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.

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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 successively 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).
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 DRS-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 hoxa1a and hoxb1b were conducted by the gain-of-function and loss-of-function approaches (McClintock et al., 2001, 2002). Over-expression of hoxa1a 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 hoxa1a affected the posterior migration of the Vth 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 parologue (non-functionalization). However, if one parologue 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 (AFIX 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 NotI 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 genome clone and ligated in frame to the egfp gene in pEFP-P1 (Clontech). From this new construct, the DNA, including the −8.0/exon 2 and egfp DNA, was excised and ligated between Apal and NotI in pEFP-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 pEFP-1 at SacI 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 Xhol and NotI, and ligated into the pGL3-Basic Vector (Promega) at the Xhol/NotI 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 EcoRV site. From
this plasmid, the 5′-region of 4.6 kb (+1.4 kb to +6.0 kb) was excised with SalI, and cloned into the SalI site at the 5′-end of the 8.0-kb genomic DNA in p5′hoxGFP (p3′/5′hoxGFP). The two downstream regions, F1 and F4 (cf. Fig. 9A), were amplified by PCR, ligated into the pGEM-T Vector (Promega), and then transferred into the ApaI/XhoI site of pZFHS70/4-EGFP-pA (kindly donated by Dr. Hiroshi Sasaki; F1-GFP, F4-GFP). To delete the RAREs in pF1/F4-GFP, inverse PCR was conducted as follows. The original plasmid was amplified by PCR using oppositely oriented 5′-phosphorylated primers that flanked the RAREs (3′-RARE1, 3′-RARE3; Figs. 1A, 2A, cf. Fig. 9A), and the products were self-ligated so that the flanked RAREs were deleted in the resulting constructs (F1ΔR-GFP, F4ΔR-GFP). When constructing plasmids throughout this work, PCR was conducted using high-fidelity LA Taq (TaKara) to amplify the DNA.

Microinjection of DNA into embryos

Plasmid DNA was purified using the Qiagen Plasmid Mini Kit (Qiagen) and linearized with appropriate restriction enzymes. DNA fragments excised from plasmid DNA or obtained by PCR were fractionated by agarose gel electrophoresis and extracted from the gel using the Qiaex II Gel Extraction Kit (Qiagen). The DNA thus prepared was solubilized in sterilized water and pressure-injected into 1-cell-stage embryos (5 pg/embryo), which were allowed to develop to appropriate stages. For co-injection experiments, the DNA fragments to be examined for their regulatory activities were mixed immediately before injection with the reporter DNA (GFP/Luc constructs) at a ratio of 3:1 (Inoue et al., 2006, 2008). Transgenic (Tg) fish lines harboring GFP constructs were established as described previously (Inoue et al., 2006).

Whole mount in situ hybridization (WMISH) and immunostaining

Digoxigenin (DIG)-labeled and fluorescein-labeled RNA probes were synthesized using T3 or T7 RNA polymerases (Stratagene) with the DIG/Fluorescin RNA Labeling Mix (Roche Diagnostic) according to the manufacturers’ protocols. WMISH was performed, essentially as described previously (Kikuta et al., 2003). When necessary, the expression of the GFP protein in embryos subjected to WMISH was examined by immunostaining using fluorescein-labeled anti-rabbit IgG antibody and anti-GFP antibody.

Microscopy

Embryos expressing GFP constructs were allowed to develop to the desired stages and observed under a fluorescence stereomicroscope (MZ FLIII, Leica) equipped with a GFP2 filter. Fluorescence images were captured with weak illumination, except for cases when a faint fluorescence needed to be observed. Confocal laser microscopy was
employed to detect the FastRed-stained krox20 mRNA and fluorescein-labeled GFP (LSM510, Carl Zeiss).

Luciferase assay

Luc constructs were injected together with the Renilla luciferase (Ren) gene (pRL-TK vector, Promega), which was driven by the HSV-TK promoter and regarded as an internal control. Expression of Luc and Ren in embryos was assayed using the Dual-Luciferase® Reporter Assay System (Promega) and the TD-20/20 Luminometer (Turner Designs) according to the manufacturers’ protocol. The regulatory DNA activities are shown as Luc activity relative to Ren activity (Luc/Ren).

Treatment of embryos with signal modifiers

For RA treatment, embryos were kept in the presence of all-trans-retinoic acid (Sigma) from 6 to 7 hpf unless specified, washed three times with water, and allowed to develop to appropriate stages. Treatment with 20 μM diethylaminobenzaldehyde (DEAB, Nakalai Tesque) was conducted from the shield stage to appropriate stages. Lithium chloride (LiCl) treatment was conducted for 10 min at the sphere stage at a concentration of 0.3 M, and treated embryos were washed three times with water and allowed to develop to appropriate stages.

mRNA synthesis and microinjection into embryos

For the synthesis of capped mRNA, the template plasmids were linearized with the appropriate restriction enzymes and transcribed with SP6 RNA polymerase, using the MEGAscript™ SP6 Kit (Ambion) according to the manufacturer’s protocol. Synthesized mRNA was pressure-injected into single blastomeres of 1- to 4-cell-stage embryos. The mRNA for egfp or lacZ was injected into embryos as a negative control.

