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Eliminating Zebrafish Pbx Proteins Reveals a Hindbrain Ground State

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Summary

The vertebrate hindbrain is divided into serially homologous segments, the rhombomeres (r). Pbx and Hox proteins are hypothesized to form heterodimeric, DNA binding transcription complexes which specify rhombomere identities. Here, we show that eliminating zebrafish Lzr/Pbx4 and Pbx2 function prevents hindbrain segmentation and causes a wholesale anterior homeotic transformation of r2–r6, to r1 identity. We demonstrate that Pbx proteins interact with Hox paralog group 1 proteins to specify segment identities broadly within the hindbrain, and that this process involves the Pbx:Hox-1-dependent induction of Fgf signals in r4. We propose that in the absence of Pbx function, r2–r6 acquire a homogeneous ground state identity, that of r1, and that Pbx proteins, functioning primarily with their Hox partners, function to modify this ground state identity during normal hindbrain development.

Introduction

During development, the vertebrate hindbrain is transiently divided into a series of seven lineage-restricted compartments, rhombomeres (r) 1–7. The rhombomeres are serially homologous, with each rhombomere having a unique identity that overlies a basic reiterated pattern visible in the patterns of neuronal differentiation (Lumsden and Keynes, 1989). Six morphological constrictions with shared molecular and histological characteristics lie between r1 and r7, while the more posterior "vagal" region of the hindbrain, sometimes referred to as r8, lies outside of the meristic series.

The morphological boundaries between rhombomeres correspond with the boundaries of expression of the Hox homeodomain proteins, which are evolutionarily conserved regulators of segment identity (Wilkinson et al., 1989). Members of *Hox* paralog groups 1–4 (*Hox-1* to *Hox-4* genes) are expressed in overlapping rhombomere-restricted domains with the most anterior *Hox* gene, *Hoxa2*, being expressed up to the r1/r2 boundary. Loss- and gain-of-function analyses have shown that Hox genes control the specification of rhombomere identities. In mouse *Hox* knockouts, individual rhombomeres are frequently lost or partially transformed to more anterior identities, with the most severe phenotypes associated with loss of *Hox-1* genes (*Hoxa1* and *Hoxb1*) which are expressed earliest of all the *Hox* genes to an anterior limit at the r3/r4 boundary (Rossel and Capecchi, 1999; Studer et al., 1998). Other *Hox* single mutant phenotypes within the hindbrain are often subtle due to the combinatorial *Hox* code that characterizes most rhombomeres, and to partial redundancy among *Hox* paralogs (Greer et al., 2000).

Hox proteins cooperate with the Pbx and Meis homeodomain proteins to achieve their DNA binding specificity (reviewed in Mann and Chan, 1996). Paired Pbx/Hox binding sites in the regulatory regions of paralog group 1–4 *Hox* genes are essential for the auto- and crossregulatory interactions that drive hindbrain *Hox* expression (Pöpperl et al., 1995; Nonchev et al., 1997; Gould et al., 1998; Manzanares et al., 2001). For example, r4-restricted expression of *Hoxb1* and *Hoxb2* depends on the direct binding of Hox-1/Pbx complexes to essential Hox/Pbx sites in the regulatory regions of these genes (Pöpperl et al., 1995; Maconochie et al., 1997). Eliminating the zygotic function of the zebrafish *lazarus* (*lzr/pbx4*) gene, which encodes the Pbx family member Lzr/Pbx4, mimics phenotypes associated with loss of individual *Hox* genes in the mouse (Pöpperl et al., 2000). However, in zygotic *lzr/pbx4* mutants, all the rhombomeres are represented, and certain of the more severe mouse *Hox* knockout phenotypes are not observed, suggesting the existence either of compensatory segment specification pathways or of other sources of Pbx activity in *lzr/pbx4* mutants. Vertebrates have multiple highly related Pbx genes, which have been hypothesized to have overlapping functions; however, redundancy between Pbx family members has not been demonstrated in vivo.

Here we show that the zebrafish *pbx* genes *lzr/pbx4* and *pbx2* function in a partially redundant manner in the specification of rhombomere identities in the hindbrain. In embryos lacking both maternal and zygotic *lzr/pbx4* and *pbx2* function, r2–r6 undergo a wholesale transformation to r1 identity. Consistent with this, cells lacking *lzr/pbx4* and *pbx2* function are unable to contribute to r2–r6 in genetic mosaics. We show directly that *lzr/pbx4* interacts genetically with the *hox-1* genes *hoxb1a* and *hoxb1b* to specify r3–r6 identities, and show evidence that this effect is partly mediated by Pbx:Hoxb1b-dependent Fgf signals from r4. We conclude that r1 is a hindbrain ground state identity: the identity conferred in the absence of Pbx activity, upon which Pbx proteins and their partners act to specify segment identities during the development of the vertebrate hindbrain.

Results

Eliminating Pbx Function in the Zebrafish Embryo

We have cloned five zebrafish *pbx* genes, *pbx1, pbx2, pbx3.1, pbx3.2*, and *pbx4* (Figure 1A) (Pöpperl et al., 2000). Of these, only *lzr/pbx4* and *pbx2* are expressed prior to 20 hr postfertilization (hpf) (Kimmel et al., 1995), during the period of hindbrain segmentation and patterning, and both are expressed ubiquitously (data not

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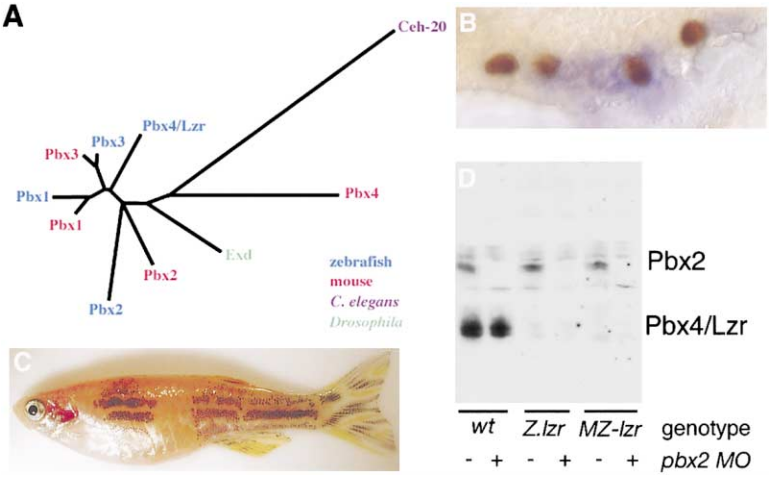

Figure 1. Eliminating Pbx Function in the Zebrafish Embryo

(A) Dendrogram of vertebrate and invertebrate Pbx proteins. Four zebrafish Pbx proteins are shown; a fifth, Pbx3.2 is very similar to Pbx3.1.

(B) Transplanted *lzr/pbx4*$^{-/-}$ blastomeres contribute to the germline in genetic mosaics. Expression of *vasa* (blue staining) in the presumptive germline of a 24 hpf mosaic zebrafish embryo colocalizes with transplanted germ cells (brown lineage label).

(C) An adult mosaic female in which *lzr/pbx4*$^{-/-}$ cells have contributed to pigment cells in an otherwise unpigmented (*nacre*$^{-/-}$) host embryo.

(D) Western blot of 18 hpf zebrafish embryo lysates using a zebrafish pan-Pbx antibody, demonstrating that Pbx proteins are eliminated in MZ*lzr; pbx2* MO embryos. Lanes 1 and 2, wild-type; lanes 3 and 4, Zlzr; lanes 5 and 6, MZlzr; lanes 1, 3 and 5, uninjected; lanes 2, 4, and 6: injected with *pbx2* morpholino. Note that by 18 hpf, maternal Lzr/Pbx4 protein is barely detectable (compare lanes 3 and 4 to lanes 5 and 6).

shown; Pöpperl et al., 2000). *pbx2* is therefore a candidate source of Pbx protein that compensates for loss of *lzr/pbx4* function in the zygotic *lzr/pbx4* mutant. A second source of Pbx function in zygotic *lzr/pbx4* mutants is maternally expressed *lzr/pbx4* mRNA, which persists until 10 hpf and therefore could function during hindbrain patterning (data not shown; Pöpperl et al., 2000).

