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Expression patterns of threespine stickleback *Hox* genes and insights into the evolution of the vertebrate body axis

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Abstract Understanding the patterning mechanisms that operate to promote differentiation of individual segments along the main body axis is an important goal of both developmental and evolutionary biology. In order to gain a better insight into the role of *Hox* genes in generating diversity of axial plans seen in vertebrates, we have cloned 11 homeobox sequences from an acanthopterygian teleost, the threespine stickleback, and analyzed the expression of 7 of these during embryogenesis. Transcripts are observed in a variety of tissues, including the neural tube, paraxial mesoderm, lateral plate mesoderm, pectoral fins, pronephric ducts, as well as some neural crest-derived structures. Anterior limits of expression in the central nervous system and paraxial mesoderm exhibited both similarities and differences to those of mouse and zebrafish homologs. In both stickleback and zebrafish embryos expression limits within the paraxial mesoderm were detected only within the trunk region in which ribs are attached to all vertebrae. The finding of this pattern in two divergent teleosts as well as in various tetrapod species supports the hypothesis that a *Hox* precode was present prior to the divergence of ray-finned and lobe-finned fishes and was subsequently used to generate different types of vertebrae in tetrapods. We also describe a dynamic pattern of expression of several stickleback *Hox* genes associated with the development of the caudal paraxial mesoderm, which suggests uncoupling of the process of segmentation from segmental identity determination. We propose that in fishes the patterning of the tail region is under the control of a separate mechanism from the trunk, which utilizes *Hox* genes in a different manner.

Key words Threespine stickleback · *Hox* genes · *Hox* code · Body axis · Tail development

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Introduction

One of the most fundamental features of development among higher metazoans is the construction of the body from serially repeated segmental units known as metameres. This phenomenon, commonly referred to as "metamerism," is thought to have played a crucial role in the evolution of more complex animals by allowing increases in body size and differentiation of body functions without seriously compromising the integration of the whole organism during periods of transition (Gerhart et al. 1982). Thus, understanding the patterning mechanisms that operate to promote differentiation of individual segments along the main body axis is an important goal of both developmental and evolutionary biology.

Studies of several chordate species have indicated that one component of this mechanism is provided by the coordinated expression of a class of developmental regulator genes known as *Hox* genes (Hunt and Krumlauf 1992). These are evidently homologs of the homeotic selector genes of *Drosophila*, with which they share a highly conserved 180 nucleotide sequence motif that encodes a 60 amino acid DNA-binding domain known as the homeodomain (Gehring et al. 1990). In chordates *Hox* genes are organized in clusters that range in number from one in amphioxus (Garcia-Fernàndez and Holland 1994) to four in mice and humans (Boncinelli et al. 1989) and seven in zebrafish (Amores et al. 1998). Within each cluster individual *Hox* genes can be assigned to one of 13 paralogous groups based on the similarity of homeodomain sequences and relative position within the cluster (Sharkey et al. 1997).

During development *Hox* genes are expressed in overlapping domains in a variety of embryonic structures in-

cluding the hindbrain (Lumsden and Krumlauf 1996) and spinal cord (Graham et al. 1989), pharyngeal arches (Krumlauf 1993), paraxial mesoderm (Dressler and Gruss 1989), digestive tract (Yokouchi et al. 1995), urogenital system (Dollé et al. 1991), and appendages (Haack and Gruss 1993). Expression of *Hox* genes is regulated primarily at the transcriptional level, and in the mesoderm and central nervous system (CNS) shows spatial and temporal colinearity with the position of genes within each cluster such that genes located toward the 3' end are expressed earlier and more anteriorly than genes lying toward the 5' end of the cluster (Duboule 1994). This generally leads to the establishment of nested domains of expression, with different regions marked by the expression of different combinations of *Hox* genes (Hunt and Krumlauf 1992). Such results, in conjunction with the phenotypes of gain- and loss-of-function genetic manipulations in the mouse have led to the general acceptance of a "*Hox* code" model that proposes that regional cellular identities are determined by the combinatorial action of groups of Hox proteins (Kessel and Gruss 1991).

The potential role of *Hox* genes in evolutionary diversification of the vertebrate body axis was first highlighted by the generation of mutant phenotypes in knockout or transgenic mice, which mimic the presumed ancestral conditions of affected skeletal structures (Lufkin et al. 1992; Rijli et al. 1993). More recently, direct comparisons of *Hox* gene expression patterns in mice, chickens, and zebrafish have demonstrated that domains of expression do evolve, in some cases in concert with morphological evolution. In amniote tetrapods anterior expression limits of various *Hox* genes are known to mark the transition points between the various types of vertebrae, suggesting that differences in axial morphology among the various species are due to the "coincident transposition" of *Hox* genes and associated vertebral elements (Burke et al. 1995). In zebrafish, however, most of the *Hox* genes have anterior limits within the trunk region (Prince et al. 1998b), and it is not established that modifications of the *Hox* code do affect axial diversity in fishes. It is probable that evolutionary divergence has also been promoted by processes of change in *Hox* gene number, as well as changes in *Hox* gene function at the level of their effects on target gene activity (Gellon and McGinnis 1998).

