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Regulation and Function of *Dbx* Genes in the Zebrafish Spinal Cord

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Abstract

Dbx homeodomain proteins are important for spinal cord dorsal/ventral patterning and the production of multiple spinal cord cell types. We have examined the regulation and function of *Dbx* genes in the zebrafish. We report that Hedgehog signaling is not required for the induction or maintenance of these genes; in the absence of Hedgehog signaling, *dbx1a/1b/2* are expanded ventrally with concomitant increases in postmitotic neurons that differentiate from this domain. Also, we find that retinoic acid signaling is not required for the induction of *Dbx* expression. Furthermore, we are the first to report that knockdown of *Dbx1* function causes a dorsal expansion of *nkx6.2*, which is thought to be the cross-repressive partner of *Dbx1* in mouse. Our data confirm that the *dbx1a/1b/2* domain in zebrafish spinal cord development behaves similarly to amniotes, while extending knowledge of *Dbx1* function in spinal cord patterning.

Keywords

Dbx; spinal cord; zebrafish; Hedgehog; retinoic acid

INTRODUCTION

Establishing correct dorsal/ventral patterning in spinal cord development is a key process in producing a functional spinal cord. Discrete domains of spinal progenitors express a code of transcription factors, and then produce moto-, inter-, and sensory neurons. Studies in a variety of vertebrate species have described the extrinsic and intrinsic cues necessary for establishing the progenitor domains. For example, *Dbx* genes encode a family of homeodomain transcription factors that define an intermediate spinal progenitor domain (Lu et al., 1992). This gene family has multiple functions in spinal cord development. In *Xenopus*, *Xdbx* inhibits neurogenesis by regulating *Xash3* expression at neural plate stages (Gershon et al., 2000), whereas in mouse and chick, studies have shown that *Dbx1/2* are necessary in spinal cord development for the production of V0/V1 interneurons (Pierani et al., 2001), radial glia, astrocytes, and oligodendrocytes (Fogarty et al., 2005).

Multiple secreted signaling pathways establish and maintain gene expression profiles of spinal cord progenitor domains (Poh et al., 2002; Chesnutt et al., 2004). The effects of Hedgehog and retinoic acid (RA) signaling in regulating *Dbx1/2* expression have been studied in mouse and chick (Pierani et al., 1999; Briscoe et al., 2001; Wijgerde et al., 2002; Novitch et al., 2003). Hedgehog is required for patterning the ventral spinal cord by either activating or repressing target genes by means of the Gli transcription factor family (Jacob and Briscoe, 2003). *Shh*^{-/-}

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mouse embryos at early stages of development exhibit ventrally expanded *Dbx1* expression (Wijgerde et al., 2002), while later in development, *Dbx1* and *Dbx2* expression are localized to the ventral midline of the spinal cord (Pierani et al., 1999; Wijgerde et al., 2002). These studies indicated that the effect of reducing Shh activity changes during development of the spinal cord and that the ventral midline expression of *Dbx1* and *Dbx2* might be indirectly caused by the ventral expansion of more dorsal progenitor domains. Additionally, it has been suggested that low levels of Hedgehog induce *Dbx* expression in intermediate domains and high levels of Hedgehog repress *Dbx* more ventrally (Briscoe et al., 2001; Wijgerde et al., 2002). However, Hedgehog signaling does not appear to be absolutely required for *Dbx* expression, because cells lacking *Smoothed* function are still capable of expressing these genes, albeit in ectopic locations (Wijgerde et al., 2002). Indirect regulation of *Dbx* expression may occur by a secondary mechanism by which Class I and Class II homeodomain genes cross-repress each other to refine the borders between different domains. The *Nkx6.2* and *Nkx6.1* transcription factors have been shown to repress *Dbx1* and *Dbx2*, respectively (Vallstedt et al., 2001). However, the expression of *Nkx6.1/6.2* has not been examined in *Dbx* mouse mutants, leaving open the possibility of reciprocal repression between the two gene families.

It has also been suggested that RA signaling is required for regulating *Dbx* expression in the intermediate spinal cord. RA is secreted from the somitic mesoderm, neural tube cells, and notochord (Solomin et al., 1998; Berggren et al., 1999; Swindell et al., 1999; Molotkova et al., 2005; Maden, 2006). Addition of RA to embryonic day (E) 10 neural explants induces the expression of *Dbx* genes (Pierani et al., 1999; Novitch et al., 2003), and blocking RA signaling from the somitic mesoderm decreases the number of *Dbx*-positive cells induced in vitro (Pierani et al., 1999). Furthermore, these studies established that the effects of Hedgehog and RA signaling on *Dbx* gene expression are independent. However, the endogenous source of RA in this process is not known, and it is not clear whether RA acts directly to induce *Dbx* expression or to counteract other signals, such as bone morphogenetic protein (BMP), that normally act to inhibit *Dbx* genes (Pierani et al., 1999; Novitch et al., 2003).

The main function of spinal progenitors is to produce various classes of postmitotic neurons. Lineage tracing in mouse reveals that *Dbx1*-positive cells predominantly give rise to *Evx1/2*⁺ interneurons in the V0 domain (Pierani et al., 2001). Overexpression of *Dbx1* by means of electroporation causes an increase in *Evx1/2*⁺ cells with a concomitant loss of *En1*⁺ neurons and no effect on the production of other ventral neurons (Pierani et al., 2001). The opposite phenotype is observed in the *Dbx1* mouse knockout, where there is a loss of *Evx1/2*⁺ interneurons with an expansion of *En1*⁺ neurons, indicating a V0 to V1 switch (Pierani et al., 2001). Further evidence suggests that *Dbx2*-positive progenitors give rise to *En1*⁺ neurons in the V1 domain (Pierani et al., 1999, 2001). While this pathway of neuronal fate specification is clearly conserved between amniotes, it is not known whether the conservation extends to other vertebrates.