Electrophoretic Mobility Shift Assay (EMSA)

The gene products for zebrafish RAR and RXR genes (raraa, and rrxrg) (Hale et al., 2006; Tallafuss et al., 2006) were synthesized in vitro using the TnT Coupled Reticulocyte Lysate System (Promega), as described previously (Parvin et al., 2008). Double-stranded oligonucleotides for RAREs were labeled with DIG using Terminal Transferase (Roche Diagnostics) and used as probes. Binding reactions, electrophoresis, and detection of the DNA–protein complexes were conducted using the DIG Gel Shift Kit, 2nd Generation (Roche Diagnostics). As a reference, a 30-bp oligo (RF-RARE), including the DR5-RARE from human RARβ (Sun et al., 2000), was used (cf. Fig. 11C).

Comparison of the genomic sequences among different vertebrate species (PipMaker analysis)

The genomic sequence flanking zebrafish hoxb1b (−20 kb to +20 kb) was compared with the corresponding regions for Hox1 genes from different species by the PipMaker analysis (http://pipmaker.bx.psu.edu/pipmaker/).
Results

Genomic organization of the zebrafish hoxb1b gene

Screening of a zebrafish genomic library by plaque hybridization using the hoxb1b cDNA as a probe gave rise to seven positive clones, all of which contained hoxb1b consisting of two exons and a single intron of 89 bp. The 5′-RACE defined the transcription initiation site 86 bp upstream to the start codon, showing that the zebrafish hoxb1b is driven by a TATA-less promoter. The clones obtained here together covered the hoxb1b gene from −8.0 to +6.4 kb. There are three repetitive sequences at −175 to −164, −156 to −148, and −121 to −111 (Fig. S1), which are similar to the Pbx–Hox binding sites present at corresponding upstream positions in the mouse/chick Hoxb1 and zebrafish hoxb1a (Marshall et al., 1994; McClintock et al., 2002). This region in mouse Hoxb1 is considered to mediate the autoregulation of Hoxb1, thus termed ARE, although the corresponding sequences in hoxb1b underwent base substitution compared with the ARE in mouse Hoxb1 (Fig. S1), as was previously noted (McClintock et al., 2002). We also found that other elements (Sos–Oct and Prep/Pbx1 sites), which are required for ARE function (Ferretti et al., 2005), also show significant base substitution in zebrafish hoxb1b. We extended this comparison to other teleosts, confirming that ARE sequences are typical in hoxb1a, whereas those for hoxb1b are not conserved, in all species examined.

McClintock et al. noted the presence of two RARE-like sequences downstream to hoxb1b (McClintock et al., 2002); a DR5-RARE at +2.6 kb and a DR2-RARE at +4.8 kb (3′-RARE2 and 3′-RARE3, respectively; Figs. 1A, 2A). Additionally, we found a DR5-RARE upstream at −2.6 kb and a DR2-type RARE downstream at +1.6 kb (5′-RARE1 and 3′-RARE1, respectively; Figs. 1A, 2A). 5′-RARE1 is reminiscent of the upstream DR5-RARE at −1.7 kb in mouse Hoxb1, which is considered to suppress expression in r3 (Studer et al., 1994). Immediately 5′ to DR5-RARE, located downstream to hoxb1a (+5.9 kb) and hoxb1b (3′-RARE2), we newly identified sequences with high similarity to conserved element 2 (CE2), which resides at similar locations near mouse Hoxa1/Hoxb1, chick Hoxb1, and human HOXA1 (Fig. 2B) and was shown to drive expression in somites and mesenchymal cells of mouse embryos (Thompson et al., 1998a,b).

It is now widely recognized that many essential transcriptional regulatory sequences were conserved during animal evolution. Thus, to find additional candidate regulatory regions of hoxb1b, we conducted an extensive comparison by PipMaker analysis between the 20-kb sequence flanking hoxb1b and the corresponding regions of the orthologous genes in other fish species, such as Takifugu, Tetraodon, and medaka fish (Figs. 1B, S2). We found four conserved sequences in the upstream region (Upstream Conserved Sequences, UCRs): UCR1–3 are conserved among hoxb1b in different teleosts, and UCR4 is conserved in zebrafish hoxb1b and hoxa1a. Downstream to hoxb1b, there are five conserved regions (Downstream Conserved Sequences, DCRs), termed DCR1–5: DCR3 is seen in zebrafish, Takifugu, and Tetraodon, and the other four regions are conserved in hoxb1b of all the fish species examined. DCR2 is additionally conserved downstream to zebrafish hoxb1a. We failed to identify significantly conserved sequences between zebrafish hoxb1b and mammalian Hoxa1/b1 (Fig. 1B). Importantly, DCR1, DCR2, and DCR4 each include one of the three RARE-like sequences described above (Figs. 1, S2).

Upstream 8.0-kb DNA drives reporter expression in the posterior hindbrain during somitogenesis

To assess the function of the upstream DNA, the region between −7968 and +787 (23 bp downstream from the 5′-end of the second exon) was ligated in frame with egfp (p5′-hoxGFP; Fig. 3). We first examined the transient expression of p5′-hoxGFP in injected embryos. Although it is inevitable that the transient expression of introduced genes in zebrafish embryos tend to be mosaic and show more or less ectopic patterns (Westerfield et al., 1992), transient GFP expression was clearly observed from the 10-somite stage, primarily in the posterior CNS, as is observed for hoxb1b (Fig. 40 vs. Fig. 4D; Table 1), and this spatially restricted expression persisted until at least 24 hpf (data not shown).

