

# Consequences of Hox gene duplication in the vertebrates: an investigation of the zebrafish Hox paralogue group 1 genes

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## SUMMARY

As a result of a whole genome duplication event in the lineage leading to teleosts, the zebrafish has seven clusters of Hox patterning genes, rather than four, as described for tetrapod vertebrates. To investigate the consequences of this genome duplication, we have carried out a detailed comparison of genes from a single *Hox* paralogue group, paralogue group (PG) 1. We have analyzed the sequences, expression patterns and potential functions of all four of the zebrafish PG1 Hox genes, and compared our data with that available for the three mouse genes. As the basic functions of Hox genes appear to be tightly constrained, comparison with mouse data has allowed us to identify specific changes in the developmental roles of Hox genes that have occurred during vertebrate evolution. We have found variation in expression patterns, amino acid sequences within functional domains, and potential gene functions both within the PG1 genes of zebrafish, and in

comparison to mouse PG1 genes. We observed novel expression patterns in the midbrain, such that zebrafish *hoxa1a* and *hoxc1a* are expressed anterior to the domain traditionally thought to be under Hox patterning control. The *hoxc1a* gene shows significant coding sequence changes in known functional domains, which correlate with a reduced capacity to cause posteriorizing transformations. Moreover, the *hoxb1* duplicate genes have differing functional capacities, suggesting divergence after duplication. We also find that an intriguing function 'shuffling' between paralogues has occurred, such that one of the zebrafish *hoxb1* duplicates, *hoxb1b*, performs the role in hindbrain patterning played in mouse by the non-orthologous *Hoxa1* gene.

Key words: Hox, Vertebrate, Zebrafish, Gene duplication, Hindbrain, Midbrain, Mauthner neurone, MLF

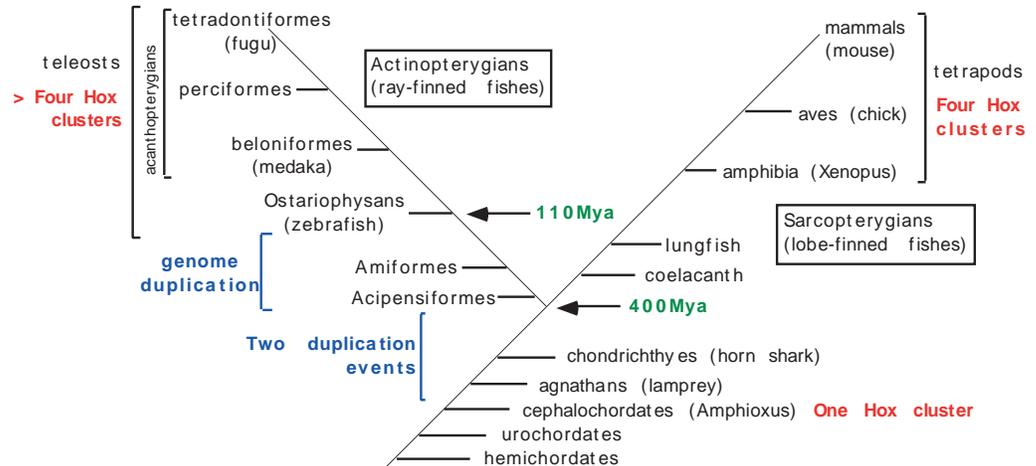
## INTRODUCTION

Clustered Hox genes encode a conserved family of transcription factors implicated in providing regional identity along the anteroposterior axis of all bilaterian animal embryos (de Rosa et al., 1999; McGinnis and Krumlauf, 1992). Invertebrates, including the cephalochordate amphioxus, have a single cluster of Hox genes (de Rosa et al., 1999; Garcia-Fernandez and Holland, 1994), but duplication events during vertebrate evolution have produced a minimum of four clusters in the jawed vertebrates, as described for mouse and human (reviewed by McGinnis and Krumlauf, 1992). A further duplication event occurred in the lineage leading to teleosts, subsequent to the divergence of the ray-finned and lobe-finned fishes about 400 million years ago (Mya) (Fig. 1; Carroll, 1988). This has resulted in a seven cluster organization for zebrafish (*Danio rerio*, an ostariophysan teleost) (Amores et al., 1998). The medaka (*Oryzias latipes*; a distantly related acanthopterygian teleost) has a similar seven cluster organization (Naruse et al., 2000), suggesting that the duplication occurred before the radiation of teleosts, which commenced about 110 Mya (Fig. 1).

Zebrafish has a minimum of 48 Hox genes, compared with the 39 described for mouse and human (Amores et al., 1998), revealing that many secondary losses of duplicated genes, including loss of an entire cluster, occurred in the lineage leading to zebrafish. Nevertheless, some duplicated Hox genes have been retained in teleosts, and the availability of duplicates may have helped to facilitate the remarkable degree of morphological variation found in modern day teleosts.

Immediately after a whole genome duplication event, the resultant gene copies are identical and thus have redundant functions. Such functional redundancy can allow eventual loss of one copy by accumulation of deleterious mutations (Wagner, 1998). However, in many instances (possibly as often as 50% of the time; Nadeau and Sankoff, 1997) duplicated genes are retained in the genome; this is believed to be due either to acquisition of novel function by one duplicate (neofunctionalization) or division of the ancestral functions between both duplicates (subfunctionalization). These events can be mediated by alterations in either coding or *cis*-regulatory sequences (discussed by Force et al., 1999; Cooke et al., 1997). Gene duplication, followed by acquisition of

**Fig. 1.** Chordate phylogeny, which illustrates evolutionary relationships and differing Hox cluster complements (from Carroll, 1988). Blue brackets indicate time spans within which particular duplication events are believed to have occurred. The times when the ray and lobe-finned fishes last shared a common ancestor, and when the ostariophysans and acanthopterygians last shared a common ancestor, are indicated in green (in millions of years ago; Mya).



novel function, is believed to have played a key role in allowing evolutionary novelties to arise (Ohno, 1970).

We wished to investigate how zebrafish Hox genes have evolved subsequent to genome duplication. We chose to focus our study on the paralogue group (PG) 1 genes, which are located at the 3' ends of the Hox clusters, because their anterior expression domains minimize potential functional redundancy with genes from other paralogue groups. In general, Hox genes show both temporal and spatial colinearity of expression, such that more 3' genes have more anterior expression limits and earlier onsets of expression than genes located more 5' within the cluster. It is at or near their anterior expression limits that Hox genes play their functional roles (reviewed by McGinnis and Krumlauf, 1992).

Mouse, and other tetrapods investigated, have three PG1 genes. The mouse *Hoxd1* gene is not expressed in the developing CNS (Frohman and Martin, 1992), and will not be discussed further. The other two genes, *Hoxa1* and *Hoxb1*, have been especially well studied; both function in patterning rhombomere 4 of the hindbrain. The rhombomeres are a transient array of segments along the anteroposterior (AP) length of the developing hindbrain (r1-r7, from A to P). 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 both expressed in presumptive r4 during gastrulation (Murphy and Hill, 1991; Barrow et al., 2000). *Hoxa1* activates *Hoxb1* expression in r4 (Studer et al., 1998), but then *Hoxa1* expression retracts posteriorly during early somite stages. *Hoxb1* expression persists in r4 under autoregulatory control (Frohman et al., 1990; Murphy and Hill, 1991; Wilkinson et al., 1989; Pöpperl et al., 1995). Loss-of-function analysis has shown that *Hoxa1* is necessary for normal formation of r4 and r5, as well as for proper segmentation of the hindbrain (Chisaka et al., 1992; Carpenter et al., 1993; Lufkin et al., 1991; Mark et al., 1993), while *Hoxb1* is required for proper r4 identity (Goddard et al., 1996; Studer et al., 1996). Interestingly, Hox PG1 genes do not display spatial colinearity, as PG2 genes are expressed more anteriorly in the hindbrains of both mouse and chick (Frasch et al., 1995; Prince and Lumsden, 1994).

As overall Hox gene function is likely to be tightly constrained within the vertebrates, we would expect to find

extensive functional conservation between the mouse and zebrafish PG1 genes. However, the genome duplication in the lineage leading to teleosts may have facilitated the modification of PG1 functions in zebrafish; these types of changes may have played a key role in the explosive radiation of teleost fishes. The zebrafish has four Hox PG1 genes, *hoxa1a*, *hoxb1a*, *hoxb1b* and *hoxc1a* (Amores et al., 1998) based on their sequences and linkage relationships. Zebrafish *hoxc1a* has no known orthologue among the tetrapods; similarly, no orthologue of *Hoxd1* has been isolated from the zebrafish. We have examined the sequences and expression patterns of these four zebrafish genes, and used a gain-of-function assay to investigate their functional capacities. We have compared our results between the four zebrafish genes, as well as with data available for mouse PG1 genes. We find differences between the PG1 genes in amino acid sequence within functional domains, in expression patterns and in functional capacities. In addition, we find evidence for an interesting function 'shuffling' between paralogues, where non-orthologous genes fulfil equivalent roles in mouse and zebrafish.

## MATERIALS AND METHODS

### Cloning and constructs

#### *hoxa1a*

PCR primers overlapping the START and STOP codons of the *hoxa1a* gene were designed from PAC# 227P6 sequence (Amores et al., 1998).

a1aSTART: ggaattcCCAAGAAGATGACACAATGAGC (*EcoRI* site underlined)

a1aSTOP: cgatgactAGCTGTTTAATTAGAGGAGTATGCC (*XbaI* site underlined)

Full-length cDNA was PCR amplified from 24-hour-old zebrafish embryo cDNA (prepared as previously described, Prince et al., 1998a) using Pwo proof reading Taq polymerase (Boehringer-Mannheim). Two alternative splice forms were isolated and subcloned into the expression vector pCS2+ (Turner and Weintraub, 1994) to generate pCS2a1a(short) and pCS2a1a(long). Each cDNA was also subcloned in frame into pCS2myc, which provides six Myc epitopes in series, to make N-terminal Myc-tagged versions.