In order to generate embryos lacking both maternal and zygotic *lzr/pbx4* function, we generated *lzr/pbx4*$^{-/-}$ germline clones by transplanting primordial germ cells from *lzr/pbx4*$^{-/-}$ donor embryos at the blastula stage to *lzr/pbx4*$^{+/+}$ host embryos. Transplanted blastomeres differentiate as primordial germ cells as determined by morphology, position, and *vasa* expression (Figure 1B). Embryos lacking both maternal and zygotic *lzr/pbx4* were generated by crossing the resulting mosaic females (Figure 1C) to *lzr/pbx4*$^{+/-}$ males; these embryos lack Lzr/pbx4 protein (Figure 1D).

To block Pbx2 function, we prevented its translation by injecting either of two overlapping but nonidentical gene-specific morpholinos (MO). This resulted in a 95% reduction in Pbx2 protein levels (Figure 1D; compare lanes 1 and 2), allowing us to estimate that MZ*lzr*; *pbx2 MO* embryos contain $<$1% of total Pbx proteins prior to 24 hpf.

Rhombomere Identities from r2 to r6 Are Lost in Embryos Lacking Pbx Function

We examined rhombomeric marker gene expression in the hindbrain of MZ*lzr*; *pbx2* MO embryos and controls (Figure 2). In wild-type embryos between 13 and 20 hpf, *mariposa* is expressed in rhombomere boundaries, *krox20* is expressed in r3 and r5, *hoxb1a* is expressed in r4, *hoxa2* is expressed at high levels in r2 and r3 and at lower levels in r4 and r5, and *val/mafB* is expressed in r5 and r6. In MZ*lzr* embryos, rhombomere boundaries in the anterior hindbrain are lost, and *krox20* and *hoxa2* expression in r3 is eliminated, as is *hoxa2* expression in r4, while *hoxb1a* expression in r4 is reduced (Figures 2B, 2E, and 2H). These phenotypes are more severe than those seen in zygotic *lzr/pbx4* mutants (Pöpperl et al., 2000 and data not shown). Depleting Pbx2 protein in otherwise wild-type embryos has no effect (data not shown) but strongly enhances the MZ*lzr* phenotype, such that boundary- and rhombomere-specific gene expression from r2 to r6 is entirely eliminated (Figures 2C, 2F, 2I, and 2L). This failure to specify rhombomere identity in the r2–r6 region stems from an early patterning defect, since neither *krox20* nor *val/mafB* expression is initiated in MZ*lzr*; *pbx2* MO embryos (Figures 2M and 2N). Importantly, however, the onset of hindbrain patterning occurs normally, since *hoxb1b* expression posterior to the presumptive r3/r4 boundary is initiated normally in MZ*lzr*; *pbx2* MO gastrula-stage embryos (Figures 2O and 2P).

Cells Lacking Pbx Function Cannot Acquire r2–r6 Identities in Genetic Mosaics

We used genetic mosaic analysis to interrogate cells lacking Pbx function as to their ability to adopt particular rhombomere identities. Wild-type cells and *pbx2 MO*-containing cells contribute throughout the hindbrain in wild-type hosts (Figure 3A). In contrast, cells from zygotic *lzr/pbx4* mutant embryos are specifically excluded from r3 and r5 (Figure 3B). Cells from MZ*lzr* donors are additionally excluded from r2 and r6 (Figure 3C). Finally, cells from MZ*lzr*; *pbx2* MO donors are excluded from a contiguous region from r2 to r6, although they contribute normally throughout r1 and more anterior regions of the brain, and to r7 and more posterior regions (Figure 3D). These observations are consistent with the marker analysis described above and confirm that the specification of hindbrain rhombomeres r2–r6 relies on Pbx function.

An Anterior Transformation to r1 Identity in Embryos Lacking Pbx Function

Since Pbx proteins are required for Hox function, and the most anterior expression of any *hox* gene in the hindbrain, that of *hoxa2*, ends at the boundary between r1 and r2, we asked whether the hindbrain of MZ*lzr*; *pbx2* MO embryos had undergone an anterior homeotic

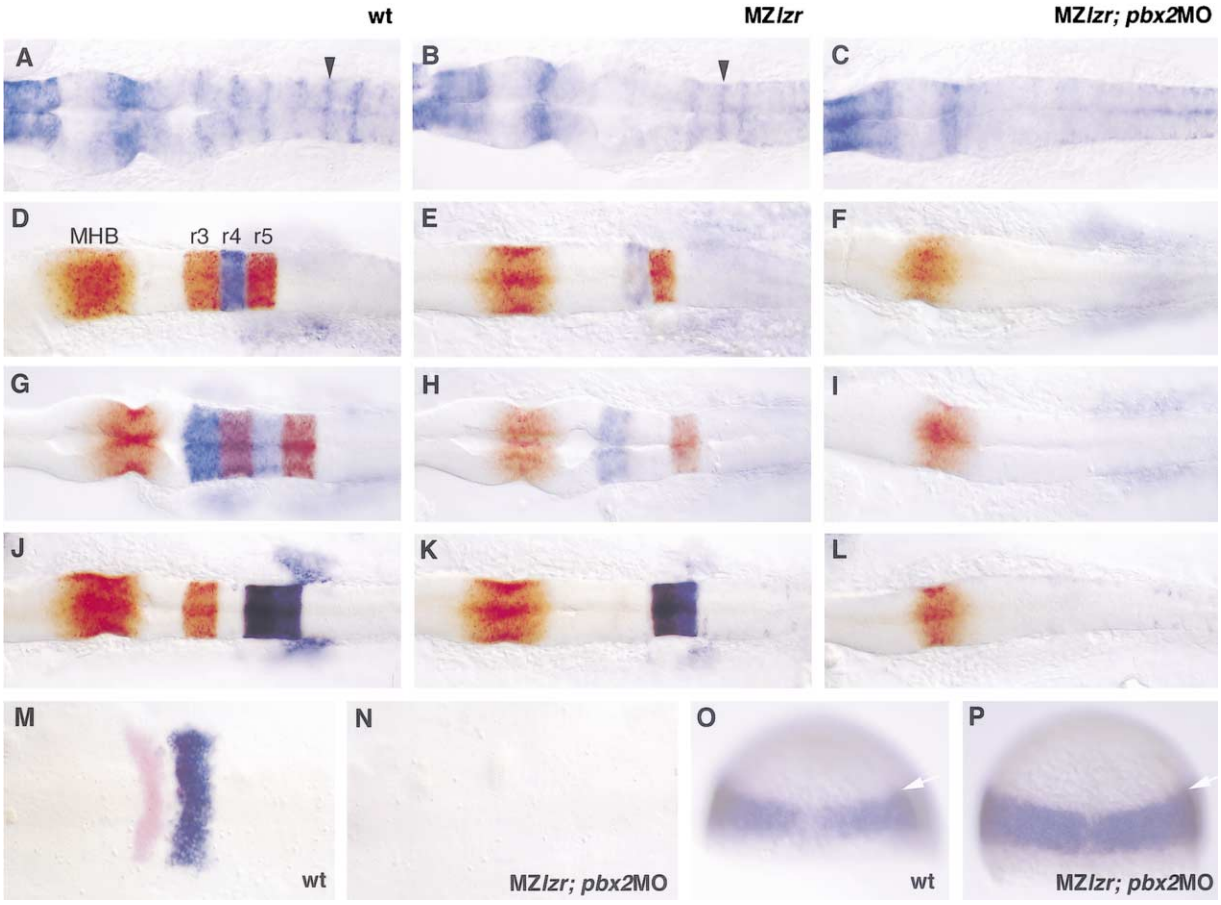

Figure 2. MZ*lzr*; *pbx2* MO Embryos Lack r2–r6 Identities

RNA in situ hybridizations of wild-type, MZ*lzr*, and MZ*lzr; pbx2* MO embryos, as shown.

