The homeodomain-containing gene Xdbx inhibits neuronal differentiation in the developing embryo

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SUMMARY

The development of the vertebrate nervous system depends upon striking a balance between differentiating neurons and neural progenitors in the early embryo. Our findings suggest that the homeodomain-containing gene Xdbx regulates this balance by maintaining neural progenitor populations within specific regions of the neuroectoderm. In posterior regions of the Xenopus embryo, Xdbx is expressed in a bilaterally symmetric stripe that lies at the middle of the mediolateral axis of the neural plate. This stripe of Xdbx expression overlaps the expression domain of the proneural basic/helix-loop-helix-containing gene, Xash3, and is juxtaposed to the expression domains of Xenopus Neurogenin related 1 and N-tubulin, markers of early neurogenesis in the embryo. Xdbx overexpression inhibits neuronal differentiation in the embryo and when co-injected with Xash3, Xdbx inhibits the ability of Xash3 to induce ectopic neurogenesis. One role of Xdbx during normal development may therefore be to restrict spatially neuronal differentiation within the neural plate, possibly by altering the neuronal differentiation function of Xash3.

Key words: Homeodomain, Neurogenesis, Neural progenitors, Xenopus, Xdbx

INTRODUCTION

Neural progenitors substantially outnumber differentiating neurons at early stages of vertebrate nervous system development. The eventual fate of these progenitors is in part linked to the timing of their differentiation as neuronal subtypes are generated at specific times during development. The temporal regulation of neuronal differentiation and the maintenance of a pool of neural progenitors therefore insure that the diversity of neuronal cell fates is generated during nervous system development. The Xenopus embryo provides a useful system for studying the factors that maintain this balance as undifferentiated progenitors and differentiating neurons are maintained in a stereotypic, regionalized pattern within the neural plate (Hartenstein, 1989, 1993). Differentiating neurons in posterior regions of the embryo form three bilaterally symmetric columns with respect to the mediolateral axis of the neural plate. These neurons, termed primary neurons, are derived exclusively from progenitors in the deep layer of the neural plate (Hartenstein, 1989). Primary neurons are bounded by domains of regionally distinct, undifferentiated neural progenitors that lie in both the superficial and deep layers of the neural plate. The specific neuronal and non-neuronal fates of these progenitors is not fully established, although maintenance of these progenitors is in part required to fuel subsequent waves of differentiation in the embryo, eventually replacing most primary neurons and contributing the majority of neurons and glia to the mature nervous system.

A number of genes are expressed broadly within the neuroectoderm that have the potential to induce ectopic neuronal differentiation when overexpressed. These positive effectors include the zinc finger transcription factor genes, Gli2, Gli3 and ZicR1 (Brewster et al., 1998; Lee et al., 1997; Marine et al., 1997; Mizuseki et al., 1998a), the HMG domain-containing gene, SoxD (Mizuseki et al., 1998b), and the homeodomain-containing gene, Xiro1 (Gomez-Skarmeta et al., 1998). What negative regulators counter the effects of these genes to maintain progenitor domains in the embryo? The transmembrane receptor, X-Notch1, may play a role in this process. X-Notch1 is expressed throughout the neural plate (Coffman et al., 1990) and, in contrast to positive effectors, the overexpression of either a constitutively active form of X-Notch1 (Chitnis et al., 1995; Coffman et al., 1993) or downstream effectors of the Notch signaling pathway (Ma et al., 1998; Wettstein et al., 1997) leads to an expansion of the neural progenitor domain within the embryo and the inhibition of neuronal differentiation.

The integration of X-Notch1 function with positive effectors to retain a balance between neurons and progenitors has been most clearly defined within the early neuronal precursors that give rise to primary neurons (for review, Chitnis, 1999). These neuronal precursors are first marked by expression of the basic/helix-loop-helix (bHLH)-containing transcription factor gene, Xenopus Neurogenin related 1 (X-Ngrnr1; Ma et al., 1996). X-Ngrnr1 induces neuronal differentiation when overexpressed in neural precursors (Ma et al., 1996). However, the neuronal differentiation function of X-Ngrnr1 is limited by lateral inhibition (Ma et al., 1996) mediated by X-Notch1 and
its ligand, X-Delta1 (Chitnis et al., 1995). Only a subset of X-Ngnr1-positive precursors escapes X-Notch inhibition and differentiates to primary neurons marked by expression of N-tubulin and other markers of neuronal differentiation (Oschwald et al., 1991). The escape from lateral inhibition is mediated by the induction of a number of other positive effectors including the HLH-containing gene, Xco-e2 (Dubois et al., 1998), the zinc finger-containing gene, X-MyT1 (Bellefroid et al., 1996) and the bHLH-containing gene, NeuroD (Chitnis and Kintner, 1996; Lee et al., 1995). The maintenance of progenitor populations within early domains of neurogenesis is therefore a finely tuned process, requiring the combined action of positive effectors, including X-Ngnr1, and negative effectors, such as X-Notch1.