We then examined GFP expression in the offspring of the founder Tg fish harboring p5′-hoxGFP. The GFP fluorescence started at the early-somite stages (4-somite) in the posterior neural plate, with a sharp anterior boundary in the hindbrain (Fig. 4P), and later became intense during somitogenesis (Fig. 4Q). By 24 hpf, however, the expression retreated to the anterior spinal cord, whereas strong expression was observed in the posterior pharyngeal arches (Fig. 4R, S). The expression described above coincides well with that of hoxb1b during somitogenesis (Fig. 4C–F). Although additional GFP expression was observed in the otic vesicle, MHB, and diencephalon, the corresponding hoxb1b expression was normally not observed, and it was unclear whether these patterns were real or ectopic.

Because it takes several hours for GFP to become mature and fluorescent (Sniegowski et al., 2005), we also examined the expression of the egfp transcript in p5′-hoxGFP Tg embryos by in situ hybridization, and found that egfp expression started earlier, at the bud stage (Fig. 4G). Comparison with krox20 expression confirmed that the anterior expression boundary of egfp coincided with that of hoxb1b during early somitogenesis (r6/r′7 boundary at the 8-somite stage, Fig. 4K, L), although the transgene expression was restricted to narrower bilateral domains. Later, egfp was expressed in the anterior spinal cord, posterior pharyngeal arches, otic vesicles, MHB, and diencephalons in accordance with the expression of GFP (Fig. 4H–J). Importantly, p5′-hoxGFP expression was never seen before the bud stage, in contrast to hoxb1b (Fig. 4A, B). We also found that p5′-hoxGFP Δint, where GFP expression is under regulation by the region from −8.0 kb to −25 bp (Fig. 3), showed indistinguishable expression (data not shown), suggesting that the intron and 5′-UTR are not essential and the upstream region plays a major regulatory role. In fact, this construct also lacked the transcription initiation site we identified, suggesting that there is an additional start site(s) further upstream.

In the zebrafish genome, the closest hox genes to hoxb1b are hoxb5b and hoxb6b, which are located −12 kb and −14 kb relative to hoxb1b, respectively (Ensembl, http://www.ensembl.org/). Since the expression of p5′-hoxGFP (and p5′-hoxGFP Δint) starts later than hoxb1b, we suspected that the 8.0-kb upstream DNA of hoxb1b actually governed the expression of these hox genes, not that of hoxb1b. However, the anterior expression boundaries of hoxb5b and hoxb6b were observed significantly more caudally in the neural plate (at the level of somite 1/2; Fig. 4M, N), as described previously (Bruce et al., 2001; Prince et al., 1998).

Together, the upstream 8.0-kb DNA contains a late enhancer of hoxb1b, which is sufficient for the spatially correct expression of hoxb1b in the posterior neural tube and pharyngeal arches during somitogenesis.

Downstream DNA is required for the early expression of hoxb1b during gastrulation

To find the early enhancer for the expression of hoxb1b in the neural plate, we placed the downstream 4.6-kb DNA, from +1.4 to +6.0 kb, at the 5′-end of the upstream 8.0-kb DNA in p5′-hoxGFP (p3′-5′-hoxGFP; Fig. 3), and examined its GFP expression in injected embryos. When examined at early somitogenesis stages (5–8 somite stages), p3′-5′-hoxGFP was expressed transiently at a much higher rate in injected embryos compared with p5′-hoxGFP (Table 1). Mosaicism was not striking (Fig. 5E), so that it was easy to recognize the expression domain, which was quite similar to that of hoxb1b in the posterior neural region (Fig. 4C, D). Importantly, transient egfp mRNA...
expression was initiated by 70% epiboly in the posterior neural plate that resembles the \textit{hoxb1b} domain at the equivalent stage (Figs. 4A, 5A). Later, transient p3\textsuperscript{5\textprime}hoxGFP expression was similar to p5\textsuperscript{5\textprime}hoxGFP expression in the posterior spinal cord, although the rate of embryos showing correct expression was again higher and the mosaicism was lower compared with GFP expression driven by the

\textbf{Fig. 3.} Structures of the reporter constructs used in the present study. The upstream 8.0-kb and downstream 4.6-kb DNA of \textit{hoxb1b} are shown with blue and pink boxes, respectively. The first exon and the 5\textsuperscript{\textprime}-end of the second exon are shown in light green, and the first intron, \textit{egfp}, firefly luciferase gene, and polyadenylation signal are shown in magenta, green, yellow, and cyan, respectively.

\textbf{Fig. 4.} Regulatory function of the \textit{hoxb1b} upstream DNA during somitogenesis. (A–F) Expression of \textit{hoxb1b} mRNA from 80% epiboly to the 20-somite stage (20-s). (G–J) Expression of 5\textprime}hoxGFP mRNA in Tg embryos from the bud stage to 30 hpf. (K–N) Expression of 5\textprime}hoxGFP (K), \textit{hoxb1b} (L), \textit{hoxb5b} (M), and \textit{hoxb6b} (N) was compared with krox20 expression in r3 and r5 by \textit{in situ} hybridization. (O–S) Transient (O) and stable (P–S) expression of 5\textprime}hoxGFP was visualized by fluorescence from 4-somite stage through 28 hpf. (A–C, E–I, K–N) Dorsal views with anterior to the top (A–C, E–I, K–N) or to the left (J, S). (D, O–R) Lateral views with anterior to the left and dorsal to the top. The anterior expression boundaries of \textit{hoxb1b} and 5\textprime}hoxGFP are shown with open arrowheads, whereas those of \textit{hoxb5b} and \textit{hoxb6b} are shown with black arrowheads. Transgene expression in the posterior pharyngeal arches, diencephalon, MHB, and otic vesicles is shown with black/white thin arrows, yellow thin arrows, open circles, and asterisks, respectively. Scale, 200 µm.
upstream 8.0-kb DNA (data not shown). Finally, we obtained essentially the same result when co-injecting p5′hoxGFP and the downstream 4.6-kb DNA (data not shown), confirming the result obtained with p3′5′hoxGFP. In zebrafish embryos, it is known that regulatory DNA can work when co-injected with the promoter-reporter DNA, which was attributed to the rapid concatemerization of DNA fragments immediately after injection into the egg cytoplasm (Stuart et al., 1988).