(A–C) *mariposa* expression (blue) in rhombomere boundaries is lost in the anterior hindbrain in MZ*lzr* and throughout the hindbrain in MZ*lzr; pbx2 MO* embryos. The arrowhead indicates the r5/r6 boundary.

(D–F) *hoxb1a* expression in r4 (blue) is reduced in MZ*lzr* embryos and is eliminated in MZ*lzr; pbx2* MO embryos.

(G–I) *hoxa2* expression, which in wild-type embryos is expressed from r2 to r5, is limited to r2 in MZ*lzr* embryos and is entirely eliminated in MZ*lzr; pbx2* MO embryos.

(J–L) *val*/mafB expression in r5 and r6 is slightly narrowed in MZlzr embryos, and is also eliminated in MZ*lzr; pbx2* MO embryos. Embryos in (A)–(L) are at 18–20 hpf. *krox20* expression in r3 and r5, and *eng3* expression surrounding the mid-hindbrain boundary (MHB) are shown in red in (D)–(L).

(M and N) Expression of *krox20* and *val*/*mafB* is never initiated in MZ*lzr; pbx2* MO embryos (shown here at 11 hpf).

(O and P) In contrast, expression of *hoxb1b* is initiated normally up to the r3/r4 boundary (arrows) in wild-type and MZ*lzr; pbx2* MO gastrulae, which are shown here at approximately 8 hpf. All embryos are in dorsal views, with anterior to the left in (A)–(N) and to the top in (O) and (P).

transformation to r1 identity. Rhombomere 1 is commonly described as the large region that extends from the midbrain-hindbrain boundary to r2. This region has an internally complex anterior-posterior organization: anteriorly, it expresses markers that extend across the midbrain-hindbrain boundary, such as *eng3* (Figure 2), and contains the neurons of the locus coeruleus and the cell bodies of the trochlear nerve; posteriorly, it expresses *fgfr3* and *ephA4a* (Figures 4A and 4D) (Sleptsova-Friedrich et al., 2001; Xu et al., 1996). Henceforth, we use the terminology of Vaage (1969) (see Discussion), referring to the *fgfr3* and *ephA4*-expressing region as r1 and the anterior region between r1 and the isthmus as r0.

We examined the expression of r0 and r1 markers in MZ*lzr*; *pbx2* MO embryos and controls. At 20 hpf, *ephA4a* is expressed weakly in r1, and strongly in r3 and r5 where it is a direct target of *krox20* (Figure 4A) (Theil et al., 1998). Progressively eliminating Pbx function eliminates the r3 and r5 domains and at the same time causes a posterior expansion of the r1 domain of *ephA4* (Figures 4A–4C). In MZ*lzr*; *pbx2* MO embryos, r1-specific *ephA4a* expression is expanded posteriorly to meet the diffuse anterior limit of *hoxb4* expression, which normally marks the r6/r7 boundary (Figure 4C). Analysis of *fgfr3*, which at 20 hpf in wild-type embryos is expressed throughout the hindbrain but at higher levels in r1, showed much the same effects. In either MZ*lzr* or MZ*lzr*; *pbx2* MO embryos, the higher-level expression domain of *fgfr3* is expanded posteriorly throughout the

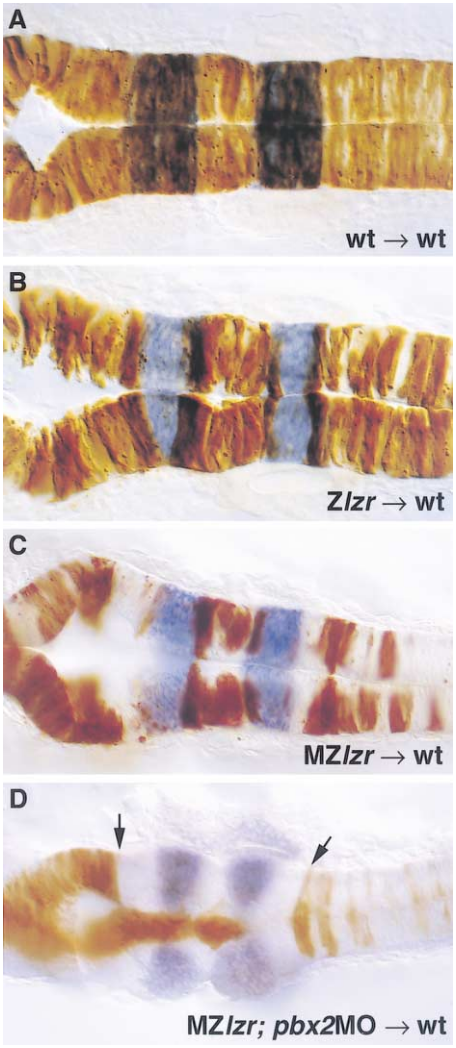

Figure 3. Cells Lacking Pbx Function Cannot Acquire r2–r6 Identities in Genetic Mosaics

(A) Lineage-labeled cells (visualized as brown staining) transplanted from a wild-type or *pbx2* MO-injected donor embryo into the presumptive hindbrain of a wild-type host embryo at the early gastrula stage (6 hpf) contribute throughout the hindbrain at 20 hpf.

(B) Donor cells lacking zyotic *lzr*/*pbx4* (brown) are excluded from r3 and r5 (indicated by *krox20* expression in blue).

(C) MZ*lzr* donor cells (brown) are further excluded from r2 and r6 (blue staining of *krox20* and neighboring segments).

(D) Finally, MZ*lzr; pbx2* MO donor-derived cells (brown) are excluded from the entire r2–r6 region (entire region surrounding blue staining of *krox20*). Excluded cells accumulate dorsally, sometimes deforming the host hindbrain. These cells are visible as out-of-focus brown staining above the host rhombomeres on the midline of the embryo shown in (D). Arrows indicate the r1/r2 and r6/r7 boundaries. All embryos are in dorsal view with anterior to the left.

hindbrain (Figures 4D–4F). Analysis of *eng3* in r0 demonstrates that r0 and r1 markers are not regulated in parallel. We note no posterior expansion of *eng3* expression (Figure 2) and conclude that r1 is the identity conferred in the absence of Pbx function.

Although the effects of eliminating Pbx function are most dramatic in the segmented region of the hindbrain, we do observe effects elsewhere. The posterior midbrain expression domains of *mariposa* and *en3* are narrowed, and the diencephalic expression domains of *ephA4* and *fgfr3* are shifted posteriorly, consistent with a role for zebrafish Pbx genes in midbrain patterning.

Homogeneous Hindbrain Neuron Identity in Embryos Lacking Pbx Function

We examined hindbrain neuronal markers in MZ*lzr*; *pbx2* MO embryos and controls. Neurons specific to r0, which include the neurons of the locus coeruleus that express *phox2a* (Guo et al., 1999) and the motor neurons of the trochlear cranial nerve (n)IV that express an *isl1*-GFP transgene (Higashijima et al., 2000) are unaffected in MZ*lzr*; *pbx2* MO embryos (Figures 5A–5D). Vagal (nX) motor neurons in the posterior hindbrain (posterior to r7) are also present, although reduced in number and disorganized (Figures 5C and 5D). In contrast, trigeminal (nV) and facial (nVII) motor neurons characteristic of r2–r6 are strongly reduced, and the few remaining neurons have unfasiculated, disorganized axons (Figure 5D, asterisks). Furthermore, *phox2a*-expressing cells which are likely to be the nascent reticulospinal neurons in r4–r6 (Guo et al., 1999) are absent at 18 hpf in MZ*lzr*; *pbx2* MO embryos (Figures 5A and 5B).