Regionalized X-Ngnr1 expression defines domains of early neurogenesis. Are there negative effectors that similarly define neural progenitor domains in the embryo? The homeodomain-containing gene Xiro3 is expressed at highest levels in neural progenitors and when overexpressed, Xiro3 inhibits neuronal differentiation in the embryo (Bellefroid et al., 1998). The Xenopus zinc finger transcription factor gene Zic2 may also play a role in patterning neuronal differentiation in the early embryo. Zic2 expression defines neural progenitor domains in the embryo and is expressed at highest levels in the lateral neural plate (Brewster et al., 1998). When overexpressed in the early embryo, Zic2 inhibits the expression of N-tubulin and other neuronal differentiation markers (Brewster et al., 1998). At the molecular level, Zic2 function may be integrated with positive regulators of neurogenesis by direct competition with Gli2 and Gli3 for DNA-binding sites (Aruga et al., 1994; Brewster et al., 1998). In addition to its ability to suppress neurogenesis within the embryo, Zic2 overexpression also results in an expansion of neural crest (Brewster et al., 1998). These findings suggest that multiple pathways converge to limit neuronal differentiation in the developing nervous system.

In order to further examine the molecular interactions that govern the patterning of regionalized neural progenitor populations, we searched for genes expressed specifically within the boundary progenitor domain at the middle of the mediolateral axis. This domain is defined at gastrula stages of development by expression of the bHLH-containing gene, Xash3 (Turner and Weintraub, 1994; Zimmerman et al., 1993). Xash3 has opposing dose-dependent effects when overexpressed in the embryo. At low doses, Xash3 induces ectopic neurogenesis in a majority of embryos while at high doses the effect of Xash3 overexpression is to inhibit neuronal differentiation (Chitnis and Kintner, 1996; Ferreiro et al., 1994). At high doses, Xash3 represses Neuronal differentiation. When taken together, our results suggest that Xdbx may function within the midpoint progenitor population to inhibit neuronal differentiation, possibly through modulating the function of Xash3.

**MATERIALS AND METHODS**

**Molecular cloning of the Xdbx gene**

A probe containing the homeodomain region of the chicken ChoxE gene (Rangini et al., 1991) was used at low stringency to screen a Xenopus stage 17 cDNA library (Kintner and Melton, 1987). A 2.1 kb cDNA clone (Xdbx) was isolated in a screen of approximately 106 plaques. The putative protein-coding region contained within this clone was sequenced in its entirety.

**In situ hybridization analyses**

The Xdbx clone was linearized with BglII and transcribed with T7 to generate a 1.6 kb antisense probe for Xdbx. Antisense probes for X-HES-5 and Hairy-2A were generated by digesting with BamHI and transcribing with T3 and T7, respectively. Probes for N-tubulin, NeuroD, X-Delta1, N-CAM, F-cadherin, Xash3, En2, Krox20 and X-Ngnr1 have all been described previously (Bradley et al., 1993; Chitnis et al., 1995; Espeh et al., 1995; Hemmati-Brivanlou et al., 1991; Krieg et al., 1989; Lee et al., 1995; Ma et al., 1996; Richter et al., 1988; Zimmerman et al., 1993).

Whole-mount in situ hybridization analyses were performed using previously described techniques (Harland, 1991; Hemmati-Brivanlou et al., 1990). For double in situ hybridizations, embryos were simultaneously hybridized with a fluorescein-substituted Xdbx probe and either a digoxigenin-substituted X-Ngnr1, N-tubulin, Xash3, Krox20 or En2 probe. Following overnight incubation with anti-fluorescein antibody at a dilution of 1:2000; embryos were washed in MAB (100 mM maleic acid/150 mM NaCl, pH 7.5) and developed using a magenta phos (3.7 ul of 50 mg/ml solution/ml)/red tetrazolium (4.5 ul of 75 mg/ml solution/ml) chromagen solution. After the reaction had developed to the correct intensity, embryos were washed in MAB and incubated in 0.1 M EDTA/MAB for 20 minutes at 65°C. Embryos were then washed twice for 10 minutes at room temperature in methanol followed by three 10 minute washes in MAB. Embryos were incubated overnight in anti-digoxigenin antibody at a dilution of 1:1000. After washing in MAB, embryos were developed with BCIP (3.7 ul of 50 mg/ml solution/ml) at 37°C. Selected embryos were dehydrated and mounted in paraplast for sectioning.

