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Evolution of Developmental Control Mechanisms

Retinoic acid-dependent establishment of positional information in the hindbrain was conserved during vertebrate evolution

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ABSTRACT

Zebrafish *hoxb1b* is expressed during epiboly in the posterior neural plate, with its anterior boundary at the prospective r4 region providing a positional cue for hindbrain formation. A similar function and expression is known for *Hoxa1* in mice, suggesting a shared regulatory mechanism for hindbrain patterning in vertebrate embryos. To understand the evolution of the regulatory mechanisms of key genes in patterning of the central nervous system, we examined how *hoxb1b* transcription is regulated in zebrafish embryos and compared the regulatory mechanisms between mammals and teleosts that have undergone an additional genome duplication. By promoter analysis, we found that the expression of the reporter gene recapitulated *hoxb1b* expression when driven in transgenic embryos by a combination of the upstream 8.0-kb DNA and downstream 4.6-kb DNA. Furthermore, reporter expression expanded anteriorly when transgenic embryos were exposed to retinoic acid (RA) or LiCl, or injected with *fgf3/8* mRNA, implicating the flanking DNA examined here in the responsiveness of *hoxb1b* to posteriorizing signals. We further identified at least two functional RA responsive elements in the downstream DNA that were shown to be major regulators of early *hoxb1b* expression during gastrulation, while the upstream DNA, which harbors repetitive sequences with apparent similarity to the autoregulatory sequence of mouse *Hoxb1*, contributed only to later *hoxb1b* expression, during somitogenesis. Possible implications in vertebrate evolution are discussed based on these findings.

Introduction

The central nervous system (CNS) of vertebrates is induced as a simple ectodermal thickening via the action of the axial mesoderm, and later this so-called neural plate is further regionalized along the anteroposterior (AP) axis. According to the two-signal hypothesis of Nieuwkoop (1999), the initial neural plate possesses the anterior characteristics of the CNS, and this primordium is succeedingly posteriorized by signals emanating from the posterior embryonic region, leading to the establishment of the AP pattern of the CNS. This further generates three brain vesicles in the anterior CNS; the forebrain, midbrain, and hindbrain.

Segmentation is an important and remarkable process during the development of the hindbrain, wherein 7–8 segments, or rhombomeres, are generated (Lumsden and Krumlauf, 1996; Moens and Prince, 2002). Several studies using zebrafish have shown that rhombomere 4 (r4) is first established in the hindbrain during gastrulation, and that it functions as a signaling center, inducing the posterior hindbrain (Maves et al., 2002; Walshe et al., 2002). Likewise, the r4 region is established early in mice, and the conspicuous roles of the two mouse paralogous group 1 *Hox* genes (*Hox1*), *Hoxa1* and *Hoxb1*, have been revealed in the establishment of r4. Both *Hox1* genes are expressed during gastrulation in the posterior neural plate, with the anterior expression boundary at the r3/r4 border (Murphy and Hill, 1991). Later, *Hoxa1* expression retreats posteriorly during somitogenesis, and is finally restricted to the spinal cord, whereas *Hoxb1* is down-regulated in r5 and the posterior hindbrain, although its expression is retained in r4. It is thought, based on the results of gene targeting and ectopic expression, that *Hoxa1* is involved in the specification of the r4 region, while *Hoxb1* is essential for the establishment of r4 identity (Carpenter et al., 1993; Mark et al., 1993; Studer et al., 1996; Zhang et al., 1994).

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Several groups have studied the regulatory mechanisms of *Hoxa1* and *Hoxb1* in mice, showing that both genes are under regulation by the retinoic acid responsive elements (RARE) located downstream of the genes. In early neuroectoderm, the expression of *Hoxa1* is dependent on the downstream DR5-type RARE at +4.7 kb (Dupé et al., 1997; Frasch et al., 1995). *Hoxa1* is induced in the neural plate via RARE during gastrulation, and then activates *Hoxb1* through the upstream autoregulatory element (ARE) containing Hox/Pbx binding sites. *Hoxb1* expression is also dependent on the downstream RARE at +3.0 kb (Huang et al., 2002; Marshall et al., 1994). Indeed, the expression of both *Hox1* genes is up-regulated by retinoic acid (RA) treatment (Maconochie et al., 1996). Retention of *Hoxb1* expression in r4 at later stages is mediated through an autoregulatory loop, which is also mediated by the upstream ARE (Pöpperl et al., 1995).

The evolutionary zebrafish counterpart of *Hoxa1* is considered to be *hoxa1a*, although it is not expressed in the hindbrain of extant zebrafish embryos, excluding the possibility that *hoxa1a* functions in r4 development (Shih et al., 2001). Zebrafish *hoxb1a* and *hoxb1b* are co-orthologues of mouse *Hoxb1,* as was shown in a comparison of the genome between the two species (Amores et al., 1998), although the expression of *hoxb1b*, which was formerly referred to as *Hoxa-1,* is highly similar to that of mouse *Hoxa1* in the neural plate (Alexandre et al., 1996; McClintock et al., 2001). It is expressed early during gastrulation in the posterior CNS with a sharp anterior expression boundary at the r3/r4 border, which rapidly retreats to the anterior spinal cord during segmentation. Likewise, *hoxb1a* expression is initiated during epiboly in the posterior CNS, and its expression also retreats posteriorly, although discrete expression is retained in r4, as with mouse *Hoxb1* (McClintock et al., 2001).