We note that the reduction in motor neurons in MZ*lzr*; *pbx2* MO embryos is less prominent at 28 hpf (compare Figure 5D to 5F). At 28 hpf in wild-type embryos, a small number of *isl1*-GFP-expressing neurons with unknown identities are present in r1 in addition to nV motor neurons in r2 and nVII motor neurons migrating through r5 (asterisks in Figure 5E) (Higashijima et al., 2000). These r1 neurons are less frequently detected at 48 hpf. It is possible that some of the *isl1*-GFP-expressing neurons in the transformed hindbrain of MZ*lzr*; *pbx2* MO have this r1 identity and are either eliminated or turn off *isl1* expression by 48 hpf.

In contrast to the reduction in branchiomotor and reticulospinal neurons, a group of neurons characteristic of r1–r3 that express an Engrailed antigen at 28 hpf (Hatta et al., 1991) (arrowheads in Figure 5E) are increased in number by 2- to 3-fold and are spread homogeneously throughout the transformed hindbrain of MZ*lzr*; *pbx2* MO embryos (Figure 5F). The precise rhombomere identity of this population of Engrailed-expressing cells, like that of the motor neurons in MZ*lzr*; *pbx2* MO embryos, is difficult to ascertain in the absence of independent markers that distinguish individual motor nuclei or interneurons. However their lack of recognizable segmental characteristics is consistent with a homogenization of hindbrain identity, while the loss of posterior neurons and the expansion of anterior neurons are consistent with a transformation to an anterior rhombomere identity.

Elucidating a Pathway for Hindbrain Patterning Downstream of Pbx

Our observation that Pbx proteins are required for the initiation of *krox20* and *val/mafB* expression was unexpected, given that Pbx is a Hox partner, and *Hox* genes, specifically the *Hox-2* and *Hox-3* paralogs, are known to be downstream targets of Krox20 and Val/Kr/MafB

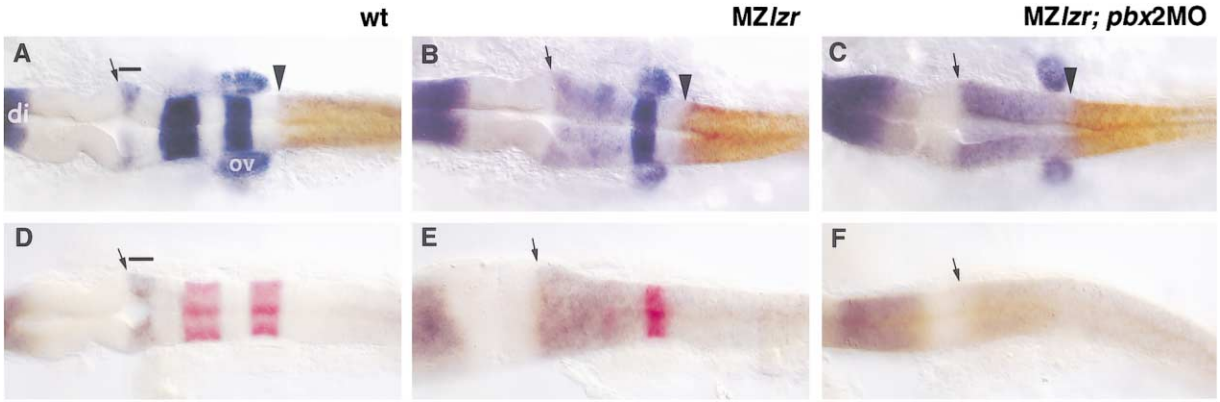

Figure 4. r2–r6 Are Transformed to r1 Identity in Embryos Lacking Pbx Function

RNA in situ hybridizations with *ephA4a* (blue) and *hoxb4* (orange; [A–C]) and *fgfr3* (blue) and *krox20* (pink; [D–F]). *ephA4a* and *fgfr3* are also expressed in the diencephalon (di), and *ephA4a* is furthermore expressed in r3 and r5, and in the otic vesicles (ov). (A and D) In wild-type embryos at 20 hpf, r1 is the narrow segment immediately anterior to r2 which expresses *ephA4a* and *fgfr3*, indicated by black bars. (B and E) In MZ*lzr* embryos, r3 is absent and r1 markers are expanded posteriorly. This extended domain of *ephA4a* expression meets the anterior limit of *hoxb4* expression, which normally marks the r6/r7 boundary. (C and F) In MZ*lzr; pbx2* MO embryos, r1 markers are expanded throughout the hindbrain while all other markers of r2–r6 identity are lost. The diencephalic expression domains of *ephA4a* and *fgfr3* are also shifted posteriorly. All embryos are in dorsal view with anterior to the left. Arrows indicate the r0/r1 boundary; arrowheads indicate the anterior limit of *hoxb4* expression at the anterior boundary of r7.

rather than their upstream regulators (Manzanares et al., 2002; Sham et al., 1993; Nonchev et al., 1996). We asked whether Pbx could interact with the earliest expressed Hox proteins, Hoxb1a and Hoxb1b, the functional counterparts of mouse Hoxb1 and Hoxa1 (McClintock et al., 2002), to specify rhombomere identities not only in r4 where these proteins are expressed but more widely in the hindbrain. Reducing Hoxb1a and Hoxb1b function using antisense morpholinos in wild-type embryos has a subtle but reproducible effect on hindbrain patterning, causing a narrowing of r4 and r5 and a compensatory expansion of r3 (Figures 6B and 6F) (McClintock et al., 2002). Elimination of zygotic *lzr/pbx4* strongly reduces r3, and to a lesser extent r4, while r5 and r6 are only slightly narrowed (Figures 6C and 6G) (Pöpperl et al., 2000). In contrast, r3–r6 are absent or very strongly reduced in zygotic *lzr/pbx4* embryos injected with *hoxb1a* and *hoxb1b* morpholinos (Figures 6D and 6H). Thus, reducing *pbx* function in zebrafish embryos reveals a requirement for Hox group 1 paralogs in the specification not only of r4 but also of r3, r5, and r6.

How do zebrafish *pbx* genes, working together with *hox-1* paralogs, contribute to the specification of r3–r6? Expression of zebrafish *val/mafB* in r5 and r6 is dependent on Fgf3 and Fgf8 signals from r4 (Maves et al., 2002; Walshe et al., 2002), so we examined *fgf3* and *fgf8* expression in MZ*lzr; pbx2 MO* embryos during early somite stages, to determine where *pbx* genes function in the genetic hierarchy specifying r5 and r6 identities. We observed that r4-specific upregulation of *fgf3* and *fgf8* expression is lost in MZ*lzr; pbx2 MO* embryos, although low-level *fgf8* expression persists throughout the transformed hindbrain (Figures 6I–6L).

We asked whether in wild-type embryos, this r4-restricted, Pbx-dependent upregulation of *fgf* expression is also dependent on Hox group 1 proteins. We found that overexpression of *hoxb1b* mRNA in wild-type embryos is sufficient to drive ectopic *fgf3* anterior to its normal transient domain of expression (Figures 6M and 6N). Furthermore, r4-specific *fgf3* expression is reduced in embryos injected with *hoxb1a* and *hoxb1b* morpholinos (Figures 6O and 6P), indicating that *fgf3* is responsive to *hoxb1b* and suggesting that *fgfs* are direct or indirect targets of *hox-1* genes in r4.

