Knockdown of duplicated zebrafish \textit{hoxb1} genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention

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SUMMARY

We have used a morpholino-based knockdown approach to investigate the functions of a pair of zebrafish Hox gene duplicates, \textit{hoxb1a} and \textit{hoxb1b}, which are expressed during development of the hindbrain. We find that the zebrafish \textit{hoxb1} duplicates have equivalent functions to mouse \textit{Hoxb1} and its paralogue \textit{Hoxal}. Thus, we have revealed a ‘function shuffling’ among genes of paralogue group 1 during the evolution of vertebrates. Like mouse \textit{Hoxb1}, zebrafish \textit{hoxb1a} is required for migration of the VIIth cranial nerve branchiomotor neurons from their point of origin in hindbrain rhombomere 4 towards the posterior. By contrast, zebrafish \textit{hoxb1b}, like mouse \textit{Hoxal}, is required for proper segmental organization of rhombomere 4 and the posterior hindbrain. Double knockdown experiments demonstrate that the zebrafish \textit{hoxb1} duplicates have partially redundant functions. However, using an RNA rescue approach, we reveal that these duplicated genes do not have interchangeable biochemical functions: only \textit{hoxb1a} can properly pattern the VIIth cranial nerve. Despite this difference in protein function, we provide evidence that the \textit{hoxb1} duplicate genes were initially maintained in the genome because of complementary degenerative mutations in defined cis-regulatory elements.

Key words: Zebrafish, Hox, Hindbrain, Rhombomere, Branchiomotor neurons, Reticulospinal neurons, Morpholino, Sub-functionalization, DDC model

INTRODUCTION

Clustered Hox genes encode a conserved family of transcription factors implicated in conferring regional identity along the anteroposterior (AP) axis of all bilaterian animal embryos (McGinnis and Krumlauf, 1992; De Rosa et al., 1999). Invertebrates have a single cluster of Hox genes (De Rosa et al., 1999; Garcia-Fernandez and Holland, 1994), but vertebrates have multiple Hox clusters as a result of paralogue group 1, possibly genome-wide, duplications during their evolutionary history. Duplication events are believed to have occurred close to the time of vertebrate origins, around 500 Mya (million years ago) (reviewed by Holland, 1999), leading to a four Hox cluster organization that has been maintained in the tetrapod vertebrates (Garcia-Fernandez and Holland, 1994). Examination of several teleost fishes has suggested that an additional, more recent, duplication event (henceforth referred to as the ‘third’ duplication) occurred early in or prior to the lineage leading to teleosts. This event has given rise to a seven Hox cluster arrangement in both zebrafish and medaka (Amores et al., 1998; Naruse et al., 2000).

The Hox cluster organizations of mouse and human have been fully described, and they share an identical arrangement of 39 genes over four clusters, termed A-D (reviewed by McGinnis and Krumlauf, 1992). Available data from \textit{Xenopus} and chick suggest that an equivalent organization is shared by all the tetrapods (Godsave et al., 1994). By contrast, Hox gene organization in the teleosts appears to be more variable (Aparicio et al., 1997; Amores et al., 1998). This variation is the result of differing patterns of gene losses within the teleosts subsequent to the third duplication event. Within the lineage leading to zebrafish, loss of many individual duplicate genes, as well as loss of one entire cluster, has produced an arrangement of 48 Hox genes arrayed over seven clusters (Amores et al., 1998). Despite the loss of many duplicates in zebrafish, there are ten instances where both products of the third Hox gene duplication have been retained.

The precise time at which the third duplication event occurred is not yet clear, although comparison of tetrapod Hox cluster organizations with those of several teleost fish species (zebrafish, pufferfish, medaka and striped bass) (Amores et al., 1998; Aparicio et al., 1997; Naruse et al., 2000) (E. Stellwag, personal communications) has allowed a broad time window to be delineated. The duplication occurred after the divergence of the ray-finned fishes (which include teleost fish) from the lobe-finned fishes (which include tetrapods), but before the radiation of the euteleosts (the group to which all the teleost species analyzed belong): i.e. between about 410 Mya and 110 Mya (Carroll, 1988) (C. Jozefowicz, J. M. M. and V. E. P., unpublished). Investigation of the mechanisms underlying
retention of specific duplicates ideally requires comparison with the ancestral, pre-duplication, condition. In the case of the zebrafish Hox genes, this is most likely to be exemplified by primitive ray-finned fishes, such as sturgeon, gar or bowfin. Unfortunately, Hox genes have yet to be studied in such primitive fish.

However, the Hox genes of the horn shark (Heterodontus francisci), a member of the cartilaginous fishes, sister group to both ray-finned and lobe-finned fishes, have been investigated (Kim et al., 2000). To date, only two horn shark Hox clusters have been described, but these clusters very closely resemble the mouse and human A and D clusters, with respect to both gene organization and sequence. These data suggest that the four Hox cluster organization of the tetrapods has changed little since the divergence of this lineage from cartilaginous fish. Thus, the mouse provides a suitable comparison group to examine the spectrum of changes that have occurred in Hox genes within the ray-finned fish lineage. We can therefore usefully compare zebrafish Hox genes to mouse Hox genes to infer the mechanisms that have facilitated retention of specific Hox gene duplicates in the zebrafish.

Gene duplication has long been thought to play an important role in evolution by providing new genetic material for selection to act upon (Ohno, 1970). Classical models have assumed that after a duplication event, one gene copy is under selection, leaving the other free to drift (reviewed by Wagner, 1998). As harmful mutations are far more likely than beneficial ones, in many cases one gene copy will accumulate deleterious changes to become a pseudogene or be completely lost (non-functionalization). In rare cases, one duplicate may acquire a key novel function (neo-functionalization), leading to preservation of both duplicates. However, vertebrate genomes appear to contain many more ancient gene duplicates than classical models would predict (Nadeau and Sankoff, 1997). Force and colleagues (Force et al., 1999; Lynch and Force, 2000) have proposed that this finding may be explained by a model of sub-functionalization. They suggest that the modular nature of eukaryotic gene enhancers may lead to a partitioning of gene functions following duplication, such that complementary expression domains are lost for each duplicate. Such changes would lead to both duplicates being necessary to preserve the function of the single ancestral gene, thus ensuring that both gene copies are retained. This has been termed the duplication-degeneration-complementation, or ‘DDC’ model. An important component of the model is that the alterations in cis-regulation underlie only the initial retention of the duplicate genes; thus the DDC model does not preclude subsequent alterations to coding sequences and protein functions.

To explore the mechanisms that underlie retention of duplicated Hox genes, we have investigated the zebrafish hoxb1a and hoxb1b genes. These genes are particularly appropriate for such a study because the function and regulation of the orthologous murine gene Hoxb1, as well as its parologue Hoxa1, have been described in great detail. In order to determine whether duplicated genes have been retained because of sub-functionalization, it is necessary to have information regarding the transcriptional regulation of the ancestral pre-duplicated gene. As Hoxb1 regulatory sequence elements have been shown to be conserved in mouse, chick and pufferfish (Marshall et al., 1994; Pöpperl et al., 1995; Langston et al., 1997), we can infer that these elements were present in the ancestral Hoxb1 gene before the third duplication event. Thus, if hoxb1a and hoxb1b were retained due to sub-functionalization, we might expect to find complementary degenerative changes in these regulatory elements.

Hoxb1, together with Hoxa1 and Hoxd1, comprise the members of mouse Hox paralogue group 1 (PG1). While Hoxd1 is not expressed during mouse hindbrain development (Frohman and Martin, 1992), Hoxb1 and Hoxa1 both play important roles in patterning this structure. The hindbrain is subdivided along its AP length during its early development into a transient array of segments termed rhombomeres (r1-r7, from anterior to posterior). Rhombomeric organization allows establishment of specific segmental identities, which facilitates proper neuronal organization in both the hindbrain and its periphery (reviewed by Lumsden and Krumlauf, 1996). Hoxa1 and Hoxb1 are co-expressed in the mouse hindbrain from the early stages of gastrulation, with an identical anterior expression limit at the presumptive r3/r4 boundary (Wilkinson et al., 1989; Frohman et al., 1990; Murphy and Hill, 1991; Barrow et al., 2000). Hoxa1 expression is very transient in r4, retracting posteriorly out of the hindbrain during early somite stages. By contrast, Hoxb1 expression is stably maintained in r4, while expression is gradually lost from r5 and r6 to leave an r4 ‘stripe’ of Hoxb1 expression. This r4 Hoxb1 domain is maintained by an autoregulatory positive-feedback mechanism, which is dependent on three defined Hox/Pbx-binding sites upstream of Hoxb1 (Pöpperl et al., 1995).