**Construction of Xdbx and GR-Xash3 constructs for injection**

All constructs for injection were subcloned into the CS2 derivative, CS2 NLS MT (Rupp et al., 1994; Turner and Weintraub, 1994), which contains an SV40 T antigen nuclear localization sequence (NLS) as well as sequences encoding 6 myc epitope tags (MT). All constructs were sequenced to insure correct protein-coding joins. Two Xdbx expression constructs (Xdbxmet and Xdbxvmet) were generated that contained the full-length protein-coding domain of Xdbx but the proteins differed in their N-terminal amino acids. These constructs yielded indistinguishable results in overexpression assays. The Xdbxmet construct includes 60 bp of 5’ Xdbx flanking sequence in addition to the normal Xdbx start codon (see Fig. 1A). The Xdbxvmet construct includes the amino acids MetGlyGlyGly N-terminal to the start site of Xdbx. Both derivatives were subcloned in frame with the downstream NLS and MT region of the CS2 vector. A homeodomain alone (XdbxHD) and a homeodomain deletion (XdbxAHD) construct
were also generated. XdbxHD contains the homeodomain coding region of Xdbx (GlyMet...SerLys) linked to two N-terminal Met codons. The XdbxHD construct is a variant of Xdbxvmet in which the homeodomain-coding region (GlyMet...SerLys) has been replaced by sequences encoding the amino acids GlyGlyGly. The VP16Xdbx construct includes sequences encoding the amino acids MetGlyGlyGly N-terminal to sequences encoding the C-terminal amino acids of herpes simplex virus VP16 (AlaPro...GlyGly) which are in turn linked to downstream sequences encoding the homeodomain of Xdbx. The En\^3Xdbx construct links sequences encoding the 298 N-terminal amino acids of Drosophila Engrailed (MetAla...LeuGly) to the Xdbx homeodomain region.

The Xash3 expression construct GR-Xash3 links the protein-coding region of Xash3 to sequences encoding the ligand-binding domain of the Glucocorticoid Receptor gene. Sequences encoding the glucocorticoid ligand-binding domain (aa 512-777; Hollenberg et al., 1985) were subcloned in frame with the downstream NLS and MT region of CS2. The Xash3 protein-coding domain (Zimmerman et al., 1993) was subcloned in frame with all of these regions, yielding a GR-NLS-MT-Xash3. In this construct, Xash3 function is hormone regulated in a manner similar to that previously reported for the related bHLH-containing gene, myoD (Kolm and Sive, 1995).

**Preparation and injection of mRNA**

The Xdbxmet, Xdbxvmet, XdbxHD, XdbxM, En\^3Xdbx and VP16Xdbx mRNAs were prepared for injection by linearizing with NotI and transcribing with SP6, X-NgnrT1, X-Notch1/ICD, X-Delta1\^STU, lacZ mRNAs were prepared for injection as previously described (Chitnis et al., 1995; Chitnis and Kintner, 1996; Ma et al., 1996; Vize et al., 1991). All mRNAs were synthesized using recommended protocols (Ambion). Yields were calculated based on incorporation of a radioactive tracer nucleotide. Embryos from either wild-type or albino Xenopus laevis were injected in a single blastomere at the 2-cell stage of development. In panels shown, 100-200 pg of the Xdbxmet or Xdbxvmet mRNA, 1 ng of XdbxHD, XdbxM, En\^3Xdbx or VP16Xdbx, 1 ng of GR-Xash3, 1 ng of X-Delta1\^STU and 1 ng of X-Notch1/ICD were injected in a volume of either 5 or 10 nl. 100 pg of lacZ mRNA was included in each injection. Injection of lower doses of Xdbx (10 or 1 pg) showed a similar phenotype but in a lower percentage of embryos. For GR-Xash3, GR-Xash3X-Delta1\^STU, GR-Xash3/Xdbxmet and GR-Xash3/X-Delta1\^STU/Xdbxmet injections, embryos were incubated in 10^{-5} M dexamethasone after stage 7 in order to activate the GR-Xash3 fusion protein. In the absence of dexamethasone, ectopic N-tubulin expression was not observed in GR-Xash3-injected embryos. Exposure of un.injected embryos to dexamethasone had no effect on patterns of N-tubulin expression. Staged embryos were fixed for 1 hour in MEMFA. For in situ hybridization, embryos were first analyzed for expression of co-injected β-gal before transfer to methanol and storage at -20°C.

**RESULTS**

**Isolation of the Xenopus Dbx gene**

The murine Dbx gene (Lu et al., 1992) is a member of the hlx family of genes encoding homeodomain-containing transcription factors. We have isolated the Xenopus homolog of this gene in a low-stringency screen of a Xenopus neurula stage cDNA library. Xdbx is homologous to Dbx throughout its protein-coding domain with an overall homology of greater than 73% (Fig. 1A; Lu et al., 1992). Within the homeodomain region, homology between Dbx and Xdbx approaches 97% (Fig. 1B) while the homology in non-homeodomain regions is approximately 68% (Fig. 1A; Lu et al., 1992). Xdbx also shares high homology with the homeodomain region of the zebrafish hlx1 gene (Fig. 1B; Fjose et al., 1994), the only domain of hlx1 for which published sequence is available. In contrast to these extensive homologies, the homology between Xdbx and other members of the hlx class of homeodomain genes is specific to the homeodomain region with no regions of homology apparent within other protein domains (Fig. 1B and data not shown). In summary, these homology comparisons suggest that Xdbx, Dbx and possibly zebrafish hlx1 represent evolutionarily conserved orthologs.