The homeodomain protein *vhnf1,* which is expressed in the hindbrain posterior to r4, lies genetically upstream of *val/mafB* in the zebrafish (Sun and Hopkins, 2001). We found that *vhnf1* expression is strongly reduced in MZ*lzr; pbx2 MO* embryos (Figures 6Q and 6R), consistent with a critical role for *Pbx* genes in the establishment of both the r4-derived signals that pattern r5 and r6 and the transcription factors that specify r5 and r6 identities in a cell-autonomous manner.

Discussion

In this work we address the function of *Pbx* genes during early development by generating embryos that lack Pbx protein during the first 24 hr of zebrafish development. We find that while the initial step in hindbrain regionalization, the onset of *hoxb1b* expression up to the r3/r4 boundary, takes place normally, all subsequent pattern within the non-vagal region of the hindbrain (r2–r6) fails to develop, and the entire region is transformed to r1 identity. From these findings, we conclude that Pbx proteins are essential for establishing r2–r6 identities, and that r1 is a hindbrain "ground state" identity. Our genetic mosaic analysis strongly supports this interpretation, since Pbx-deficient cells cannot contribute to any wild-type rhombomere from r2–r6.

Interactions between members of the vertebrate Hox and Pbx homeodomain protein families are essential for Hox function both during embryogenesis and hematopoeisis. In the developing vertebrate hindbrain, bipartite Hox/Pbx binding sites in the regulatory regions of

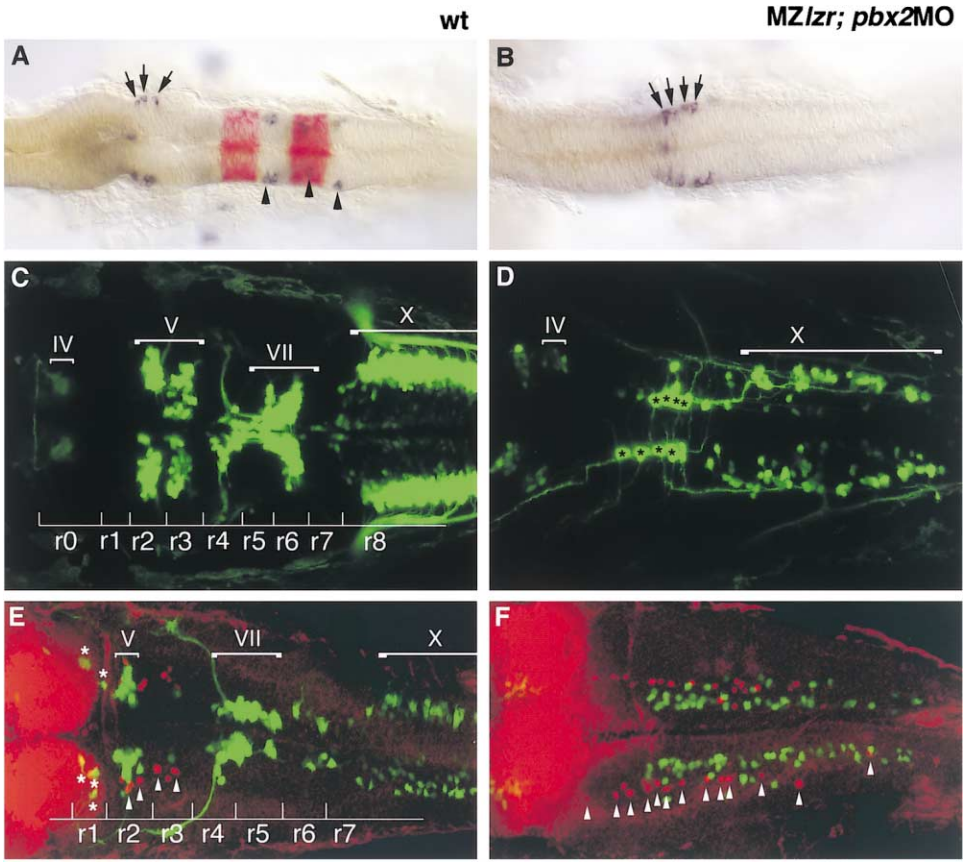

Figure 5. Hindbrain Neuron Identity Is Homogenized in Embryos Lacking Pbx Function

(A, C, and E) Wild-type embryos; (B, D, and F) MZ*lzr; pbx2* MO embryos.

(A and B) RNA in situ hybridizations with *phox2a* (blue) and *krox20* (red) at 20 hpf showing that the neurons of the locus coeruleus, which differentiate in r0 (arrows), are unaffected in MZ*lzr; pbx2* MO embryos. Arrowheads indicate hindbrain *phox2a*-expressing cells in r4–r6 which are absent in MZ*lzr; pbx2* MO embryos.

(C) Cranial motor neurons (*isl1*-GFP expressing, green) are segmentally organized in 48 hpf wild-type embryos. The motor nuclei of cranial nerves IV (trochlear, in r0), V (trigeminal, in r2 and r3), VII (facial, primarily in r6), and X (vagal, poterior to r7) are indicated. Rhombomeres are also indicated (r).

(D) In MZ*lzr*; *pbx2* MO embryos, nIV are normal and nX neurons are present but reduced; however, very few motor neurons are detected in the intervening region (asterisks).

(E) Overlay of cranial motor neurons (*isl1*-GFP, green) and Engrailed staining (4D9 antibody) at 28 hpf. Motor neurons of cranial nerves are indicated as in (A) and (B). At this earlier stage, nIV neurons are not yet detectable, but a set of unidentified motor neurons are present in r1 (white asterisks; Higashijima et al., 2000). Eng-expressing neurons differentiate in r1–r3 (red staining; white arrowheads; Hatta et al., 1991). In this dorsal view, the r1 neurons are ventral to the broad Eng domain that extends through r0, and so in this confocal projection, they are not distinguishable.

(F) In MZ*lzr; pbx2* MO embryos, *isl1*-GFP and Eng-expressing neurons are found homogeneously throughout the transformed region of the hindbrain (white arrowheads). All embryos are in dorsal view with anterior to the left.

Hox genes are essential for the auto-, para-, and cross-regulatory interactions that contribute to the elaboration of segment-restricted Hox gene expression (Pöpperl et al., 1995; Maconochie et al., 1997; Manzanares et al., 2001; Gould et al., 1998). Furthermore, zebrafish *lzr/pbx4* is essential for *hox* gene function in *hox* overexpression experiments (Pöpperl et al., 2000; K.L. Cooper and C.B.M., unpublished data).

Below, we describe a model for how zebrafish *pbx* genes, interacting primarily with paralog group 1 and 2 *hox* genes, specify regional identities throughout all but the most posterior hindbrain. However, we note that Pbx and Hox proteins can and do function independently of one another in some contexts. In *Drosophila*, repression of *sal* expression in the haltere by Ubx is independent of Exd (Galant et al., 2002), while in vertebrates, reporter expression driven by a Hoxb4 autoregulatory element is only weakly dependent on Pbx (Gould et al., 1998). Conversely, functions for Pbx proteins have been described that involve interactions with the ParaHox protein Pdx1 (Dutta et al., 2001) or the non-Hox proteins Eng (Peltenburg and Murre, 1996) and MyoD (Knoepfler et al., 1999). Indeed, a marked shortening of the midbrain we observe in MZ*lzr; pbx2 MO* embryos suggests that zebrafish Pbx proteins have functions that are independent of Hox proteins. While it is unlikely that disrupting the functions of known non-Hox Pbx partners is responsible for the transformed hindbrain phenotype of MZ*lzr; pbx2 MO* embryos, we cannot rule out the possibility that other, as yet unidentified Pbx partners contribute to the anterior homeotic transformation we observe in MZ*lzr; pbx2 MO* embryos.