Mutant analysis of mouse Hoxa1 and Hoxb1 has revealed that these two paralogs play divergent, but partially redundant, roles in patterning the hindbrain. The prime function of the Hoxb1 gene is to confer proper r4 identity, as loss of Hoxb1 function results in major alterations to the r4-derived facial (VIIth) motoneurons (Goddard et al., 1996; Studer et al., 1996; Gauf et al., 2000). By contrast, loss of Hoxa1 function causes a radical reduction in the AP extent of r4 and r5, with an accompanying reduction in the size of the adjacent otic vesicle (Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Mark et al., 1993). Hoxa1 also plays a role in setting the appropriate anterior expression limit of Hoxb1 (Barrow et al., 2000): in the absence of Hoxb1 function the anterior limit of Hoxb1 is shifted towards the posterior, accompanied by a concomitant posterior shift of the r3 territory. Hoxa1 function is thus required for the most anterior component of normal Hoxb1 expression, acting through the three 5’ Hox/Pbx-binding sites that are also required for autoregulation (DiRocco et al., 1997).

Analysis of mice mutant for both Hoxa1 and Hoxb1 has shown that the functions of these two paralogues are synergistic (Studer et al., 1998; Rossel and Capocci, 1999); in double null mutants the loss of posterior hindbrain territory is greatly exacerbated. Gain-of-function experiments with mouse Hoxa1 and chick Hoxb1 have shown that these PG1 genes have similar functional capacities when ectopically expressed (Zhang et al., 1994; Bell et al., 1999). Based on these findings, Rossel and Capocci (Rossel and Capocci, 1999) have suggested that the primary difference between murine Hoxal and Hoxb1 lies not in their coding sequences, but in the precise regulation of expression of the two genes. However, this cannot be concluded without directly demonstrating that the two gene products have equivalent biochemical functions. Such a demonstration has been performed for Hoxa3 and
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Hoxd3, using ‘knock-in’ experiments, and in this particular case, the two paralogues have equivalent functional capacities (Greer et al., 2000).

In the zebrafish there are a total of four PG1 genes: \textit{hoxa1a}, \textit{hoxb1a}, \textit{hoxb1b} and \textit{hoxc1a}. These genes have been unambiguously assigned to their appropriate clusters based on both sequence and linkage analysis (Amores et al., 1998; McClintock et al., 2001). The zebrafish ortholog of mouse \textit{Hoxa1}, zebrafish \textit{hoxa1a}, is not expressed in presumptive r4, and thus cannot play a role in early patterning of this hindbrain territory (McClintock et al., 2001; Shih et al., 2001). The \textit{hoxb1} duplicate genes, \textit{hoxb1a} and \textit{hoxb1b}, are both expressed in rhombomere 4 of the hindbrain (Alexandre et al., 1996; Prince et al., 1998a). Intriguingly, \textit{hoxb1a} and \textit{hoxb1b} have expression profiles that are similar to those of mouse \textit{Hoxb1} and \textit{Hoxa1}, respectively (McClintock et al., 2001), although zebrafish \textit{hoxb1a} lacks the early gastrula stage expression shown by mouse \textit{Hoxb1}. Our gain-of-function experiments with each of the zebrafish \textit{hoxb1} duplicates showed that either has the capacity to repattern r2 to an r4 phenotype (McClintock et al., 2001). Taken together, our previous findings suggested that zebrafish \textit{hoxb1a} and \textit{hoxb1b} could be the functional equivalents of mouse \textit{Hoxb1} and \textit{Hoxa1}, respectively.

We have used a morpholino-based ‘knockdown’ approach to test directly the functions of the zebrafish \textit{hoxb1} duplicates. Our results demonstrate that zebrafish \textit{hoxb1a} and \textit{hoxb1b} play similar roles to mouse \textit{Hoxb1} and \textit{Hoxa1}, revealing that a ‘function shuffling’ among paralogues has occurred during vertebrate evolution. Although we find that the zebrafish \textit{hoxb1} duplicates have partially redundant roles, a series of rescue experiments reveals that the proteins do not have completely interchangeable biochemical functions. In spite of this difference in protein function, we provide evidence that initial retention of the duplicates occurred via sub-functionalization of defined \textit{cis}-regulatory elements, as predicted by the DDC model.

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\textbf{MATERIALS AND METHODS}
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\textbf{Micro-injections}
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Antisense ‘morpholinos’ were designed by Gene Tools to target the \textit{hoxb1a} and \textit{hoxb1b} genes:

\begin{itemize}
\item MOb1a: 5′GGAACGTGCTCATACGCAATTA
\item MOb1b: 5′AATTCATGGTTGACTGACCAAGC
\end{itemize}

(The complement of the start of translation is underlined.)

(Approximately 100 nl of morpholino was injected at the yolk/blastoderm interface at the one- to four-cell stage, at concentrations ranging from 0.5 mg/ml to 4 mg/ml in phenol red buffer (0.25% phenol red, 120 mM KCl, 20 mM Hepes-NaOH pH 7.5). A second \textit{hoxb1b} morpholino, complementary to 5′ UTR sequences but not incorporating the ATG start of translation, was also designed. This reagent did not produce any phenotype when injected alone.

Synthetic capped mRNAs were micro-injected alone or together with morpholinos, in phenol red buffer. mRNA was produced from linearized DNA templates using the Ambion Megascript kit according to manufacturer’s instructions. mRNAs were generated from the previously described pCS2\textit{hoxb1a}, pCS2\textit{hoxb1b} and pCS2\textit{AmphHox1-1} constructs (McCintock et al., 2001). In addition, a pCS2\textit{mouseHox1} construct was generated by cloning full-length mouse \textit{Hoxb1}-coding sequence (Accession Number, NM 008266) into pCS2+ (Turner and Weintraub, 1994).

N-terminal Myc-tagged pCS2\textit{mycb1a} and pCS2\textit{mycb1b} constructs were generated for the production of ‘rescue’ mRNAs. Each rescue construct had 6 N-terminal Myc epitopes provided by pCS2\textit{myc} (Turner and Weintraub, 1994) and no Hox 5′ UTR sequence was incorporated, such that Myc was fused directly to the ATG, to avoid sequence complementary to the morpholinos. The pCS2\textit{mycb1a} construct retained 12 nucleotides that are complementary to MOb1a and the pCS2\textit{mycb1b} construct retained seven nucleotides that are complementary to MOb1b. However, it has previously been determined that morpholinos act most efficiently when binding at, or immediately upstream of, the start of translation (www.gene-tools.com/). Thus, the N-terminal tags ensured that the first codon of the Hox protein lay internally within the transcript and thus translation was unlikely to be affected by any residual annealing of the morpholino.

To confirm that the morpholinos were capable of blocking translation of the target Hox genes, we also generated expression constructs based on pCS2GFP (Turner and Weintraub, 1994), in which the 5′ UTR plus approximately the first 300 bp of coding sequence of each \textit{hoxb1} duplicate gene was fused in frame to GFP using standard PCR-based cloning. Injection of 40 μg/ml of mRNA generated from each of these constructs produced prominent fluorescence, which was assayed at 8-9 hours of development. We then used co-injections to test the ability of each \textit{hoxb1} morpholino to knock down expression of the corresponding mRNA. We found that 1 mg/ml of MOb1a or 4 mg/ml of MOb1b (the concentrations used in our functional experiments) completely abrogated fluorescence of the target mRNA, such that injected embryos were indistinguishable from un.injected embryos (data not shown). An unrelated control morpholino had no effect on the fluorescence levels from either construct. These control experiments confirmed that the morpholinos were able to knock down the target Hox genes.

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\textbf{In situ hybridization analysis}
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In situ hybridization was performed as previously described (Prince et al., 1998a). In situ probes for the following genes were used: \textit{krox20} (Oxtoby and Jowett, 1993), \textit{mariposa} (Moens et al., 1996), \textit{islet1} (Inoue et al., 1994), \textit{hoxb1a} (Prince et al., 1998a), \textit{hoxb1b} (McCintock et al., 2001) and \textit{hoxb4} (Prince et al., 1998b). Images were photographed on a Zeiss Axioskop using 100 ASA Ektachrome film.

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\textbf{Retrograde labeling}
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Reticulospinal neurons were revealed by retrograde labeling from the spinal cord at 5 days of larval development as previously described (Alexandre et al., 1996). Labeled brains were visualized by confocal microscopy.

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\textbf{Confocal and fluorescence microscopy}
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Confocal analysis was performed on a Zeiss LSM510 scanning confocal microscope. Fluorescence and bright-field images were photographed on a Zeiss Axioskop using 1600 ASA Ektachrome film, images were merged using Adobe Photoshop 5.5.