**Expression of Xdbx during Xenopus development**

The expression of Xdbx during Xenopus development was characterized using whole-mount in situ hybridization techniques. The expression of Xdbx appears neural specific. Xdbx expression is first detected in neural plate stage embryos within two domains (Fig. 2A). In anterior regions of the embryo, Xdbx expression is detected within a zone at the midline of the neural plate (Fig. 2A, arrowhead, C) while, in posterior regions, Xdbx is expressed in a bilaterally symmetric stripe that lies at the middle of the mediolateral axis (Fig. 2A and B, arrow). This pattern of Xdbx expression is maintained throughout neural fold stages of development.

Following neural tube closure, Xdbx is maintained in posterior regions of the embryo at the midpoint of the dorsosventral axis of the neural tube (Fig. 2D, arrow, E). The expression domain apparent at the anterior midline of the neural plate (Fig. 2A, arrowhead), the presumptive ventral region of the neural tube, is not detectable following neural tube closure. At stages intermediate to stage 20 and stage 28, an anteroposterior striped pattern of Xdbx appears within anterior regions of the embryo (data not shown). By stage 28, these stripes have coalesced to yield a uniform zone of Xdbx expression (Fig. 2F). In anterior regions, Xdbx expression is largely specific to the dorsal neural tube (Fig. 2G). Expression in more posterior regions of the embryo remains specific to the midpoint of the dorsosventral axis (Fig. 2H). This midposition expression is restricted to the ventricular zone suggesting that Xdbx expression is specific to mitotic progenitors within the developing nervous system (Fig. 2H).

**Mapping Xdbx expression with respect to the anteroposterior and mediolateral axes**

In order to gain a more detailed view of the pattern of Xdbx expression in early neural development, we compared Xdbx expression to that of other regionalized neural markers. The Xdbx expression boundaries in anterior regions of the developing nervous system were mapped relative to Krox20 and Engrailed 2 (En2) using double-label, whole-mount in situ hybridization techniques. En2 is expressed specifically at the midbrain-hindbrain boundary in the developing nervous system (Hemmati-Brivanlou et al., 1991). In embryos double-labeled with En2 and Xdbx, the anterior domain of Xdbx expression lies immediately rostral to the En2 domain (Fig. 3A, Xdbx, arrowhead; En2, asterisk).

The posterior striped domain of Xdbx expression was compared to that of Krox20, a gene expressed specifically within rhombomeres 3 and 5 of the developing hindbrain (Bradley et al., 1993). The anterior limit of the striped domain overlaps the rhombomere 5 domain of Krox20 expression indicating that the bilaterally symmetric stripe of Xdbx...
expression is specific to the posterior hindbrain and spinal cord (Fig. 3B, Xdbx; arrow; Krox20, asterisks).

The posterior domain of Xdbx expression was also mapped relative to the mediolateral domains of Xash3, X-Ngnr1 and N-tubulin expression. Xash3 is expressed at the apparent midpoint of the mediolateral axis commencing at mid-gastrula stages of development (Zimmerman et al., 1993). Comparison of Xdbx and Xash3 expression indicates that Xdbx overlaps the Xash3 expression domain at the middle of the mediolateral axis although Xash3 expression extends to a more medial region of the axis (Fig. 3C,E, Xdbx; arrow; Xash3, gray arrow; superficial light blue staining in E is background reactivity resulting from prolonged chromagenic incubation). In contrast, comparison of Xdbx expression to that of X-Ngnr1 and N-tubulin at the neural plate stage of development indicates juxtaposed patterns of expression. X-Ngnr1 is expressed in three symmetric domains of neuronal precursors that correlate with sites of primary neurogenesis (Ma et al., 1996). N-tubulin is expressed subsequent to X-Ngnr1 within these domains and is specific to differentiating primary neurons (Oschwald et al., 1991; Richter et al., 1988). Comparison of Xdbx expression to that of X-Ngnr1 and N-tubulin indicates that, within the posterior neural plate, Xdbx expression juxtaposes the two most medial zones of X-Ngnr1 and N-tubulin expression (Fig. 3D,F,G; Xdbx; arrow; X-Ngnr1 and N-tub, asterisks). Our findings suggest that Xdbx is not expressed within primary neurons or their precursors but rather is expressed together with Xash3 in neural progenitors within the neural plate.

Negative crossregulation of Xdbx and X-Ngnr1 expression

The finding that Xdbx expression forms a boundary between domains of X-Ngnr1 expression suggested that these genes might negatively regulate each other at a transcriptional level. We therefore examined the expression of X-Ngnr1 in embryos injected with Xdbx as well as the expression of Xdbx in embryos injected with X-Ngnr1. In both sets of injected embryos, there was a reciprocal relationship between the expression of the Xdbx and X-Ngnr1 genes. In embryos overexpressing Xdbx, X-Ngnr1 expression was downregulated (30/40 embryos, Fig. 3H) while, in embryos overexpressing X-Ngnr1, endogenous Xdbx expression was downregulated (8/11 embryos, Fig. 3I).

