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Conserved co-regulation and promoter sharing of *hoxb3a* and *hoxb4a* in zebrafish

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Abstract

The expression of zebrafish *hoxb3a* and *hoxb4a* has been found to be mediated through five transcripts, *hoxb3a* transcripts I–III and *hoxb4a* transcripts I–II, driven by four promoters. A 'master' promoter, located about 2 kb downstream of *hoxb5a*, controls transcription of a pre-mRNA comprising exon sequences of both genes. This unique gene structure is proposed to provide a novel mechanism to ensure overlapping, tissue-specific expression of both genes in the posterior hindbrain and spinal cord. Transgenic approaches were used to analyze the functions of zebrafish *hoxb3a*/*hoxb4a* promoters and enhancer sequences containing regions of homology that were previously identified by comparative genomics. Two neural enhancers were shown to establish specific anterior expression borders within the hindbrain and mediate expression in defined neuronal populations derived from hindbrain rhombomeres (r) 5 to 8, suggesting a late role of the genes in neuronal cell lineage specification. Species comparison showed that the zebrafish *hoxb3a* r5 and r6 enhancer corresponded to a sequence within the mouse HoxA cluster controlling activity of *Hoxa3* in r5 and r6, whereas a homologous region within the HoxB cluster activated *Hoxb3* expression but limited to r5. We conclude that the similarity of *hoxb3a*/*Hoxa3* regulatory mechanisms reflect the shared descent of both genes from a single ancestral paralog group 3 gene.
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Introduction

Hox genes encode a family of evolutionarily conserved transcription factors with important functions in axis specification and patterning of the central nervous system. The vertebrate Hox genes consist of 13 paralog groups, which are arranged in clusters on different chromosomes. In tetrapods, 39 genes are found in 4 clusters (A–D), whereas teleosts have more than 4 clusters as a consequence of a whole genome duplication event early in their lineage (reviewed by Hurley et al., 2005; Jaillon et al., 2005). The zebrafish has a total of 48 Hox genes, with some duplicate genes retained, but the majority of genes present as singletons (Amores et al., 1998). Hox expression patterns are co-linear, with the location of a given gene within the cluster reflecting its expression domain and phase along the AP axis of the embryo (Lewis, 1989). Thus, more 3′ located genes are expressed both more anteriorly and earlier than more 5′ located genes.

The axial Hox code also correlates with hindbrain regionalization (for reviews, see Lumsden and Krumlauf, 1996; Moens and Prince, 2002). Shortly after the neural tube has formed, the region that gives rise to the hindbrain becomes subdivided into seven or eight segments along its AP axis. These rhombomeres (r) represent domains of defined gene expression and neuronal cell types. For example, Hox3 paralog genes are expressed in r5, r6, and

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posterior, whereas the Hox4 paralog genes act in r7, r8, and posterior (Prince et al., 1998; Whiting et al., 1991). The role of specific individual Hox genes in rhombomere specification has been investigated in some detail (Bell et al., 1999; Cooper et al., 2003; Kiecker and Lumsden, 2005; McClintock et al., 2002). Further, the combinatorial function of entire paralog groups of Hox genes has been investigated (Gaufo et al., 2003; McNulty et al., 2005). Recently, several studies have shown that vertebrate Hox genes function in the specification of defined neuronal populations within the hindbrain and spinal cord, indicating a role of the genes in neuronal cell lineage specification (Briscoe and Wilkinson, 2004; Dasen et al., 2005; Gaufo et al., 2003; McNulty et al., 2005; Tarchini et al., 2005). For example, a combination of Hox3 paralogous genes serves to specify the motoneuron population of r5 and r6 (Gaufo et al., 2003; Guidato et al., 2003). In the mouse, the identity of noradrenergic sensory neurons is controlled by a distinct combination of Hox genes throughout r3–r5. These neurons require *Hoxa2* activity in r3, *Hoxb1* activity in r4, and the combinatorial function of *Hoxa3*/*Hoxb3* in r5 (Gaufo et al., 2004). Hox genes are also found to be expressed in the adult brain (Cillo et al., 2001; Greer and Capecchi, 2002; Oberdick et al., 1998; Sarno et al., 2005) and have been reported to control the establishment of neuronal circuits in the brain stem (del Toro et al., 2001).