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\textbf{Immunohistochemistry}
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The following antibodies were used for whole-mount immunohistochemistry as previously described (Prince et al., 1998b): \textit{3A10} antibody recognizes the Mauthner neurons (Furley et al., 1990); \textit{RMO44} antibody recognizes a subset of RS neurons, including the Mauthners (Pöpperl et al., 2000).

\begin{center}
\textbf{RESULTS}
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\textbf{Loss-of-function of zebrafish \textit{hoxb1a} differentially affects two classes of rhombomere-specific neurons}

The zebrafish \textit{hoxb1a} gene has a stable r4 expression domain, similar to the later expression of mouse \textit{Hoxb1}. This conserved
phenotypes of mouse Hoxb1 null mutants, led us to hypothesize that hoxb1a is necessary for normal r4 identity. To test directly the function of hoxb1a, we used a morpholino-based knockdown approach. Morpholinos are stabilized antisense oligos that have been shown to block effectively and specifically translation of mRNAs in both Xenopus and zebrafish embryos (Heasman et al., 2000; Nasevicius and Ekker, 2000). Using a GFP-hoxb1a fusion construct, we confirmed that the morpholino targeted to hoxb1a (MOb1a) was able to efficiently block translation of ectopic hoxb1a message (see Materials and Methods). We then examined hindbrain segmental identity in embryos where hoxb1a function was knocked down, by assaying the specification and behavior of two classes of neurons with rhombomere-specific identities.

In the zebrafish, both the branchiomotor (BM) neurons of the cranial nerves and the reticulospinal (RS) interneurons have a rhombomere-specific disposition and thus provide markers of segmental identity. The organization of BM nerves is generally well conserved between zebrafish and mouse (Chandrasekhar et al., 1997). Each BM nerve consists of groups of neuronal cell bodies that lie in clusters within specific rhombomeres, projecting axons that fasciculate and leave the hindbrain at defined exit points to innervate the muscles of the adjacent pharyngeal arches. Thus, the facial (VIIth) nerve has cell bodies that differentiate in r4 and r5, and project axons out of r4 to innervate the second pharyngeal arch. The cell bodies of these VIIth nerve BM neurons undergo a characteristic migration towards the posterior, along a medial path immediately adjacent to the floorplate, followed by a short lateral migration, to ultimately form nuclei in r6 and r7 (Fig. 1) (Chandrasekhar et al., 1997; Higashijima et al., 2000). These VIIth nerve cells differentiate at around the 16-hour stage, commence their posterior migration at about the 19-hour stage, and begin to arrive at their r6 and r7 destinations by 24 hours. By the 36-hour stage, the VIIth nerve neurons have reached their final positions. The BM neurons characteristic of r2 and r3, the neurons of the Vth (trigeminal) nerve, have a very different organization. The cell bodies of the Vth nerve differentiate as a major anterior group in r2 (Va) and a smaller, later differentiating, posterior group in r3 (Vp); neither of these groups undergoes any migration towards the posterior (Fig. 1) (Chandrasekhar et al., 1997; Higashijima et al., 2000). However, both the r2 and r3 groups of Vth nerve cell bodies migrate laterally within their rhombomeres of origin. By 24 hours for r2, and 30 hours for r3, the Vth nerve BM neurons are distributed in a mediolateral array that is not immediately adjacent to the floor plate; by 36 hours, these BM neurons have completed their lateral migration. Both r2 and r3 neurons project axons to an exit point in r2 to innervate the adjacent first pharyngeal arch.

The second major class of neurons that displays rhombomere specific identity is the reticulospinal (RS) interneurons, which provide the major route through which the brain communicates with the spinal cord to control locomotion. Zebrafish RS neurons form a ladder-like array along the AP extent of the hindbrain and comprise distinct cell types, characteristic to each rhombomere, recognizable by size, mediolateral location and axonal projection (Metcalfe et al., 1986; Hanneman et al., 1988) (see Fig. 4A). By contrast, the RS neurons of the mouse are far more complex and do not display a simple ladder-like array (Auclair et al., 1999). In zebrafish, r4 is characterized by several distinct RS neurons: the large, contralaterally projecting Mauthner neurons that lie one on each side of the midline, clusters of smaller medially located Mi cells, and cells of the lateral vestibular nuclei (Metcalfe et al., 1986; Mendelson, 1986a; Mendelson, 1986b; Hanneman et al., 1988).

To investigate the function of hoxb1a in patterning these r4-specific neurons, we micro-injected embryos at the one- to four-cell stage with a morpholino (MO) targeted to hoxb1a (MOb1a). We assayed the character of the BM neurons by using embryos transgenic for an islet1 reporter construct, which directs green fluorescent protein (GFP) expression specifically within the cell bodies and projections of the branchiomotor nerves (Higashijima et al., 2000), or by in situ hybridization to islet1 mRNA (Fig. 1). We found that over the range of concentrations tested (0.5-4 mg/ml) almost every MO-injected islet1 transgenic embryo exhibited profound and specific alterations to the facial (VIIth) nerve, as assessed by fluorescence or confocal microscopy (116 out of 117 embryos assayed). Our primary finding was that hoxb1a function is necessary for normal posterior migration of facial (VIIth) nerve cell bodies, as has been shown for murine Hoxb1 (Goddart et al., 1996; Studer et al., 1996).

We examined MOb1a-injected embryos over a range of stages up to 5 days, and found them to have normal gross morphology; our molecular analysis showed that overall organization of the hindbrain was also normal (data not shown). No alterations were found in the r2- and r3-derived Vth (trigeminal) nerve neurons, nor in the Xth (vagal) nerve neurons that lie posterior to r7. However, the cell bodies at the r4 level showed a spatial distribution remarkably similar to those of the r2-characteristic cell bodies of the Vth nerve (Fig. 1). Thus, the cells were located more laterally within r4 than normal, and did not undergo their characteristic posterior migration behavior. Rather, a cluster of cells formed in r4, with a similar shape and mediolateral location to the r2 characteristic Vth nerve cells (Fig. 1A-H). Even after 5 days of development, the VIIth nerve cell bodies remained in r4 rather than migrating posteriorly towards r6 and r7 (data not shown). Transplantation of neurons between wild-type and morpholino-injected embryos has demonstrated that the normal migration of VIIth nerve neurons requires cell-autonomous function of hoxb1a (K. L. Cooper, V. E. P. and C. B. Moens, unpublished).

As the hoxb1a-deficient embryos showed major alterations in VIIth nerve cell body location, we used confocal microscopy to trace their axon projections in islet1 transgenic embryos at the 48-hour and 72-hour stages (Fig. 1G-J). In wild-type 48-hour stage embryos, the Vth nerve axons, which exit the hindbrain via r2, have formed a single fasciculated bundle that projects along the posterior of the eye into the first pharyngeal arch (Fig. 1I). The VIIth nerve axons course anteriorly from their cell bodies, which lie in r6 and r7 at this stage, to exit the hindbrain via r4. These axons project ventrally into the second pharyngeal arch, their pathway bifurcating ventrally (Fig. 1I). GFP-positive VIIth nerve/octavolateralis efferent (OLE) neurons are located in close proximity to the VIIth nerve neurons, and their axons follow the same tract as the VIIth
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nerve before turning posteriorly into the otic region (Higashijima et al., 2000) (Fig. 1I, red arrowhead). In 48-hour MOb1a-injected embryos, the axons of both the Vth and VIIth nerves follow similar trajectories to those of wild-type embryos, projecting into the first and second pharyngeal arches, respectively (n=10; Fig. 1J). However, the VIIIth/OLe axon tract was absent from the injected embryos, with just a few unfasciculated axons entering the otic region on occasion...
Fig. 2. Maintenance of zebrafish hoxb1a transcription requires Hoxb1a protein.
Transcription of endogenous hoxb1a was assayed by whole-mount in situ hybridization in uninjected control embryos (left-hand panels) or in embryos injected with 1 ng/µl MOb1a (right-hand panels). (A,B) 24 hour embryos: (A) hoxb1a is expressed at high levels in r4 of control embryos; (B) in the MOb1a-injected embryos, there is a reduction in transcript levels in the medial part of the neural tube. (C,D) At 36 hours, loss of medial transcription in response to MOb1a is more severe. (E,F) Transverse sections (planes indicated by red arrows in C,D, dorsal towards the top) encompassing the whole of r4 were hand cut from 36 hour embryos post in situ.

(n=10; Fig. II). At the 72-hour stage, the BM neuron axons have projected further ventrally along their pathways. At this later stage, we again found that the axon pathways within the pharyngeal arches of MOb1a-injected embryos did not differ from those of wild type, but that the VIIIth/OLe tract was absent (n=10; data not shown).