In order to obtain a more complete understanding of the development and evolution of chordate morphology, as well as to test the generality of observations made in model species, it is thus important to obtain more information on the structure of *Hox* clusters and the expression patterns of individual *Hox* genes in species that are representative of the complete range of anatomical diversity in chordates. In this contribution we describe some features of *Hox* gene expression in an acanthopterygian teleost, the threespine stickleback *Gasterosteus aculeatus*. Acanthopterygian fishes are by far the most diverse and successful group of teleosts, comprising roughly 50% of extant fish species, most of which live in the sea (Nelson 1984). The second largest group of teleosts, the ostariophysin fishes, including the zebrafish, are more dominant in fresh water and could utilize *Hox* genes differently. Information on the sequence and cluster organization of *Hox* genes is available for one acanthopterygian fish, the Japanese pufferfish *Fugu rubripes* (Aparicio et al. 1997), but in situ hybridization experiments have not been reported. Here we show that in the threespine stickleback *Hox* genes appear to be utilized in a very similar manner to those of zebrafish (Prince et al. 1998a, 1998b), although some subtle differences are apparent, particularly in the rhombomeres and pharyngeal arches. We discuss several features of the expression in various structures, particularly during morphogenesis of the pectoral fins and caudal somites, in light of their implications for the ontogeny of these tissues in various vertebrates.

Materials and methods

Cloning of homeobox sequences

Genomic DNA used to amplify homeobox sequences was prepared from muscles and internal organs of a threespine stickleback female by standard proteinase K digestion/phenol-chloroform extraction followed by ethanol precipitation using the procedure for zebrafish described in Westerfield (1993). PCR was performed using two sets of degenerate oligonucleotide primers. The common reverse primer, 5'-CAT(A/G)CG(C/G)CG(A/G)TT(T/C)TG(A/G)-AACGA-3' was designed to recognize the WFQNRRM motif that is close to the C-terminus of most Hox homeodomains. The primer 5'-CGGAA(A/G)AAGCG(A/C)TG(T/C)CC-3' is specific for the RKKRCP motif found at positions 2–7 in homeodomains of group 9, 10, and 11 genes of tetrapods, but led to the cloning of only two stickleback homeoboxes, clone 06 (*Hoxc-9*) and clone 04 (*Hoxa-10*; see Fig. 1). The remaining clones were obtained with a forward primer, 5'-GA(A/G)CT(A/G)GAGA A(A/G)GAGTT-3', designed for the ELEKEF motif present at positions 15–20 in most Hox homeodomains. PCR was performed with an Ericomp twinblock thermal cycler using *Pfu* polymerase (Stratagene) to reduce the error rate, with the following conditions: 5 min denaturation at 95°C, followed by 30 cycles of 90 s at 95°C, 1 min at 55°C and 1 min at 72°C, followed by 7 min at 72°C. PCR products were purified and cloned into the *Srf*I site of the pCR-Script vector (Stratagene) by blunt end ligation. Positive clones were identified, and plasmid mass preparations made using anion-exchange resins (Qiagen).

Sequence analysis and assignment of the identity of clones

Selected PCR clones were sequenced manually on both strands initially with a Sequenase 2.0 DNA sequencing kit (US Biochemical) and later by rhodamine-dye labeled automated sequencing on an ABI 377 (Perkin-Elmer). Nucleotide sequences were conceptually translated into amino acid sequences and compared with corresponding portions of homeodomain sequences from *Hox* genes of the Japanese pufferfish *F. rubripes* (Aparicio et al. 1997). The identity of clones was determined by inspection of groupings in an evolutionary tree constructed using the neighbor-joining algorithm (Saitou and Nei 1987) with p distance (proportion of various amino acids between two compared sequences; for application of this method to *Hox* cluster genes see Zhang and Nei 1996), as implemented in MEGA phylogenetic analysis software package (Kumar et al. 1993). Statistical significance of the clustering in the resulting tree was estimated by bootstrap analysis (Felsenstein 1985) with 1000 independent replications. Additional comparisons with mouse or zebrafish genes did not significantly alter the basic con-

clusions drawn from the comparison with *Fugu* sequences (data not shown).

Whole-mount in situ hybridization

Embryos used for the whole-mount in situ hybridization were prepared by artificial fertilization and raised at a constant temperature of 18°C as described by Ahn (1998). Staging was performed under a Stemi 2000 Zeiss dissection microscope, following conventions used for the description of zebrafish development (Kimmel et al. 1995). Detailed descriptions of stages are presented by Swarup (1958) and Ahn (1998).