In Tg embryos harboring p3′5′hoxGFP, the fluorescence appeared at the 95% epiboly stage (Fig. 5F), and mRNA expression was detected at the 70%-epiboly stage (Fig. 5B), in the posterior neural plate, as with hoxb1b (Fig. 4A). Later during somitogenesis, p3′5′hoxGFP was expressed in the posterior neural plate/tube and posterior pharyngeal arches (Fig. 5C, D, G–L), in addition to several seemingly ectopic regions, as was observed for p5′hoxGFP. The precise position of the anterior expression boundary of p3′5′hoxGFP was examined using confocal laser scanning microscopy by comparison with the krox20 expression in r3 and r5, locating the primary boundary at the r3/r4 border, as reported for hoxb1b (McClintock et al., 2001), with weak expression in r3 (Fig. 5M–O). Confocal microscopy also showed that the transgene expression recapitulated hoxb1b expression in the notochord (Fig. 5P, see also Fig. 5H) (McClintock et al., 2001).

Thus, the presence of the upstream 8.0-kb DNA and the downstream 4.6-kb DNA is sufficient to recapitulate hoxb1b expression during development, at least until the end of somitogenesis. Of note, the downstream 4.6-kb DNA was required for the early expression of hoxb1b during epiboly in the posterior neural plate. Furthermore, as described below, the downstream 4.6-kb DNA drove the expression of GFP under regulation by a heterologous promoter, showing that the 4.6-kb DNA is sufficient for early expression. The higher expression rate and lower mosaicism of p3′5′hoxGFP, compared with p5′hoxGFP, at later stages also suggests that the downstream 4.6-kb DNA can enhance the regulatory activity of the upstream DNA during somitogenesis.

### Table 1

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<sup>a</sup> Transient expression of GFP constructs in injected embryos were scored by fluorescence at early somite stages (5–8 somite stages).

<sup>b</sup> Numbers of embryos showing GFP fluorescence. Percentages relative to alive embryos are shown in parentheses.

<sup>c</sup> Weak expression with no apparent spatial specificity.

<sup>d</sup> Highly mosaic expression with restriction to the posterior neural tube.

<sup>e</sup> Intense expression in the neural tube with distinct anterior boundaries and little mosaicism within the hindbrain.

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Fig. 5. Early expression of hoxb1b in the posterior neural plate is driven by the downstream DNA. (A–D) Expression of 3′5′hoxGFP was visualized by WMISH using DIG-labeled probe in injected embryos (transient expression, A) or in Tg embryos (B–D). (E–P) Expression of 3′5′hoxGFP was detected as fluorescence under a stereomicroscope (E–L) or by confocal microscopy (M–P) in injected embryos (E or in Tg embryos (F–P). (M–O) Expression of krox20 in r3 and r5 (M) was detected in a Tg embryo expressing 3′5′hoxGFP (N). Their merged image (O) shows that the anterior expression boundary of 3′5′hoxGFP coincides with the r3/r4 boundary. (A–D, H, K, M–O) Dorsal views with anterior to the top (A–D, H, M–O) or to the left (K). (E–G, I, J, L, P) Lateral views with anterior to the left. Anterior expression boundaries of 3′5′hoxGFP are shown with open arrowheads. The expression in the posterior hindbrain (F), pharyngeal arches, notochord, MHB otic vesicles, and diencephalon (J, L) are shown with a curve, white thin arrows, open thick arrows, open circles, asterisks, and yellow thin arrows, respectively. Scale, 200 µm.
Transcriptional regulation of \textit{hoxb1b} by RA, Wnt, and Fgf signals

According to the two-step model of Nieuwkoop, the neural plate is posteriorized by signals emanating from the posterior region after being induced by organizer signals, and several factors have been proposed as candidates for posteriorizing signals, including RA, FGF, and Wnt. It is well known that the expression of \textit{Hox} genes are regulated by RA, and in at least some of the \textit{Hox} genes, RA responsive elements (RAREs) in the flanking regions have been implicated in RA regulation (Maconochie et al., 1996; Glover et al., 2006). In zebrafish embryos, \textit{hoxb1b} expression is expanded anteriorly by treatment with RA, Wnt, and FGF signals, and down-regulated by antagonizing reagents of these signals, as would be expected from their hypothetical roles (Kudoh et al., 2002).