Our confocal analysis showed that the axonal projections of the hoxb1a-deficient VIIth nerve neurons did not differ from those of wild-type embryos, revealing that the axons of the mislocated neurons remained capable of responding to the pathfinding cues used by normal VIIth nerve axons. We conclude that when hoxb1a function is knocked down, the r4-derived BM neurons lose the capacity to migrate posteriorly, yet their axons project normally into the second pharyngeal arch. We also conclude that hoxb1a function is required for establishment of the VIIth/OLe axon tract. However, as the individual neurons of the VIIth and VIIIth nerves cannot be distinguished, we are unable to determine whether VIIth nerve neurons are absent from MOb1a-injected specimens, or whether they fail to migrate posteriorly, as do VIIth nerve neurons.

By contrast, injection of MOb1a did not cause any identifiable change to the appearance of the reticulospinal (RS) neurons, as assayed by immunohistochemistry (3A10 antibody; n=17) or retrograde labeling from the spinal cord (n=9, data not shown). The lack of alterations to RS neurons suggests that hoxb1a function is not necessary for proper differentiation, localization or axonal projection of this class of early born interneurons. However, we have previously demonstrated that mis-expression of hoxb1a, or other PG1 genes, is sufficient to induce differentiation of ectopic r4-characteristic neurons, including Mauthner neurons, at the r2 level (McClintock et al., 2001). The Mauthner neurons are born at approximately 7.5 hours (Mendelson, 1986a), shortly after the onset of hoxb1b expression (Alexandre et al., 1996), but shortly before the onset of hoxb1a expression (Prince et al., 1998a). These results suggest that the earlier expressed hoxb1b gene may be responsible for RS neuron specification in r4.

Loss of Hoxb1a protein causes down-regulation of hoxb1a transcription

In the mouse, Hoxb1 expression is maintained at high levels in r4 by an autoregulatory feedback loop (Pöpperl et al., 1995). Our previous gain-of-function analysis has shown that ectopic hoxb1a can induce transcription of the endogenous hoxb1a gene in rhombomere 2 (and in other anterior regions of the embryo when introduced at high concentrations) (McClintock et al., 2001), suggesting that zebrafish hoxb1a is subject to a similar autoregulatory mechanism. We therefore investigated hoxb1a mRNA levels in MOb1a-injected embryos. Using in situ hybridization, we found that hoxb1a mRNA levels were equivalent in control and MO-injected embryos up to the 20-hour stage (n=9; data not shown), but by the 24-hour stage there was significant reduction in hoxb1a mRNA levels in MO-injected embryos (n=15; Fig. 2A,B). Levels of hoxb1a transcript were reduced still further at the 36-hour stage, particularly in the most medial part of the neural keel (n=16; Fig. 2C-F), consistent with the idea that zebrafish hoxb1a is indeed subject to autoregulatory control. In wild-type embryos, hoxb1a expression levels up-regulate in r4 at around 11 hours of development, shortly after the onset of expression (McClintock et al., 2001). Yet, our morpholino injection results suggest that hoxb1a autoregulation is not important until after 20 hours of development. However, we cannot preclude an earlier role for Hoxb1a protein in maintenance of hoxb1a transcription: the MOb1a morpholino acts by binding directly to hoxb1a mRNA and may therefore act to stabilize the message.

Our injection experiments with MOB1a have revealed that zebrafish Hoxb1a function is required both for proper migration of VIIth nerve BM neurons and for proper transcriptional regulation of hoxb1a. In the mouse Hoxb1 knockout, similar inhibition of migration of VIIth nerve
Fig. 3. Loss-of-function of hoxb1 duplicate genes causes alterations in segmental organization of the posterior hindbrain. (A–C) In situ hybridization with krox20 (a marker for r3 and r5) and hoxb4 (expressed in r7 and posterior) at 20 hours, anterior towards the top, rhombomeres r3–r6 and their AP extent are indicated (red double-headed arrows). (A) Wild-type control; (B) embryo injected with 4 mg/ml MOβ1b, note reduction in AP extent of r4, r5 and r6 and expansion of r3; (C) Embryo co-injected with 1 mg/ml MOβ1a + 4 mg/ml MOβ1b, note exacerbation of reduced size of r4 and r6, and further expansion of r3 towards the posterior. (D–F) Bright-field lateral views of live embryos at the 24-hour stage, anterior towards the left. The AP extent of the otic vesicles is indicated by white bars. (D) Wild-type embryo; (E) MOβ1b-injected embryo; (F) MOβ1a+MOβ1b-injected embryo. Arrows indicate otoliths.

Table 1. Loss-of-function of hoxb1 duplicate genes alters segmental organization of the hindbrain

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rhombomere 3 (μm)</th>
<th>Rhombomere 4 (μm)</th>
<th>Rhombomere 5 (μm)</th>
<th>Rhombomere 6 (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>52.7 (s.d. 2.6)</td>
<td>47.5 (s.d. 3.7)</td>
<td>57.4 (s.d. 3.5)</td>
<td>41.2 (s.d. 2.1)</td>
</tr>
<tr>
<td>MOβ1b</td>
<td>66.8 (s.d. 6.6)</td>
<td>36.5 (s.d. 5.5)</td>
<td>50.6 (s.d. 3.7)</td>
<td>31.8 (s.d. 3.7)</td>
</tr>
<tr>
<td>MOβ1a+β1b</td>
<td>73.5 (s.d. 5.5)</td>
<td>24.0 (s.d. 6.2)</td>
<td>46.8 (s.d. 3.1)</td>
<td>27.5 (s.d. 2.1)</td>
</tr>
<tr>
<td>RNA rescue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOβ1b + hoxb1b mRNA</td>
<td>-</td>
<td>49.0 (s.d. 5.3)</td>
<td>52.1 (s.d. 4.5)</td>
<td>37.1 (s.d. 2.1)</td>
</tr>
<tr>
<td>MOβ1b + hoxb1a mRNA</td>
<td>-</td>
<td>50.6 (s.d. 3.8)</td>
<td>55.3 (s.d. 2.9)</td>
<td>36.5 (s.d. 6.9)</td>
</tr>
</tbody>
</table>

In situ hybridization with probes for krox20 and hoxb4 was performed on 20-hour stage embryos to allow visualization of r3, r4, r5 and r6. A minimum of 20 samples was used for each measurement. Images in dorsal view were digitized, transferred to Adobe Photoshop and rhombomeres measured in micrometers. Measurements were taken at a halfway point between the midline and the lateral margin of the rhombomere. The standard deviation (s.d.) of each set of measurements is noted, as is the percentage change with respect to wild-type control embryo measurements. Student’s t-test confirmed that the MOβ1b or MOβ1a+MOβ1b morpholino-treated rhombomeres had statistically different AP lengths from wild-type rhombomeres (for each rhombomere, P<0.0001).
(n=7) or by 3A10 antibody stain (n=30), suggesting that hoxb1b function is not required for proper RS neuron specification.

**Knockdown of both hoxb1 duplicates leads to alterations in reticulospinal neuron disposition and exacerbates segmentation defects**

As some, but not all, r4-specific characters were affected by knockdown of hoxb1a or hoxb1b individually, we wished to determine whether the duplicate genes might have some redundant functions. To test this hypothesis, we co-injected both MOb1a and MOb1b. In response to knockdown of both hoxb1 duplicate genes, we observed significant alterations to the RS neurons in r4 and posteriorly, using both immunohistochemistry (RMO44 antibody, n=74) and retrograde labeling (n=19). In uninjected control embryos, we observed bilateral contralaterally projecting r4-specific Mauthner neurons in 100% of embryos using either technique (RMO44, n=30; retrograde labeling n=16). By contrast, in injected embryos we found that Mauthner neurons were absent, either unilaterally (43%) or bilaterally (39%) (Fig. 4B-D). In cases where unilateral Mauthners remained, they were frequently displaced a short distance towards the posterior (Fig. 4B).

We also observed ectopic neurons at the r4 level, which were smaller than Mauthner neurons and did not project contralaterally; between one and three of these cells was observed on each side of the hindbrain, lying just lateral to the medial Mi neurons (Fig. 4B-D, ectopic cells indicated by asterisks). Our analysis of three-dimensional confocal projections and confocal z-series, revealed that the ectopic neurons lay ventrally within r4, at a similar dorsoventral level to the Mauthner neurons of uninjected specimens. In three specimens, ectopic r4 neurons and a Mauthner neuron were present together in the same hemisegment, and at the equivalent dorsoventral level; this finding suggests that the ectopic r4 neurons are unlikely to form via additional cell divisions of a Mauthner neuron precursor cell. Co-injected embryos also showed a radically reduced number of r4-specific lateral vestibular neurons, as revealed by retrograde labeling (between 0 and 4 cells were labeled, in comparison with approximately 16 cells in wild-type specimens) (Fig. 4A-D). This may reflect loss of the vestibular neurons, but could also reflect an inability to label the cells because of changes in their projections. As the RMO44 antibody does not recognize these cells we cannot distinguish between these possibilities. More posteriorly, we observed that the r5 RS neurons tended to be distributed more broadly than normal. This altered distribution included a spreading out of the cells along the AP axis. We also occasionally observed an individual cell lying more lateral than normal within r5, although the average number of r5 cells remained constant (Fig. 4B-D). It should be noted that by the 5 day stage, when retrograde labeling was performed, that there are no independent markers of rhombomere identity available. Thus, as r5 is reduced in AP extent by knockdown of hoxb1b, we cannot rule out the possibility that the r5 RS neurons spread into adjacent rhombomeric territories.