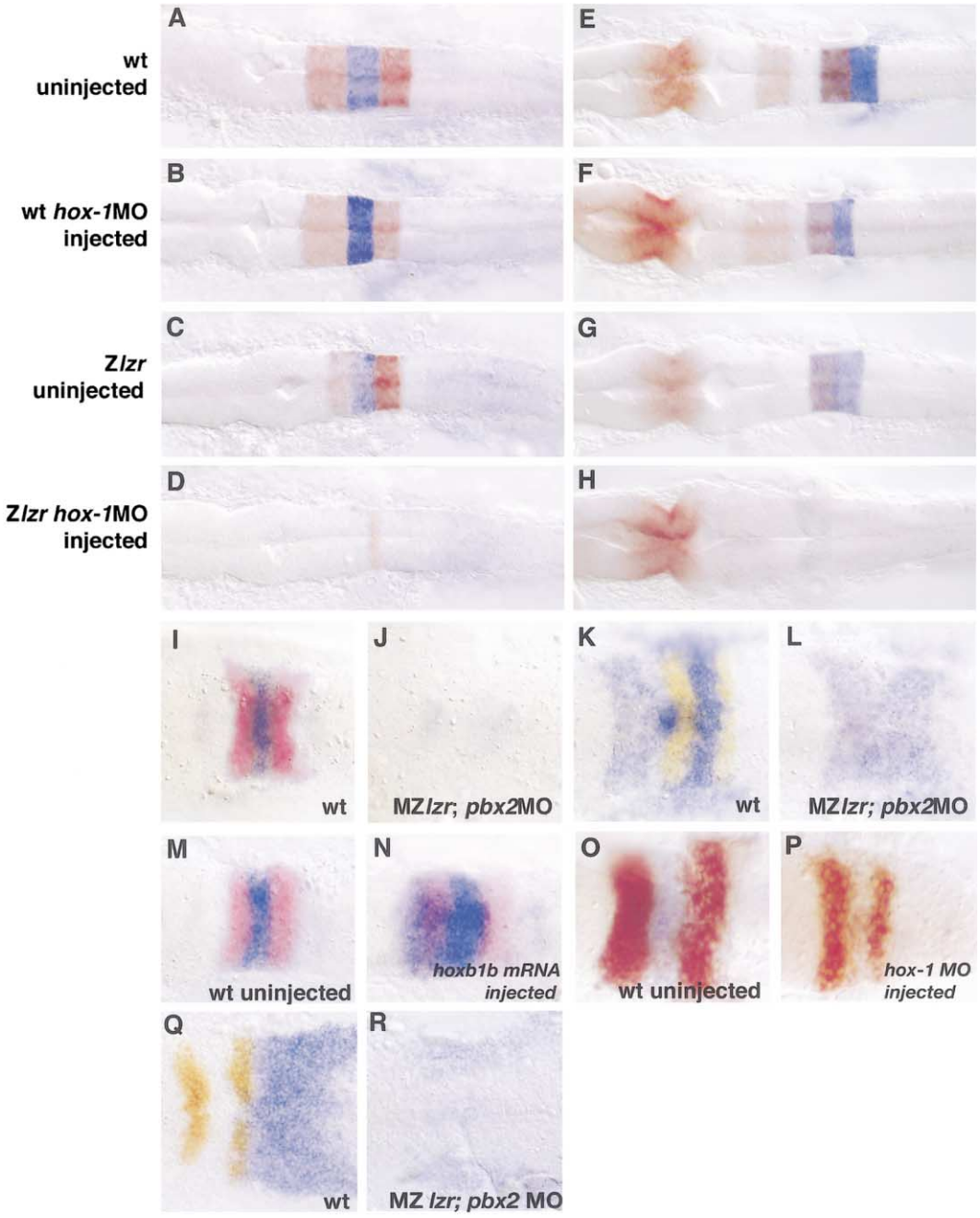

Figure 6. *Pbx* Interacts with *Hox-1* Genes to Pattern the Hindbrain

(A–H) *Pbx* and *Hox-1* genes synergize in the specification of r3–r6 identities. Shown are expression of *hoxb1a* (blue staining in r4 in [A]–[D]) and *val/mafB* (blue staining in r5 and r6 in [E]–[H]). *krox20* in r3 and r5 in (A)–(H) and *eng3* surrounding the mid-hindbrain junction in (E)–(H) are in red. Wild-type embryos (A, B, E, and F) and zygotic *lzr/pbx4*$^{-/-}$ embryos (C, D, G, and H) were uninjected (A, C, E, and G) or injected with *hoxb1a* and *hoxb1b* gene-specific morpholinos (B, D, F, and H). While hindbrain patterning is only mildly affected in *Zlzr* and wild-type, *hox-1* MO-injected embryos, expression of r3–r6 markers are strongly reduced or eliminated in *Zlzr*; *hox-1* MO embryos.

(I–P) We examined expression of *fgf3/8* that is shown in blue, while *krox20* is in red/orange to demarcate the positions of r3 and r5 in each panel. (I and J) Expression of *fgf3* in r4 at 11.5 hpf in wild-type (I) is lost in MZ*lzr; pbx2* MO (J) embryos. (K and L) Expression of *fgf8* (blue staining) is transiently upregulated in r4 during early somite stages (11 hpf) in wild-type (K) but not MZ*lzr; pbx2* MO embryos (L). (M and N) *fgf3* expression (blue staining) which is normally restricted to r4 at 11.5 hpf (M) is expanded in wild-type embryos injected with *hoxb1b* mRNA (N). (O and P) *fgf3* expression in r4 is reduced in 11 hpf embryos injected with *hoxb1a* and *hoxb1b* morpholinos. (Q and R) *vhnf1* is expressed posterior to the r4/r5 boundary in wild-type embryos (Q); this expression is strongly reduced in MZ*lzr; pbx2* MO embryos (R).

Specification of Rhombomere Identities by Pbx

Interactions between Pbx and Hox-1 paralogs (*hoxa1* and *hoxb1* in the mouse, *hoxb1a* and *hoxb1b* in the zebrafish) directly specify r4 identity via essential Hox-1/Pbx binding sites in the regulatory regions of target genes (Pöpperl et al., 1995; reviewed in Nonchev et al.,

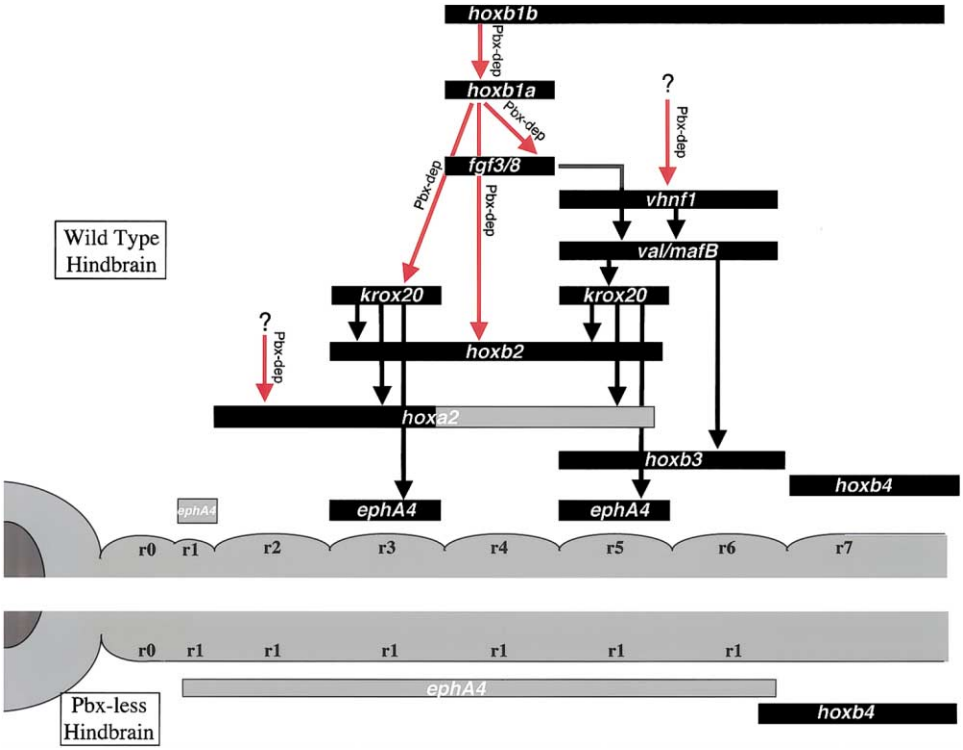

Figure 7. A Proposed Model of Pbx-Dependent Events in Zebrafish Hindbrain Patterning