To determine whether the response of \textit{hoxb1b} to these signals is also mediated by the genomic regions included in p3’-5’\textit{hoxGFP}, we examined the expression of p3’-5’\textit{hoxGFP} in embryos treated with reagents affecting the AP patterning of the embryo. When p3’-5’\textit{hoxGFP}-Tg embryos were treated with RA at the shield stage, \textit{egfp} expression significantly expanded to reach the anterior end at the 80% epiboly stage, as was shown by WMISH (Fig. 6C, D), and this RA effect on the transgene was indistinguishable from that on \textit{hoxb1b} (Fig. 6A, B) (Kudoh et al., 2002). The anterior expansion of p3’-5’\textit{hoxGFP} was confirmed by the anterior expansion of GFP expression at the 8-somite stage in similarly treated Tg embryos (Fig. 6L, J). Anterior expansion was also observed for p5’\textit{hoxGFP}, though much less extensively (Fig. 6K, L), showing that the main responsive element to RA resides within the downstream DNA. When embryos were...
treated with DEAB, which is considered to inhibit retinaldehyde dehydrogenase and suppress RA synthesis (Perz-Edwards et al., 2001), both the transgene and hoxb1b receded to the same extent in the medial region during epiboly (Fig. 6E–H), while lateral expression was little affected for both genes. A similar recession was observed by GFP fluorescence at the bud stage (Fig. 6Q, R). The responsiveness of p3′-hoxGFP to RA gradually declined during epiboly and was finally lost by the 3-somite stage (Fig. 6M–P). Consistent with this, DEAB had little effect on p3′-hoxGFP expression at the 10-somite stage (Fig. 6S, T), suggesting that RA responsiveness is confined to the epiboly stage.

When embryos were subjected to treatment with LiCl at the blastula stage, which is known to posteriorize embryos in contrast to its dorsalizing effect during cleavage (Kudoh et al., 2002; Stachel et al., 2001), both the transgene and hoxGFP expression receded halfway towards the animal pole, exactly like hoxb1b expression in similarly treated embryos (Fig. 7A–D; Kudoh et al., 2002). In contrast, when we injected into p3′-hoxGFP-Tg embryos the mRNA for zebrafish dkk1, which encodes a Wnt antagonist (Hashimoto et al., 2000), transgene expression receded significantly posteriorly, like hoxb1b expression in similarly treated embryos (Fig. 7E–H). Interestingly, hoxb1b expression not only receded but was also suppressed medially as a result of Wnt inhibition, while no such additional suppression in the medial region was obtained in p3′-hoxGFP-Tg embryos. Finally, when embryos carrying p3′-hoxGFP was injected with the mRNA for fgf3, anterior expansion of transgene expression was observed as with hoxb1b in embryos over-expressing fgf3 (Fig. 7I–L).

Taken together, the caudalizing effects of the RA, Wnt, and Fgf signals through the anterior expansion of posterior neural plate. The data also suggest differences in transcriptional level. The data also suggest differences in transcriptional level.

Downstream DNA region drives transcription in the posterior neural plate independent of the upstream DNA and hoxb1b promoter

To determine whether the regulatory activity of the downstream 4.6-kb DNA depends on the functions of the upstream DNA and/or the hoxb1b promoter, we examined the expression of GFP under regulation of the heat shock promoter (hsp-GFP) of zebrafish hsp70 (Halloran et al., 2000; Inoue et al., 2006) when co-injected with the downstream 4.6-kb DNA (cf. Fig. 9A), and found that hsp-GFP was expressed in the posterior neural plate from the epiboly stage under regulation by the 4.6-kb DNA, as with p3′-hoxGFP (Figs. 8A, B, S3A). Since the 4.6-kb DNA activated a heterologous promoter (heat shock promoter) in a temporally and spatially specific manner, we concluded that the regulatory activity of the downstream DNA was largely independent of the upstream or promoter DNA of the gene.

Localization of the cis-elements to two downstream conserved regions

To locate the downstream regulatory region(s), we subdivided the downstream region from +1.4 to +6.4 kb, which includes the 4.6-kb DNA with four conserved sequences (DCR1–4), into five overlapping subregions (F1–F5; Fig. 9A), which we amplified by PCR and co-injected with p5′hoxGFP into embryos. At the early somitogenesis stage, F1 and F4 significantly enhanced the correct expression of p5′hoxGFP, whereas F2 and F3 had marginal effects and F5 did not enhance p5′hoxGFP expression at all (Figs. 8C–H, 9B). Thus, it seems likely that F1 and F4 contain regulatory elements of hoxb1b that are important for the correct expression of hoxb1b in early embryos. Further analysis using p5′hoxGFP as a reporter showed that the two regions from +1352 to +1687 (336 bp, F1–1) and from +4682 to +5001 (320 bp, F4–2) were responsible for the full activity (Figs. 8I, J, S3D,E). The two conserved regions, DCR1 and DCR4, are completely

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Fig. 7. Downstream region mediates the Wnt and Fgf signal in hoxb1b regulation. Wild-type embryos (A, B, E, F, I, J) or 3′-hoxGFP Tg embryos (C, D, G, H, K, L) were treated with LiCl as specified (A–D) or injected with mRNA for the specified genes (E–H, 150 pg/embryo; I–L, 75 pg/embryo) (E–L), and examined for 3′-hoxGFP mRNA expression by WISH. Anterior expression boundaries of the transgene are marked with open arrowheads. Numbers of embryos showing the expression pattern represented by the photos vs. numbers of stained embryos are shown at the bottom-right. Scale, 200 µm.
Fig. 8. Localization of the regulatory activities in the downstream region of hoxb1b. The downstream 4.6-kb DNA (A, B) or its subfragments (D–L) were co-injected with 5′-hoxGFP DNA (D–J) or hsp-GFP (A, B, K, L) into embryos, which were observed at 80% epiboly (A), bud stage (L), or 5–8 somite stages (B–K). When 5′-hoxGFP (C) or hsp-GFP (not shown) was injected alone, little expression was observed at this stage. Anterior expression boundaries of the transgene are marked with open arrowheads (B) The ectopic expression sometimes seen in the mesendoderm is marked with an asterisk.