#### *hoxb1a*

The 5' end of the gene was isolated by RACE-PCR, from a primer

within our previous 3' RACE clone (Prince et al., 1998a). This sequence was then used to generate a PCR primer overlapping the START codon and full-length cDNA amplified and subcloned as described above to generate pCS2b1a and pCS2mycb1a.

b1aSTART: cgggaattCCGCCATTAATTGCGTATGG (*EcoRI* site underlined)

b1aSTOP: gctctagaCACATTTGATCAGTGCACCC (*XbaI* site underlined)

(In this construct, approximately 40 nucleotides of 3'UTR sequence were incorporated downstream of the STOP codon.)

#### hoxb1b

Published sequence (Alexandre et al., 1996) was used to design PCR primers to isolate full-length coding sequence as described above.

b1bSTART: ccgctcgaGCTTGGTCAAGCAATGAATTC (*XhoI* site underlined)

b1bSTOP: gctctagaTTAGTAGTTCTAATGCGACAC (*XbaI* site underlined)

(In this construct an additional 100 bp of 3' UTR was incorporated downstream of the STOP codon.) We found seven individual discrepancies at the nucleotide level with the previously reported sequence for *hoxb1b* (Alexandre et al., 1996); these are single nucleotide changes/deletions and they lead to one amino acid change in the N-terminal part of the protein and a run of 11 different amino acids C-terminal to the homeodomain. The sequence we report within this problematic region shares several conserved amino acids with *hoxb1a*. pCS2b1b and pCS2mycb1b were generated as described above. In addition, a mutagenized control version (pCS2b1bMUT) was generated by changing the amino acid at position 50 within the homeodomain (Q257 to E257) using the Stratagene Quickchange kit according to manufacturer's instructions. A similar approach was previously used to mutagenize the homeobox gene *Xotx2* (Pannese et al., 1995) resulting in loss of DNA binding activity of the protein product and loss of function. As expected, mRNA from pCS2b1bMUT produced no phenotype after injection.

#### hoxc1a

The 5' end of the gene was isolated by RACE-PCR from a primer within the 3' sequence (kindly provided by Angel Amores). The 5' sequence was used to generate a PCR primer overlapping the START codon and full-length cDNA amplified and subcloned into pCS2+ as described above. Primer sequences were:

c1aSTART: gctctagaTGCGGTCATGAATTCTTAT (*XbaI* site underlined);

c1aSTOP: cgggatccCGCTTTTAATTTTCACGTG (*BamHI* site underlined).

Presence of additional amino acids between the hexapeptide and homeodomain, in comparison with other vertebrate PG1 genes, suggested absence of an intron; this finding was confirmed by PCR amplification of this region from genomic DNA. An N-terminal Myc-tagged version of *hoxc1a* was also constructed (pCS2mycc1a) as described above, but using a c1aSTOP primer that includes an *XbaI* site rather than a *BamHI* site.

All constructs were completely sequenced in both directions to ensure correct frame and sequence. For each cDNA the presumed START of translation lies downstream of an inframe STOP codon.

#### Micro-injections

Synthetic capped mRNAs were produced from linearized DNA templates using the Ambion Megascript kit according to manufacturer's instructions. mRNA was suspended in water + 1% rhodamine dextran (Molecular Probes) at concentrations between 10 and 100 ng/μl and approximately 1 nl pressure-injected into the cytoplasm of one- to two-cell stage embryos.

#### Assays for protein production

Synthetic mRNAs were tested for their ability to produce a protein

product of the expected size by in vitro translation using a Promega reticulocyte lysate kit followed by SDS-PAGE electrophoresis. Amersham 'rainbow' markers were used to assess molecular weight. To assay protein production in vivo, 15-30 embryos were injected with Myc-tagged constructs and protein extracts from these embryos used in Western blot analysis. The injected embryos were harvested at the 20-22 hour stage, dissected from the yolk, and protein extracted using NP40 lysis buffer with protease inhibitors (as previously described; MacNicol et al., 1993). Approximately 10 μg of total extracted protein (as assayed by a Lowry reaction) was then electrophoresed on a 10% SDS-PAGE gel, electroblotted to nitrocellulose filters and the filters probed for Myc protein using the monoclonal 9E10 anti-Myc antibody (Roth et al., 1991). Detection was completed with the ECL chemiluminescent kit (Amersham).

#### Immunochemistry

The following antibodies were used for whole-mount immunochemistry as previously described (Prince et al., 1998b): 3A10 antibody (Furley et al., 1990), 9E10 anti-myc antibody (Roth et al., 1991) and HNK-1 (Sigma).

#### Retrograde labelling

Reticulospinal neurones were revealed by retrograde labelling from the spinal cord at 5 days of larval development. Labelling was performed as previously described (Alexandre et al., 1996). Labelled brains were visualized by confocal microscopy using a Zeiss LSM510 confocal microscope and a 25× water immersion objective.

#### In situ hybridization

In situ hybridization was performed as previously described (Prince et al., 1998a). In situ probes for the following genes were used: *krox-20* (Oxtoby and Jowett, 1993), *mariposa* (Moens et al., 1996), *islet-1* (Inoue et al., 1994), *ephA4* (Xu et al., 1995) and *hoxb1a* (Prince et al., 1998a). In addition, full-length coding sequences of *hoxb1b*, *hoxa1a* and *hoxc1a* (as described above) were subcloned into pBluescript (Stratagene) for generation of in situ probes. The probe for *hoxa1a* recognizes both splice variants of the gene.

## RESULTS

### Sequence and domain structure of the four zebrafish paralogue group 1 genes

Vertebrate paralogue group (PG) 1 genes are distinguishable from *Hox* genes in other paralogue groups by three main criteria. PG1 genes lie most 3' within *Hox* clusters, they have seven diagnostic residues in the homeodomain (Sharkey et al., 1997), which probably confer DNA binding specificity, and they have a conserved hexapeptide sequence, WMKVKR, that lies 19 or 20 amino acids N-terminal of the homeodomain. We compared the amino-acid sequences of the zebrafish PG1 gene products with those of mouse and the single amphioxus PG1 gene, *AmphiHox-1* (Fig. 2A). We found that *hoxa1a*, *hoxb1a* and *hoxb1b* possess all the PG1 gene-specific features. However, *hoxc1a* has two amino acid substitutions in PG1 diagnostic homeodomain residues, as well as two amino acid substitutions in the hexapeptide. Although the *hoxc1a* gene is physically adjacent to *hoxc3a* (Amores et al., 1998), it encodes none of the seven PG2-specific residues, confirming that it has been correctly assigned to PG1.

Comparison of cDNA and genomic sequences revealed differences in the intron/exon structure of the zebrafish PG1 genes in comparison with those of mouse and human (Fig. 2B). In general, all reported vertebrate PG1 genes have a single

**A**

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mHoxa-1 1 -----MNSFLEVP-ILGSDGSG---TCSARAYPSDHGITTFQS---CAVSANS CGGD
zfHoxa1a 1 -----MSFLLDFSSISGGGSGSGGSCSVRAFHGDHGLSTFQS---CAVRLNSCGSD
zfHoxb1a 1 ---MDSSRNMSFLEVP-ICNRCTNA---YSPKAGY-HLLDQAFPGPPHTGHASDYNAD
zfHoxb1b 1 ---MNSYLDYI-TYNRGSNT---YSSKVG-CFPVEQBYL---SACASTNSYIPE
mHoxb-1 1 ---MDYNRMSFLEVP-LCNRGPSA---YSAPT-----SFPD---CSAPAVDSYAGE
amphiHox1 1 MEQMIDTARMNSYVDYS-LCNGDQNT---CSPRSYQDYGVPAQYS---CANNVDRHYTM
mHoxd-1 1 -----MSSYLEYV-SCAAGGGS-----GGVGG---DVLAFAP---KFCADAREVALQ
zfHoxc1a 1 -----MNSYHGFRDITSLFIKGYHA---TGMVRLHQDSTADFP---SREDVNNNEADF

mHoxa-1 46 DRFLVGRGVQ-IGSPHHHHHHHHHPQTATYQTSNGLGISYSHSS-CGPSYGAQNFSAF
zfHoxa1a 51 ERFMSN----ISS---QDVINSQPQAGSYQSGFTLSITYSAH---PSYGTQSFCTG
zfHoxb1a 52 GRLYVGGSNPPTAAQAQHONGIYAHQHQN---QTGMGLTYGGT---GTTSYGTQACA-
zfHoxb1b 45 GRP-VGGN---TFTSAPHETHGTSYAQIQSQ---PFLHNDVMGKT---GHSNFCKQTRFP
mHoxb-1 43 SRY---GGG---LPSALQQNSG---YFVQQPP---SSLGVSPSP---APSGYAPAACN-
amphiHox1 44 GPN---G-Q---LPPSAG---PGPG---PVPGPS---PYDPPVIMPNG---DPQNFYTSYNH
mHoxd-1 41 PAFPLQNGDGAFAVSCCLPLATAR---PTPSPAG-PAQSPVQPAAPRYAPCTLEGAVERG
zfHoxc1a 49 LQY-----HFTNLICATATG-----ACSKP---CEDPGRANSTR---PFPQNDQFYR