For whole-mount in situ hybridization, embryos were collected at regular intervals from "50% epiboly" (middle gastrula) to the "vitelline vein at inner eye" (late organogenesis) stages. They were rinsed briefly in fresh 10% Hank's saline and fixed overnight in MEMFA (Harland 1991) at 4°C. Embryos were then rinsed twice in 1× phosphate-buffered solution and dehydrated in 100% methanol before storage at –20°C in fresh 100% methanol. Digoxygenin-labeled riboprobes were synthesized from cloned PCR products using the Genius 4 nonradioactive RNA labeling kit (Boehringer-Mannheim) following manufacturer's instructions except for the use of T3 instead of SP6 RNA polymerase. The in situ hybridization protocol was similar to that used for *Xenopus* embryos (Harland 1991), with the following modifications: (a) dechorionation was performed manually after rehydration using a pair of Hamilton 5 watchmaker's forceps; (b) acetylation was performed after postfixation in 4% paraformaldehyde; (c) embryos were cleared and mounted in 70% glycerol solution. Staining was allowed to develop for up to 24 h, and documented using a Zeiss Axiovert 100 inverted microscope connected to an Optronics 3 CCD video-camera system. Images displayed on the video screen were captured by Scion image 1.59 frame grabber software (Scion Co.) and processed using Adobe Photoshop 3.0 (Adobe Co.).

Results

Cloning of homeobox sequences from the threespine stickleback

Sequencing of 20 different randomly chosen clones generated from a pool of PCR-amplified genomic stickleback DNA has led to the identification of at least ten different homeobox sequences (Fig. 1). Four of these were

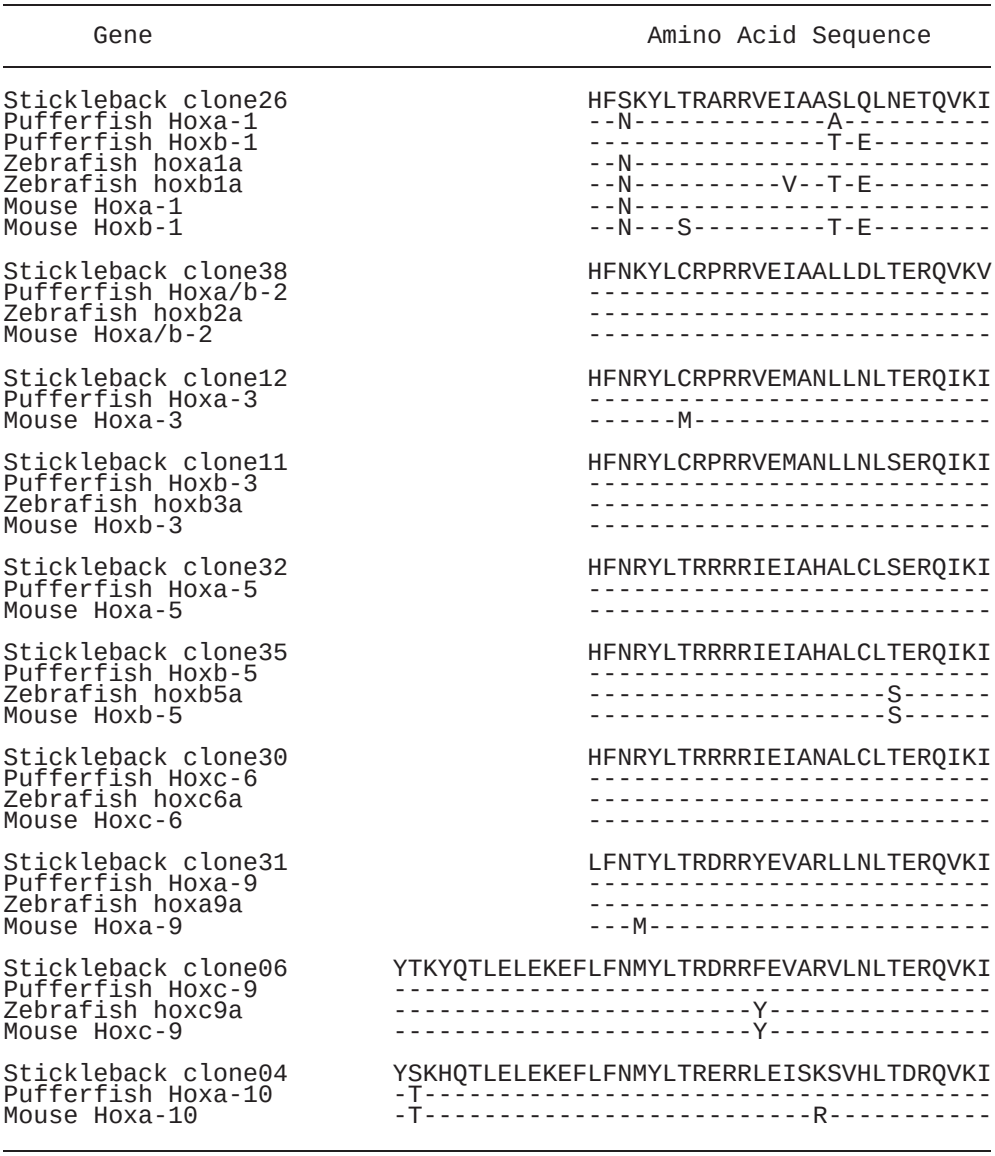

Fig. 1 Amino acid sequence comparison between threespine stickleback *Hox* clones and their putative homologs in pufferfish, zebrafish, and mouse. Sequences of zebrafish homologs are compiled from Prince et al. (1998a, 1998b), Amores et al. (1998), and the references cited therein, using revised nomenclature for zebrafish *Hox* genes (Amores et al. 1998). Mouse and pufferfish sequences are taken from public sequence databases and Aparicio et al. (1997), respectively