Responsiveness of hoxb1b to RA is mediated by the two downstream conserved regions

It is well known that mouse Hoxa1/Hoxb1 and zebrafish hoxb1a/hoxb1b are regulated by RA (Glover et al., 2006; Kudoh et al., 2002; Woltering and Durston, 2008), and functional RARE sequences have been implicated in the regulation of mouse Hoxa1 and Hoxb1. Since RAREs are also seen in the flanking DNA of hoxb1b (5′-RARE1 and 3′-RARE1/2/3), its responsiveness to RA was estimated quantitatively using a luciferase assay. When the Luc gene is under regulation by the upstream 8.0-kb DNA, together with the endogenous promoter (p5’-hoxLuc), little enhancement effect was observed when embryos were treated at the shield and bud stages, consistent with the marginal expression of F1-GFP and F4-GFP, which had been confirmed to be expressed in the posterior neural region (Fig. 10B–D, F–H), when the two RAREs were disrupted (F1ΔR-GFP and F4ΔR-GFP; Fig. 10A). As a result, disruption of the RAREs abrogated GFP expression (Fig. 10E, I), demonstrating that the regulatory functions of F1 and F4 depend on the RAREs as expected.

RAR/RXR complexes can specifically bind to the RAREs in the downstream DNA

We finally examined the binding activity of the two RAREs with RAR/RXR, which is the trans-acting factor mediating the RA signal (Bastien and Rochette-Egly, 2004). In EMSA, DIG-labeled oligos containing 3′-RARE1 (D1R) and 3′-RARE3 (D4R) showed significant mobility shifts in the presence of RAR/RXR, demonstrating the formation of RARE-RAR/RXR complexes (Fig. 11A, C). This complex formation was disrupted by excess amounts of unlabeled cognate or another RARE-containing oligo and the reference RARE oligo (RF-RARE) that was confirmed to specifically bind with RAR/RXR (Fig. 11A, C) (Parvin et al., 2008). Furthermore, when base substitution was introduced into the RARE consensus sequences (D1m and D4m), the competition activities of D1R/3′-RARE1 and D4R/3′-RARE3 were reduced, confirming specificity of RAR/RXR binding to the two RAREs downstream to hoxb1b (Fig. 11B, C).

Discussion

Expression of hoxb1b is regulated by the genomic region from −8.0 kb to +6.0 kb

The present study showed that the expression of zebrafish hoxb1b in the developing CNS, after mid-gastrula through at least the end of somitogenesis, could be recapitulated by the flanking DNA from −8.0 to +6.0 kb. The data further suggest that the regulation of hoxb1b by the three well-known caudalizing signals, RA, Wnt, and Fgf, and its patterning function in CNS development are mediated by the flanking DNA.

Transcriptional regulation by the upstream DNA of hoxb1b

We show here that the upstream 8.0-kb region drives expression in the posterior CNS only after gastrulation (Fig. 12). Although this
activation stage is significantly later than that of hoxb1b, the anterior boundary of the expression driven by this region coincided with that of hoxb1b, not with those of hoxb5b and hoxb6b, arguing that the 8.0-kb region is responsible for hoxb1b regulation. To our knowledge, this is the first report showing Hox1 regulation in the CNS by the upstream DNA, although its possible functions in hoxb1b regulation remain to be further examined.

There is a sequence immediately upstream to hoxb1b with partial similarity to the ARE of Hoxb1 that activates transcription by an autoregulatory loop (Ferretti et al., 2005; Pöpperl et al., 1995) and contributes to the maintenance of Hoxb1 expression in r4. Importantly, the upstream DNA that includes this ARE-like sequence did not drive specific expression in r4 in the present study, confirming that this sequence apparently lost its function, as previously suggested (McClintock et al., 2002).

The RARE identified upstream to hoxb1b is reminiscent of the RARE at a similar site for Hoxb1 that activates transcription by an autoregulatory loop (Ferretti et al., 2005; Pöpperl et al., 1995) and contributes to the maintenance of Hoxb1 expression in r4. Importantly, the upstream DNA that includes this ARE-like sequence did not drive specific expression in r4 in the present study, confirming that this sequence apparently lost its function, as previously suggested (McClintock et al., 2002).

Fig. 9. Localization of the regulatory activities in the downstream region of hoxb1b by quantitative analyses of the reporter gene expression. (A) Subregions downstream to hoxb1b that were amplified by PCR using specific primers (Table S1) and examined for their regulatory functions in the neural plate. The exons of hoxb1b, RAREs, and DCRs are shown with dark grey boxes, ovals, and light grey boxes, respectively. The subregions with significant regulatory activities are shown with thick lines. (B) Embryos co-injected with one of the 5 downstream subregions (F1–F5) and p5′hoxGFP were scored for the GFP expression at 3–10 somite stages. Embryos with mosaic but posteriorly restricted GFP expression in the neural plate and those with less mosaic and more intense posterior expression with distinct anterior boundaries relative to live injected embryos are shown in light grey and dark grey bars, respectively. Numbers above respective bars show the numbers of injected live embryos. (C–E) p5′hoxLuc (5′-Luc) were co-injected with the downstream 4.6-kb DNA (C, D) or its subregions (E), and examined for luciferase expression 1 h after RA treatment conducted at the shield (C, E) or bud (D) stage. Data for control and RA treatment are shown with light grey and stippled bars, respectively. Ordinates represent Luc/Ren values (C, D) or the Luc/Ren values in RA-treated embryos relative to those in untreated embryos (E). All the data are from three or more experiments, and the vertical bars represent standard errors of means.