In zebrafish, three genes comprise the *Dbx* family: *dbx1a*, *dbx1b*, and *dbx2* (Seo et al., 1999). Phylogenetic analysis suggests that *dbx1a* and *dbx1b* represent duplicate orthologs of the amniote *Dbx1* gene. The zebrafish *dbx2* gene is related, although not definitively orthologous, to amniote *Dbx2* (Seo et al., 1999). Overexpression of *dbx1a* causes malformation of brain ventricles, resulting in a fused brain and defects in neuron clusters and axon projections in the forebrain (Hjorth et al., 2002). Knockdown of *dbx1a* by morpholino injection leads to altered hindbrain morphogenesis but does not affect patterning of this structure. Whereas these studies collectively reveal varied functions for *Dbx* genes throughout the central nervous system, detailed characterization of the establishment, regulation, maintenance, and progeny produced by *Dbx*-positive cells in the zebrafish spinal cord has not been described.

In the current study, we investigate *Dbx* regulation and function in zebrafish. First, we examine the expression of all three *Dbx* genes at multiple developmental stages and determine their spatial relationship with each other, as well as additional homeodomain genes. We show that blocking Hedgehog signaling causes a ventral expansion of all three *Dbx* genes and an increased number of two subclasses of interneurons, whereas RA signaling is necessary only for the maintenance of *dbx1a* expression. Lastly, we examine the consequences of *dbx1a/1b* loss-of-function and demonstrate that the progenitor marker *nkx6.2* is dorsally expanded into *dbx1a/1b* territory, resulting in a loss of interneurons produced from the *dbx1a/1b* domain. Together, our data suggest that *Dbx* gene expression and function are evolutionarily conserved between zebrafish and amniotes, and that neither Hedgehog nor RA signaling is required for induction of *Dbx* expression in vivo.

RESULTS

Characterization of *Dbx* Gene Expression in Zebrafish Spinal Progenitors

Expression of *dbx1a* in the zebrafish spinal cord has been reported to begin around 13 hours postfertilization (hpf; Fjose et al., 1994). We were interested in comparing expression patterns of *dbx1a* (Fig. 1A-E), *dbx1b* (Fig. 1F-J), and *dbx2* (Fig. 1K-O) at multiple time points during spinal cord development. Although their expression domain occupies a constant two- to three-cell-diameter stripe in the intermediate spinal cord, there are differences in the timing of expression between *Dbx* genes. Only *dbx1a* and *dbx1b* are expressed at 15 hpf (Fig. 1A,F,K), while *dbx2* expression does not begin until 24 hpf (Fig. 1M) and does not span the entire rostral/caudal axis until 28 hpf (data not shown). By 48 hpf, *dbx1a* (Fig. 1D) and *dbx1b* (Fig. 1I) are still present, while *dbx2* (Fig. 1N) is no longer expressed. The perdurance of *dbx1a/1b* expression at this late stage of spinal cord development indicates that *dbx1a/1b* may contribute not only to the production of neurons but also glia as observed in late stages of mouse spinal cord development (Fogarty et al., 2005). One day later in development at 72 hpf, *dbx1b* (Fig. 1J) is the only family member expressed. These data suggest that, although *dbx1a/1b/2* occupy similar territory in the spinal cord, their regulation during development is quite different. Furthermore, we noticed that *dbx1a* is the only family member to be expressed in presumptive postmitotic neurons based on position and morphology (Fig. 1B,C; Fjose et al., 1994), similar to results from mouse where *Dbx1* expression persists in neurons for a brief time (Pierani et al., 1999).

Next, we examined the relationship of the *dbx1a* domain to other spinal progenitor genes. Double in situ hybridization shows that the dorsal boundary of *dbx1a* expression is adjacent to *pax3* (Fig. 2A-A'') and *pax7* (Fig. 2B-B''). We found that *dbx1a* completely overlaps with *dbx1b* (Fig. 2C-C'') at 24 hpf and is expressed within the *dbx2* domain (Fig. 2D-D'') at 28 hpf. We also found that the ventral boundary of *dbx1a* is adjacent to *nkx6.1* (Fig. 2E-E'') and *nkx6.2* (Fig. 2F-F'') and that there are multiple cell diameters between *dbx1a* and *olig2* (Fig. 2G-G'') and *nkx2.2* (Fig. 2H-H'') expression. These results illustrate that spinal progenitor gene expression patterns, in relation to the *Dbx* domain, are largely conserved between zebrafish and amniotes.

Hedgehog Signaling Is Required for Spatial Localization, but Not for Induction of *Dbx* Gene Expression

We were interested in determining if *Dbx* expression required Hedgehog signaling for either its induction or maintenance. We treated embryos with cyclopamine to block Smoothed function (Cooper et al., 1998) beginning at either 50% epiboly (5 hpf) or 18 hpf and then performed in situ hybridization for multiple progenitor markers at 24 or 28 hpf. To determine the effectiveness of our cyclopamine treatments, we examined *ptc1* expression (Fig. 3A-B'), which is a direct target of Hedgehog (Marigo and Tabin, 1996). In cyclopamine-treated

embryos, detectable *ptc1* expression was abolished (Fig. 3A',B'), indicating that we had effectively blocked all Hedgehog signaling (Goodrich and Scott, 1998). As another control, we analyzed the expression of spinal cord progenitor markers that are down-regulated in zebrafish when Hedgehog signaling is blocked. Consistent with previous studies, expression of *nkx6.1* (Fig. 3C,C'; Cheesman et al., 2004) and *nkx6.2* (Fig. 3E,E'; Guner and Karlstrom, 2007) were abolished in embryos treated with cyclopamine at 50% epiboly, whereas dimethyl sulfoxide (DMSO) -treated embryos displayed normal expression of these markers. Interestingly, when we applied cyclopamine at 18 hpf and fixed at 24 hpf, low levels of *nkx6.1* (Fig. 3D,D') and *nkx6.2* (Fig. 3F,F') were present, indicating that Hedgehog is necessary not only for induction but also for maintenance of their expression.