Expression domains are denoted by rectangles, with lighter shading corresponding to lower levels of expression. We have labeled Pbx-dependent events with red arrows. Known regulatory interactions which are independent of Pbx function are labeled with black arrows. In temporal order, from top to bottom, *hoxb1b* (the functional counterpart of mouse *Hoxa1*) is expressed up to the presumptive r3/r4 border and is required for the upregulation of *hoxb1a* (the functional counterpart of mouse *Hoxb1*) in r4. *Hox-1* paralogs cooperate with Pbx proteins to upregulate expression of *fgf3* and *fgf8* in r4, which in turn induce expression of *val/Kr* in r5/r6. *Hox-1:Pbx* interactions also contribute to r3 specification, possibly also through induction of Fgf signaling in r4. The expression of *hoxa2* in r2 and *vhnf1* in r5/r6 are also both dependent on Pbx function; however, the partners with which Pbx interacts in these regulatory events remain obscure (denoted by question mark). In the absence of Pbx proteins, r1 expression of *ephA4a* is expanded posteriorly to a point beyond the otic vesicle, meeting that of *hoxb4*, and the hindbrain reverts to a "ground state identity" as shown in the bottom of the schematic.

1997), and in mouse *Hoxa1*$^{-/-}$*;b1*$^{-/-}$ double mutants, r4 identity is not specified (Rossel and Capecchi, 1999; Studer et al., 1998). In the zebrafish, morpholino knockdown of *hoxb1a* and *hoxb1b* do not prevent r4 specification (Figure 6) (McClintock et al., 2002); however, our results argue that this is likely due to the failure of the morpholinos to completely eliminate gene function rather than due to a basic difference in the mechanism of *hox-1* function in mouse and zebrafish, since we have found that reducing *hoxb1a* and *hoxb1b* function in a zygotic *lzr/pbx4* background prevents r4 specification (Figure 6). Furthermore, reducing *pbx* activity uncovers a role for *hox-1* genes in specifying pattern more broadly in the hindbrain, since *krox20* expression in r3 and r5 and *val/mafB* expression in r5 and r6 are also virtually eliminated in *Zlzr; hox-1* MO-injected embryos.

Our observations suggest that Hox-1 proteins, together with their Pbx partners, function at the top of the hindbrain patterning hierarchy, possibly specifying rhombomere identities through the regulation of signals derived from r4. Indeed, previous work in the mouse demonstrated that *hoxa1*, which is never expressed in r3, nevertheless contributes non-cell autonomously to its specification (Helmbacher et al., 1998). Recently, *fgf3* and *fgf8* were shown to be expressed in r4 in zebrafish and to be required for the specification of r5 and r6 (Maves et al., 2002; Walshe et al., 2002). We have found that in zebrafish embryos lacking *pbx2* and *pbx4* function, upregulation of Fgf signals (Fgf3 and Fgf8) in r4 fails to occur (Figure 6). We also show that *hox-1* genes are both required and sufficient to drive *fgf3* expression in r4. These observations, together with previous work in the zebrafish and mouse, lead us to a model for hindbrain patterning in the zebrafish (Figure 7). In this model, Pbx interacts with Hox-1 genes to specify r4 identity including the upregulation of *hoxb1a, hoxa2*, *fgf3*, and *fgf8*. Fgf3 and Fgf8 in turn signal to the surrounding rhombomeres, contributing to the establishment of r3, r5, and r6 identity.

It is important to note, however, that Pbx function in hindbrain development is not limited to driving the expression of r4-derived patterning signals. If this were the case, mutant cells would be able to acquire r2–r6 identities in a wild-type host. However, our mosaic analysis demonstrates unambiguously that Pbx is required cell autonomously in r2–r6 since mutant cells cannot contribute to, and as a result are excluded from, these rhombomeres in a wild-type host. Thus we propose that Pbx is required at multiple levels of the hindbrain patterning hierarchy, first with Hox-1 proteins in r4 but then

subsequently with other Hox or as yet unidentified non-Hox partners to specify aspects of r2–r6 identity in a cell-autonomous manner. Consistent with this, Hox-3 expression in r5 and r6 has been shown to be maintained by a Pbx-dependent autoregulatory loop (Manzanares et al., 2001), and Hoxa2, which requires Pbx function in zebrafish (K.L. Cooper and C.B.M., unpublished data) has been shown to contribute to r2 and r3 specification in the mouse (Gavalas et al., 1997; Barrow et al., 2000).

Pbx Gene Function in Zebrafish and Mice

Four Pbx genes have been cloned in mice and five in zebrafish. In the mouse, *Pbx1, Pbx2*, and *Pbx3* are all expressed during hindbrain patterning (H. Pöpperl, personal communication), and *Pbx1* mutants have late embryonic pharyngeal arch malformations, severe anemia, and pancreatic hypoplasia that, although consistent with a role in mediating either *Hox* or *Pdx1* gene function, do not reveal the breadth of Hox function in embryonic hindbrain patterning (Selleri et al., 2001). In zebrafish, only *pbx2* and *pbx4* are expressed ubiquitously during the first 24 hr of development, while *pbx1* and *pbx3* are expressed only after 24 hr and in cell-type-restricted patterns (H. Pöpperl, A.J.W., and C.B.M., unpublished data; Pöpperl et al., 2000). We have observed that the four zebrafish *pbx* genes are functionally equivalent in that each can efficiently rescue the *lzr/pbx4* mutant phenotype when overexpressed (data not shown). It seems likely that two or more of the mouse *Pbx* genes may be similarly redundant and that disruption of multiple mouse *Pbx* genes would be expected to approximate the phenotype that we describe here.

Pbx-Independent Events in Zebrafish Hindbrain Development: the Vagal Hindbrain

In contrast to the strong effects we see on patterning in r2–r6 in MZ*lzr*; *pbx2 MO* embryos, patterning of the posterior-most vagal region of the hindbrain is relatively mildly affected. The anterior limit of *hoxb4* expression is diffuse; however, levels of *hoxb4* expression are normal. Vagal motor neurons characteristic of the posterior hindbrain are also present, although reduced in number. Most strikingly, cells lacking Pbx function are able to contribute normally to the hindbrain posterior to the r6/r7 boundary, suggesting that they are able to acquire the cell surface characteristics of the posterior-most hindbrain. Our observation that posterior hindbrain identities are less strongly affected by loss of Pbx function is unexpected given that the Hox genes that are expressed in this region are strongly auto- and crossregulated, and the required autoregulatory elements contain essential Pbx:Hox binding sites (Gould et al., 1998). However, we note that in a cross-species reporter assay designed to test the Pbx dependence of such a Hoxb4 autoregulatory element, reporter expression was only partially suppressed in Exd mutant flies (Gould et al., 1998), suggesting that Hox-4 may be partially Pbx independent in vertebrates. Other critical Hox-4 targets may be similarly Pbx independent. Another explanation is that aspects of posterior hindbrain patterning may not be strictly dependent on Hox function. The vagal hindbrain is outside of the meristic series, forming a nonsegmented transition zone between the segmental region of the hindbrain and the spinal cord. The robust ability of MZ*lzr*; *pbx2 MO* cells to contribute to this region in genetic mosaics may reflect a reduced importance of cell sorting for the maintenance of identity in this region of the hindbrain.