Our results reveal that the presence of at least one normal hoxb1 duplicate is required to confer appropriate identity to the RS neurons in r4 and more posteriorly. The r4-specific Mauthner neurons are born at about 7.5 hours, and the r5 RS neurons at about 9.5 hours (Mendelson, 1986a). The hoxb1b gene is first expressed at 6 hours (early gastrulation) (Alexandre et al., 1996; McClintock et al., 2001), before the time at which the r4 Mauthner neurons are born. However, the hoxb1a gene is not expressed until about 2 hours after their birthday (Prince et al., 1998a). Nevertheless, either hoxb1b or hoxb1a has the ability to allow normal Mauthner neuron differentiation. This finding suggests that Mauthner cell identity is not necessarily established at the time the neurons undergo their final cell division, but rather can remain labile for several hours.

In the mouse, loss-of-function of both Hoxb1 and Hoxa1 exacerbates the segmentation phenotypes associated with

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**Fig. 4.** The hoxb1 duplicates have redundant functions in RS neuron specification. Retrograde labeling of 5-day larvae, merged confocal images, anterior towards the top, rhombomeres are numbered. (A) Wild-type control, note the bilateral r4-specific Mauthner neurons (M), the more lateral vestibular neurons (nV), and the smaller, laterally located r2-specific rol2 neurons. (B-D) Larvae injected with MOb1a at 1 mg/ml and MOb1b at 4 mg/ml. Note loss of Mauthners (•) and their replacement with smaller more medially located cells. In B, a unilateral Mauthner remains, although it is displaced posteriorly (M'). In C, ectopic lateral cells at the r5/6 level are indicated (red arrowhead).
RNA rescue experiments confirm specificity of morpholino function

To confirm that the MO phenotypes were caused by loss-of-function of the individual Hox genes, we performed rescue experiments using co-injection of mRNAs that encoded the knocked-down gene products. To prevent the MOs from blocking translation of the ectopically introduced transcripts, we used N-terminal Myc-tagged constructs with no Hox gene 5'UTR sequences present (see Materials and Methods). We found that these constructs produced transcripts that function identically to untagged transcripts in a gain-of-function assay (McClintock et al., 2001).

We found that co-injection of 1 mg/ml of MOb1a with 15 or 20 μg/ml of hoxb1a mRNA (generated from pcS2mycb1a) at the one-cell stage led to highly efficient rescue of the loss-of-function phenotype. In the presence of MOb1a alone, nearly 100% of specimens lost normal migration of the facial (VIIth nerve) BM neurons (as revealed by islet1 expression, Fig. 5A,C). By contrast, in the co-injected embryos the VIIth nerve neurons showed migration towards the posterior (97%; Fig. 5B,D). At earlier developmental stages (12 and 15 hours, data not shown) we observed similar altered sizes of r3 through r6 in response to knockdown of both hoxb1 genes, suggesting that mis-specification of rhombomere identity occurred from the very earliest stages of hindbrain regionalization.

The embryos co-injected with MOb1a and hoxb1a mRNA also showed the previously described gain-of-function phenotype (McClintock et al., 2001). Thus, the r2-specific trigeminal (Vth) nerve neurons showed alterations from their normal phenotype, with a more medial localization characteristic of facial (VIIth) nerve neurons (indicated as VII', Fig. 5B,D) (McClintock et al., 2001). Other aspects of the gain-of-function phenotype at the r2 level, such as ectopic r4-characteristic Mauthner neurons and activation of hoxb1a transcription, were also observed (data not shown). These results confirm that exogenous hoxb1a is sufficient to rescue the loss of normal VIIth nerve neuron migration that results from MOb1a injection, verifying that this phenotype is a result of loss-of-function of hoxb1a.

Fig. 5. Morpholino generated loss-of-function phenotypes are rescued by ectopic protein. (A-D) Rescue of MOb1a phenotype, disposition of BM neurons is revealed with islet1: (A,B) islet-GFP transgenics: (C,D) islet1 in situ hybridization at 28 hours, anterior towards the left. (A,C) Embryos injected with MOb1a alone at 1 mg/ml, note lack of migration of r4-derived BM neurons (VII). (B,D) Embryos co-injected with MOb1a at 1 mg/ml and hoxb1a mRNA at 15 μg/ml, note rescue of migration of r4-derived neurons (VII), plus posteriorizing transformation of r2-derived neurons (VII'). (E-H) Rescue of MOb1b phenotype, embryos at 20 hours, anterior towards the top, krox20 in situ hybridization. (E) Uninjected wild-type control, note approximately equal AP extents of r3, r4 and r5; r4 size indicated with double-headed arrow. (F) hoxb1b mRNA injected (20 μg/ml) embryo, note no change in r4 size but increase in r3 AP extent, as we have previously described for gain-of-function experiments (McClintock et al., 2001). (G) MOb1b injected (4 mg/ml) embryo, note shift in r3/4 boundary towards the posterior, resulting in a significant reduction in AP extent of r4, together with increase in AP extent of r3. (H) Embryo co-injected with hoxb1b mRNA plus MOb1b, note rescue of r4 AP extent to wild-type proportions, together with increased size of r3 AP extent as seen with RNA alone.
findings demonstrate that ectopically provided Hoxb1a is sufficient to cause gain-of-function phenotypes in the absence of endogenous Hoxb1a function.

We similarly tested whether hoxb1b mRNA can efficiently rescue the MOb1b phenotype. In embryos co-injected with mRNA and MOb1b, we found that the AP lengths of r4 through r6 increased towards the wild-type measurements (Table 1; Fig. 5H). This confirmed that exogenous Hoxb1b is sufficient to rescue the segmentation phenotype that results from injection of MOb1b, and verified that this phenotype results from loss-of-function of hoxb1b. The co-injected embryos also showed the previously described gain-of-function phenotypes, which include an increase in the AP extent of r3 caused by a shift of the r2/3 boundary towards the anterior (McClintock et al., 2001) (Fig. 5F,H).

The hoxb1a MO knockdown phenotype cannot be rescued by the hoxb1b duplicate gene, but the hoxb1b MO knockdown phenotype can be rescued by hoxb1a

Paralogous Hox genes often show partially redundant functions, suggesting that paralogues can have equivalent biochemical functions. This has been demonstrated for two mouse Hox PG3 genes, which have been shown to act synergistically (Condie and Capecchi, 1994); the mouse Hoxa3-coding sequence can functionally substitute for the Hoxd3-coding sequence (Greer et al., 2000). Nevertheless, individual null mutants for either mouse Hoxa3 or Hoxd3 have phenotypes that affect completely independent structures (Condie and Capecchi, 1993; Manley and Capecchi, 1995).

Thus, the differences in the patterning functions of these two genes must be mediated at the level of cis-regulation, rather than by differences between the proteins. We therefore wished to investigate whether the zebrafish hoxb1 duplicate genes also share equivalent biochemical functions.

To test whether hoxb1b has equivalent functional capacities to hoxb1a, we attempted to rescue the hoxb1a knock-down phenotype by co-injection of hoxb1b mRNA. When MOb1a (1 mg/ml) was co-injected with hoxb1b mRNA (at 20 or 40 μg/ml), concentrations that produce a high incidence of gain-of-function phenotypes when injected alone; Fig. 6A,C), we saw neither rescue of the hoxb1a knock-down phenotype nor a gain-of-function phenotype with regard to the BM neurons (Fig. 6B,D). Thus, islet1 positive neurons did not migrate posteriorly from r4 in co-injected islet1 transgenic embryos (100%; n=16), nor in non-transgenic embryos assayed by in situ hybridization for islet1 (100%; n=32); the r2 and r3 located Vth nerve neurons also appeared wild-type in these co-injected embryos. A trivial explanation for these results would be that Hoxb1b protein is less stable than Hoxb1a protein, and is therefore not present at a sufficient concentration to compensate for lack of Hoxb1a at the stage when BM neurons begin to migrate. However, we have previously shown by western blot analysis that mis-expressed Myc-tagged Hoxb1a and Hoxb1b proteins are present at equivalent concentrations in 20- to 22-hour stage embryos (McClintock et al., 2001), shortly after the onset of VIIth nerve neuronal migration at around 19 hours.