We then examined expression of *Dbx* genes after cyclopamine application to determine whether Hedgehog was required for their induction (Fig. 3G,G',I,I',K,K'). All DMSO-treated embryos showed wild-type expression of *dbx1a/1b/2* (Fig. 3G,I,K) whereas cyclopamine-treated embryos displayed a ventral expansion of all three genes (Fig. 3G',I',K'). Therefore, Hedgehog signaling is dispensable for the induction of these genes but it also prevents their expression in the ventral spinal cord. Our results differ from a previous study, which shows a more subtle ventral expansion of *dbx1a* in the *smoothened* mutant zebrafish (Guner and Karlstrom, 2007). This difference is likely due to the presence of maternally supplied *Smoothened* protein in the mutant, allowing for Hedgehog signaling to partially repress *dbx1a* from the most ventral extent of the spinal cord. In our study the cyclopamine treatments block both maternal and zygotic *Smoothened* function.

We next asked if Hedgehog was required for the maintenance of the *Dbx* genes by applying cyclopamine at 18 hpf after the genes have been induced in the spinal cord (Fig. 3H,H',J,J',L,L'). Although *dbx2* is not expressed throughout the entire spinal cord at this time of development, it is present at low levels in the very rostral spinal cord segments. We found that all three *Dbx* genes are still expressed and are slightly ventrally expanded, but not to the extent observed in the 50% epiboly-24 hpf treatment. Therefore, we concluded that Hedgehog function is not required for *Dbx* maintenance, but functions at later stages to restrict expression from the ventral spinal cord.

We were also interested in determining the state of the dorsal boundary of *Dbx* gene expression after cyclopamine treatment. Previous work has shown that *Msx1/2* homeodomain proteins can repress *Dbx* gene expression (Timmer et al., 2002). We examined expression of a homologous gene, *msxc*, in zebrafish and found its expression was unaffected by blocking Hedgehog signaling at 50% epiboly (Fig. 3M,M'). This result is consistent with the unaffected dorsal boundary of *dbx1a/1b* expression (Fig. 3G',I'), but not with the apparent ventral shift of the *dbx2* dorsal boundary (Fig. 3K'), suggesting that other factors can also repress expression of *dbx2*. Of interest, we did observe ventral expansion of *pax3* (Fig. 3O,O'), but not *pax7* (Fig. 3Q,Q'), into the *dbx1a/1b* domain resulting in overlap that is normally not present (see Fig. 2A-A"). These data differ from those presented previously, which show that *pax3* expression was unaffected in *smoothened* mutants (Guner and Karlstrom, 2007). When cyclopamine was added at 18 hpf, we did not observe a shift in *msxc* (Fig. 3N,N'), *pax3* (Fig. 3P,P'), or *pax7* (Fig. 3R,R'), indicating that Hedgehog is not required for maintaining the expression domains of any of these genes. Cumulatively, these results suggest that *Dbx* expression does not directly require Hedgehog signaling, and the ventral *Dbx* boundary is set indirectly by Hedgehog signaling while the dorsal limit is set by another signal such as BMPs.

Effects on *Dbx* Gene Expression Lead to Changes in the Specification of Postmitotic Neurons

The results from blocking Hedgehog signaling indicate a general dorsalization of the spinal cord as observed by a loss of *nkx6.1/6.2* and ventral expansion of *dbx1a/1b/2* and *pax3*. We

expect that the expansion of the *Dbx* progenitor domain would also produce increases in the postmitotic neurons produced from this domain. Previous studies in mouse have shown that *En1*⁺ (V1) interneurons are produced from the *Dbx2* domain (Pierani et al., 1999, 2001) and *Pax2.1*⁺ (V0/V1) interneurons are produced from the *Dbx1/2* domain (Burrill et al., 1997). In zebrafish, *dbx1a/1b/2* are expressed in overlapping domains (see Fig. 2C-C'', D-D''), whereas *Dbx1* and *Dbx2* in mouse occupy separate domains at equivalent developmental time. Therefore, when we blocked Hedgehog signaling from 50% epiboly, we hypothesized that the number of *en1b*⁺ and *pax2.1*⁺ interneurons would be increased (Fig. 4). We counted individual cells expressing each marker in the same five spinal cord segments of multiple DMSO-treated embryos and compared them with cyclopamine-treated embryos. We found that the average number of *en1b*⁺ (Fig. 4A,C,E) and *pax2.1*⁺ (Fig. 4B,D,F) cells was increased from 5.67 (n = 12) to 8.75 (n = 12; $P < 0.0001$) and 5.80 (n = 15) to 8.60 (n = 15) ($P < 0.0001$), respectively. Of interest, the additional neurons appeared to be located near their normal location rather than expanded ventrally, indicating that Hedgehog-independent factors may regulate their final position, or that not all *Dbx*-positive progenitors are competent to produce these neurons. We nonetheless conclude from these results that the increased number of *en1b*⁺ and *pax2.1*⁺ interneurons correlates with the expansion of *dbx1a/1b/2* expression.