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Fig. 1. Xdbx encodes an hlx class homeodomain-containing protein. (A) The Xdbx sequence and putative protein-coding domain are shown. The homeodomain region is underlined. The Xdbx sequence is available through GenBank (accession number AF253504). (B) Comparison of the Xdbx homeodomain to other members of the hlx homeodomain family. Dashes indicate conserved residues. The genes listed include zebrafish hlx1 (Fjose et al., 1994), murine Dbx (Lu et al., 1992), chick ChoxE (Rangetti et al., 1991), murine Dbx2 (Shoji et al., 1996), murine Hlx (Allen et al., 1991) and Drosophila H2.0 (Barad et al., 1988).
Effects of Xdbx overexpression on patterns of neurogenesis within the neural plate

Our finding that Xdbx is expressed in neural progenitors and that Xdbx overexpression downregulates expression of X-Ngnr1, a positive effector of neurogenesis, suggested that Xdbx might play the opposing role of inhibiting neurogenesis within neural progenitors. Embryos injected with Xdbx were therefore characterized for expression of the neuronal differentiation marker N-tubulin, which marks zones of primary neurogenesis in the embryo (Oschwald et al., 1991; Richter et al., 1988). In response to Xdbx overexpression, N-tubulin expression was downregulated at the neural plate stage of development (44/51 embryos; Fig. 4A).

Additional markers of neuronal differentiation were also investigated in Xdbx-injected embryos. Expression of the bHLH-containing gene NeuroD and the neurogenic gene X-Delta1 preceede and then overlap domains of N-tubulin expression during normal development (Chitnis et al., 1995; Lee et al., 1995). In embryos overexpressing Xdbx, NeuroD and X-Delta1 expression was downregulated (NeuroD, 5/5 embryos; X-Delta1, 6/9 embryos; Fig. 4B and data not shown). As N-tubulin, NeuroD and X-Delta1 expression is positively correlated with neuronal differentiation during normal development, these data support an opposing role for Xdbx in inhibiting neurogenesis within the developing embryo.

The homeodomain region of Xdbx is sufficient for function

In order to investigate the functional domains of the Xdbx protein, we constructed two variants of Xdbx, XdbxHD and XdbxΔHD, and tested their function in Xenopus embryos. XdbxHD encodes only the homeodomain region of the Xdbx protein and XdbxΔHD contains a specific deletion of the same homeodomain region. In response to XdbxHD overexpression, N-tubulin expression was downregulated at the neural plate stage (16/35 embryos; Fig. 4C). In contrast, embryos overexpressing XdbxΔHD showed normal patterns of N-tubulin expression (17/19 embryos; data not shown). These findings suggest that the homeodomain region of Xdbx is both required and sufficient for inhibiting neuronal differentiation in the embryo. However, the inhibition observed following XdbxHD overexpression was not as efficient as that observed following overexpression of the full-length Xdbx protein-coding domain (compare 45% for XdbxHD versus 86% for Xdbx) suggesting that other regions of the protein cooperate to enhance the function of the homeodomain region.

In order to assess whether the inhibitory effects of Xdbx on neuronal differentiation were mediated either via transcriptional activation or repression, we linked either a heterologous transcriptional repressor (EnR; Han and Manley, 1993; Jaynes and O'Farrell, 1991; Smith and Jaynes, 1996) or a heterologous transcriptional activator (VP16; Sadowski et al., 1993; Lee et al., 1995). In embryos overexpressing Xdbx or EnRXdbx, 5/5 embryos; EnRXdbx, 11/26 embryos; data not shown). These results suggest that the function of the Xdbx homeodomain is dominant to heterologous repressor or activator regions.

Xdbx overexpression does not disrupt the early regionalization of the neuroectodermd

Xdbx overexpression inhibits a number of neuronal differentiation markers including N-tubulin, X-Ngnr1 and NeuroD. We next investigated the expression of neural markers not specifically associated with differentiating neurons in order to determine the range of Xdbx function. N-CAM is expressed in both progenitor and differentiated neuron populations (Krieg et al., 1989). In contrast to neuronal differentiation markers, expression of N-CAM was not inhibited in embryos overexpressing Xdbx (10/11 embryos; Fig. 4D). Some embryos exhibited lower levels of N-CAM expression but, unlike the complete absence of N-tubulin expression in response to Xdbx overexpression, N-CAM expression always remained detectable (Fig. 4D). In addition, the overexpression of Xdbx resulted in a lateral expansion of the N-CAM-positive domain in a minority of embryos (3/11 embryos; data not shown).

We also examined the expression of Xash3 and F-cadherin,
two markers expressed with Xdbx within the midpoint neural progenitor population. Both Xash3 and F-cadherin initiate expression at gastrula stages of development, prior to the onset of Xdbx expression in the embryo (Espeseth et al., 1995; Zimmerman et al., 1993). In embryos overexpressing Xdbx, normal expression of Xash3 and F-cadherin was noted in a majority of embryos (Xash3, 7/10 embryos; F-cadherin, 20/20 embryo, Fig. 4E,F). Taken together, these overexpression results suggest that Xdbx does not affect the early regionalization of the neuroectoderm but instead specifically inhibits neuronal differentiation within the developing neural plate.