Although co-injection of hoxb1b mRNA and MOb1a did not alter r2-specific BM neurons, we did observe other gain-of-function phenotypes associated with ectopic Hoxb1b. Thus, ectopic Mauthner neurons formed at the r2 level (40%; n=43), as previously described for injection of hoxb1b mRNA alone (McClintock et al., 2001). This result is consistent with our demonstration that either duplicate is sufficient to allow production of Mauthner neurons. We also found that co-injection of hoxb1b mRNA and MOb1a led to ectopic transcription of hoxb1a at the r2 level at the 20-hour stage (92%; n=36), again as observed in response to hoxb1b mRNA alone (McClintock et al., 2001). This hoxb1a transcription was downregulated at both the r2 and r4 levels by the 36-hour stage (data not shown), consistent with an increasing requirement for an autoregulatory feedback loop to maintain hoxb1a transcription. These gain-of-function phenotypes confirmed that hoxb1b mRNA was functional in the co-injections. We can therefore conclude that Hoxb1a and Hoxb1b do not have equivalent biochemical functions: only Hoxb1a is capable of mediating migration of the VIIth nerve BM neurons.

We also attempted rescue of the hoxb1a MO phenotype using other PG1 gene mRNAs. In each case, we used an RNA concentration that produced gain-of-function BM neuron phenotypes in at least 70% of embryos when injected alone. We found that mouse Hoxb1 mRNA efficiently rescued the MOb1a phenotype (85%, n=39; data not shown). The single amphioxus PG1 gene, AmphiHox-1, also rescued the hoxb1a knockdowns.
albeit with lower efficiency (25%, \( n=25 \); data not shown). In each case, co-injection of both MO\(\text{b}1\)a and the mRNA led to rescue of the lack of VIIth nerve BM neuron migration, as well as gain-of-function phenotypes at the r2 level. Thus, unlike hox\(\text{b}1\)b, the mouse Hox\(\text{b}1\) or Amph\(\text{h}1\) RNA do have the capacity to substitute functionally for hox\(\text{b}1\)a. The results of our experiments to rescue the hox\(\text{b}1\)a knockdown phenotype reveal evolutionary conservation of the capacity of Hox PG1 genes to pattern BM neurons. However, they also reveal that Hox paralogues are not necessarily functionally interchangeable: in this case the products of the duplicate genes, hox\(\text{b}1\)a and hox\(\text{b}1\)b, do not have equivalent function.

In complementary experiments, we tested the capacity of PG1 genes other than hox\(\text{b}1\)a to rescue the MO\(\text{b}1\)a knockdown phenotype. We found that zebrafish hox\(\text{b}1\)a mRNA (\( n=20 \); Table 1) or mouse Hox\(\text{b}1\) mRNA (\( n=20 \), data not shown), could efficiently rescue the reduction in size of rhombomeres 4-6 that results from the knockdown of hox\(\text{b}1\)b. However, if mis-expressed hox\(\text{b}1\)a/Hox\(\text{b}1\) resulted in extensive upregulation of hox\(\text{b}1\)b transcription, ectopic hox\(\text{b}1\)b RNA could theoretically cause a rescue by titrating out the morpholino targeted against hox\(\text{b}1\)b. To test this possibility, we investigated the expression pattern, and levels, of hox\(\text{b}1\)b in hox\(\text{b}1\)a mRNA-injected embryos. We detected no differences between injected and wild-type embryos by in situ hybridization for hox\(\text{b}1\)b (using two-color double in situ with the r3/r5 marker krox20, at 10 hours (\( n=30 \)) and 12 hours (\( n=30 \) (data not shown)). Thus, we conclude that either zebrafish hox\(\text{b}1\)a, or mouse Hox\(\text{b}1\), has the capacity to allow proper segmental organization of the posterior hindbrain in the absence of hox\(\text{b}1\)a.

Our demonstration that hox\(\text{b}1\)a can rescue the defect in hindbrain segmentation is consistent with our finding that knockdown of both hox\(\text{b}1\) duplicates disrupts segmentation more profoundly than knockdown of hox\(\text{b}1\)b alone. Thus, although only hox\(\text{b}1\)a is capable of allowing proper VIIth nerve BM neuron migration, the two zebrafish hox\(\text{b}1\) duplicates share the capacity to allow the hindbrain to segment properly. However, in the normal situation, endogenous hox\(\text{b}1\)a is not able to compensate fully for knockdown of hox\(\text{b}1\)b, and therefore the MO\(\text{b}1\)a alone causes significant reduction in the sizes of posterior rhombomeres. We suggest that this reflects either the later expression onset of hox\(\text{b}1\)a or limiting concentrations of hox\(\text{b}1\)a protein in a normal embryo, rather than any differences in the capacity of the two duplicate gene products to confer proper segmental organization to the posterior hindbrain. Thus, while hox\(\text{b}1\)b is unable to allow migration of r4 BM neurons, because of differences at the protein level, the lesser role for hox\(\text{b}1\)a in hindbrain segmentation appears to be solely a function of differential regulation.

**Regulatory sequence analysis suggests sub-functionalization of the hox\(\text{b}1\) duplicate genes**

The spatial and temporal expression patterns of zebrafish hox\(\text{b}1\)a and hox\(\text{b}1\)b together resemble the expression pattern of mouse Hox\(\text{b}1\). Thus, the hox\(\text{b}1\)b expression pattern is similar to the early, gastrula phase of mouse Hox\(\text{b}1\) (and Hox\(\text{a}1\)) expression, while the hox\(\text{b}1\)a expression pattern is similar to the later, stable r4 expression of mouse Hox\(\text{b}1\). The cis-regulatory elements controlling transcription of mouse Hox\(\text{b}1\) in the neuroectoderm have been well defined, and have been shown to be conserved in mouse, chick and pufferfish (Marshall et al., 1994; Pöpperl et al., 1995; Langston et al., 1997). The early phase of mouse Hox\(\text{b}1\) expression, during gastrulation, is dependent on retinoic acid signaling through a retinoic acid response element (RARE) located 3’ of the coding sequence (Studer et al., 1998). By contrast, the stable r4 expression domain of Hox\(\text{b}1\) is maintained via a 5’ autoregulatory control element (Pöpperl et al., 1995). The sequences of the regulatory elements of zebrafish hox\(\text{b}1\)a and hox\(\text{b}1\)b are available from the zebrafish genome sequencing group at the Sanger Institute. We have therefore been able to analyze these sequences for the presence of RAREs and
autoregulatory elements similar to those described for other vertebrate Hoxb1 genes, as well as to compare the complete regulatory sequences of the two duplicate genes (Fig. 7).

RAREs consist of two direct repeat sequences, (A/G)G(G/T)C (X)n (A/G)G(G/T) C, where (X)n represents the number of nucleotides separating the repeats. The mouse Hoxb1 RARE that mediates early neuroectodermal expression lies about 3 kb downstream of the translational start and has two nucleotides separating the repeats (DR2 RARE). A DR5 RARE is also present 3′ of Hoxb1, about 7 kb downstream, but this sequence is important primarily for proper transcription in the developing gut (Huang et al., 1998).

Early neural expression of Hoxal is also dependent on a RARE located 3′ of the coding sequence (Langston et al., 1997; Due et al., 1997). The dependence of both Hoxal and Hoxb1 transcription on retinoid signaling explains the very similar early expression domains of these mouse PG1 Hox genes in r4 and more posteriorly. In zebrafish hoxb1b, we have found a DR3 RARE sequence in the 3′ regulatory sequences lying 2459 bp downstream from the start of translation (GGTTCACgtgAGTTCA), as well as a DR2 RARE lying 4752 bp downstream (GGTTCAGGGTTC). Thus, similar to the murine Hoxal gene, hoxb1b has two RAREs, one or both of which may be important for mediating the early neuroectodermal expression phase. By contrast, the hoxb1a duplicate lacks the early expression shown by hoxb1b or mouse Hoxb1. In hoxb1a, only one intact RARE sequence is present within 10 kb downstream of the end of the coding sequence. This is a consensus DR3 RARE (GGTTCACacagAGTTCA), which lies approximately 6 kb downstream from the start of hoxb1a translation. Thus, both mouse Hoxb1 and zebrafish hoxb1b have two RARE elements in their 3′ regulatory sequence, while hoxb1a has only one, with a location and spacing between the half elements similar to the gut enhancer of Hoxb1. We therefore suggest that loss of a DR3 RARE may be sufficient to explain the absence of gastrula stage neural expression of hoxb1a.