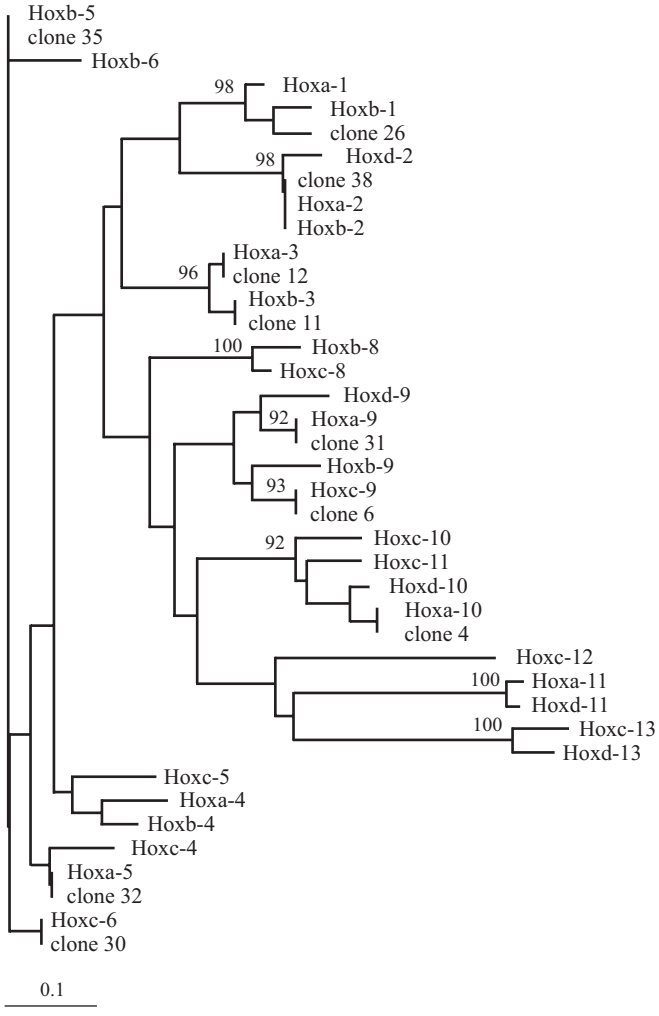

Fig. 2 Neighbor-joining tree showing evolutionary relationships based on degree of sequence similarities between putative amino acid sequences of stickleback clones and corresponding regions of pufferfish *Hox* genes. Only positions 21–47 of the homeodomains were used in the construction of the tree. Bootstrap values greater than 90% are shown on the respective nodes. The tree is presented as unrooted. Genes belonging to the paralogous group 7 are not shown in the tree due to the lack of group 7 genes in *Fugu Hox* clusters (Aparicio et al. 1997)

represented by two separate clones that were either identical or differed only by up to three nucleotide substitutions, which we consider to be likely to be due to PCR-induced sequence errors or allelic polymorphisms (see Misof and Wagner 1996), although it is still possible that some of the duplicate clones actually derive from paralogous genes.

In general, the stickleback clones exhibited more similarity at the amino acid sequence level to Japanese pufferfish (*F. rubripes*) sequences than to those of zebrafish or mouse *Hox* genes (Fig. 1), which agrees well with the known evolutionary relationships among these three species. In order to obtain preliminary paralogous group assignments for the cloned sequences, an evolutionary tree was constructed based on the similarity of the amino acid sequences of the *G. aculeatus* and *F. rubripes* homeodomains using the neighbor-joining method (Saitou and Nei 1987). Examination of the topology of the resulting tree indicated that members of paralogous groups 1, 2, 3, 5, 6, 9, and 10 from three different *Hox* clusters (*Hoxa*, *Hoxb*, and *Hoxc*) are likely to be represented (Fig. 2) although bootstrap support for clusters including *Hox* genes belonging to groups 4–6 was weak while that for the other clusters was strong. Nevertheless, the majority of clones have completely identical amino acid sequences to those of particular *Fugu Hox* genes (Fig. 1), and even adopting conservative criteria (retaining only those clusters with bootstrap values of at least 90) the data are consistent with the presence in the threespine stickleback of multiple members of anterior (clones 26, 38, 11, and 12), middle (clones 32, 35, and 30), and posterior (clones 31, 06, and 04) groups of *Hox* genes that are distributed on at least two *Hox* clusters (*Hoxa* and *Hoxc*).