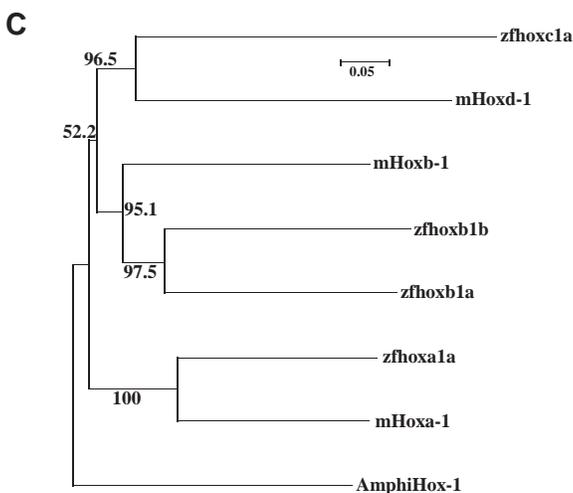
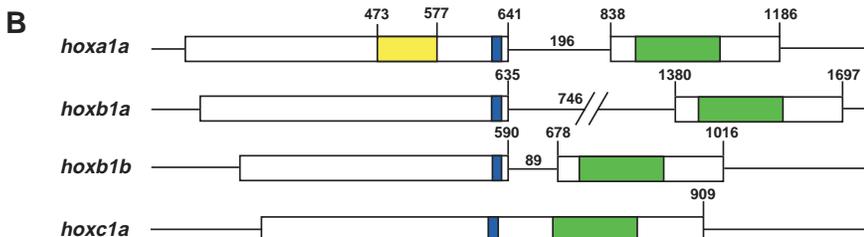
mHoxa-1 104 YGPYGLNQEADVSGGYP---PCAPAVYSGNLSTPMVQHSHHHHGYAGGTV---GSPQYIHH
zfHoxa1a 98 YNHVALNQDVESSVSFP---QCGLVYSGNISSTVVQHRHRRHGYSSGNVHLHQFQYVGA
zfHoxb1a 106 NSDYAQHQYFIPNPEQDMYHSSGFSSTNAS-PHYGSMAGAYCGAQAQVPA-APYQHHG-
zfHoxb1b 95 HSDYTG-HQHVLTQADHMLRQLSPGFVSVNMG-ANIGTYSSENC-RPGVSA-SHVQSY--
mHoxb-1 88 -PSYGPSQYYSVQSEB---DGSYFHPSSYG-AQLGGLPDSYG---AGVGS-GPYVFPQP
amphiHox1 98 YSHPG-GHHMSNGYGTN---NHAAMYSGVSG-RACRFLLVVQLRNERIV---APPLDLS-
mHoxd-1 97 AAPASAAEYGLGSGAFDFPGALGRAADEGGAHVRYATSAVSFGSGLSLLQVDFAAF
zfHoxc1a 90 PVHFNRPQVPSFQSCRE---QGLSRKGFSPSDFRTFRTSSAHCELGSPVNN---TQTEVR--

mHoxa-1 159 -SYGQ---EQQTLLALAT-YNNSLSPHSHASHQEA RSPAS ET---SSPAQTFDWMKVKRNP
zfHoxa1a 156 -TYGNSDQANLTFVAGCSNPLSPLHVPHDACCSPLEBDG---VPTGQTFDWMKVKRNP
zfHoxb1a 163 -CEQDQ---HQRAYSQG---TYADL SASQGT EKDDTDQ-----PFPKTFDWMKVKRNP
zfHoxb1b 149 -AYGE---PEFHGCG---SFSKYQVSDSDSKTN---IKQPTFDWMKVKRNP
mHoxb-1 140 -PYGT---EQATTFAS---AYDLLSEDKSPSCSEPS---TLTPRTFDWMKVKRNP
amphiHox1 149 -QYGY---MHHTG---QDPMIST---TCNPPAP---SPPVATYDWMKVKRNP
mHoxd-1 157 GEPQPPACLKEPADG-HPGFPQTVPAPG-ACPKPASPTSSLPAAHSTFEWVKVRNP
zfHoxc1a 143 -TYDG-----PVRHS---AVEDDNTGHGALNLTNET---LHSGKTFEWMVKRNP

mHoxa-1 212 KTGKVGEGYVY-----GQPNVTRNFTTKQLTELEKEFHNKYLTRARRV
zfHoxa1a 212 KTGKAGEYFG-----GQPNVTRNFTTKQLTELEKEFHNKYLTRARRV
zfHoxb1a 210 KTGKVAEYGL-----GQPNVTRNFTTKQLTELEKEFHNKYLTRARRV
zfHoxb1b 195 KTKVVAEYGIH-----GQNIIRNFTTKQLTELEKEFHNKYLTRARRV
mHoxb-1 187 KTKVSEGLGL-----GAPGLRNTFTTKQLTELEKEFHNKYLTRARRV
amphiHox1 190 RTGKPEYGFYTT-----SGPNNRNTFTTKQLTELEKEFHNKYLTRARRV
mHoxd-1 215 KKSGLSEYGAT-----SPPSALRNTFTTKQLTELEKEFHNKYLTRARRV
zfHoxc1a 187 RAAKIQLKCS DRELKTNHGRDSD EPTSSGSRNTFTTKQLTELEKEFHNKYLTRARRV
** *

mHoxa-1 257 EIAASLQLNETQVKIWFQNRMRKQKKREKEG-LLPISPATPPGSDKTEESSSEKSSPSPS
zfHoxa1a 257 EIAASLQLNETQVKIWFQNRMRKQKKREKEG-LLPKSLSEKQKDGLEKTEDASBKSPSPS
zfHoxb1a 254 EIAATLELNETQVKIWFQNRMRKQKKREKEG-LAPASST---SSKDLEDQ-SDHTSTSSP
zfHoxb1b 240 EVAATLELNETQVKIWFQNRMRKQKKREKEG-TAPVIRK---VTLCSSGQNDHSTSSP
mHoxb-1 231 EIAATLELNETQVKIWFQNRMRKQKKREKEGGRMPAGPPG---CPKEAAGDASDQACTSP
amphiHox1 236 EIAAALNLNETQVKIWFQNRMRKQKKREKEN-----G---FSTPGS--GGSPAG
mHoxd-1 260 EIANCLQLNDTQVKIWFQNRMRKQKKREKEG-LLATAAS---VASIKLRSGETSPKSGRN
zfHoxc1a 247 EIANPLQLSETQVKIWFQNRMRKQKKMLREG-LAQG---L--MLISGCEDESKSGRD
* *

mHoxa-1 316 APSPASSTSDTLTTS
zfHoxa1a 316 TSPSPS-TVEAYSSN-
zfHoxb1a 309 EASPSDSS-----
zfHoxb1b 296 GASPTSDSSTAI----
mHoxb-1 289 EASPSSTIS-----
amphiHox1 280 EDSPSKST-----
mHoxd-1 317 LGSPSQAQEPS-----
zfHoxc1a 298 CSSPD-----
    
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intron located between the hexapeptide and homeodomain. The *hoxa1a* gene has a splice variant that results when a small exon N-terminal to the hexapeptide is spliced out (Fig. 2B); this variant maintains the open reading frame and thus encodes a smaller protein that retains a functional homeodomain. As described below, we found that the long and short forms of *hoxa1a* have identical functional capacity in our gain-of-function assay. Splice variants have also been described for mouse and human *Hoxa1* (Baron et al., 1987; Hong et al., 1995), however, in these cases the additional splice results in a frameshift that renders the homeodomain nonfunctional. The *hoxc1a* gene has no intron and, as a consequence, the spacing between the hexapeptide and homeodomain is extended to 35 amino acids rather than the 19-20 amino acids found in all other vertebrate PG1 gene products reported (or the 21 found in *AmphiHox-1*). It has previously been suggested that such alterations to the length of the linker between the hexapeptide and homeodomain may alter normal interactions with Pbx co-factors (Scott, 1999). Taken together with the amino acid changes in the hexapeptide noted earlier, our results suggest that the ability of *hoxc1a* to interact with Pbx co-factors may be impaired.

A neighbour-joining tree showing the phylogenetic relationships between the PG1 genes

**Fig. 2.** Hox paralogue group 1 genes: sequence and functional domains. (A) Clustal X (Thompson et al., 1997) alignment of amino acid sequences of zebrafish, mouse and amphioxus Hox paralogue group 1 genes. Conceptual translations of the four zebrafish Hox PG1 genes are compared with mouse *Hoxa1*, *Hoxb1* and *Hoxd1* and *AmphiHox-1*. Identical residues in red, conserved changes in blue. Hexapeptide and homeodomain are overlined in blue and green, respectively; note the unusually long linker region between the hexapeptide and homeodomain regions of *hoxc1a*. The diagnostic PG1 residues (Sharkey et al., 1997) are indicated with asterisks (below sequence). The 2/7

diagnostic residues not conserved in *hoxc1a* are indicated with black rather than red asterisks. (B) Schematic of intron/exon structure for the four zebrafish PG1 genes, drawn to scale; hexapeptide and homeodomain are indicated in blue and green, respectively, alternatively spliced exon is indicated in yellow. Based on comparison to genomic sequences (GenBank Accession Numbers AF071243, AF071251, U40995, AF071263). Note that *hoxc1a* has no intron, as confirmed by PCR on genomic DNA. Numbers indicate intron/exon boundaries with respect to the start of translation; the length of the primary intron is also indicated. The following coding sequences have been placed on the EMBL database: *hoxc1a*, Accession Number AJ306432; *hoxb1b*, Accession Number, AJ306433; and *hoxa1a*, Accession Numbers AJ306430 and AJ306431. (C) Neighbour-joining tree to show the phylogenetic relationships between Hox PG1 genes (based on Clustal X alignment in A; displayed using NJ-Plot; Perriere and Gouy, 1996), bootstrap values based on 1000 replicates are shown; scale bar refers to branch lengths. The tree suggests that mouse *Hoxd1* and zebrafish *hoxc1a* group together; however, the long branch lengths imply that these genes are more distantly related.

