5'-GCTCTAGACTGATATAAAGTTAACACT-3') which bracketed the open reading frame and possessed restriction sites for StuI and XbaI, respectively, to facilitate directional cloning. Concentrations of the xArx2 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 fluorescence under UV light, and to the required stages. The uninjected side served as a contralateral control.

Loss-of-function assays were conducted similarly using fluoresceinated control morpholino oligonucleotides (xArx2-MO) (Gene Tools) (5'-GATCGAGAGAAGCTCG CACGAGTGTCTGG-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'-GCTCTACGT CATTCAATTTTA-3').

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: xBx1 (N. Papalopulu), xArx20 (D. Wilkinson), xArx (H. El-Hodiri), xOtx2 (I. Blitz), xOtx1 (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'-CGGATCAGGCTT CATTGGCTCTAG-3' and reverse 5'-AAGGCTGAA CATTTCAAGTCG-3') that contained Stul and Xbal 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 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 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 (Winklbauer et al., 2001). A construct containing a xArx2 morpholino-equivalent site was created in the pCS2-Myc vector. The oligonucleotides (5'-GATCCACGGACATCC TGCAGCCCAGCA-3' and 5'-GAGCTGCCGTCGAAGA GATCTGGGGCGAGG-3'), which contained restriction sites for BamHI and ClaI, 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'-GATCTTGAGACGTCGAGCTCA
GCAATG-3' and 5'-CGCAATGCTAGCTGCAGACTGCTGCT-CAA-3'). Subsequently, xArx2 was directionally cloned into these constructs using primers (forward 5'-GAA GGCTCATAGGGGCGCACTACCA-3' and reverse 5'-GCTCATAGCTCGATAAAAATTC-CAA-3'), which contained restriction sites for StuI and XbaI, respectively. xArx2-containing constructs included pCG2-myv-Arx, pCG2-myv-Arx with the xArx2-MO-equivalent site, and pCG2-myv-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.

ACKNOWLEDGMENTS

The authors thank the colleagues listed within the methods section for supplying probes. They also thank Martin Downenowicz and Amanda Ellwood for their technical contributions to preliminary studies. The work was supported by NSERC USRA fellowship (to A.E.), NSERC doctoral scholarship (to F.K.S.), University of Windsor Summer Fellowship (to M.D.), and Ontario Graduate Scholarship (to M.W.).

LITERATURE CITED


xARX regulates forebrain development


AQ1: Please confirm that all author names are OK and are set with first, surname last.

AQ2: Please note that reference “Harland, 1991” cited in the text is not given in the reference list. Kindly provide details of the same.

AQ3: Kindly provide the complete page numbers for reference “Abbott, 2003.”