In addition, we were able to isolate one further clone from a stickleback genomic DNA library by low-stringency plaque hybridization with the clone 04 as the probe. The sequence of this clone indicates complete identity with the mouse Hoxd-9 protein for 11 residues upstream of the homeodomain, but the homeodomain differs from *Fugu* Hoxd-9 at nine residues, and the C-terminal portion diverges from the zebrafish ortholog over the last five residues (D. Ahn, unpublished observation). In a sequence comparison using the entire homeodomain sequence, this clone was found to cluster with the group 9 *Hox* genes of *Fugu*, most strongly with *Hoxc-9* (data not shown). Since the sequence of this clone is very different from that of clone 31 (*Hoxa-9* homolog) and, more importantly, from clone 06 (*Hoxc-9* homolog), this clone is likely to be derived from a third stickleback *Hox* cluster. At present it is not clear whether it is from cluster b, d, or another cluster analogous to those recently documented in killifish and zebrafish (Amores et al. 1998; Misof and Wagner 1996).

Expression of stickleback *Hox* genes during embryogenesis

Transcription of threespine stickleback *Hox* genes during embryogenesis was examined by whole-mount in situ hybridization (Fig. 3), using selected cloned PCR homeobox fragments as templates for synthesis of digoxygenin-labeled RNA probes. Most probes performed well, with good signal-to-noise ratios and high repeatability from experiment to experiment. For most of the genes, expression was clearly recognizable by the five-somite stage, although the more anterior genes began to show expression much earlier than this. Below we describe the expression patterns of representative stickeback *Hox* genes by tissue, starting with a general description of mRNA expression within the CNS and pharyngial arches, then in the paraxial mesoderm, and finally in the pectoral fin buds. For convenience as well as to facilitate comparison with expression profiles of potential homo-

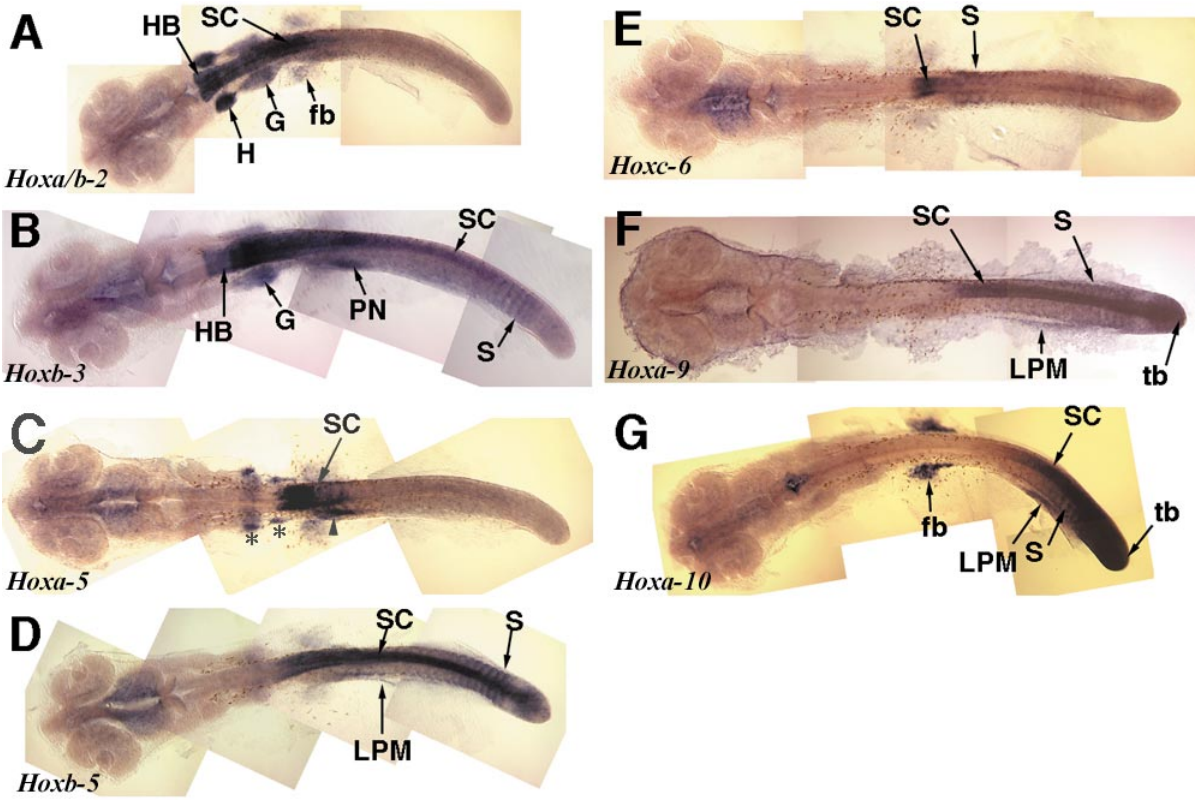

Fig. 3A–G Expression of *Hox* genes in 20-somite stage three-spine stickleback embryos. **A** *Hoxa/b-2*. **B** *Hoxb-3*. **C** *Hoxa-5*. **D** *Hoxb-5*. E *Hoxc-6*. **F** *Hoxa-9*. **G** *Hoxa-10*. *HB* Hindbrain; *SC* spinal cord; *H* hyoid arch primordium; *G* gill arch primordium; *fb* pectoral fin bud; *PN* pronephric duct; *S* somite; *LPM*lateral plate mesoderm; *tb* tailbud. *Arrowhead* and *stars* in **C** mark expression of *Hoxa-5* in a pair of ventromedial stripes of cells of unknown identity and pairs of neural-crest derived cells, respectively

logs in other species we refer to each individual clone using names of *Fugu Hox* genes that have the highest degree of amino acid sequence similarity (Fig. 1).