Downstream DNA is primarily responsible for hoxb1b regulation

The presence of the downstream 4.6-kb DNA, in addition to the upstream DNA, temporally and spatially recapitulated hoxb1b expression from the epiboly stage. Furthermore, the 4.6-kb DNA had similar regulatory activities on egfp under regulation by the heat shock promoter, showing that the downstream DNA was sufficient for hoxb1b regulation in early embryos, and that this function was independent of the endogenous promoter (Fig. 12). Since the
downstream DNA correctly regulated the transcription when placed 5′ to the upstream 8.0-kb DNA in p3′5′hoxGFP (Fig. 3), the downstream DNA does not depend on its position, as is known for typical enhancers. In addition, the upstream DNA did not affect the regulatory function of the downstream DNA, showing that there is no need to insulate hoxb1b from the influence of the upstream DNA. Meanwhile, since hoxb5b and more upstream hoxbb genes are not expressed during epiboly in the CNS, the upstream hoxbb genes may be functionally insulated from the downstream DNA.

Further reporter assays showed that the regulatory function of the downstream 4.6-kb DNA resided primarily in the two regulatory regions that are both highly conserved among hoxb1b of different teleosts (DCR1 and DCR4). In particular, RA responsiveness was largely mediated by the downstream DNA through the two DR2-type RAREs in these conserved regions. We further confirmed by deletion analysis that these two RAREs were essential for hoxb1b expression in the flanking DNA. Consistent with these findings, in mouse embryos, early expression of both Hoxa1 and Hoxb1 is governed by the downstream RAREs (Dupé et al., 1997; Langston et al., 1997; Marshall et al., 1994).

Interestingly, we found that RA responsiveness was high at the shield stage, but declined after the bud stage. This shows that the spatial regulation of hoxb1b is determined by the RA level in the hindbrain in early gastrulae, but it is likely that other mechanisms probably take over its regulation after epiboly. It is now clear that the expression of zebrafish hoxb1b and mouse Hoxa1 in the neural plate is governed primarily by RA, although how the anterior boundary is positioned is still to be determined. It is possible that the RARE may interpret the gradient of RA as a morphogen, leading to the establishment of the anterior boundary, but another model is based on dynamic and region-specific expression of different cyp26 genes (Hernandez et al., 2007; White et al., 2007). To address this, it will be helpful to use the GFP construct we engineered here, because it faithfully recapitulates hoxb1b expression and GFP expression can be readily visualized in transparent zebrafish embryos where RA distribution is intentionally modified.

Another conserved sequence (DCR2), which was found among hoxb1b in different teleosts and even downstream of zebrafish hoxb1a, also includes a DR5-type RARE sequence (3′-RARE2). Although reporter assays failed to demonstrate its regulatory function in the present study, the sequence similarity (Fig. 2A) and close association with CE2 suggest that 3′-RARE2 corresponds to the DR5-type RARE downstream to murine Hoxb1. This RARE was shown to be responsible for Hoxb1 expression in the developing gut, which might have been missed under our experimental condition (Huang et al., 1998). The possible involvement of DCR4 and DCR5 in other aspects of endogenous hoxb1b expression (Alexandre et al., 1996) is a subject of future study.

Evolution of the Hox1 genes in vertebrates

It is well-known that there are four Hox clusters in tetrapods, which are often ascribed to the two rounds of genome-wide duplication that occurred in the common ancestors of vertebrates (675 mya; Vandeveer et al., 2004), whereas 7 to 8 clusters are present in the genome of most teleost species (Crow et al., 2006). The
expansion of the teleost Hox cluster is usually explained by the putative fish specific genome duplication (FSGD) that occurred 320 mya, between basal ray-finned fish and basal teleosts (Vandepoele et al., 2004). Structural comparison of the Hox clusters, as well as the analysis of expression patterns and functions in mice and zebrafish embryos, suggested that hoxb1a and hoxb1b of zebrafish, which were probably derived from Hoxb1 by FSGD, acquired the functions of Hoxb1 and Hoxa1, respectively, for the development of the hindbrain, especially r4 formation. In contrast, teleost hoxa1a, which is the direct descendent of Hoxa1, apparently lost its function in hindbrain formation. This functional shuffling was recently explained by the DDC model (Force et al., 1999; McClintock et al., 2002).

In the tetrapod case, Hoxa1 and Hoxb1 both possess an enhancer that drives expression in the posterior neural plate in early embryos with its anterior border at the r3/r4 boundary, and Hoxb1 is additionally regulated by the upstream ARE sequence. It was recently suggested that the early posterior enhancer and ARE of the original Hoxb1 were passed to the two descendent paralogues, hoxa1a and hoxb1b, but the ARE was disrupted only in hoxb1b, leading to the present expression patterns of the two paralogues (McClintock et al., 2002). It is likely that the posterior enhancer in the original Hoxa1 of the ancient vertebrate was lost in the teleost lineage, in contrast to tetrapods, as a result of the generation of hoxa1a that plays roles similar to those of Hoxa1, resulting in a loss of the expression and function of hoxa1a in the developing hindbrain.