The regulation of Hox gene expression and function is highly complex and in details still poorly understood. Auto/cross-regulation as well as enhancer sharing and post-transcriptional splice mechanisms have been described (Brend et al., 2003; Gavalas et al., 2003; Manzanares et al., 2001; Sharpe et al., 1998; Tarchini et al., 2005). It has also been shown that chromatin remodeling has an important role in the precise activation and co-linear expression of these genes (Chambeyron and Bickmore, 2004; Chambeyron et al., 2005; Kmita and Duboule, 2003). Hox gene regulation is further controlled by global control regions (GCR) outside of the cluster, where several *cis*-regulatory elements act on a set of Hox genes in defined cell types (Spitz et al., 2003, 2005). GCRs as well as local enhancers are evolutionarily conserved and can be identified by homology searches in distantly related species. Large genomic sequences around the genes(s) of interest can be aligned using bioinformatic tools such as VISTA or PIPMAKER (Frazer et al., 2004; Schwartz et al., 2000; Wasserman and Sandelin, 2004). This approach, termed phylogenetic footprinting, allows the detection of regions of conservation within non-coding sequences (Bejerano et al., 2004; Boffelli et al., 2003; Frazer et al., 2003; Lenhard et al., 2003; Santini et al., 2003). Blocks of conserved non-coding regions (CNRs) are also termed CNEs (E for elements), CNSs (S for sequences), or CNGs (NG for non-genic). Conserved non-coding sequences display relatively low rates of sequence evolution compared to surrounding neutrally evolving genomic regions (Dermitzakis et al., 2005). Conservation of non-coding sequences suggests selective pressure and therefore function, for instance as enhancers, silencers, promoters, or regulatory micro-RNAs in the context of the surrounding genes (de la Calle-Mustienes et al., 2005; Frazer et al., 2004; Hardison, 2000; Müller et al., 2002; Nobrega et al., 2003; Tanzer et al., 2005), a fact that can be tested in transient reporter gene studies (de la Calle-Mustienes et al., 2005; Dickmeis et al., 2004; Müller et al., 1999; Woolfe et al., 2005).

A phylogenetic footprint and structural analysis of the *hoxb3a*/*hoxb4a* cluster sequence was performed previously to detect conserved sequences that may function to control *hoxb3a* and *hoxb4a* expression in the developing hindbrain (Hadrys et al., 2004). By aligning Hox cluster sequences of zebrafish, pufferfish, mouse, and human, we detected 22 blocks of conserved non-coding regions downstream of *Hoxb5*. Some of these CNRs corresponded to enhancers previously described in the mouse. Because neither *hoxb3a* nor *hoxb4a* has a duplicate within the zebrafish genome, there is no sub-functionalization that may complicate the comparative interpretation of enhancer/promoter studies.

Here we describe the genomic loci of the zebrafish *hoxb3a* and *hoxb4a* genes, the transcripts generated from them, and sharing of exon and promoter sequences between them. Enhancers and promoters were functionally identified by testing the regulatory potential of conserved non-coding sequences through transient EGFP reporter gene expression. *Hoxb4a* regulation was further studied using an enhancer trap insertion just downstream of *hoxb4a*. The results presented here complement known features of *Hoxb3* and *Hoxb4* regulation from mouse and chick and thus reveal evolutionarily conserved mechanisms, but also uncover important new aspects of the transcriptional and post-transcriptional regulation of both genes in zebrafish, including the differential use of several promoters and the resulting expression of different proteins. A newly identified master promoter might control the synthesis of a transcript containing all exons of *hoxb3a*/*hoxb4a* and may also control expression of the micro-RNA *miR-10b* (Tanzer and Stadler, 2004; Wienholds et al., 2005), which is located within the nested gene structures, about 8 kb downstream of *hoxb3a*/*hoxb4a* exon 1 and 1.8 kb upstream of *hoxb4a* exon 2, and is expressed in the domain of promoter activity. Our data further suggest that *hoxb3a* and *hoxb4a* have roles in defined neuronal cell lineages within hindbrain rhombomeres 5 through 7 and 8.

Materials and methods

Maintenance of zebrafish

Zebrafish were kept in a 14-h light/10-h dark cycle at 28.4°C and were fed with commercial flake food and brine shrimp. Single pairs were mated for embryo production. Fertilized embryos were collected at specific stages for injections and/or in situ hybridization.

Screening of zebrafish EST databases

BLAST searches of zebrafish EST databases available from the zebrafish genome project were performed using *hoxb3a* exon 1 sequences for alignments. Two previously not assigned sequences were found to correspond to a new type of *hoxb4a* transcript (database entries ZF-101-P00023_DEPE-F_MO9 (EST traces) and CK680990). These sequences were assembled and this *hoxb4a* transcript (type II; Fig. 1A) has been deposited in the Third Party Annotation section of DDBJ/EMBL/GenBank databases under the accession number TPA: BK005804.

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