Expression in the central nervous system and pharyngeal arches

Similar to observations in other vertebrates, several of the stickleback *Hox* genes are expressed in the hindbrain and spinal cord with sharp anterior limits. The most rostral expression was detected for *Hoxa/b-2* (clone 38), which is expressed at very high levels in the second, third, and fourth rhombomeres (abbreviated r2, r3, and r4), and at somewhat lower levels in the remaining rhombomeres and anterior spinal cord back to the level of the seventh somite (Fig. 3A). This gene is also expressed strongly in the primordia of the hyoid arch and the five gill arches (Fig. 4A). Expression of this gene remains strong up to the stage at which the vitelline vein reaches the level of the inner eye, at which point expression in the hyoid and gill cartilages is strongest in ventral-most elements (Fig. 4C). *Hoxb-3* (clone 11) is also expressed in the hindbrain, starting in r4 and extending caudally within the neural tube to a sharp boundary at the level of the last pair of somites (Fig. 3B). The strongest expression is detected in r7, which is flanked by a weaker expression in r6 and r8 (Fig. 4B). Expression in the pharyngeal region is restricted to the gill arches, being stronger in more posterior arches (Fig. 4D).

Two genes have anterior expression limits in the posterior hindbrain. *Hoxa-5* (clone 30) is unusual in having two separate domains in the CNS, the first within the caudal hindbrain and the second beginning at the level of the first somite and extending caudally only as far as the level of the fifth somite. Transcript is also detected in the lateral regions of most posterior pharyngeal arch primordia, and in a cluster of cells lying just anterior to the first somite (Fig. 3C). *Hoxb-5* (clone 35) shares the same anterior limit in the hindbrain as clone 30, but a fairly uniform level of expression is maintained throughout the spinal cord except for a slight decrease in staining intensity near the posterior border adjacent to the last pair of somites (Fig. 3D).

Hoxc-6 (clone 30), *Hoxa-9* (clone 31), and *Hoxa-10* (clone 04) all have anterior limits of transcription in the rostral spinal cord and detectable expression throughout the remainder of the CNS. *Hoxc-6* is strong in the spinal cord at the level of the second and third somites and weak elsewhere (Fig. 3E). *Hoxa-9* expression commences at the level of the third somite and is notable for a pattern of three parallel rows of strongly expressing cells in the midline and dorsolateral regions of the spinal cord (Fig. 3F). *Hoxa-10* has the anterior limit at the level of somite seven, followed by much stronger expression in the posterior spinal cord and tailbud (Fig. 3G).