The Hindbrain Ground State

In this work, we have made an important distinction between r1 and r0, with r1 being the narrow, *ephA4a, fgfr3*-expressing domain immediately anterior to r2, and r0 being the larger *engrailed*-expressing domain between r1 and the mid-hindbrain junction. A similar distinction was previously hypothesized based on morphological criteria in the chick (Vaage, 1969), and more recent molecular analysis has suggested that the r1-r0 distinction may indeed be a common feature of vertebrate embryos since *fgfr3* is expressed in a similar domain in the chick (Walshe and Mason, 2000). Importantly, r1 is the posterior-most region of the neural tube which does not express any *hox* genes; however, it is competent to respond to Hox-encoded patterning information (Jungbluth et al., 1999). Taken together with the strong evidence presented here and by others for physical and genetic interactions between Pbx and Hox proteins, we propose that the primary function of Pbx genes during hindbrain patterning is to facilitate Hox function, and that r1 is a hindbrain ground state: the default fate established in the absence of Pbx:Hox activity.

How is r1 established? The inference from this work is that the hindbrain ground state identity presages, and is modified by, Hox gene function. However, our r1 markers *ephA4a* and *fgfr3* are expressed relatively late during hindbrain development. The identification of earlier r1 markers will allow us to better understand the timing and mechanism of r1 development. If the posterior limit of r1 is defined by Hox gene expression, recent work has suggested that its anterior boundary is positioned in response to signals from the mid-hindbrain junction. In *ace/fgf8* zebrafish mutants, r0 is lost together with its resident neurons, while r1 is expanded anteriorly (Guo et al., 1999; Sleptsova-Friedrich et al., 2001).

What is neuronal architecture of the ground state rhombomere? The transformed hindbrain of MZ*lzr; pbx2* MO embryos contains a homogeneous array of *isl1*-expressing motor neurons and Eng-expressing neurons. *Isl1*-GFP-expressing motor neurons and Eng-expressing neurons are present in r1 in wild-type embryos, so the pattern we see in MZ*lzr; pbx2* MO embryos is not inconsistent with the transformation to r1 identity we see based on marker gene expression and genetic mosaic analysis. However, we are unable to unambiguously assign specific rhombomere identities to the neurons in the transformed hindbrain of MZ*lzr; pbx2* MO embryos.

What is the fate of the ground state rhombomere? Recent lineage analysis of neuroepithelial cell fates in the anterior hindbrain of the zebrafish embryo identified distinct fates for cells derived from the upper rhombic lip and lower rhombic lip territories (Koster and Fraser, 2001). Analysis of *ephA4a* expression at later stages suggests that r1 corresponds with the anterior-most limit of the lower rhombic lip, while r0 corresponds with the upper rhombic lip (data not shown). Although further

lineage analysis from earlier stages is required, these results suggest that r1 and r0 indeed have distinct fates within the developing brain.

Conclusions

We have shown that the zebrafish *pbx* genes *lzr/pbx4* and *pbx2* are essential for the specification of rhombomere identities in the hindbrain. In embryos lacking *lzr/pbx4* and *pbx2* function, r2–r6 are transformed to r1 identity. Consistent with this, cells lacking *lzr/pbx4* and *pbx2* function are unable to contribute to r2–r6 in genetic mosaics. We have shown directly that *lzr/pbx4* interacts genetically with the *hox* paralog group 1 genes *hoxb1a* and *hoxb1b* to specify r3–r6 identities, and we show evidence that this effect is partly mediated by Pbx:Hoxb1b-dependent Fgf signals from r4. Whether interactions between Pbx and other Hox or non-Hox partners contribute to rhombomere specification, and in particular to the specification of r2, remain to be determined. We conclude that r1 is a hindbrain ground state identity: the identity conferred in the absence of Pbx:Hox activity. In so doing, we resurrect an important distinction between r1, the ground state rhombomere, and r0, the more anterior "isthmic" rhombomere originally described by Vaage (1969). Future investigations should reveal whether r1, like r2–r6, constitutes a developmental compartment with distinct fates in the developing brain.

Experimental Procedures

cDNA Cloning, mRNA Overexpression, and Western Blotting

We cloned three zebrafish *pbx* genes (*pbx3.1*, *pbx3.2*, and *pbx1*) by degenerate PCR. *pbx2* was identified in the zebrafish EST database. All of these were identical to zebrafish *pbx* genes cloned by Dr. H. Pöpperl (personal communication). *hoxb1b*, *pbx2*, and control *lzr/pbx4* mRNAs were transcribed according to manufacturer's specifications (Ambion) and injected into one-cell stage embryos derived from a *lzr/pbx4*$^{+/-}$ intercross. mRNA concentration was determined by gel electrophoresis and diluted to 100 ng/μl. Rescue of *lzr/pbx4* phenotype was determined by *krox20* in situ hybridization and subsequent genotyping. Western blotting was performed as described (Waskiewicz et al., 2001).

Germline Mosaics

Mosaic females containing *lzr/pbx4*$^{-/-}$ germline clones were generated by isochronic transplantation of rhodamine-dextran labeled blastomeres from 4 to 5 hpf donor embryos derived from a *lzr/pbx4*$^{+/-}$ *x lzr/pbx4*$^{+/-}$ intercross to unpigmented *lzr/pbx4*$^{+/+}$*; nacre*$^{-/-}$ host embryos. 18% (17/96) of mosaic females had greater than 1% contribution of *lzr/pbx4*$^{-/-}$ cells to their germline, and of these, 8 had greater than 20% contribution, as determined by the proportion of pigmented offspring. Prescreening for the presence of donor-derived cells with characteristic primordial germ cell (PGC) morphology and position increased the frequency of germline transmission (37% [7/19] PGC^{+} mosaics were germline). Injection of a nanos morpholino (Koprunner et al., 2001) into the host embryo, intended to reduce the contribution of host cells to the germline, increased the proportion of donor-derived cells in the germline (average of 36% in nanos MO-injected germline mosaics versus 10% in uninjected germline mosaics).

Morpholino Injections

To eliminate both *lzr/pbx4* and *pbx2* function, embryos from natural matings between germline mosaic females and *lzr/pbx4*$^{+/-}$ males were injected with 0.5 ng of either of two *pbx2* morpholinos (MO; TGATGGGCTGCTGCGGCTGAACAT or CCGTTGCCTGTGATGGGC TGCTGCG). Injection with a mutant *pbx2* MO (TGATAGGCAGCTTC GGTTGCGACAT) that has 20/25 identical nucleotides to wild-type *pbx2* MO, caused no decrease in *krox20* expression in either zygotic *lzr/pbx4* or MZ*lzr* embryos and no enhancement of those phenotypes. Hox-1 function was reduced by injecting 4 ng of a combination of *hoxb1a* and *hoxb1b* MOs as described (McClintock et al., 2002) except that a second nonoverlapping *hoxb1b* MO (ACCAAG CAAAATTGATTAAGCAGGG) was also included.

RNA In Situ Hybridization, Antibody Staining, and Genetic Mosaics

Two-color RNA in situ hybridizations were performed essentially as described (Prince et al., 1998) except that in some cases Iodo-Nitrotetrazolium Violet (Sigma) was used as a substrate for the alkaline phosphatase-conjugated anti-fluorescein antibody. Branchiomotor neurons were visualized with the *isl-1*-GFP transgene (Higashijima et al., 2000). The presence of Engrailed antigen was assayed using the 4D9 monoclonal antibody (Hatta et al., 1991; Patel et al., 1989). Gastrula stage transplants were carried out as described (Moens and Fritz, 1999).

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