If the hoxb1 duplicate genes were retained because of sub-functionalization, we would expect to find degeneration of a separate cis-regulatory module in hoxb1. As the hoxb1 duplicates differ in the duration of their expression in r4, we examined their regulatory sequences for autoregulatory elements; such an element is crucial for stable r4 expression in mouse. The mouse Hoxb1 autoregulatory element consists of three conserved sequence repeats, which lie between 200 and 300 bp upstream of the translational start (Fig. 7A), and are bound by Hoxb1 protein together with a Pbx co-factor. Mutations in these sequences are sufficient to disrupt stable r4 expression of mouse Hoxb1, with alterations to repeat 3 causing the most severe defects; furthermore, oligomerized repeat 3 is sufficient to drive autoregulation (Pöpperl et al., 1995). Our inspection of the genomic sequence lying 5′ to hoxb1a has revealed that all three of these Hox/Pbx-binding sites are conserved in the zebrafish (Fig. 7A), consistent with our demonstration that hoxb1a is subject to autoregulatory control. However, the 5′ regulatory sequence of hoxb1b shows alterations in each one of these repeats, with repeat 3 having the most extensive changes (Fig. 7A).

Despite these distinct changes in the hoxb1b Hox/Pbx-binding sites, there remains extensive homology between the 5′ regulatory sequences of hoxb1a and hoxb1b (Fig. 7B). Disruption of the mouse Hoxb1 Hox/Pbx-binding site by targeted point mutations (Pöpperl et al., 1995) suggests that the changes seen in zebrafish hoxb1b are sufficient to explain the lack of a stable r4 expression domain for this gene. The complementary degeneration of cis-regulatory elements for the two hoxb1 duplicate genes, such that hoxb1a has lost a RARE element and hoxb1b has lost autoregulatory elements, is consistent with the DDC model, and suggests that the initial retention of the duplicates was dependent upon sub-functionalization.

DISCUSSION

Our knockdown experiments have shown that the zebrafish hoxb1a gene is required for proper migration of the neurons of the r4-derived facial (VIIth) nerve, whereas the hoxb1b gene is required for proper formation of r4 and more posterior rhombomere territories. These roles are very similar to those of the mouse Hoxb1 and Hoxal genes, respectively, revealing that ‘function shuffling’ has occurred during evolution of the vertebrate PG1 genes. Nevertheless, inspection of cis-regulatory sequences strongly suggests that initial retention of the hoxb1a and hoxb1b duplicate genes relied upon sub-functionalization. We have also found that there is significant functional redundancy between the zebrafish hoxb1 duplicate gene products: either duplicate is sufficient for Mauthner neuron differentiation, and either duplicate is capable of rescuing the segmental defects caused by knockdown of hoxb1b. Nevertheless, the two gene products do not have equivalent biochemical functions, as only the hoxb1a gene has the capacity to allow proper migration of VIIth nerve neurons. Thus, the hoxb1a and hoxb1b gene products have diverged in biochemical function since their duplication. However, we suggest that this divergence probably occurred subsequent to initial sub-functionalization of the two genes, because of complementary cis-regulatory mutations.

Zebrafish hoxb1 duplicate genes are required for proper hindbrain segmental organization and rhombomere identity

We have previously proposed, based on indirect evidence, that zebrafish hoxb1a and hoxb1b might be the functional equivalents of mouse Hoxb1 and Hoxal, respectively (McClintock et al., 2001). In this study, we made use of morpholino-based knockdown technology to test this hypothesis directly. In strong support of our hypothesis, we find that the knockdowns of hoxb1a and hoxb1b do indeed share many properties with the knockouts of mouse Hoxb1 and Hoxal.

The primary phenotype caused by loss-of-function of zebrafish hoxb1a or mouse Hoxb1 is loss of normal VIIth cranial nerve patterning. We find that in the absence of zebrafish Hoxb1a protein, the r4-derived BM neurons that would normally comprise the motor component of the facial (VIIth) nerve do not undergo their characteristic posterior migration, but rather remain at the r4 level, similar to the behavior of Vth nerve neurons in r2. These results suggest that in response to hoxb1a knockdown, the VIIth nerve neurons have either lost their normal identity and undergone an anteriorizing homeotic transformation, or have simply lost their capacity to migrate. In support of the latter hypothesis, we find that in hoxb1a knockdown zebrafish, the r4-derived
BM neurons retain their usual axonal trajectory, exiting from r4 to enter the second pharyngeal arch. Nevertheless, we suggest that the hoxb1a-deficient r4 neurons have taken on Vth nerve identity, but that they retain the ability to respond to local VIIth nerve axon pathfinding cues. Consistent with this idea, Guthrie and colleagues have shown using a transplantation approach that when chick Vth nerve neurons are placed posterior to their normal location, they too can project to the second pharyngeal arch, rather than pathfinding anteriorly to their normal target (Guthrie and Lumsden, 1992; Warrilow and Guthrie, 1999). Thus, axons of Vth nerve neurons are capable of responding to the pathfinding cues normally used by VIIth nerve neurons. Until distinct molecular markers are available to allow the neurons of the zebrafish Vth and VIIth nerves to be unambiguously distinguished, it will not be possible to discriminate between a requirement for hoxb1a to confer neuronal identity, versus a requirement for hoxb1a to confer neuronal identity, versus a requirement for hoxb1a to confer neuronal identity, versus a requirement for hoxb1a in migration of the r4-derived BM neurons.

When zebrafish hoxb1a is knocked down, transcription of hoxb1a is progressively downregulated, revealing positive autoregulation. In mouse, a direct autoregulatory feedback mechanism has been documented, mediated through three defined Hox/Pbx-binding sites upstream of the Hoxb1 regulatory elements (Pöpperl et al., 1995). An identical set of regulatory elements is present upstream of zebrafish hoxb1a (Fig. 7), suggesting that an equivalent direct feedback mechanism underlies positive autoregulation of hoxb1a. Taken together, our results are consistent with the hypothesis that zebrafish hoxb1a is the functional equivalent of mouse Hoxb1. The only obvious difference between the loss-of-function phenotypes is that Hoxb1 knockout mice show significant progressive reduction in the number of islet-positive r4-derived BM cells due to apoptotic cell death (Gaufo et al., 2000). By contrast, we have not observed any obvious loss of islet positive cells in zebrafish lacking hoxb1a function.

The primary phenotype caused by knocking down zebrafish hoxb1b is altered segmental organization of the posterior half of the hindbrain. When zebrafish hoxb1b is knocked down, we find that rhombomeres 4, 5 and 6 are all significantly reduced in AP extent, as is the adjacent otic vesicle. The hoxb1b knock-down phenotype is similar to that caused by mutations in mouse Hoxal (Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Mark et al., 1993), although both the different mouse Hoxal mutants investigated show more severe defects. Interestingly, the reduction we observe in AP extent of rhombomeres 4-6 does not result in discernable changes to neuronal identity within these rhombomeres. This finding suggests that precise placement of rhombomere boundaries is not absolutely required for proper rhombomere identity to be attained.

Knockdown of both zebrafish hoxb1 duplicates produces a phenotype that is again comparable with that of double mouse mutants for both Hoxal and Hoxb1 (Goddard et al., 1998; Studer et al., 1998), where the r4 and r5 territories become yet further reduced. In these embryos, we do find changes to rhombomere identity: there are alterations to the reticulospinal neurons in r4-r6, including absence of the r4-specific Mauthner neurons. Importantly, we have observed reduced rhombomere sizes in hoxb1a/loxhoxb1b-deficient zebrafish embryos from the earliest stages at which molecular markers can discriminate rhombomeric territories. This suggests a fundamental change in allocation of the hindbrain primordium to specific rhombomeric domains, and, in turn, argues against the alternative model of a progressive reduction in rhombomere sizes, either as a result of reduced cell division rates, or of increased cell death. Our preliminary TUNEL analyses have not revealed any major waves of cell death in the hindbrains of hoxb1a/loxhoxb1b-deficient zebrafish embryos (J. M. M. and V. E. P., unpublished).

As morpholinos may not be able to block translation completely, it is possible that the different phenotypes that result from mouse and zebrafish loss-of-function experiments are caused by incomplete knockdown of the zebrafish genes. However, we propose that the milder zebrafish phenotypes may instead reflect the contrasting manners in which mouse and zebrafish embryos respond to the presence of cells with inappropriate identity within the CNS. We hypothesize that zebrafish embryos are tolerant of mis-patterned cells within the CNS, whereas these cells tend to be eliminated by apoptosis in mouse. There is some precedent for this model: the mouse kreisler mutant loses cells posterior to r4 as a result of cell death (McKay et al., 1994), whereas zebrafish mutant for valentino, the zebrafish homolog of kreisler, were not found to show unusual levels of apoptosis (Moens et al., 1996). These different types of responses to mis-patterned cells may explain the retention of unmigrated islet-positive BM neurons in the hoxb1a knockdown zebrafish, while equivalent cells are eventually eliminated by apoptosis from the Hoxb1 mutant mouse. Tolerance of mis-patterned cells may also help to explain why Hoxal mutant mice lose a larger proportion of the posterior hindbrain than do hoxb1b knock-down zebrafish.