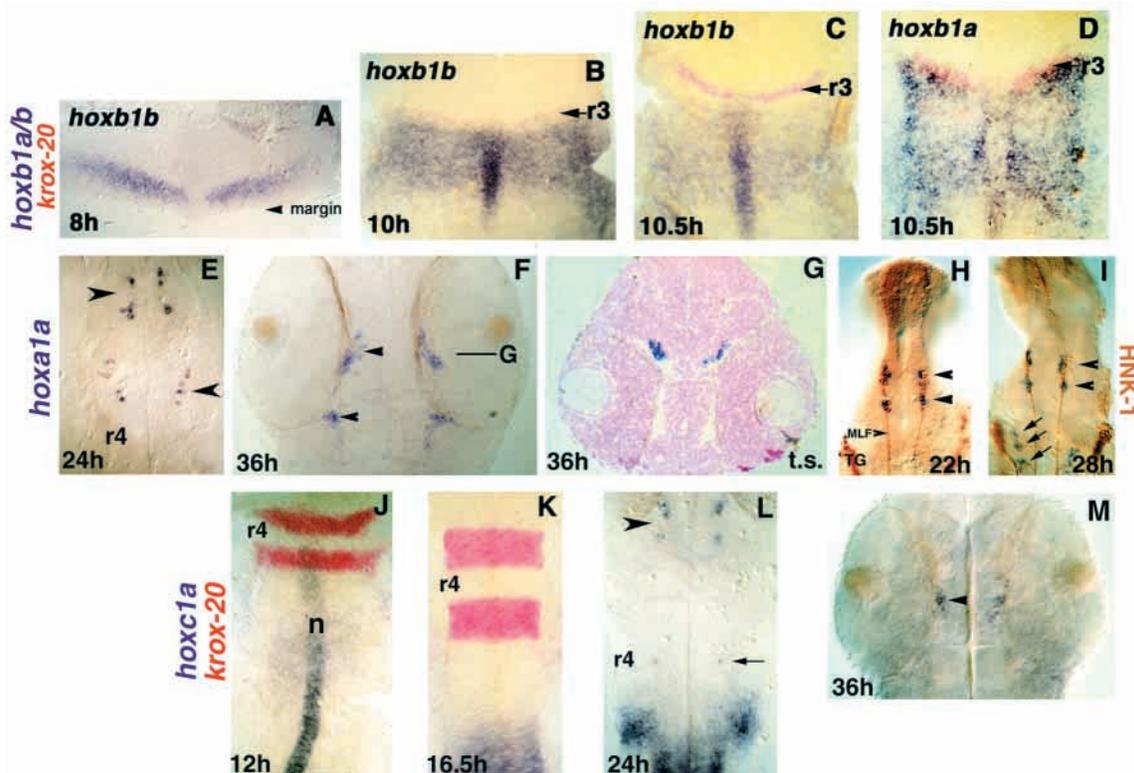
(Fig. 2C) indicates that zebrafish *hoxa1a* is most closely related to mouse *Hoxa1* (supported by a bootstrap value of 100%), and the two zebrafish *hoxb1* duplicate genes are most closely related to mouse *Hoxb1* (bootstrap value 95%). However, we show below that two orthologous genes, zebrafish *hoxa1a* and mouse *Hoxa1*, have evolved very different developmental functions in their respective species.

### Zebrafish *hoxa1a* and *hoxc1a* are expressed in ventral midbrain neurones

One of the ways that gene function can evolve is by alterations in spatial or temporal patterns of gene expression. To determine whether such events have occurred in the zebrafish, we examined expression profiles of the Hox PG1 genes between gastrulation and 36 hours (h) of development (Fig. 3). In the mouse, *Hoxa1* is expressed transiently in r4, where it activates *Hoxb1* expression and then recedes posteriorly; *Hoxb1* expression is then maintained in r4 by autoregulation. We found that *hoxb1b* has the earliest onset of expression of the zebrafish PG1 genes, early in gastrulation (Fig. 3A) in

presumptive r4 (Alexandre et al., 1996; note that in this study *hoxb1b* was referred to as *hoxa1*). We have confirmed this localization at tail bud stage (10 h) by double in situ hybridization with the r3/r5 marker *krox-20* (Oxtoby and Jowett, 1993; Fig. 3B). Shortly after this stage, the *hoxb1b* expression domain in the CNS recedes posteriorly (Fig. 3C; Alexandre et al., 1996). As we previously reported (Prince et al., 1998a), the *hoxb1a* gene has a slightly later onset of expression at approximately 9 h, with an anterior limit at the r3/r4 boundary (Prince et al., 1998a; Fig. 3D). Expression of *hoxb1a* within r4 is upregulated shortly after the onset of expression (Fig. 3D), and this high level expression domain is retained at all stages examined; such prolonged r4-specific expression is a feature conserved with the *Hoxb1* genes of all vertebrates investigated (Prince et al., 1998a). Zebrafish *hoxb1a* has a similar expression profile to mouse *Hoxb1*, but the duplicate gene, *hoxb1b*, is expressed in a similar manner to mouse *Hoxa1*. This suggests that zebrafish *hoxb1b* may play a similar developmental role to mouse *Hoxa1*.

If zebrafish *hoxb1b* has an expression pattern equivalent to



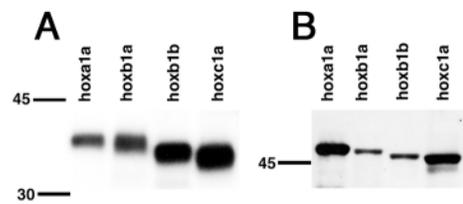
**Fig. 3.** Whole-mount in situ hybridization analysis of zebrafish PG1 genes. Two-colour double in situ hybridization shows Hox genes in purple, plus *krox-20* in red as a marker of r3 and r5. All embryos are mounted with dorsal side towards reader and anterior upwards. Rhombomere (r) numbers as indicated. (A-D) The *hoxb1b* and *hoxb1a* genes both have early expression domains in r4. (A) *hoxb1b* expression at 80% epiboly (8 hours, h) lies in bilateral epiblast domains above the margin. (B) At tailbud stage (10 h) *hoxb1b* expression is localized to r4 abutting early *krox-20* expression in r3. (C) By the one-somite stage (10.5 h) *hoxb1b* expression has already started to retreat posteriorly and is absent from r4. (D) *hoxb1a* expression at the equivalent stage (one somite) is already upregulated in r4. (E-I) *hoxa1a* is expressed in an anterior subpopulation of neurones. (E) At 24 h *hoxa1a* expression is localized to discrete bilateral clusters of cells in the anterior hindbrain and ventral midbrain (arrowheads). (F) 36 h; expression is now localized to cell clusters in the midbrain, medial to the eyes, and r1 (arrowheads). (G) 3.5  $\mu$ m transverse section (t.s.) through plane indicated in F. (H) HNK-1 antibody staining reveals cell bodies of the nMLF (arrowheads), the MLF axon tract and the trigeminal ganglia (TG) at 22 h. *hoxa1a* expression colocalizes to the nMLF. (I) *hoxa1a*-expressing cells continue to colocalize with HNK-1-positive neurones in the nMLF (arrowheads) at 28 h, arrows indicate *hoxa1a*-expressing cells in the anterior hindbrain. (J-M) *hoxc1a* expression: (J) at 12 h in notochord (n); (K) at 16.5 h in CNS (anterior limit at spinal cord/hindbrain junction); (L) at 24 h in bilateral cell clusters in the ventral midbrain (arrowhead) and Mauthner neurones (arrow); (M) at 36 h in cells medial to eyes (arrowhead), similar to *hoxa1a*.

that of mouse *Hoxa1*, how is zebrafish *hoxa1a*, the orthologue of mouse *Hoxa1*, expressed? We find that *hoxa1a* is expressed in an entirely different manner to mouse *Hoxa1*. The *hoxa1a* gene has a late onset of expression (20 h; Fig. 3E, data not shown), and is only expressed in small bilateral cell clusters located in both the ventral midbrain and (laterally) in the anterior three rhombomeres of the hindbrain (Fig. 3E-I). The midbrain expression sites lie ventrolaterally within the neural tube, as revealed by section analysis (Fig. 3G). Double-labelling with HNK-1 antibody, a marker of early differentiating primary neurones, shows that *hoxa1a* expression in the midbrain is closely associated with the neurones of the nucleus of the medial longitudinal fasciculus (nMLF; arrowheads Fig. 3H,I; Wilson et al., 1990). The expression of *hoxa1a* is very different from that of any murine PG1 gene, and may reflect a neofunctionalization in zebrafish, or alternatively, may be a primitive vertebrate characteristic that has been lost in the mouse. The location of the expression domain, anterior of PG2 gene expression domains, in principle restores spatial colinearity.

The fourth gene, *hoxc1a*, has no tetrapod orthologue, and shows several amino-acid sequence differences from the other three zebrafish PG1 genes in known functional domains. The *hoxc1a* gene does not have a broad expression domain in r4. Rather, it shows early expression more posteriorly within the CNS and also in the notochord (similar to *hoxb1a* and *hoxb1b*; Fig. 3B-D; Prince et al., 1998b). In addition, *hoxc1a* also has later localized expression in midbrain and hindbrain neurones (similar to *hoxa1a*). Expression commences at 10.5 h in the notochord, with an anterior limit that lies ventral to r4, similar to *hoxb1b* (Fig. 3C,J, data not shown) and this domain is retained until 14 h (data not shown). Low-level expression in the overlying CNS is observed from 11 h, with an anterior limit at approximately the hindbrain/spinal cord transition point (data not shown, Fig. 3K). By 24 h, there is expression in the two bilateral Mauthner neurones in r4 (arrow, Fig. 3L; confirmed by double-staining with the 3A10 antibody; data not shown), and in bilateral clusters of cells in the ventral midbrain (Fig. 3L,M; arrowheads). As the expression levels of *hoxa1a* and *hoxc1a* are not high enough to allow two-colour double in situ analysis, we used comparisons of embryos probed with each gene individually, or with both genes together, to show that midbrain expression of *hoxc1a* and *hoxa1a* appears to localize to the same cell population (data not shown).

### Functional analysis of zebrafish PG1 genes

Having established that the zebrafish PG1 genes show significant differences in expression profiles to their murine counterparts, we wished to assess and compare the functional capacities of the four genes. To this end we took a gain-of-function approach. In general, ectopic expression of a *Hox* gene anterior to its normal expression site leads to a 'posteriorizing' homeotic transformation, where an anterior structure takes on properties characteristic of a more posterior structure (reviewed by McGinnis and Krumlauf, 1992). Conveniently, the hindbrain region has a wealth of both molecular and neuroanatomical markers that enable AP identity to be easily assessed. These include the reticulospinal (RS) neurones, which form a ladder-like array along the AP extent of the hindbrain, with specific cell types characteristic to each rhombomere recognizable by size, mediolateral



**Fig. 4.** Efficiency of Hox protein translation. (A) In vitro translation products of synthetic PG1 Hox gene mRNAs analyzed by SDS-PAGE electrophoresis. Protein products of the expected size are efficiently produced in vitro. Predicted sizes: long form of *hoxa1a*, 36 kDa; *hoxb1a*, 35 kDa; *hoxb1b*, 34 kDa; long form of *hoxc1a*, 34 kDa. Molecular weight marker sizes in kDa are indicated. (B) Western blot analysis of Myc-tagged Hox proteins synthesized in vivo after micro-injection of 50 ng/ $\mu$ l concentrations of each PG1 mRNA. Lysates of whole embryos were prepared at the 20-22 hour stage and 10  $\mu$ g of total extracted protein electrophoresed and blotted. As expected, Myc-tagged proteins (six Myc-epitopes) are approximately 10 kDa larger than untagged versions. Molecular weight marker sizes in kDa are indicated.

location and projection type (Metcalf et al., 1986; Hanneman et al., 1988; see Fig. 5A). For example, the large, contralaterally projecting Mauthner is an RS neurone characteristic of r4. Similarly, the branchiomotor (BM) neurones of the cranial nerves have a rhombomere-specific disposition. Each motor nerve consists of groups of neuronal cell bodies that lie in clusters within specific rhombomeres, projecting axons that fasciculate to leave the hindbrain at defined exit points. Thus, the trigeminal (Vth) cranial nerve has BM neurone cell bodies that differentiate in r2 and r3 and project axons out of r2 to innervate the first pharyngeal arch. 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. However, unlike the trigeminal nerve, the facial nerve cell bodies subsequently undergo a characteristic posteriorward migration to lie ultimately in r6 and r7 (Chandrasekhar et al., 1997; Higashijima et al., 2000). Using detailed analysis of neuroanatomical and molecular markers in our gain-of-function assays, we have revealed differences in the functional capacities of the four PG1 genes.