One possible cause of an increase in postmitotic neurons is that blocking Hedgehog signaling could generally affect neurogenesis in the spinal cord. To assay for changes in neurogenesis, we used transgenic zebrafish expressing green fluorescent protein (GFP) under the control of the pan-neuronal *huC* promoter (Park et al., 2000). At 24 hpf, the number of GFP-positive neurons per 12- μ m section was not significantly affected ($P > 0.4$) in cyclopamine-treated embryos (6.0 ± 0.3 [SEM], n = 12) compared with DMSO-treated controls (6.4 ± 0.5 [SEM], n = 12; Fig. 4G,H). Thus, *en1b*⁺ and *pax2.1*⁺ interneurons were expanded independently from the total number of neurons. These results support our conclusion that expansion of the *dbx1a/1b/2* domain directly causes an increase in specific classes of postmitotic neurons. Furthermore, we conclude that the *dbx1a/1b/2* domain in zebrafish may produce *en1b*⁺ and *pax2.1*⁺ interneurons as it does in mouse.

RA Signaling Is Not Required for Induction of *Dbx* Gene Expression

Next, we were interested in examining the influence of another important signal, RA, on the establishment of the *Dbx* domain. Previous data have suggested that RA is required for *Dbx* expression in other vertebrates (Pierani et al., 1999; Novitsch et al., 2003). To block RA signaling in embryos, we used 4-(diethylamino)-benzaldehyde (DEAB), which has been shown to inhibit retinaldehyde dehydrogenase and subsequently the synthesis of RA (Perz-Edwards et al., 2001; Hamade et al., 2006; Kopinke et al., 2006). Embryos were treated with DEAB at 50% epiboly to test whether RA is required for the induction of *Dbx* expression. To determine the effectiveness of the DEAB treatment, we performed two analyses. After application of 50 μ M DEAB in DMSO to wild-type and *Tg(isll:GFP)^{rw0}* embryos, we performed in situ hybridization using *krox20* (Fig. 5A,B) and imaged cranial nuclei expressing GFP (Fig. 5C,D). DEAB-treated embryos exhibited loss of *krox20* expression in rhombomere 5 (Fig. 5B), and loss of the vagal nuclei in the hindbrain of *Tg(isll:GFP)^{rw0}* transgenics (Fig. 5D). Both of these results are consistent with previous studies and indicate that we effectively inhibited RA signaling (Begemann et al., 2004; Maves and Kimmel, 2005).

Next, we examined the expression of the *Dbx* genes after DEAB treatment (Fig. 5E-P'). Because RA has previously been shown to induce *Dbx* expression in neural explants, we analyzed expression at both 18 hpf and 24 hpf. At 18 hpf, *dbx1a* and *dbx1b* expression was unaffected in DEAB embryos (Fig. 5E,F,I,J), suggesting that RA signaling is not required for inducing expression of these genes. However, we found that *dbx2* expression was prematurely expressed at 18 hpf (Fig. 5M,N'). When we analyzed embryos at 24 hpf, we observed lack of

dbx1a expression in DEAB-treated embryos (Fig. 5F,F), indicating that RA signaling may be required for the maintenance of this gene. In contrast, *dbx1b* and *dbx2* expression were present in DEAB-treated embryos (Fig. 5K,L,O,P), although *dbx2* expression spanned the entire rostral/caudal axis of the spinal cord unlike in DMSO-treated embryos (not shown). Collectively, these results suggest that RA signaling does play some role in regulating *Dbx* expression but is not required for the induction of these genes in vivo.

Although DEAB has been suggested to provide the most complete block of RA signaling possible in zebrafish (Begemann et al., 2004), our data contrasted with a previous study in chick using pharmacological agents to block RA receptors (Pierani et al., 1999). Therefore, we treated embryos with the pan-RA receptor inhibitor BMS493, which has also been used previously in zebrafish to block RA signaling (Grandel et al., 2002; Begemann et al., 2004). Embryos were soaked in 10 μ m BMS493, beginning at 50% epiboly and fixed at either 18 or 24 hpf, and we analyzed mRNA expression of all three *Dbx* family members. At 18 hpf and 24 hpf, the dorsal/ventral position of *dbx1a*, *dbx1b*, and *dbx2* expression was unaffected, while at 24 hpf, *dbx2* was prematurely expressed throughout the entire rostral/caudal axis of the embryo (not shown). We did not observe a loss of *dbx1a* expression at 24 hpf or premature expression of *dbx2* at 18 hpf after BMS493 treatment, which we believe is due to a less complete block of RA signaling compared with DEAB (Begemann et al., 2004). Nevertheless, the similar results obtained with antagonists of both ligand and receptors support the conclusion that RA is not required for *Dbx* induction.

***dbx1a* and *dbx1b* Function Are Required for Specification of Intermediate Spinal Progenitors**

The overlapping expression of *dbx1a* and *dbx1b* indicate that both genes may act redundantly in spinal cord development. We were interested in determining how the loss of *dbx1a/1b* would affect spinal cord patterning because Class I and Class II homeodomain proteins often cross-repress each other to sharpen the boundaries between adjacent progenitor domains. Prior research has shown that, in the *Nkx6.2* knockout mouse, *Dbx1* expands ventrally, suggesting that these genes may mutually repress each other (Vallstedt et al., 2001). However, it has not been shown in any system if the loss of *Dbx1* causes the dorsal expansion of *Nkx6.2*. We hypothesized that, if *nkx6.2* and *dbx1a/1b* were cross-repressive partners in zebrafish, we would observe a dorsal expansion of *nkx6.2* in *dbx1a/1b* morphants.

To test the combined function of *dbx1a* and *dbx1b*, we simultaneously knocked down zygotic transcription with splice-blocking morpholino oligonucleotides for both genes (Fig. 6). Reverse transcriptase-polymerase chain reaction (RT-PCR) showed partial block of splicing at a concentration of 7 ng/nl for *dbx1a* (Fig. 6A) and 2.5 ng/nl for *dbx1b* (Fig. 6B). Due to an early requirement for *dbx1a/1b*, embryos did not survive past gastrulation when we injected higher doses of morpholino; therefore, we analyzed a hypomorphic phenotype in the double morphants. Compared with 24 hpf stage-matched wild-type embryos (Fig. 6C), *dbx1a/1b* morphants have a shorter rostral/caudal axis (Fig. 6D). We also observed a defect in hindbrain morphogenesis similar to Hjorth et al. (2002; data not shown). As a control for morpholino specificity, we injected each morpholino individually and compared the resulting phenotypes to double morphants.