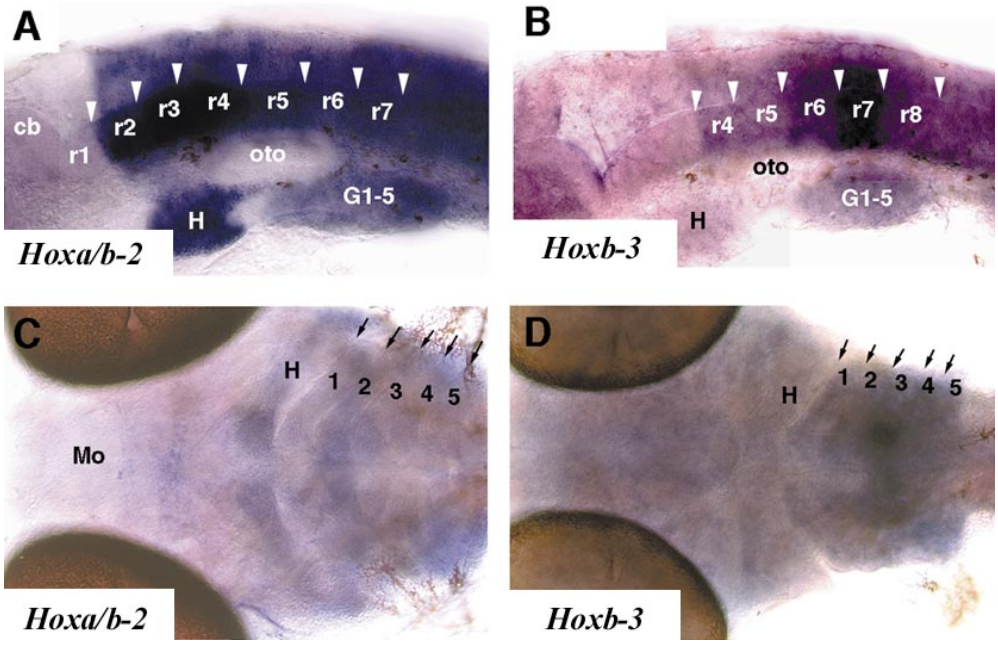

Fig. 4A–D Expression of stickleback *Hox* genes in the hindbrain and pharyngeal arches. **A**, **B** 20-somite stage, lateral views with anterior to the *left*. **C** "Vitelline vein at inner eye" stage. **D** "Vitelline vein at lens" stage, ventral views with anterior to the *left*. In threespine stickleback embryos, the otocyst (*oto*) spans the complete length of the fourth, fifth, and sixth rhombomeres (*r4*, *r5*, *r6*). Note the presence of prominent bulges corresponding to each rhombomere. r1/r2/r3 boundaries are usually a lot less clear than the others. **A** *Hoxa/b-2*. **B** *Hoxb-3*. *cb* Cerebellum; *r1–r7* rhombomeres 1–7; *H* hyoid arech primordium; *G1–G5* primordia for gill arches 1–5. **C** *Hoxa/b-2*; expression in differentiating pharyngeal arches. *H* Hyoid arch; *1–5* gill arches 1–5. Each of the clefts separating individual arches are marked by *arrows*. *Mo* mouth. **D** *Hoxb-3*; expression is limited to the gill arches and is somewhat stronger in posterior arches. The dark patch in the branchial region is a shadow created by persistently strong expression of *Hoxb-3* in the hindbrain

Expression in the paraxial mesoderm

Six of the stickleback *Hox* genes that we examined are transcribed in the somitic paraxial mesoderm. For two of them, *Hoxa/b-2* and *Hoxb-3*, expression is maintained only transiently, leaving no trace in mature somites. By contrast, the other four genes, *Hoxb-5*, *Hoxc-6*, *Hoxa-9*, and *Hoxa-10*, are seen to undergo stereotypic changes in expression patterns that are intimately linked to the formation and differentiation of individual somites. Detailed description of this early expression pattern is provided in the companion paper for *Hoxb-5*, *Hoxa-9*, and *Hoxa-10* (Ahn and Gibson 1999).

Except for *Hoxb-3*, which is expressed in the anterior pronephric ducts, the mesodermally transcribed stickleback genes are also expressed in the adjacent lateral plate mesoderm with anterior limits at the same level (*Hoxa/b-2*, *Hoxc-6*) or one (*Hoxa-9* and *Hoxa-10*) or two (*Hoxb-5*) somites posterior to the level of the limit in the somitic mesoderm, which itself is two to three somites posterior to the limit of expression in the CNS. In most embryos somites 4, 5, 7, and 9 appear to be the most anterior expression limits for *Hoxb-5*, *Hoxc-6*, *Hoxa-9*, and *Hoxa-10*, respectively. These transcription profiles are in good agreement with those recently described for zebrafish *Hox* genes (Prince et al. 1998b), in that the anterior limits all lie within the presumptive trunk portion of the vertebral axis.

Expression associated with tail morphogenesis

Cells in the tail region were seen to undergo complex temporal changes in level of expression of the four *Hox* genes that are expressed throughout the segmentation period: *Hoxb-3*, *Hoxb-5*, *Hoxa-9*, and *Hoxa-10*. Five domains showing different combinations of expression of these genes, named 1–5 from posterior to anterior, can be recognized within the paraxial mesoderm of the tail region. Although these are presented as spatially distinct in Fig. 5, they also represent a temporal sequence of changes that cells at a given axial level move through. The whole pattern moves along the body axis in concert as embryogenesis proceeds, but these changes do not affect the anterior limits of expression that are set prior to the formation of the tail, as shown in the accompanying contribution (Ahn and Gibson 1999).

Domain 1 occupies the presomitic mesoderm at the most posterior tip of the tail and consists of cells in the tail bud that express high levels of *Hoxa-9* and *Hoxa-10* but not *Hoxb-3* or *Hoxb-5*. This domain is also known to express a variety of signaling molecules including members of the Wnt family in zebrafish embryos (Blader et al. 1996; Krauss et al. 1992) and contains cells that are mitotically highly active. Domain 2 lies adjacent to the most recently differentiated portion of the notochord and neural tube and transiently expresses *Hoxb-5*, *Hoxa-9*, and *Hoxa-10* but not *Hoxb-3*. In zebrafish another sig-