Xdbx function appears independent of X-Notch1 and Xiro3 activity

The inhibition of neuronal differentiation markers in response to Xdbx overexpression is similar to the previously characterized phenotype in embryos in which the X-Notch1 signaling cascade has been activated (Chitnis et al., 1995; Coffman et al., 1993). We therefore examined the effects of activated Notch signaling on Xdbx expression. Although Xdbx and X-Notch1 have a similar overexpression phenotype, X-Notch1 signaling does not activate Xdbx expression. In fact, Xdbx expression was inhibited in embryos that overexpressed a constitutively active form of the X-Notch1 receptor (8/10 embryos; Fig. 5A). We also examined Xdbx expression in embryos in which the Notch signaling cascade was disrupted via overexpression of a dominant negative variant of X-Delta1, X-Delta1 STU. In embryos overexpressing X-Delta1STU an increased density of neuronal differentiation as marked by N-tubulin expression was noted in domains of primary neurogenesis as previously reported (Chitnis et al., 1995 and data not shown). Normal patterns of Xdbx expression were observed in these embryos (5/5 embryos; Fig. 5B).

We next examined downstream mediators of the Notch pathway in embryos overexpressing Xdbx, including Hairy-2A and X-HES-5. The Hairy-2A gene is normally expressed at the boundaries of the neural plate (Turner and Weintraub, 1994) while X-HES-5 is expressed in bilaterally symmetric stripes along the mediolateral axis of the neuroectoderm that apparently overlap domains of X-Delta1 expression (P. Wilson and K. Z., unpublished data). In Xdbx-injected embryos, neither Hairy-2A nor X-HES-5 expression was positively
Xdbx inhibits Xash3 function

The ability of Xdbx to inhibit neurogenesis when overexpressed is correlated with its normal expression in the midpoint neural progenitor domain. This domain also expresses Xash3, a bHLH-containing transcription factor that when overexpressed can drive neurogenesis in the embryo in a dose-dependent manner (Chitnis and Kintner, 1996; Ferreiro et al., 1994). In order to investigate the ability of Xdbx to modulate the neuronal differentiation function of Xash3, we assayed their integrated effects on neuronal differentiation. For these experiments, we used a Xash3 fusion construct that linked the Xash3 protein-coding domain to the ligand-binding domain of the Glucocorticoid Receptor gene (Hollenberg et al., 1985). In this construct, Xash3 function is dependent upon hormone addition and thus all embryos shown were incubated in dexamethasone (see Materials and Methods). GR-Xash3 overexpression led to ectopic neurogenesis (12/28 embryos; Fig. 6A) in a number of embryos as previously reported for Xash3 (Chitnis and Kintner, 1996). However, when GR-Xash3 and Xdbx were co-injected into the embryo, no ectopic neurogenesis was observed (0/21 embryos; Fig. 6B). In addition, normal patterns of primary neurogenesis were disrupted in GR-Xash3/Xdbx-injected embryos (19/21 embryos; Fig. 6B).

In a subset of embryos, the effect of GR-Xash3 overexpression was to inhibit rather than to promote neuronal differentiation (8/17 embryos decrease N-tubulin; 6/17 embryos increase N-tubulin; Fig. 6A,C) suggesting the possibility that Xdbx functioned in cooperation with Xash3 to inhibit neuronal differentiation. The ability of Xash3 to limit neurogenesis in the embryo depends upon X-Delta1-mediated activation of the Notch pathway (Chitnis and Kintner, 1996). The inhibition of X-Delta1 function in Xash3-expressing embryos by co-injection of the dominant negative Delta variant X-Delta1STU relieves inhibition and results in a dense pattern of ectopic neurogenesis in the majority of embryos (Chitnis and Kintner, 1996; 12/18 embryos increase N-tubulin; 2/18 embryos decrease N-tubulin; Fig. 6D). In contrast, the co-injection of Xdbx together with GR-Xash3 and X-Delta1STU results in the inhibition of N-tubulin expression in the majority of embryos (11/17 embryos decrease N-tubulin; 2/17 increase N-tubulin; Fig. 6E). Thus Xdbx does not positively cooperate with Xash3 via Notch activation to inhibit neurogenesis but rather appears to activate an independent pathway to alter the neuronal differentiation function of Xash3.

DISCUSSION

The homeobox motif is common to a large number of genes
that play important roles during vertebrate development (Gehring et al., 1994). Our current studies have focused on a single, evolutionarily conserved member of the hlx class of homeodomain-containing genes, Xdbx. The expression of Xdbx is restricted with respect to both the anteroposterior and mediolateral axes of the neural plate, indicating a regionally specific role in nervous system development. Overexpression studies in Xenopus embryos indicate that Xdbx inhibits neuronal differentiation suggesting that it may function to restrict neurogenesis temporally and spatially within the developing nervous system.