In the spinal cord, we observed a dorsal expansion of *nkx6.2* expression in *dbx1a/1b* morphants, confirming that *dbx1a/1b* function is necessary to repress *nkx6.2* from intermediate domains (Fig. 6E,F). Single *dbx1a* or *dbx1b* morphants had no change in *nkx6.2* expression (not shown), indicating that the dorsal shift of *nkx6.2* is specific to the simultaneous loss of both genes produced by the morpholinos. In contrast, we found that *msxc* expression was unaffected in *dbx1a/1b* morphants (Fig. 6G,H), consistent with previous data indicating that *Dbx* genes are unable to repress *Msx* expression (Timmer et al., 2002). We also did not observe any defects in *pax3* expression (not shown), suggesting that other factors such as BMP and Wnt signaling

may restrict the expression of dorsal progenitor markers (Chesnutt et al., 2004; Ille et al., 2006; Zechner et al., 2006).

Next, we were interested in determining how the loss of *dbx1a/1b* function affected the production of postmitotic interneurons (Fig. 7). As a control, we performed in situ hybridization at 24 hpf for *isl1*, a marker of primary motoneurons in the ventral spinal cord. As expected, the mean number of *isl1*⁺ neurons within somite segments 8-18 did not significantly differ ($P > 0.5$) between wild-type (22.90, $n = 10$) and *dbx1a/1b* morphants (22.60, $n = 10$; Fig. 7A-C). We also examined *en1b*⁺ and *pax2.1*⁺ interneurons, which we hypothesized differentiate from the *dbx1a/1b* domain (Thaeron et al., 2000). *en1b*⁺ interneurons were significantly decreased ($P < 0.0001$) in *dbx1a/1b* morphants (11.44, $n = 9$) compared with wild-type (16.90, $n = 10$; Fig. 7D-F). *pax2.1*⁺ interneurons were similarly decreased ($P < 0.001$) in *dbx1a/1b* morphants (16.89, $n = 9$) compared with wild-type (27.50, $n = 8$; Fig. 7G-I). At 24 hpf, there was no significant change in the number of *en1b*⁺ interneurons in single *dbx1a* or *dbx1b* morphants (data not shown), again indicating that the phenotype is specific to the simultaneous loss of both genes.

As with our cyclopamine treatments, we were interested in determining if changes in the number of *en1b*⁺ and *pax2.1*⁺ interneurons after *dbx1a/1b* morpholino injections were secondary to a general effect on neurogenesis. We did not observe a change in the number of Hu-expressing cells per 12- μ m section ($P > 0.5$) in *dbx1a/1b* morphants (7.2 ± 0.3 [SEM], $n = 12$) compared with wild-type (7.3 ± 0.4 [SEM], $n = 12$). This result, combined with the consistent number of *isl1*⁺ neurons, suggest that the effects on *en1b*⁺ and *pax2.1*⁺ interneurons were a direct consequence of eliminating *dbx1a/1b* function in progenitors.

DISCUSSION

Properly establishing and maintaining the *Dbx* progenitor domain is important for spinal cord development, but the mechanisms underlying regulation of *Dbx* expression and the function of these genes has not been well studied in zebrafish. Here, we have characterized the expression of *dbx1a*, *dbx1b*, and *dbx2* throughout spinal cord development (Fig. 1). We found that the spatial relationship of the *Dbx* genes to other known progenitor markers is well conserved in zebrafish compared with amniotes (Fig. 2). Our data indicate that blocking Hedgehog signaling does not affect the induction or maintenance of *dbx1a/1b/2* expression, but instead leads to expansion of these genes into the ventral spinal cord (Fig. 3). The patterning defects when Hedgehog was blocked were evident not only in the progenitor domain but also in postmitotic neurons (Fig. 4). We observed an increase in *en1b*⁺ and *pax2.1*⁺ interneurons in cyclopamine-treated embryos, indicating that (1) the *Dbx* domain produces *en1b*⁺ and *pax2.1*⁺ interneurons and (2) by blocking Hedgehog signaling, we have dorsalized the spinal cord. We determined that blocking RA signaling does not affect the induction of *dbx1a* and *dbx1b* but causes premature expression of *dbx2* (Fig. 5). Also, it is possible that RA is specifically important for the maintenance of *dbx1a* expression. Lastly, we investigated the developmental consequence of knocking down *dbx1a/1b* gene function (Fig. 6). We found ventral spinal cord patterning defects demonstrated by the dorsal expansion of *nkx6.2* into the *dbx1a/1b* domain, while dorsal progenitor expression was unaffected. Also, we observed decreases in the number of subtype-specific interneurons believed to differentiate from the *dbx1a/1b* domain (Fig. 7). Together our data indicate an evolutionarily conserved role for *Dbx* genes in spinal cord patterning, and lead to an open question regarding the nature of the inductive signal for *Dbx* expression.

Conservation of *Dbx* Gene Expression in Zebrafish

We have determined that expression of the *Dbx* genes during spinal cord development remains consistent from their onset when the spinal cord is being formed to the time when they are no

longer present. The *dbx1a* and *dbx1b* genes most likely represent duplicate orthologs of the single *Dbx1* gene in mouse and chick (Seo et al., 1999). Zebrafish *dbx2* is less conserved in terms of onset of expression, although the territory it occupies is quite similar to, although a bit wider than, *dbx1a* and *dbx1b*. In fact, sequence comparison between the three genes indicates *dbx2* is the most divergent (Seo et al., 1999). Recent reports in zebrafish have shown that many of the spinal progenitor genes have conserved spatial relationships (Cheesman et al., 2004; Lewis et al., 2005; Guner and Karlstrom, 2007). We have demonstrated that the relationship of *Dbx* expression to other progenitor genes is evolutionarily conserved in zebrafish, which we find intriguing given the size difference of the zebrafish spinal cord compared with amniotes.