Ectopic expression of the four genes was achieved by micro-injection of synthetic mRNAs into one- or two-cell stage embryos. To ensure that equivalent concentrations of each mRNA produced similar amounts of protein, we examined translation levels of each mRNA (Fig. 4). We found that proteins of the appropriate size were translated in vitro (Fig. 4A). Equivalent mRNA concentrations of N-terminal *myc*-tagged constructs were then injected into embryos, and protein extracted at 20-22 h of development, a stage when both neuroanatomical and molecular markers of hindbrain identity have become established. Western blot analysis showed that proteins translated in vivo from injected PG1 mRNAs were present within embryos at similar levels during the stages when hindbrain identity is imparted (Fig. 4B). For *hoxb1b* and *hoxc1a* the functional capacity of the *myc*-tagged versions was confirmed (Table 1). The minor variation in protein levels produced by the four constructs did not correlate at all with differences in function of the mRNAs. We also performed Myc immunodetection on whole-mount specimens (data not shown), which revealed that the four constructs produced

stable Myc-tagged protein localized to the nuclei of numerous cell types throughout the embryo.

Injection of a mutated *hoxb1b* construct, with a single alteration in the homeodomain expected to prevent DNA binding (see Methods), did not cause any phenotype. This control confirmed that the PG1 proteins were mediating their phenotypic effects via DNA binding, as expected for transcription factors.

### ***hoxb1b* can repattern r2 to an r4 phenotype: a posteriorizing homeotic transformation**

The *hoxb1b* gene is normally expressed transiently in presumptive r4, before receding towards the posterior (Fig. 3). We found that micro-injection of 50 ng/μl concentrations of *hoxb1b* caused a very well-defined homeotic transformation, where r2 took on multiple neuroanatomical and molecular properties characteristic of r4 (Fig. 5; Table 1). Ectopic r4-characteristic RS neurones were revealed at the r2 level by retrograde labelling from the spinal cord (Metcalf et al., 1986; Alexandre et al., 1996; Fig. 5C,D, Table 1). These neurones were sometimes observed bilaterally in r2, but more often unilaterally, presumably because the mRNA tended to become localized to one side of the embryo after injection (Blader et al., 1997). Ectopic RS neurones included Mauthners (as also revealed using 3A10 antibody; insets Fig. 5B,D; Table 1), consistent with a previous report (Alexandre et al., 1996). Extending this previous work, we also observed additional classes of r4-characteristic RS neurones at the r2 level: medial (MiM and MiV) cells, and laterally located vestibular nuclei (Fig. 5C,D). Ectopic r4-characteristic BM neurones were visualized by their expression of *islet-1* (Fig. 5E,F; Chandrasekhar et al., 1998). The Vth nerve cell bodies have a characteristic lateral location in discrete clusters in r2 and r3, whereas the VIIth nerve cell bodies lie medially and migrate from their point of origin in r4 and r5 towards the posterior (Fig. 5E). Mis-expression of *hoxb1b* caused r4-characteristic medially located *islet-1*-expressing BM neurones to form in r2, and generally also in r1 (Fig. 5F), suggesting that transformed BM cells may inappropriately migrate towards the anterior.

Having observed a posteriorizing transformation of the hindbrain, as assayed by alterations in differentiated neuronal cell types, we wished to analyze the initial specification of

rhombomere identity in *hoxb1b*-injected embryos at earlier stages. The *hoxb1a* gene provides the only available r4-specific molecular marker in zebrafish (Fig. 5G). As predicted for a transformation of r2 to r4 identity, we noted ectopic expression of *hoxb1a* at the r2 level in *hoxb1b* injected embryos (Fig. 5H; Table 1). Rarely (less than 10% of cases), we observed ectopic expression of *hoxb1a* not only at the r2 level, but also in the r3 territory, which produced a single broad domain of expression. However, we did not observe *hoxb1a* expression anterior to r2 in response to micro-injection of even a 100 ng/μl concentration of *hoxb1b*.

Generally, the ectopic domain at the r2 level was shorter from anterior to posterior than the endogenous r4 domain (Fig. 5H), suggesting that the transformed 'r2' territory is reduced in AP extent. Concomitantly, we find a variable degree of expansion in the AP extent of the r3 territory, as assessed by *krox20* expression (Fig. 5H, data not shown), or *ephA4* expression (data not shown; Xu et al., 1995). These changes in molecular identity of the rhombomeres were accompanied by shifts in the location of rhombomere boundaries, as assessed by expression of the boundary marker *mariposa* (Fig. 5I,J; Moens et al., 1996).

We conclude that ectopic *hoxb1b* can cause a complete transformation of r2 to an r4 identity, as assayed by both neuroanatomical and molecular markers. Furthermore, we suggest that the neuroanatomical changes resulting from ectopic expression of *hoxb1b* are linked to an early switch in molecular identity, as shown by r2 expression of *hoxb1a*. However, no alterations in hindbrain identity were observed anterior to r2, suggesting that *hoxb1b* does not have the capacity to posteriorize structures that lie further anterior.

### **Mis-expression of *hoxb1a* causes posteriorizing transformations of r2 and more anterior structures**

The *hoxb1a* gene is expressed from early stages in r4 (Prince et al., 1998a; Prince et al., 1998b), but unlike *hoxb1b*, which has only transient r4 expression, *hoxb1a* expression is maintained at this level until at least 36 h. This prolonged *hoxb1a* expression in r4 suggests that it plays an important role in imparting r4 identity. We found that at low mRNA concentrations (25 ng/μl) *hoxb1a* mis-expression generally caused an identical phenotype to *hoxb1b* mis-expression,

**Table 1. Percentages of phenotypes produced after injection of Hox genes**

Phenotype	Construct				
	<i>hoxb1b</i>	<i>hoxb1a</i>	<i>hoxa1a</i>	<i>hoxc1a</i> *	<i>Amphi-Hox1</i>
Ectopic Mauthners (3A10)	51% (279)‡	45% (53)	50% (95)	33% (57)	77% (22)
r4-characteristic RS neurones at r2 level (retrograde labelling)	60% (78)	89% (27)	67% (9)	–	–
<i>hoxb1a</i> expression in r2	69% (174)	76% (96)§	100% (14)§	87% (32)¶	100% (48)§
r4-like branchiomotor neurones at r2 level ( <i>isl-1</i> )	50% (32)	69% (93)	66% (30)	63% (19)	–
Expanded r3 territory ( <i>krox-20/mar</i> expression)	80% (111)	24% (41)	–	–	–

The percentage of embryos injected with a particular construct that showed each phenotype is followed by the number of experimental embryos tested (*n*). Any marker or technique used to visualize a particular phenotype is indicated in parentheses. Figures in the table reflect grouping of results from embryos injected with *hoxb1b* mRNA at 50 and 100 ng/μl, *hoxb1a* mRNA at 25 and 50 ng/μl, *hoxa1a* at 25 and 50 ng/μl, *hoxc1a* at 50 ng/μl only, and *AmphiHox-1* at 10 and 25 ng/μl.

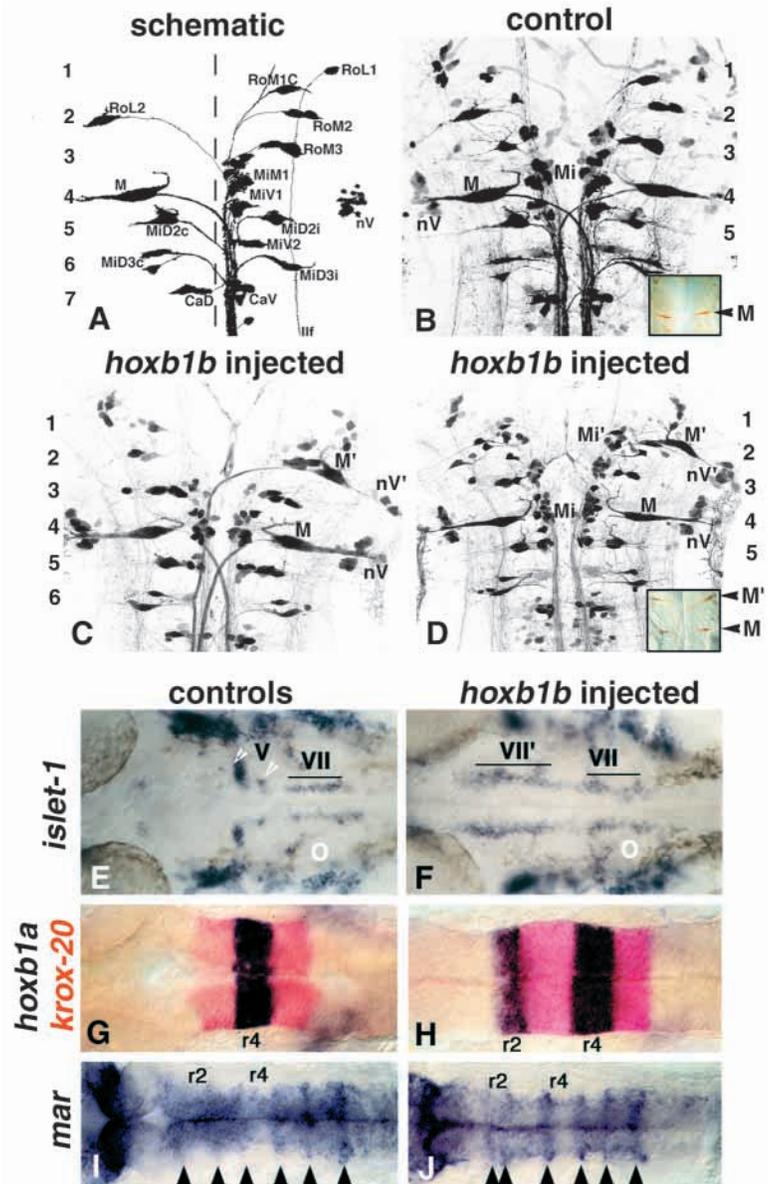
\*These embryos were injected with pCS2hoxc1amyc, to allow direct comparison with pCS2hoxb1bmyc (in constructs with identical Kozak sequences) and with results of western analysis; Fig. 4.