Pattern of Xdbx expression during development

Expression of the Xdbx gene initiates at the neural plate stage of development and appears neural specific. At early stages, Xdbx expression is regionally restricted both within anterior and posterior domains of the embryo. Within anterior regions, Xdbx is expressed at the midline of the mediolateral axis of the neuroectoderm in a zone immediately adjacent to the domain of En2 expression. Based on the fate map of the Xenopus neural plate (Eagleson and Harris, 1990), this region will later give rise to diencephalic derivatives but as Xdbx expression is transient within this zone the more specific fate of these progenitors remains unknown.

Xdbx is also expressed in longitudinal stripes that first define the middle of the mediolateral axis of the neural plate and subsequently define the midpoint of the dorsoventral axis of the neural tube. This regional expression is shared with murine Dbx and zebrafish hlx1 (Fjose et al., 1994; Lu et al., 1992) as well as with more divergent members of this family, including murine Dbx2 and chicken ChoxE (Rungini et al., 1991; Shoji et al., 1996). The transient expression of Xdbx within ventricular zone progenitors in the frog precludes tracking the differentiated fate of the Xdbx-expressing progenitors. However, recent work in both mouse and chick suggests that Dbx1 and Dbx2 progenitors are bound for an interneuron fate (Matise et al., 1999; Pierani et al., 1999). Expression studies in the chick spinal cord indicate that the Dbx1 protein is transiently co-localized with two differentiated markers of V0 interneurons, Lim1/2 and Evx1/2 (Pierani et al., 1999).

Moreover, the downregulation of Dbx1 expression is correlated with a loss of V0 interneurons while downregulation of Dbx2 expression correlates with a loss of the V1 interneuron population (Pierani et al., 1999).

The role of the Xdbx homeodomain region

The homeodomain region of Xdbx is both required and sufficient to inhibit N-tubulin expression when overexpressed in the embryo indicating the importance of this domain in Xdbx function. The homeodomain region facilitates the DNA-binding properties of the homeodomain class of proteins (for review, Gehring et al., 1994). Recent studies have shown that the DNA target specificities of homeodomain-containing proteins can be altered by protein-protein interactions (Mann and Affolter, 1998; Wilson and Desplan, 1995). Sites of protein-protein interaction have been mapped both within homeodomain as well as non-homeodomain regions of proteins. In addition, these interactions occur both with other homeodomain-containing proteins (for review, Mann and Affolter, 1998; Wilson and Desplan, 1995) as well as with other classes of transcription factors, including zinc finger and bHLH-containing proteins (Johnson et al., 1997; Lee et al., 1998). The dominance of the Xdbx homeodomain to both a heterologous activator (VP16) as well as a heterologous repressor (EnR) that we observe in overexpression assays might be explained by interactions mediated by the homeodomain with additional dominantly acting transcriptional regulatory proteins. The activity of the homeodomain region of Xdbx in functional assays, albeit lower than the activity of the wild-type protein, therefore provides a starting point for further study of both the Xdbx-DNA and/or Xdbx-protein interactions that govern functional activity.

Xdbx may act to refine patterns of neurogenesis within the neural plate

The spatially restricted expression of the proneural bHLH gene X-Ngnr1 is important for the stereotypic pattern of primary neurogenesis during normal development as X-Ngnr1 misexpression drives neurogenesis throughout the neural plate (Ma et al., 1996). In contrast, Xdbx overexpression has the opposite effect of suppressing neurogenesis throughout the neural plate. We have noted a reciprocal transcriptional relationship between the expression of these genes. X-Ngnr1 and Xdbx are normally expressed in juxtaposed domains within the neural plate and our findings indicate that the overexpression of one gene has a negative effect upon expression of the other. This negative reciprocal transcriptional regulation may shape the expression borders of each gene during normal development and the negative regulation of X-Ngnr1 expression by Xdbx may in turn play a role in inhibiting neurogenesis within the midpoint progenitor population at early stages of nervous system development.

Xdbx expression is also downregulated by the overexpression of Notch and Xiro3, genes that inhibit rather than promote neuronal differentiation in the embryo. Why is Xdbx downregulated both by genes that promote as well as inhibit neuronal differentiation? As X-Ngnr1 and Xiro3 induce X-Delta1 (Bellefroid et al., 1998; Ma et al., 1996) expression, it is possible that the downregulation of Xdbx in embryos overexpressing X-Ngnr1 and Xiro3 is an indirect consequence of Notch activation. Alternatively, the downregulation of Xdbx in response to both genes that promote an early neural progenitor state as well in response to genes that promote neuronal differentiation may reflect the transient expression pattern of Xdbx during normal development. Xdbx is not expressed in differentiated neurons and in neural progenitors, Xdbx expression initiates subsequent to Xash3 and F-cadherin (Epseseth et al., 1995; Zimmerman et al., 1993), suggesting the possibility that Xdbx expression specifically marks an intermediate stage in the differentiation of these progenitors.