Comparison of mouse Hoxa1/Hoxb1 and zebrafish hoxb1b in terms of conserved cis-elements

There is a DR5-RARE downstream to mouse Hoxa1, which drives expression in the neuroectoderm (Dupé et al., 1997; Langston et al., 1997). Downstream to mouse Hoxb1, there are a DR2-RARE and a DR5-RARE, which govern the expression in the early neural plate and digestive tract, respectively (Fig. 1A) (Huang et al., 1998; Marshall et al., 1994), and another functional DR2-RARE is present upstream to Hoxb1 (Studer et al., 1994). In the present study, we showed four possible RARE sequences in total near hoxb1b (5′-RARE1 and 3′-RARE1–3). In addition, we found a sequence highly similar to CE2, which also resides at similar locations near amniote Hoxa1/b1 and was shown to regulate expression in somites and mesenchymal cells of mouse embryos (Thompson et al., 1998a,b). A similar distribution of RARE and CE2 sequences near the mouse Hoxa1/b1 and zebrafish hoxb1b suggests that the transcriptional regulatory mechanism is conserved in these genes.

Of note, comparison of the distribution of RAREs and other possible cis-elements demonstrated the similarity of hoxb1b to mouse Hoxa1 despite the functional similarity to Hoxa1; both hoxb1b and Hoxa1 have a RARE and ARE-like sequence in the upstream region, and DR2/DR5-RARE and CE2 sequences in the downstream region. This probably reflects the derivation of hoxb1b from Hoxb1 during early teleost evolution. However, as described above, it seems that the ARE was disrupted only in hoxb1b. This ARE disruption should have led to the alteration of hoxb1b expression from the pattern seen in Hoxb1 to that of Hoxa1, which then probably lead to functional redundancy between hoxb1b and hoxa1a in hindbrain development. The result was probably elimination of cis-elements for hindbrain expression,
such as RAREs, only in hoxa1a. In the present study, we noticed that hoxb1b in other teleosts also possessed deviated ARE-like sequences, and salmon hoxa1 also lacks expression in the hindbrain (Mungpakdee et al., 2008), suggesting that the disruption of ARE occurred early in teleost evolution, soon after the FSGD. Meanwhile, hoxa1a is expressed in the ventral regions of the midbrain and anterior hindbrain after the pharyngula stage (Shih et al., 2001), and it seems likely that hoxa1a involvement in the development of these regions has allowed this gene to be retained in the zebrafish genome. Similar disruption of one of duplicated cis-regions was reported for the fugu hoxa2(a)/hoxa2(b) co-orthologue pair of Hoxa2, where the r4-specific enhancer of the original Hoxa2 lost its activity by base substitution, resulting in a situation where only one of the hoxa2 co-orthologues (hoxa2(b)) now retains the r4-specific enhancer (Tümpel et al., 2006).

Whatever the detail of the molecular evolution of the Hoxa1/b1 genes in vertebrates, the combined expression patterns of mouse Hoxa1 and Hoxb1 in the early hindbrain are very similar to those of hoxa1a, hoxb1a, and hoxb1b in zebrafish embryos. This seems to be why the dynamic alteration of the cis-elements in teleosts seems to have been allowed after the FSGD.

Disruption of the integrity of the hoxb gene cluster during evolution

Among the two hoxb duplicate clusters in zebrafish, the hoxb cluster retains the original cluster organization (10 genes), whereas the hoxbb cluster has extensively lost hox genes, resulting in only four genes, compared with 10 genes in the mammalian Hoxb cluster. It has been proposed that the homeobox cluster is held together in chordates by the existence of interdigitated control regions (Mulley et al., 2006). Under such a situation, genomic alterations, such as inversion and translocation, will be detrimental to the entire regulatory system of the gene cluster. In the case of the hoxbb cluster, the intergenic region between hoxb1b and hoxb5b should have lost hoxb2b, hoxb3b, and hoxb4b. It is likely that there were multiple interdigitated cis-elements for these lost genes in the ancient hoxb cluster, which has likely contributed to the maintenance of the tetrapod Hoxb cluster. The possible reduction of this selective pressure after the FSGD probably led to large-scale deletion in one of the duplicate clusters, giving rise to the present simplified hoxb cluster. Once the integrity of the hoxbb cluster was destroyed and the functions of hoxbb2/b3/b4/b5 were lost, the selective pressures should have become operative again in the other hoxb cluster (hoxba cluster), resulting in maintenance of the organization of this cluster. Such relief from the selective pressures maintaining cluster integrity probably explains the extensive reduction of Hox genes after the FSGD (39 Hox genes in mice vs. 49 Hox genes in zebrafish).

Comparative approach toward understanding the gene regulatory mechanism

Genomic sequence data are now available for many animal species, allowing for identification of conserved non-coding sequences throughout the genome, many of which have turned out to be important regulatory sequences in animal development (Sandelin et al., 2004; Woolfe et al., 2005; Inoue et al., 2008). Indeed, in the present study we have shown the functional importance of a portion of the conserved sequences in the vicinity of hoxb1b. As to the remaining conserved sequences, more detailed study is needed to reveal their roles at different stages and/or in more restricted regions. It is becoming widely accepted that changes in the cis-regulatory elements of genes were more important for driving animal evolution than alterations of the gene products (Carroll, 2008; Olho, 1970). The functional shuffling of hoxa1a, hoxb1a, and hoxb1b during teleost evolution is one example showing the importance of the changes in gene regulation in animal development. In this study, we analyzed hoxb1b regulation by the combination of a comparative genomics approach and functional analysis of the flanking DNA, revealing the evolution of the regulatory mechanism for duplicated Hox1 genes. Finally, we emphasize that, since teleosts underwent an additional genome-wide duplication after divergence from the other vertebrate lineages, the comparison of the regulatory mechanism between teleosts and other vertebrates will provide valuable information for understanding the role of gene duplication in animal evolution.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.10.011.

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