Functional analyses of zebrafish *hoxb1a* and *hoxb1b* were conducted by the gain-of-function and loss-of-function approaches (McClintock et al., 2001, 2002). Over-expression of *hoxb1a* and *hoxb1b* was shown to cause similar effects; r2 acquired r4 characteristics, such as the appearance of the reticulospinal neuron. Meanwhile, the functional knockdown of *hoxb1b* led to expansion of r3 at the expense of r4–6, whereas the knockdown of *hoxb1a* affected the posterior migration of the VIIth cranial nerve branchiomotor neurons from r4 in the posterior hindbrain. Together, it seems likely that zebrafish *hoxb1a* and *hoxb1b* functionally correspond to mouse *Hoxb1* and *Hoxa1*, respectively, and that *hoxa1a* lost its function in hindbrain patterning during evolution.

In zebrafish embryos, *hoxb1b* expression is anteriorly expanded by treatment activating the RA, FGF, and Wnt signals (Alexandre et al., 1996; Kudoh et al., 2002), which are the most promising candidates for the posteriorizing signals identified to date (Sasai and De Robertis, 1997). Indeed, in late zebrafish blastulae, *fgf3*, *fgf8*, and *wnt8* are expressed posteriorly at the blastoderm margin (Fürthauer et al., 1997; Kelly et al., 1995; Koshida et al., 2002; Phillips et al., 2001). Additionally, the gene for Raldh2, which catalyzes RA biogenesis, is expressed in the posterior mesoderm, whereas *cyp26*, which encodes the RA degrading enzyme, is expressed in the anterior ectoderm (Begemann et al., 2001; Grandel et al., 2002). Kudoh et al. further showed that the FGF and Wnt signals are mediated by the RA signal when regulating *hoxb1b* expression, suggesting a pivotal role for RA in the patterning of the neuroectoderm (Kudoh et al., 2002), consistent with the regulation of mouse *Hoxa1*/*b1* genes by RA/RAREs.

Prince and collaborators suggested that the functional shuffling among *Hox1* genes during vertebrate evolution could be explained by the duplication–degeneration–complementation (DDC) model (McClintock et al., 2002), which was originally proposed by Force et al. (Force et al., 1999). According to this model, when a given gene is duplicated, the resulting genes are redundant, usually leading to a loss of one paralogue (non-functionalization). However, if one paralogue acquires a new function (neo-functionalization) or two paralogues share the functions of the original gene (sub-functionalization), they will be retained within the genome. This model also suggests that such genomic evolution can be driven by alterations in the regulatory regions of the genes, as described in the Discussion. However, to test the applicability of the DDC model to the evolution of *Hox1* genes in zebrafish, a detailed comparison of the transcriptional regulation should be conducted between mouse *Hoxa1*/*b1* and zebrafish *hoxb1a*/*b1b*.

In the present study, to clarify the evolution of the regulatory mechanism of *Hox1* genes that has allowed teleosts and mammals to cope with the constraints of hindbrain patterning, we performed promoter analysis of *hoxb1b* and compared the regulatory mechanisms of *Hox1* between mammals and fish. Our data show that the expression of zebrafish *hoxb1b* is primarily regulated by downstream DNA, including functional RAREs, while the upstream ARE-like region has lost its regulatory function. We also identified regulatory functions in the upstream DNA of *hoxb1b* that have not been found in the *Hox1* gene of other vertebrates. These data shed light on the evolution of positional information in the hindbrain during vertebrate evolution.

Materials and methods

Animals

Adult zebrafish (*Danio rerio*) were maintained at 27 °C in a 14-h light/10-h dark cycle. Embryos were raised at 28.5 °C to appropriate stages. Morphological features and hours post-fertilization (hpf) were used to stage embryos (Kimmel et al., 1995).

Cloning of the genomic DNA for hoxb1b

Screening of a zebrafish genomic phage library (λFIX II, 1×10^6 independent clones) was performed by plaque hybridization using the *hoxb1b* cDNA as a probe (Alexandre et al., 1996). Genomic DNA from the positive clones obtained were excised from the purified phage DNA with *Not*I and subcloned into pBluescript II SK(+).

Determination of the transcription initiation site

Total RNA purified from 24-hpf embryos was subjected to 5′-rapid amplification of the cDNA ends for *hoxb1b* using the 5′ RACE system for Rapid Amplification of cDNA Ends (Gibco BRL) according to the manufacturer's protocol. The cDNA obtained was ligated into pUC19, and the 14 clones randomly chosen were subjected to sequencing, leading to determination of the *hoxb1b* transcriptional start site. Positions around the *hoxb1b* gene referred to hereafter are relative to this site.

Construction of the plasmids

Genomic DNA, including the upstream 8.0-kb DNA, first exon, first intron, and 5′-terminal 12 bp of exon 2 (−8.0/exon 2), was excised from the genomic clone and ligated in frame to the *egfp* gene in pEGFP-N1 (Clontech). From this new construct, the DNA, including the −8.0/exon 2 and *egfp* DNA, was excised and ligated between *Apa*I and *Not*I in pEGFP-1 (Clontech), from which the *egfp* DNA had been removed in advance, giving rise to a new GFP construct (p5′hoxGFP). To exclude the exon–intron DNA, the upstream DNA from −8.0 kb to −25 bp was cloned into pEGFP-1 at *Sac*I in a forward orientation (p5′ hoxΔInt). For the quantitative analysis of transcriptional regulation using the firefly luciferase (Luc) gene, the *hoxb1b*-derived DNA in p5′ hoxGFP (−8.0/exon 2) was excised with *Xho*I and *Nco*I, and ligated into the pGL3-Basic Vector (Promega) at the *Xho*I/*Nco*I site (p5′ hoxLuc).

The downstream 4.9-kb DNA of *hoxb1b* from +1.4 kb to +6.3 kb was amplified from the genomic clone by polymerase chain reaction (PCR), and subcloned into pBluescript II SK + at the *Eco*RV site. From