‡Of the 279 embryos injected, 85 were injected with pCS2hoxb1bmyc. A similar percentage of each class of embryos showed ectopic Mauthners.

§The ectopic *hoxb1a* expression in these embryos was generally confined to r2 only, in response to 25 ng/μl concentrations of *hoxb1a* and *hoxa1a* mRNAs, but often extended beyond the r2 territory, either into r3 or more anteriorly into mid- and forebrain (e.g. Fig. 6E,H), in response to the 50 ng/μl concentrations of *hoxb1a* and *hoxa1a* mRNAs, or in response to either concentration of *AmphiHox-1* mRNA.

¶The ectopic *hoxb1a* expression in these embryos was in small, often laterally confined domains at the anterior r2 level (e.g. Fig. 6J).

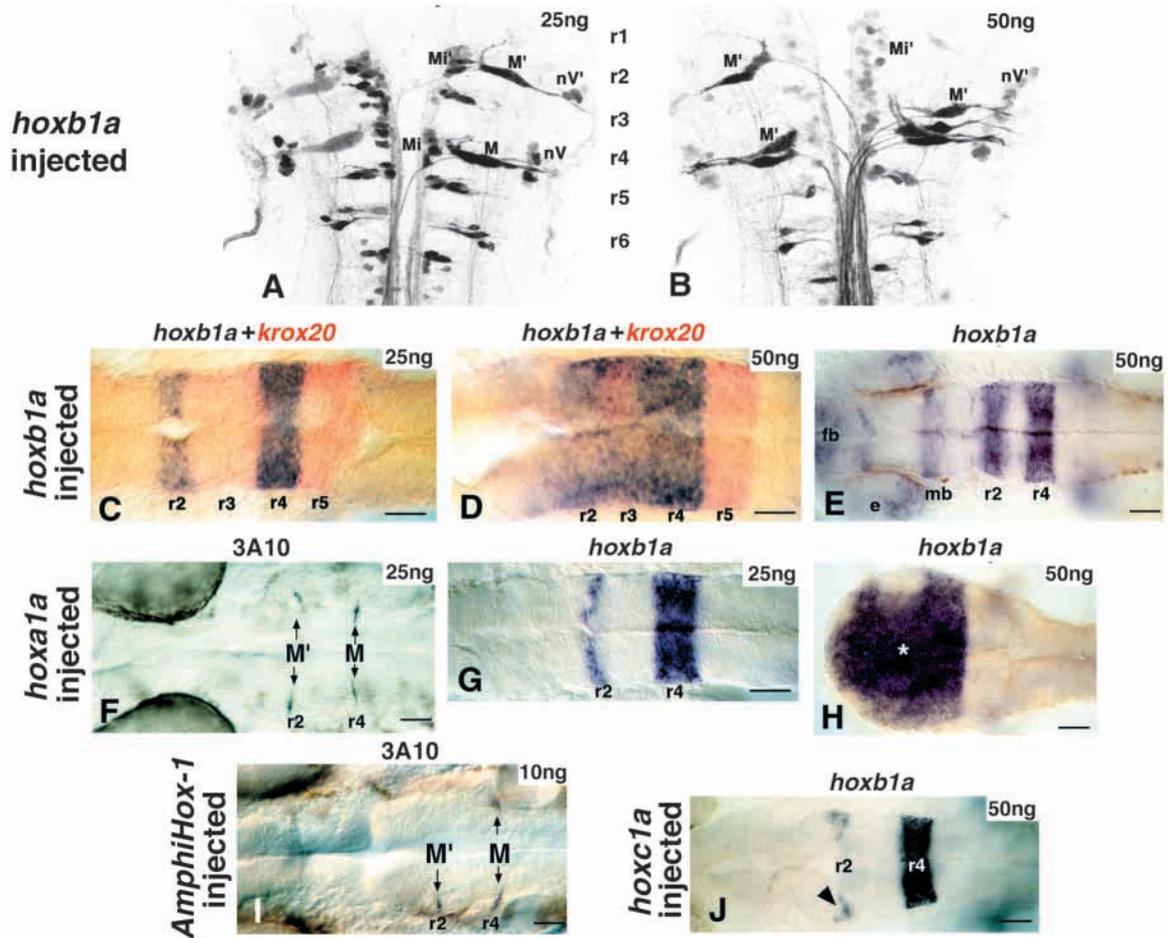
**Fig. 5.** Disposition of neuroanatomical and molecular markers reveals that mis-expression of *hoxb1b* transforms r2 to an r4 phenotype. (A–D) Retrograde labelling from the spinal cord of 5-day-old larvae reveals the disposition of the RS neurones (anterior to the top). (A) The subset of neurones labelled from the right hand side of the spinal cord only is schematized and labelled: M, Mauthner; nV, nucleus of the vestibular formation; MiM1, middle medial 1 cells; MiV1, middle ventral 1 cells; RoL2, rostral lateral 2 cells; llf, lateral longitudinal fascicle. Other abbreviations as previously designated (Hanneman et al., 1988). The locations of the rhombomeres relative to the RS neurones are indicated on the left-hand side. (B) Retrograde labelling of a normal 5-day-old larva. Mi, MiM1 plus MiV1 cells; rhombomeres numbered on right-hand side. Inset, 3A10 antibody staining reveals the r4 Mauthner neurones, M. (C,D) Mis-expression of *hoxb1b* leads to formation of the r4 characteristic M, Mi and nV cells at the r2 level; ectopic neurones are indicated by '. These examples show unilateral duplications, less frequently bilateral duplications were observed, as shown in the inset in (D) by 3A10 antibody staining to reveal ectopic Mauthner neurones at the r2 level, M'. (E–J) Whole-mount in situ hybridization analysis of injected embryos, mounted with dorsal side uppermost and anterior to the left; in each case an unmanipulated control embryo is shown on the left-hand side and a *hoxb1b*-injected embryo at the same stage on the right-hand side. (E,F) 28 h; (G–J) 19 h; (E) *islet1* expression labels cell bodies of the branchiomotor neurones. The trigeminal (Vth nerve) cell bodies have a lateral location, show intense labelling, and are subdivided into a major anterior (r2) population and a minor posterior (r3) population (white arrowheads). The facial (VIIth nerve) cell bodies lie more medially, show lower level expression, and form an anterior-posterior array through r4–r6 at this stage (bracket). (F) *hoxb1b*-injected embryo, *islet1* expression shows medial facial-like neurones (VII') at the level of r2 and r1, and possibly extending into the midbrain. O, otic vesicle. (G) In wild-type embryos, *hoxb1a* (blue) is expressed in r4 and *krox-20* (red) in r3 and r5. (H) In *hoxb1b*-injected embryos there is ectopic *hoxb1a* (blue) expression at the r2 level, note expansion of the r3 *krox-20* territory (red). (I) *mar* is expressed at elevated levels in rhombomere boundaries (arrowheads). (J) In *hoxb1b*-injected embryos, *mar* boundary staining reveals expansion of the r3 territory at the expense of the r2 territory.



whereby r2 takes on properties characteristic of r4 (Table 1). Thus, retrograde labelling (Fig. 6A) and immunocytochemistry with the 3A10 antibody (data not shown) revealed ectopic r4-characteristic Mauthner, medial and lateral vestibular neurones at the r2 level. We also assessed the expression patterns of molecular markers of hindbrain identity in response to ectopic *hoxb1a* expression, including *hoxb1a* itself, using a riboprobe specific to the 3'UTR of the endogenous *hoxb1a* gene (Table 1). Micro-injection of 25 ng/ $\mu$ l concentrations of *hoxb1a* mRNA resulted in an r2 domain of endogenous *hoxb1a* expression (Fig. 6C), again similar to the effects of *hoxb1b* mis-expression. Furthermore, *hoxb1a* is activated in r2 in response to ectopic *hoxb1b* and may therefore mediate some or all of the phenotypic effects of *hoxb1b*.