Hedgehog Signaling Is Not Required for *Dbx* Gene Induction

Previous studies have offered conflicting evidence as to whether *Dbx* expression absolutely requires a Hedgehog signal for induction (Pierani et al., 1999; Briscoe et al., 2001). However, cell-autonomous loss of all Hedgehog signaling by removal of *Smoothed* function leads to a shift in position, but not complete absence, of *Dbx* genes (Wijgerde et al., 2002). This experiment suggests that Hedgehog acts to set the position of *Dbx*-positive progenitors, but is not absolutely required for expression of these genes. Our data support this model, as cyclopamine-treated zebrafish embryos exhibit a ventral expansion of *Dbx* genes but not loss of expression (Fig. 8A). Although our controls indicate that we have effectively blocked Hedgehog signaling, we cannot rule out the possibility that very low remaining levels of Hedgehog induce *Dbx* in the normal domain. Further studies in embryos lacking maternal and zygotic *Smoothed* function could help resolve this issue. Regardless, the data suggest that Hedgehog may act through *Nkx6* genes to indirectly limit the ventral boundary of *Dbx* expression. The difference between our results and previous studies with respect to the dorsal boundary of *dbx1a/1b* expression may be explained by variability in the timing or extent of signals such as BMP and Wnt, which could act to exclude *Dbx* from more dorsal spinal regions. In the mouse, loss of *Smoothed* function may allow endogenous BMP or Wnt signals to induce genes such as *Msx1/2* and, thus, inhibit *Dbx* expression. In contrast, BMP or Wnt activity may not penetrate as far ventrally in zebrafish embryos at 24 hpf, resulting in preservation of the dorsal *dbx1a/1b* boundary. Interestingly, the dorsal boundary of *dbx2* expression was shifted ventrally after cyclopamine treatment, suggesting that regulation of this later-expressed gene may be differentially sensitive to dorsal signals. The ventral shift observed in *pax3* expression makes it a candidate for *dbx2* regulation, and intriguingly, simultaneous knockout of *Pax3* and *Pax7* in mouse produces a phenotype consistent with expanded *Dbx2* expression (Mansouri and Gruss, 1998).

RA May Act Indirectly in Regulating *Dbx* Expression

Previous literature shows that application of RA to nascent neural tube explants can induce *Dbx* expression, whereas antagonists of RA added to the culture decreased the ability of adjacent tissues to induce *Dbx1/2*-positive cells (Pierani et al., 1999). In addition, expression of dominant-negative RA receptors in spinal progenitors leads to cell autonomous reduction of *Dbx* expression (Novitch et al., 2003). In contrast, our data suggest that RA signaling is not required for the induction of *Dbx* genes in the zebrafish spinal cord. One possible explanation for these results is that RA acts indirectly to antagonize dorsal BMP or Wnt signals, which themselves limit *Dbx* expression. This model is supported by the previous explant studies, in which addition of RA simultaneously led to induction of *Dbx* and repression of *Pax7* (Pierani et al., 1999). If these dorsal signals are not playing an identical role in the zebrafish spinal cord before 24 hpf, then RA signaling would be unnecessary for *Dbx* induction. Our results also indicate that blocking RA signaling can lead to premature *dbx2* expression in the spinal cord. The mechanism underlying this premature induction is not clear, but it is possible that RA can regulate the temporal progression of progenitor gene expression in the spinal cord. The

differential effects of blocking RA signaling on the *Dbx* genes could be due to divergent upstream regulatory elements or compensatory mechanisms within the *Dbx* family. Additional time-dependent studies are necessary to further explore these possibilities.

***Dbx* Function Is Important for Intermediate Spinal Cord Development**

Loss-of-function studies have been performed for *dbx1a* function in hindbrain development (Hjorth et al., 2002) but function of zebrafish *Dbx* genes in the spinal cord had not been examined. Our *dbx1a/1b* loss-of-function results indicate that similar phenotypes occur in the zebrafish as compared with the *Dbx1* mouse knockout (Pierani et al., 2001). We also observed a dorsal expansion of *nkx6.2* into the territory normally occupied by *dbx1a/1b*. This finding is the first evidence that *dbx1* antagonizes *nkx6.2* expression, suggesting that these genes are cross-repressive partners (Fig. 8B). We also determined that, in the absence of *dbx1a/1b*, there is a concomitant loss of interneurons produced from this progenitor domain (Fig. 8B). It would be interesting in the future to determine whether there is redundancy or compensation for the loss of *dbx1a/1b* function by *dbx2*. Furthermore, our analysis was limited to the 24 hpf time point, because we were investigating the patterning and differentiation function of *dbx1a/1b*. Later analysis, at 48 hpf for example, would be useful to determine whether this domain also contributes to the production of glia as observed in mouse (Fogarty et al., 2005).

***Dbx* Expression May Represent a “Default” Fate for Spinal Progenitors**

The *Dbx* expression domain lies in a region where multiple signaling pathways are converging. In the intermediate spinal cord, Hedgehog concentrations are quite low (Poh et al., 2002; Jacob and Briscoe, 2003) and it is clear that these low levels are necessary to prevent *Nkx6* genes from being expressed in these cells. Low levels of BMP are also important to position the dorsal boundary of *Dbx* expression (Timmer et al., 2002), and RA is required in an unknown way to allow *Dbx* expression, possibly by antagonizing these dorsal signals (Pierani et al., 1999; Novitsch et al., 2003). Cumulatively, these data suggest that *Dbx* expression may occur in the absence of any dorsalizing or ventralizing signals and, thus, may represent a “default” state of spinal progenitors. Interestingly, *Dbx1*-expressing progenitors uniquely produce the three major cell types of the spinal cord: neurons, astrocytes, and oligodendrocytes, and thus represent the most multipotent progenitor population in the tissue (Fogarty et al., 2005). Additional work is necessary to determine whether any inductive molecules are required to induce *Dbx* expression in vivo, or if instead, it is simply sufficient to remove all Hedgehog, Wnt, and BMP signaling.