What role does Xdbx play in molecular interactions at the midpoint of the mediolateral axis?

One of the earliest markers of the midpoint of the neuroectoderm is the proneural bHLH gene, Xash3 (Zimmerman et al., 1993). Overexpression studies indicate that Xash3 can act as a dose-dependent positive effector of neurogenesis (Chitnis and Kintner, 1996). The eventual neuronal or non-neuronal fate of Xash3-expressing progenitors is not known in the embryo; however, it is clear that neuronal differentiation is not observed in domains of Xash3 expression at early stages of development. What factors modulate Xash3
function to inhibit neuronal differentiation. Activated X-Notch1 signaling may play a role in shaping Xash3 function during development. Co-injection experiments indicate that Xash3-expressing progenitors remain responsive to activated Notch signaling (Chitnis and Kintner, 1996) and overexpression experiments indicate that Xash3 induces expression of the Notch ligand, X-Delta1. As activated X-Notch1 results in the inhibition of neuronal differentiation (Chitnis et al., 1995), the responsiveness of Xash3-expressing cells to Notch may play a role in inhibiting the differentiation of Xash3-positive progenitors.

Our current analyses suggest that Xdbx may play a distinct role in controlling Xash3 function within the midpoint progenitors of the posterior neural plate. Overexpression of Xdbx does not downregulate the expression of Xash3 and thus Xdbx does not inhibit Xash3 at a transcriptional level. However, Xdbx is capable of altering the neuronal differentiation function of Xash3. When co-injected into the embryo, Xdbx inhibits the ectopic neuronal differentiation that results when Xash3 is injected alone. Given the ability of Xash3 to both promote and inhibit neuronal differentiation when overexpressed, this alteration in the neuronal differentiation function of Xash3 could result either from an inhibition or an enhancement of Xash3 function. We favor the hypothesis that Xdbx has a negative rather than a cooperative effect on Xash3 function based on our finding that Xash3 function is independent of lateral inhibition, the mechanism responsible for the high dose, inhibitory effects of Xash3 on neuronal differentiation.

The overlap in Xash3 and Xdbx expression is not complete even at the midpoint of the axis suggesting that these genes may interact with additional genes in other regions of the embryo. In this regard, recent characterization of Dbx1 and Dbx2 expression in chick indicates that Dxb2 is expressed in a broader domain with respect to the dorsoventral axis of the spinal cord than is Dbx1 (Pierani et al., 1999). The isolation of additional Dbx homologs in Xenopus may therefore identify a related gene that more completely overlaps the Xash3 expression domain, particularly at the midpoint of the mediolateral axis of the developing neural plate.

What is the mechanism by which Xdbx mediates altered Xash3 function? Our current analyses indicate that Xdbx overexpression inhibits the expression of X-Ngrn1 and NeuroD, genes with direct links to neuronal differentiation in the early embryo. Thus the ability of Xdbx to alter Xash3 function may depend upon its ability to either directly or indirectly inhibit the expression of X-Ngrn1 and/or NeuroD within zones of Xash3 expression. Xash3 overexpression induces the ectopic expression of NeuroD within neural and non-neural ectodermal progenitors (Kanekar et al., 1997) and thus NeuroD appears to be a downstream target of Xash3. The fact that Xash3 and NeuroD are not normally co-expressed in neural plate progenitors therefore suggests that functions such as those described for Xdbx limit Xash3 target regulation at early stages of normal development. The regulation of Xash3 function by Xdbx could result either from competition for transcriptional targets, activation of reciprocal targets and/or direct protein-protein interactions such as those outlined above for homeodomain-containing proteins.

Conclusions

Although a subset of neurons differentiate at the neural plate stage of development, the majority of neurons and glia of the mature Xenopus nervous system derive at later time points from the neural progenitor populations of the neural plate stage embryo. The integration of positive and negative inducers likely creates a balance between neuronal differentiation and progenitor cell maintenance throughout the neural plate. Recently several pairings of positive and negative inducers have been identified within vertebrate embryos, including Gli/Zic2 (Brewster et al., 1998), Xash3/Notch (Chitnis and Kintner, 1996) and X-Ngrn1/Notch (Ma et al., 1996). In these pairings, one gene is widely expressed (Gli and Notch; Brewster et al., 1998; Coffman et al., 1990; Marine et al., 1997) while the other is expressed specifically within neural progenitors (Zic2 and Xash3; Brewster et al., 1998; Zimmerman et al., 1993). Xdbx and Xash3 therefore represent a novel pairing in that each gene is expressed specifically within a domain of neural progenitors. The integrated functions of regionalized pairs such as Xash3 and Xdbx may complement previously defined mechanisms and represent another level of control in the maintenance of progenitor populations in the early embryo. The maintenance of neural progenitors for later differentiation therefore appears to depend on the additive effects of a number of independent molecular pathways.

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