Upon injection of higher concentrations of mRNA (50 ng/ $\mu$ l), we observed additional effects of *hoxb1a* mis-expression, which we interpret as posteriorizing transformations of structures anterior to r2. These alterations

included production of multiple ectopic Mauthner and Mi neurones (e.g. Fig. 6B), which formed in a variety of locations including r4 itself and at more anterior locations from r3 through to the midbrain. Similarly, injection of 50 ng/ $\mu$ l concentrations of *hoxb1a* mRNA resulted in much more extensive expression of endogenous *hoxb1a*, anterior to its usual location, in a variety of different patterns, including (1) expression in a single broad territory encompassing, at minimum, r2, r3 and r4 (e.g. Fig. 6D), (2) ectopic expression in r2 and in an additional 'stripe' within the midbrain (e.g. Fig. 6E), and (3) ectopic expression in a broad domain reaching up to the anterior limit of the embryo (data not shown). Embryos in the first and second classes often also showed ectopic *hoxb1a* expression in one or both eyes (Fig. 6E). Embryos from the third class invariably had profound truncations of anterior structures, such that the eyes were largely or completely absent. Double labelling of truncated embryos with the 3A10 antibody revealed multiple randomly projecting Mauthner neurones



**Fig. 6.** Mis-expression of *hoxb1a*, *hoxa1a* or *AmphiHox-1* causes extensive posteriorizing transformations, but mis-expression of *hoxc1a* causes reduced transformations. The concentration of mRNA injected (in ng/ $\mu$ l) is indicated in the top right-hand corner of each panel. (A,B) *hoxb1a*-injected embryos, retrograde labelling from the spinal cord, rhombomeres as numbered, ectopic neurones are indicated by '. (A) Bilateral transformation of r2 to an r4-like character, note duplications of M, Mi and nV (vestibular nuclei) neurones. (B) Example with a more extensive posteriorizing transformation, multiple Mauthner neurones have formed at the r2-r4 levels; there are also unilateral ectopic Mi cells extending through r1-r4. (C-E) *hoxb1a*-injected embryos assayed for expression of endogenous *hoxb1a* (blue) and *krox20* (red, C,D only), 19 h, anterior towards left. (C) Example with *hoxb1a* expression at the r2 level; note also expansion of the *krox20* positive r3 territory. (D) Example with more extensive ectopic expression of endogenous *hoxb1a*, expression is present in r1-r3 in addition to the r4 domain. (E) Example with ectopic *hoxb1a* expression in r2, midbrain (mb), forebrain (fb) and eyes (e). (F) *hoxa1a* mis-expression; example of bilateral ectopic Mauthner neurones (M') in r2 revealed with 3A10 antibody at 28 h. (G,H) *hoxa1a*-injected embryos assayed for expression of *hoxb1a*. (G) Note ectopic expression of *hoxb1a* at the r2 level and enlargement of the r3 territory. (H) Note extensive anterior truncation accompanied by expansive ectopic *hoxb1a* expression (asterisk). (I) *AmphiHox-1* mis-expression, example of a unilateral ectopic Mauthner neurone (M') in r2 revealed with 3A10 antibody. (J) *hoxc1a*-injected embryo assayed for expression of *hoxb1a*, note small, lateral domains of ectopic expression of *hoxb1a* at the anterior r2 level (arrowhead). Scale bars: 50  $\mu$ m.

within the *hoxb1a* expression domain; in some cases as many as ten individual Mauthner neurones could be identified (data not shown). Embryos in these second and third classes were never observed in response to injection of *hoxb1b*, although similar amounts of protein were produced by equivalent concentrations of *hoxb1a* and *hoxb1b* mRNAs (Fig. 4). Our results suggest that *hoxb1a* preferentially affects r2, but can also act to posteriorize more anterior midbrain and forebrain structures. This capacity is very different from that of *hoxb1b*, whose action does not extend anterior to r2.

#### ***Hoxa1a* and *AmphiHox-1* have similar functional capacities to *hoxb1a***

The zebrafish *hoxa1a* gene has an expression pattern

inconsistent with a normal role in r4 patterning. However, this gene is the orthologue of the mouse *Hoxa1* gene, which has been implicated in r4 patterning by both loss- and gain-of-function analysis. We therefore wished to test whether zebrafish *hoxa1a* would act in a similar manner to mouse *Hoxa1* in a gain-of-function analysis, or alternatively, in an entirely different manner related to its disparate expression pattern. We found that *hoxa1a*, in either short or long splice-forms, acts in a similar manner to *hoxb1a* (Fig. 6F-H, Table 1). Thus, in response to low concentrations of *hoxa1a* mRNA (25 ng/ $\mu$ l) r2 is posteriorized to an r4 phenotype, as revealed by ectopic Mauthners (Fig. 6F) and *hoxb1a* expression (Fig. 6G) at the r2 level. In response to higher concentrations (50 ng/ $\mu$ l) we observed more extensive posteriorizing transformations,

where ectopic Mauthners (data not shown) and *hoxbla* expression (Fig. 6H) are distributed more broadly anterior to r4, in association with severe truncations of the anterior of the embryo (Fig. 6H). Thus, despite not normally being expressed in r4, the *hoxa1a* gene has the capacity to produce aspects of r4 pattern in more anterior structures, showing that it shares functional properties with its murine orthologue.

We additionally tested the single amphioxus PG1 gene, *AmphiHox-1*, in our mis-expression assay. Like the zebrafish PG1 Hox genes, this gene also had the capacity to induce posteriorizing transformations (Fig. 6I; Table 1). Even at the lowest concentration injected (10 ng/μl), mRNA encoding *AmphiHox-1* frequently caused anterior truncations and extensive ectopic *hoxbla* expression (data not shown), similar to the effects of *hoxbla* mis-expression. It is interesting that the amphioxus PG1 gene can perform an r4 patterning role in the zebrafish despite the very different anatomy of these species; for example, there is no obvious rhombomeric organization in the amphioxus brain.

In support of a specific conservation of PG1 gene function across species, the capacity to transform anterior structures towards an r4 phenotype does not extend to Hox genes outside of PG1. Similar effects have not been demonstrated in response to mis-expression of PG2, PG3 or PG5 genes (Yan et al., 1998; M. P. Hunter and V. E. P., unpublished; Bruce et al., 2001).

#### ***hoxc1a* has a reduced capacity to cause a posteriorizing transformation**

In comparison to the other PG1 genes, *hoxc1a* showed a reduced ability to cause posteriorizing transformations as assayed by most, but not all, of our markers. Specifically, micro-injection of 50 ng/μl concentrations of *hoxc1a* mRNA produced ectopic r2 Mauthner neurones in only 33% of experimental embryos (Table 1). Moreover, although we observed ectopic *hoxbla* expression in response to *hoxc1a* (Table 1), the ectopic *hoxbla* domains were narrow and often located only laterally within anterior r2 (compare Fig. 6J with Figs 5H, 6C,H); more expansive domains that extend beyond the r2 territory were never observed. In contrast, the percentage of embryos with r4 characteristic BM neurones at the r2 level was approximately equivalent to the percentage found after injection of an equivalent concentration of *hoxb1b*. Reduced functional capacity of *hoxc1a* in comparison with other genes does not reflect lower levels of protein production; rather, an equivalent amount of *hoxc1a* mRNA produced somewhat higher concentrations of protein than the *hoxb1b* and *hoxbla* constructs (Fig. 4B). Overall, our results suggest that the multiple changes in the amino acid sequence of the *hoxc1a* protein may have disabled some, but not all, functions of the gene. Furthermore, the differential effects of ectopic *hoxc1a* expression on RS and BM neurone patterning suggest that these two sets of hindbrain neurones may have different and independent patterning requirements.

## **DISCUSSION**

The Hox genes have provided us with an ideal system to explore the evolution of key developmental genes after a genome duplication event. As a result of the functional constraints acting on these conserved genes, we can identify common PG1 gene

functions over very large evolutionary distances. However, we have identified a number of interesting differences in the zebrafish PG1 genes in comparison with those of the mouse, at both the *cis*-regulatory and the coding sequence levels. These changes include differences in gene expression patterns, coding sequences and functional capacities. We also describe an interesting instance of function ‘shuffling’ between paralogues, where non-orthologous genes are used for equivalent purposes in zebrafish and mouse. We discuss each of these differences, and their implications, in detail below.

#### **Zebrafish Hox genes have unusual midbrain expression**

Vertebrate Hox genes generally show spatial and temporal colinearity of expression (McGinnis and Krumlauf, 1992). One exception to spatial colinearity is that mouse and chick PG1 genes have anterior expression limits that lie posterior to those of PG2 genes (Frasch et al, 1995; Prince and Lumsden, 1994). Zebrafish *hoxa1a* and *hoxc1a* have expression in small discrete populations of ventral neurones within the midbrain, which, in principle, restores spatial colinearity. However, this expression is radically different from that of other Hox genes during CNS development, being confined to small groups of neurones rather than extending over broad domains. This suggests that midbrain expression may reflect a release of these Hox genes from global cluster regulation (reviewed by Duboule, 1998). In support of this alternative, the late onset of *hoxa1a* expression, approximately 9 hours after the onset of *hoxa2b* or *hoxa3a* expression, is inconsistent with temporal colinearity. Dissociation from global control mechanisms may in turn be facilitated by the location of the genes at the far 3' ends of the clusters.

There are precedents for such escape from cluster regulation in *Drosophila*. For example, *zen* is a Hox-like gene located within the Homeotic complex, which nevertheless has a dorsally localized extra-embryonic expression pattern unlike that of any Hox gene (Falciani et al., 1996). Similarly, *fushi tarazu* is a Hox-like gene (Telford, 2000) that lies within the Homeotic complex, yet is expressed with a pair-rule pattern, and later in developing neurones (Doe et al., 1988). Zebrafish *hoxa1a* and *hoxc1a* may similarly have taken on a role in later neuronal patterning. At present, our findings do not allow us to determine whether midbrain Hox expression represents a conservation of spatial colinearity, or alternatively an escape from cluster-based regulatory mechanisms. However, our data do shed some light on the evolutionary origins of Hox gene midbrain expression.

Hox midbrain expression could either represent a derived characteristic of zebrafish, or instead may reflect a more primitive vertebrate condition. In support of the hypothesis that midbrain expression is derived, no such expression has been reported for murine PG1 genes (Frohman et al., 1990; Murphy and Hill, 1991; Wilkinson et al., 1989). However, we propose that despite the mouse data, midbrain expression is likely to have an ancient origin within the vertebrates. We have observed midbrain expression of *hoxa1a* in the distantly related teleost medaka (C. Jozefowicz and V. E. P., unpublished), suggesting that this expression is a common feature of the teleosts. Furthermore, our finding of midbrain expression for PG1 genes from both *hoxa* and *hoxc* clusters suggests that midbrain-like expression may have existed before the initial Hox cluster

duplications associated with vertebrate origins (Garcia-Fernandez and Holland, 1994). Consistent with the hypothesis that midbrain expression is an ancestral vertebrate characteristic, there has been one report of midbrain expression in a sarcopterygian vertebrate, *Xenopus laevis* (Kolm and Sive, 1995). This suggests that midbrain expression may have been present in the common ancestor of the sarcopterygians (lobe-finned fishes) and actinopterygians (ray-finned fishes). It should be noted that expression of *Xenopus Hoxa1* has not yet been explored with single-cell resolution, and thus the precise relationship between this expression and the discrete neuronal expression domains we observe in zebrafish remains unclear. Nevertheless, all of these data suggest that lack of midbrain expression in mouse may be a derived characteristic, and it will be of interest to investigate *Hoxa1* expression in additional sarcopterygians such as chick, as well as in the chondrichthyan fishes (sharks and rays) that pre-date the actinopterygian/sarcopterygian divide.