**Redundant and unique functions of hoxb1 duplicate genes**

We have demonstrated that the zebrafish hoxb1 duplicates share some redundant functions. Although there are segmentation defects in the absence of Hoxb1b, these are worsened by removal of hoxb1a, and conversely can be rescued by providing additional hoxb1a. Thus, either gene product can function to allow proper hindbrain segmentation if provided at the appropriate time at sufficient levels. Similarly, either hoxb1 duplicate is sufficient to allow differentiation of r4-characteristic Mauthner neurons; only when the functions of both duplicates are disrupted are these cells lost.

We have also established that hoxb1a has a unique function, not shared by hoxb1b. Only hoxb1a has the capacity to allow proper migration of the VIIth nerve BM neurons. Thus, the duplicates do not have completely interchangeable biochemical functions. This non-equivalence of function for the hoxb1a and hoxb1b proteins was previously suggested by our gain-of-function experiments, where we demonstrated that ectopic hoxb1a has the capacity to cause more extensive posteriorizing transformations than hoxb1b (McClintock et al., 2001). The role of Hoxb1 genes in BM neuron migration is very likely to be an ancestral one, as it is shared by the mouse Hoxb1 gene (Goddard et al., 1996; Studer et al., 1996); mouse Hoxb1 is also able to facilitate migration of zebrafish neurons, as we have shown in our rescue experiments. While the capacity to confer proper Mauthner neuron specification has been retained by both zebrafish hoxb1 duplicates, the ability to facilitate proper BM neuron migration has been lost by zebrafish hoxb1b. Our alignment of the protein sequences of zebrafish Hoxb1a and Hoxb1b, and mouse Hoxb1 has not revealed any obvious regions of identity that are specific to
those proteins that allow migration. However, future domain swapping experiments between zebrafish Hoxb1a and Hoxb1b should allow us to establish which domains of Hoxb1a are required for BM neuron migration.

The divergence in functions of Hoxb1a and Hoxb1b proteins is in contrast to the retention of equivalent function demonstrated by mouse Hox paralogues from group 3. Two of the mouse PG3 genes, Hoxa3 and Hoxd3, have coding sequences that are functionally interchangeable: when the coding sequence of one is replaced with that of the other, in the normal genomic context, no abnormalities can be detected in the resultant animals (Greer et al., 2000). The two zebrafish Hoxb1 genes are the result of a duplication event that occurred within the lineage leading to teleosts, a maximum of 410 Mya, whereas the duplication events that produced the tetrapod Hox paralogues are thought to have occurred earlier in vertebrate origins, about 500 Mya (Carroll, 1988; Holland, 1999). Nevertheless, despite their more recent origin, the zebrafish Hoxb1 proteins have diverged to a greater extent than have the mouse Hox PG3 proteins.

**Hox PG1 gene evolution**

We have made use of available data from mouse and other vertebrates to develop a model to explain the mechanism of retention of the zebrafish hoxb1 duplicate genes. The DDC model (Force et al., 1999; Lynch and Force, 2000) predicts that complementary degenerative changes to duplicate genes are likely to occur within modular cis-regulatory elements, allowing independent changes in a subset of expression domains. In the case of the Hoxb1 gene, extensive comparative studies have revealed that specific regulatory modules were present in the common ancestor of tetrapods and teleosts (Marshall et al., 1994; Pöpperl et al., 1995; Langston et al., 1997). In accordance with the DDC model, we find that different conserved regulatory modules have degenerated in each of the two zebrafish hoxb1 gene duplicates. Thus, hoxb1b, but not hoxb1a, has acquired changes to the autoregulatory elements responsible for stable r4 expression, while hoxb1a, but not hoxb1b, has lost a RARE that may be necessary for early neural expression (Fig. 8). These complementary changes are likely to have been sufficient to allow preservation of the two genes as postulated by the DDC model.

Nevertheless, the results of our experiments suggest that the functions of the two hoxb1 gene products have also diverged significantly, such that hoxb1b has lost the ability to mediate migration of VIIth nerve BM neurons. Thus, in an alternative model, sub-functionalization of the duplicates could have resulted from complementary changes in the cis-regulatory elements of hoxb1a, and in the coding sequence of hoxb1b. According to this scenario, the hoxb1a gene lost the capacity to perform a gastrula stage role in hindbrain segmentation due to loss of a RARE element, exactly as posited above. However, the hoxb1b gene lost the capacity to perform a later role in r4 patterning not as a result of changes to its cis-regulatory

**Fig. 8.** Model outlining the evolutionary mechanism of Hox PG1 gene ‘function shuffling’. The cis-regulatory elements characterized for the mouse and human Hoxa1 and Hoxb1 genes ([3’ RAREs (blue), Hox/Pbx binding sites (red)]) are assumed to be present in the ancestral, pre-‘third’-duplication, condition. We also postulate the presence of a regulatory domain directing midbrain expression of Hoxa1 (purple), although no such domain has yet been characterized. The duplication event in the lineage leading to teleosts produced redundant copies of both Hoxa1 and Hoxb1 in an ancestor of the zebrafish. The hoxa1b duplicate was eventually lost by accumulation of deleterious mutations (‘non-functionalization’) as predicted by classical models. By contrast, the hoxb1a and hoxb1b genes accumulated complementary degenerative changes in their cis-regulatory elements, such that hoxb1a lost early RARE-mediated expression and hoxb1b lost autoregulation. This led to retention of the duplicate genes, as both were required to maintain the expression pattern and function of the single Hoxb1 ancestral gene (sub-functionalization), as predicted by the DDC model. As hoxa1a and hoxb1b shared similar coding sequences and expression patterns, these two genes were now functionally redundant with respect to a role during gastrulation in setting up segmental organization of the hindbrain. These non-orthologous genes were thus able to go through another ‘sub-functionalization’ event, such that hoxa1a lost its early RARE-mediated expression, which was retained by hoxb1b. Thus, hoxb1b became essential for proper hindbrain segmentation, the role played in the ancestral state by Hoxa1. Retention of the hoxa1a gene in the lineage leading to zebrafish was presumably dependent on a function that was not redundant with hoxb1b, possibly a role in midbrain patterning. We term this rearrangement of PG1 gene roles ‘function shuffling’.
elements, but rather as a result of changes to its coding sequence. In this model, the alterations to the autoregulatory elements of hoxb1b would have happened subsequent to the changes in the coding sequence.

We favor the model that the complementary changes in cis-regulatory elements led to initial retention of the duplicate genes (Fig. 8), and that this was followed by a secondary loss of the capacity of Hoxb1b to mediate VIIth nerve neuron migration. Subsequent to initial sub-functionalization of duplicates, functional constraints on each individual gene may be relaxed, allowing alterations in coding sequences to occur. Thus, once hoxb1b lost late r4 expression, domains of the protein necessary only for VIIth nerve neuron migration would have been free to change.

The alterations in both cis-regulation and coding sequences, regardless of which occurred first, have ultimately allowed zebrafish hoxb1a to retain the primary ancestral role of Hoxb1, while the zebrafish hoxb1b gene plays the role that is played by Hoxal in mouse (and presumably in other tetrapods). In addition, expression of the zebrafish hoxa1 ortholog, hoxa1a, has been lost in the hindbrain (McClintock et al., 2001; Shih et al., 2001). We propose that an intermediate evolutionary phase of functional redundancy between a hoxa1 gene and hoxb1b (Fig. 8) has allowed loss of the early expression of hoxa1a because of degenerative changes in the 3’ RARE sequences. The early role of the hoxa1 gene, in hindbrain segmentation, was then taken over by hoxb1b. We have termed this rearrangement of functions amongst paralogous genes ‘function shuffling’. This phenomenon may prove to be a common consequence of the gene duplications that have characterized vertebrate evolution.

Gene duplications are thought to provide new genetic material that can ultimately allow evolutionary novelties to arise. In the case of Hox PG1, it appears that duplication has allowed at least one of the genes, hoxb1b, to acquire alterations to its coding sequence such that the biochemical function of the gene product has changed. In addition, both hoxb1 duplicates have undergone changes in cis-regulatory sequences that may have important functional ramifications. Our data highlight the importance of both coding sequence and cis-regulatory changes after duplication of key developmental control genes. As we come to understand the consequences of other specific examples of gene duplications, we can begin to evaluate whether gene duplication has in fact been instrumental in the evolution of vertebrate body plans.

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