EXPERIMENTAL PROCEDURES

Fish Strains and Staging

Embryos were obtained from natural spawning of wild-type (AB*) or *Tg(isll:GFP)^{rw0}* (Higashijima et al., 2000) zebrafish lines. All developmental stages in this study are reported in hours postfertilization at 28.5°C, according to Kimmel et al., (1995).

Morpholino Injections

The *dbx1a* and *dbx1b* splice-blocking morpholino antisense oligonucleotides were obtained from Gene Tools, LLC. Morpholino sequences are as follows: *dbx1a*, 5'-AAACACTTACCTTTCTTTGGTGACG-3'; *dbx1b*, 5'-CTGATGACGATGCTATAAAAAAAT-3'. Both *Dbx* morpholinos were simultaneously injected into one-cell stage wild-type zebrafish embryos at concentrations of 7 ng/nl and 2.5 ng/nl for *dbx1a* and *dbx1b*, respectively.

RT-PCR

Seventy-five wild-type embryos and *dbx1a* or *dbx1b* morphants at 24 hpf were used for preparing RNA. Total RNA was isolated using Trizol (Invitrogen) reagent and was reverse transcribed by random hexamers using the Superscript First Strand Synthesis Kit (Invitrogen) following the manufacturer's protocol. PCR was performed for 30 cycles using an annealing temperature of 57°C (*dbx1a*) and 60°C (*dbx1b*), and reactions were visualized on 1% agarose gels in TAE. The spliced product for *dbx1a* is 191 bp and unspliced product is 243 bp. The spliced product in *dbx1b* is 194 bp, and the unspliced product is approximately 1.7 kb.

In Situ Hybridization

Probe synthesis and in situ hybridization were performed as described elsewhere (Oxtoby and Jowett, 1993). Single and double in situ hybridizations were carried out using digoxigenin- or fluorescein-labeled antisense RNA probes (Jowett, 2001) and visualized using BM Purple (Roche) and Fast Red (Roche). The following RNA probes were made by our lab from DNA plasmids: *dbx1a*, *dbx1b*, *dbx2*, *msxc*, and *nkx6.2*. We were given the following DNA plasmids: *en1b* (Higashijima et al., 2004), *krox20* (Oxtoby and Jowett, 1993), *nkx2.2* (Barth and Wilson, 1995), *nkx6.1* (Cheesman et al., 2004), *pax2.1* (Krauss et al., 1991), *pax3* (Seo et al., 1998), *pax7* (Seo et al., 1998), *ptc1* (Concordet et al., 1996), and *olig2* (Park et al., 2002). For whole-mount photography after all staining methods, embryos were deyolked and mounted laterally or cross-sectioned on a cryostat. Whole-mount and cryosectioned embryos were imaged on a compound microscope.

Cyclopamine Treatments

A 10 mM stock solution of cyclopamine (Toronto Research Chemicals) dissolved in DMSO (Fisher) was diluted to 100 µM in embryo medium. Embryos were soaked in cyclopamine beginning at either 50% epiboly (5 hpf) or 18 hpf and fixed at 24 hpf or 28 hpf overnight in 4% paraformaldehyde.

DEAB Treatments

A 10-mM stock solution of DEAB (Sigma-Aldrich) dissolved in DMSO was diluted to 50 µM in embryo medium. Embryos were soaked in DEAB beginning at either 50% epiboly and fixed at 18 hpf, 24 hpf, or 28 hpf overnight in 4% paraformaldehyde.

BMS493 Treatments

A stock solution of BMS493 (gift from V. Prince) dissolved in DMSO was diluted to 10 µM in embryo medium. Embryos were soaked in DEAB beginning at 50% epiboly and fixed at either 18 hpf or 24 hpf overnight in 4% paraformaldehyde.

Analysis of Hu-Positive Neurons

Postmitotic neurons were counted in 12 nonconsecutive 12-µm cryosections, either by direct GFP fluorescence using Tg(*elavl3:EGFP*)^{z8} embryos (Park et al., 2000), or by immunofluorescence with anti-HuC/D antibody (Molecular Probes) used at 1:1,000. Staining of transgenic embryos with the antibody showed complete co-labeling of all GFP-positive neurons.

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Fig. 1.

Expression of *dbx1a/1b/2* during spinal cord development. Spinal cord cross-sections are shown at five different stages of development for *dbx1a*, *dbx1b*, and *dbx2*. **A-E:** *dbx1a* expression is present at 15 hours postfertilization (hpf) through 48 hpf and is absent at 72 hpf. The territory that *dbx1a* occupies does not change appreciably throughout time, although at 48 hpf (D) *dbx1a* is no longer expressed in postmitotic neurons. **F-J:** *dbx1b* expression is very similar to *dbx1a* expression, with the exception that it is present at low levels at 72 hpf. **K-O:** *dbx2* expression differs from *dbx1a/1b*. It is not expressed in the spinal cord until 24 hpf and only in rostral segments at that time and is down-regulated by 48 hpf.

Fig. 2.