### ***hoxc1a* has reduced functional capacities correlating with altered sequence**

We have observed that the *hoxc1a* gene shows a reduced capacity to transform some, but not all, aspects of r2 identity towards an r4 phenotype. In comparison with other PG1 genes, the ability of *hoxc1a* to activate *hoxb1a* expression, or induce ectopic RS neurones, is significantly reduced. However, the capacity to repattern BM neurones is not, suggesting that a subset of PG1 functions may be altered in *hoxc1a*. Transcriptional activation of *hoxb1a* is likely to require both a Hox PG1 gene product and a Pbx co-factor protein. A Hox/Pbx heterodimer probably binds to regulatory elements upstream of *hoxb1a*, by analogy with mouse *Hoxb1* regulatory mechanisms (Pöpperl et al., 1995; Studer et al., 1998). The *hoxc1a*-coding sequence has significant sequence differences from the other PG1 genes that are likely to preclude efficient interaction with Pbx co-factors. Specifically, the hexapeptide motif, which has been shown to interact directly with Pbx co-factors (Passner et al., 1999; Piper et al., 1999), has two amino acid changes in *Hoxc1a* relative to all other vertebrate PG1 proteins examined. In addition, the *hoxc1a* protein has a significantly extended linker between the hexapeptide and homeodomain, and the recently solved structure of the human Hoxb1-Pbx1 heterodimer bound to DNA (Piper et al., 1999) has led to the explicit prediction that alterations in the length of this linker will alter the ability of a Hox protein to interact with its Pbx co-factor (Scott, 1999). Thus, we propose that the loss of some *Hoxc1a* functional capacities is a result of reduced ability to interact with Pbx co-factors. However, the retention of ability to repattern BM neurones may imply that some normal functions of *Hoxc1a* are Pbx independent.

It is interesting to note that the *hoxc1a* gene of the acanthopterygian teleost pufferfish, *Fugu rubripes*, does not encode an open reading frame, indicating that *hoxc1a* function is dispensable in at least one teleost (Aparicio et al., 1997). Thus, it is possible that zebrafish *hoxc1a*, like the *Fugu* gene, is in the process of being lost. Together these data suggest that teleost Hox gene complements are not yet fixed.

### **Zebrafish *hoxb1b* is the functional equivalent of murine *Hoxa1***

We hypothesize that zebrafish *hoxb1b* fulfills the

developmental role played in mouse by the *Hoxa1* gene. The expression patterns of these two genes are remarkably similar: both have very transient early expression in presumptive r4, then recede posteriorly. Furthermore, we find that *hoxb1b* mis-expression can confer r4 identity upon r2. Together, our findings suggest that *hoxb1b* plays a normal role in the development of r4.

Mutant analyses have shown that mouse *Hoxa1* is required for normal r4 and r5 formation (Chisaka et al., 1992; Carpenter et al., 1993; Lufkin et al., 1991; Mark et al., 1993), and that *Hoxa1* is required to set the appropriate anterior boundary of *Hoxb1* expression (Barrow et al., 2000). Once *Hoxb1* is activated in r4, its expression is maintained by auto-regulation (Pöpperl et al., 1995). Both Hoxa1 and Hoxb1 protein can heterodimerize with a Pbx co-factor to bind a defined Hox/Pbx-binding site 5' of *Hoxb1* to activate its transcription (Pöpperl et al., 1995; Studer et al., 1998). Consistent with these regulatory relationships, when *Hoxa1* is globally mis-expressed in transgenic mice, *Hoxb1* is ectopically expressed at the r2 level (Zhang et al., 1994). We find evidence of a similar functional hierarchy between two PG1 genes in the zebrafish: mis-expression of zebrafish *hoxb1b* leads to ectopic activation of *hoxb1a* in r2. Thus, we propose that during normal development Hoxb1b initially activates *hoxb1a* expression at the r4 level, and that *hoxb1a* expression is then maintained by an autoregulatory mechanism. The hypothesis that the regulation of *Hoxb1* orthologues has been conserved during evolution is further supported by the presence of Hox/Pbx-binding sites 5' of the chicken and *Fugu Hoxb1* genes (Pöpperl et al., 1995).

The observation that ectopic expression of *hoxb1b* causes r2 to take on an r4 fate without altering more anterior structures suggests that additional factors, present only in r2, are required for the transformation. Candidate factors include Pbx and Meis family co-factor proteins. Meis family co-factors bind defined regulatory elements that lie close to the sites bound by Hox and Pbx proteins; trimeric complexes of Meis, Hox and Pbx then form (reviewed by Mann and Affolter, 1998). Interestingly, Pöpperl et al. (2000) have recently shown that zebrafish Pbx protein levels are reduced anterior to the r1/2 boundary. In addition, we have recently described expression of zebrafish *meis2.1* in r2 of early somite stage embryos (Zerucha and Prince, 2001), suggesting that *Meis2.1* may provide the co-factor that allows activity of *hoxb1b* in r2. Consistent with the hypothesis that co-factors are limiting the extent of the posteriorizing transformation in response to *hoxb1b*, a recent report indicates that mis-expression of *hoxb1b*, together with *meis3*, leads to much more extensive posteriorizing transformations than those mediated by *hoxb1b* alone (Vlachakis et al., 2001).

### **The *hoxb1a* and *hoxb1b* duplicates have different functional capacities**

We find that ectopic expression of low levels of *hoxb1a* mRNA (25 ng/μl concentrations) can cause homeotic transformations similar to those mediated by *hoxb1b*, where r2 takes on properties of r4. However, we also find that in response to higher levels of *hoxb1a* mRNA (50 ng/μl concentrations), structures anterior to r2 are transformed to an r4 phenotype. In some instances, the anterior of the embryo is severely truncated, and all structures anterior to r4 take on r4 identity,

as revealed by massive expansion of *hoxb1a* expression and production of multiple Mauthner neurones. Interestingly, these experimental embryos have a very similar phenotype to embryos in which *meis3* and *hoxb1b* are both mis-expressed (Vlachakis et al., 2001), suggesting that the capacity of *hoxb1a* to mediate more extensive transformations than *hoxb1b* may reflect a reduced requirement for a Meis-family co-factor.

We also find that *hoxa1a* and the single amphioxus PG1 gene, *AmphiHox-1*, have similar functional capacities to *hoxb1a*: ectopic expression can transform anterior structures to an r4 identity. These findings suggest that although *hoxa1a* is not normally expressed in r4, and amphioxus does not have an r4-like structure, the similar coding sequences of these different PG1 genes allow them to regulate the same set of downstream target genes in our assay, leading to an equivalent transformation event. Interestingly, *AmphiHox-1* mRNA causes severe posteriorizing transformations, together with truncations of anterior structures, at significantly lower concentrations than *hoxb1a* or *hoxa1a* mRNA. This may merely be a consequence of greater protein production or stability, but could reflect intrinsic higher activity levels of the single amphioxus PG1 protein in comparison with any one of the four zebrafish PG1 proteins. As *hoxb1a* transcription is activated in response to mis-expression of any one of the other PG1 genes, it remains unclear whether the phenotypic consequences of PG1 gene mis-expression are always mediated directly by the individual Hox proteins produced by the injected mRNAs, or whether some effects are mediated indirectly via Hoxb1a protein. In principle, the effects of all ectopic PG1 proteins may simply reflect an ability to trigger an autoregulatory feedback loop that maintains *hoxb1a* expression. A similar situation has been described for the *Drosophila deformed* gene, which can be activated by ectopic expression of an orthologue, the human HOXD4 gene, through a regulatory element that is normally used for autoregulation (McGinnis et al., 1990). Correspondingly, the conserved regulatory elements from human HOXD4 can confer a *deformed* expression pattern within fly embryos (Maliki et al., 1992).

Our gain-of-function assay did not allow investigation of the normal developmental role of *hoxa1a*. The *hoxa1a*-positive midbrain neurones lie within or near the HNK-1-positive nMLF. Differentiated cells can first be recognized in this nucleus at around 16 hours of development (Wilson et al., 1990), earlier than the stage at which we first detect Hox gene expression in the midbrain. It is thus possible that Hox gene expression plays a role in the maturation of this nucleus, but unlikely that it is involved in neuronal specification. Consistent with this idea, we have not observed formation of ectopic HNK-1 positive neurones in response to ectopic Hox PG1 gene expression.

### Zebrafish PG1 gene evolution has involved function 'shuffling'

Our data strongly suggest that zebrafish *hoxb1b* and *hoxb1a* perform equivalent functions to mouse *Hoxa1* and *Hoxb1*, respectively. To explain this surprising finding we suggest an evolutionary model in which mouse and zebrafish have used different genes to the same end. We propose that following duplication of the four ancestral *Hox* clusters to an eight cluster arrangement, the *hoxb* duplicates would both have had an r4 expression domain. As the coding regions of *Hoxa1* and *Hoxb1*

genes are very similar (and potentially functionally interchangeable), minor changes in regulation of one of the two *hoxb1* duplicates during evolution would allow this gene to fulfil the early r4 patterning role provided in mouse by *Hoxa1*. According to this scenario, *hoxa1a* was then free to lose its r4 expression domain because the early r4 patterning role was fulfilled by *hoxb1b*.

A similar use of related genes for equivalent purposes has been described for the zebrafish Bmp genes; the zebrafish *bmp2b* gene appears to be functionally equivalent to the non-orthologous *Xenopus Bmp4* gene in dorsoventral patterning of the gastrula (Nguyen et al., 1998). Such function 'shuffling' among closely related zebrafish genes may prove to be a common consequence of the genome duplication event that occurred in a teleost ancestor. Further comparative studies should help to shed light on how genes, and ultimately gene networks, have evolved during the radiation of the vertebrates.

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### Note added in proof

During review of this manuscript a description of the expression pattern of zebrafish *hoxa1a* was published (Shih et al., 2001).

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