Relationship of *dbx1a* to other spinal cord patterning genes. **A-H''**: Spinal cord cross-sections showing double in situ hybridization at 24 hours postfertilization (hpf; *dbx2* 28 hpf). In each panel patterning genes are in blue (A-H), *dbx1a* is shown in red (A'-H'), and merged images are shown in (A''-H''). *dbx1a* expression does not overlap with expression of *pax3* (A-A''), *pax7* (B-B''), *nkx6.1* (E-E''), *nkx6.2* (F-F''), *olig2* (G-G''), or *nkx2.2* (H-H''). *dbx1a* completely overlaps with *dbx1b* (C-C'') and is expressed within the domain of *dbx2* (D-D'').

Fig. 3.

Analysis of patterning defects in cyclopamine-treated embryos. Spinal cord cross-sections of embryos treated with dimethyl sulfoxide (DMSO) or cyclopamine at 50% epiboly or 18 hours postfertilization (hpf) are shown. **A-F**: The 100 μ M cyclopamine blocked expression of *ptc1* (A-B"), *nkx6.1* (C-D"), and *nkx6.2* (E-F") under both treatment conditions. **G,I,K**: Expression of *dbx1a* (G, G"), *dbx1b* (I, I"), and *dbx2* (K, K") expands ventrally after cyclopamine treatments beginning at 50% epiboly compared with DMSO controls. **H,J,L**: Similarly, after cyclopamine treatments beginning at 18 hpf, *dbx1a* (H,H"), *dbx1b* (J,J"), and *dbx2* (L,L") expand slightly ventrally. **M-P**: The expression of *msxc* (M-N") is unaffected at either stage of treatment, whereas *pax3* (O,O") expression expands ventrally into the *dbx1a/1b* domain

when treated at 50% epiboly, but no expansion is observed in the 18-24 hpf treatment (P,P").
Q,R: In contrast, *pax7* (Q-R") expression does not expand ventrally in either treatment.

Fig. 4. *en1b*⁺ and *evx1*⁺ interneurons increase in cyclopamine-treated embryos. A-D: Lateral whole-mount views of subtype-specific postmitotic marker are shown with rostral to the left at 24 hours postfertilization (hpf). **A-F:** *en1b*⁺ (A,C,E) and *pax2.1*⁺ (B,D,F) interneurons are increased in cyclopamine-treated embryos. Error bars represent SEM. **G,H:** Representative sections of green fluorescent protein (GFP) expression in *Tg(elavl3:EGFP)^{zf8}* embryos are shown. There is no change in the number of Hu-positive neurons after cyclopamine treatment.

Fig. 5. Analysis patterning defects in 4-(diethylamino)-benzaldehyde (DEAB) -treated embryos. **A,B:** Dorsal view of 24 hours postfertilization (hpf) embryos showing *krox20* mRNA expression. Dimethyl sulfoxide (DMSO) -treated embryos show *krox20* expression in rhombomeres 3 and 5 and DEAB-treated embryos only show expression in rhombomere 3. **C,D:** dorsal view of green fluorescent protein (GFP) expression in cranial nuclei in *Tg (isl1:GFP)^{rw0}* embryos at 24 hpf. DMSO-treated embryos have normal GFP expression, and DEAB-treated embryos have loss of the vagal (X) nuclei and abnormal positioning of the VIIIth cranial nuclei. **E-P:** Spinal cord cross-sections of embryos treated with DMSO or DEAB. *dbx1a* mRNA expression is unaffected when embryos are fixed at 18 hpf (E,F) but by 24 hpf, progenitor expression and often postmitotic neuron expression of *dbx1a* is lost (G,H). *dbx1b*

expression is unaffected at 18 hpf (I,J) and 24 hpf (K,L) in DEAB-treated embryos. *dbx2* expression is prematurely induced at 18 hpf in DEAB-treated embryos (M,N) and is maintained at 24 hpf (O,P).

Fig. 6. *dbx1a* and *dbx1b* morpholinos cause spinal cord patterning defects. **A,B:** RT-PCR for *dbx1a* (A) and *dbx1b* (B) in morphants show unsplliced products (asterisk in each gel) at a concentration of 7 ng/nl for *dbx1a* and 2.5 ng/nl for *dbx1b*. Both morpholinos were injected simultaneously to produce *dbx1a/1b* loss-of-function embryos. **C,D:** Whole-mount views of a wild-type (C) and *dbx1a/1b* morphant (D) embryo at 24 hours postfertilization (hpf) with rostral to the left. *dbx1a/1b* morphants have a shortened rostral/caudal axis and hindbrain morphology defects. **E-H:** Spinal cord cross-sections of wild-type and *dbx1a/1b* morphants at 24 hpf. **E,F:** The *nkx6.2* expression is expanded dorsally in *dbx1a/1b* morphants compared with wild-type. **G,H:** The *msxc* expression is unaffected in *dbx1a/1b* morphants.

Fig. 7. *dbx1a/1b* knockdown causes postmitotic neuron defects. Postmitotic neuron analysis of *dbx1a/1b* morphants. Lateral whole-mount views of subtype-specific postmitotic marker are shown with rostral to the left at 24 hours postfertilization (hpf). **A-C:** *isl*⁺ motoneurons are unaffected in *dbx1a/1b* morphants. **D-I:** *en1b*⁺ (D-F) and *pax2.1*⁺ (G-I) interneurons are decreased in *dbx1a/1b* morphants. Error bars represent SEM.

Fig. 8.

Summary of Hedgehog regulation and loss-of-function studies. **A:** Effects on spinal cord development after blocking Hedgehog signaling. The *nkx6.2* expression is lost, and *dbx1a/1b* expression expands ventrally. The dorsal boundaries of *dbx1a/1b* and *msxc* expression are unaffected. In addition, postmitotic neurons from the *Dbx* domain are expanded. **B:** Effects on spinal cord development after *dbx1a* and *dbx1b* are knocked down. In morphants, *nkx6.2* expression expands dorsally while the ventral limit of *msxc* expression is unaffected. Also, postmitotic neurons produced from the *Dbx* domain are reduced in morphants.