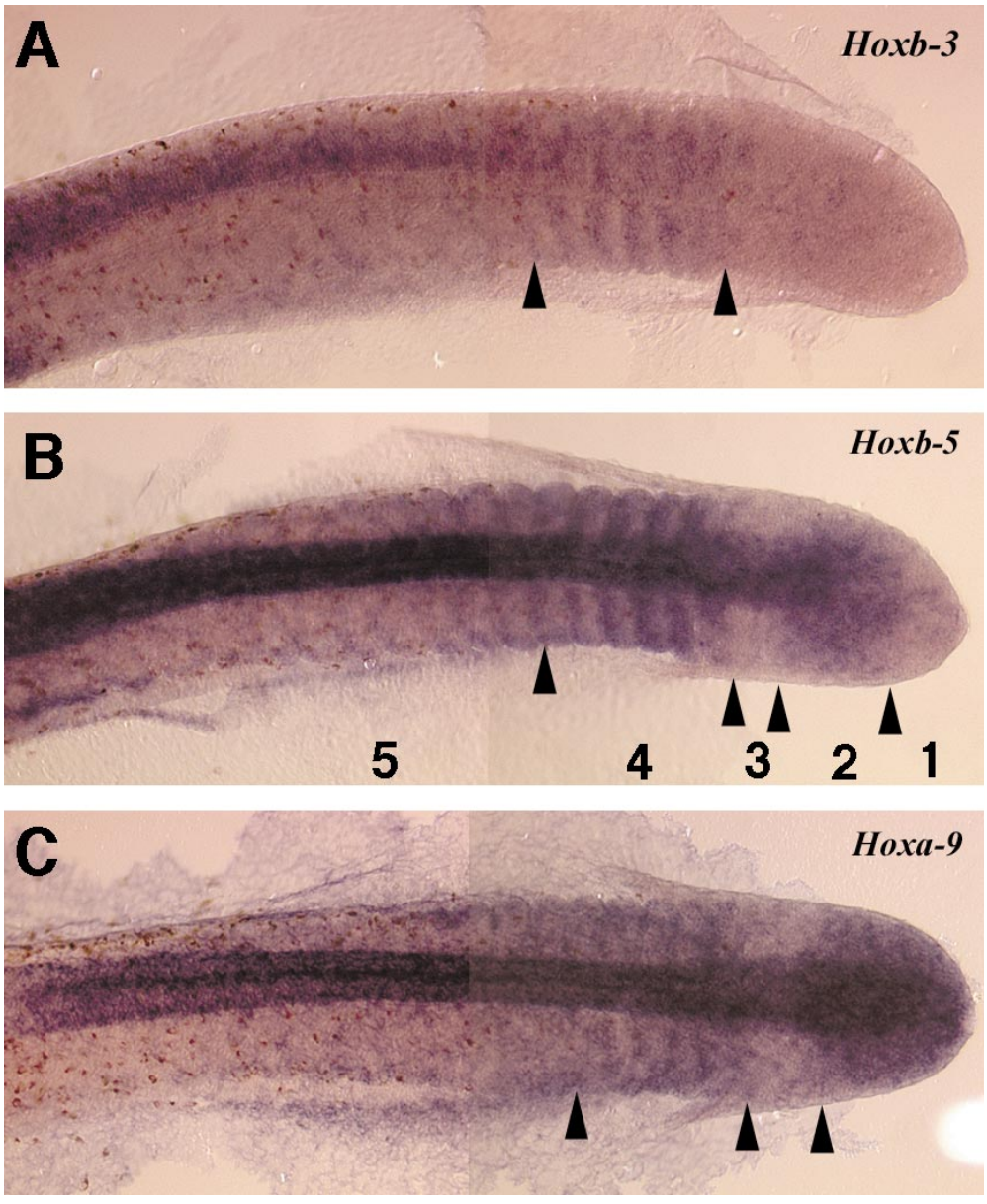

Fig. 5A–C Expression of stickleback *Hox* genes and functional domains within the paraxial mesoderm. **A** *Hoxb-3*. **B** *Hoxb-5*. **C** *Hoxa-9*. All embryos are at approximately the 20-somite stage and are shown in a dorsal to dorsolateral view with anterior to the *left*. Expression pattern of *Hoxa-10* is similar to *Hoxa-9*. The five functional domains that can be envisaged in the paraxial mesoderm based on differential expression of *Hox* genes are discussed in the text. The boundary of each domain is marked by *arrowheads* in **B**. Note the absence of expression of *Hoxb-3* in domains 1, 2, 3, and 5; and of *Hoxb-5* in domains 1 and 3, contrasted with the presence of expression of *Hoxa-9* in domain 1

naling molecule, Sonic hedgehog (Shh), is strongly expressed in the notochord cells of this region during caudal somitogenesis (Krauss et al. 1993). The rest of the presomitic mesoderm lying anterior to domain 2 constitutes domain 3. It is striking that stickleback *Hox* genes show either no (*Hoxb-5*, *Hoxa-9*) or weak (*Hoxa-10*) expression in domain 3, which contrasts with the activation of regulatory genes proposed to be involved in segmental prepatterning of the presomitic mesoderm, such as *her1* (Müller et al. 1996) and *deltaD* (Dornseifer et al. 1997) in the equivalent cells of the zebrafish.

Domain 4 includes the five most recently formed somites and the anterior half of the next soon-to-be-formed somite. Transcription of each of the four stickleback *Hox* genes is reinitiated (or strongly upregulated) in the anterior half of each somite or in the cells lying immediately posterior to the somitogenic furrow. This results in the six dorsoventral stripes seen in lateral views (Fig. 3B, D, F, G). These stripes were never detected prior to furrow formation, suggesting an intimate connection between this process and reinitiation of *Hox* gene expression. The remaining portion of the paraxial mesoderm, including all of the fully formed somites, constitutes domain 5, in which expression is restricted to a subset of ventrolateral cells close to the surface of each somite.

Expression in the pectoral fin bud

Expression of stickleback *Hox* genes in the developing pectoral fins during the later stages of embryogenesis is broadly consistent with what has been described for other vertebrates. *Hoxc-6* transcription is restricted to the anterior portion of the pectoral fin bud, possibly with an

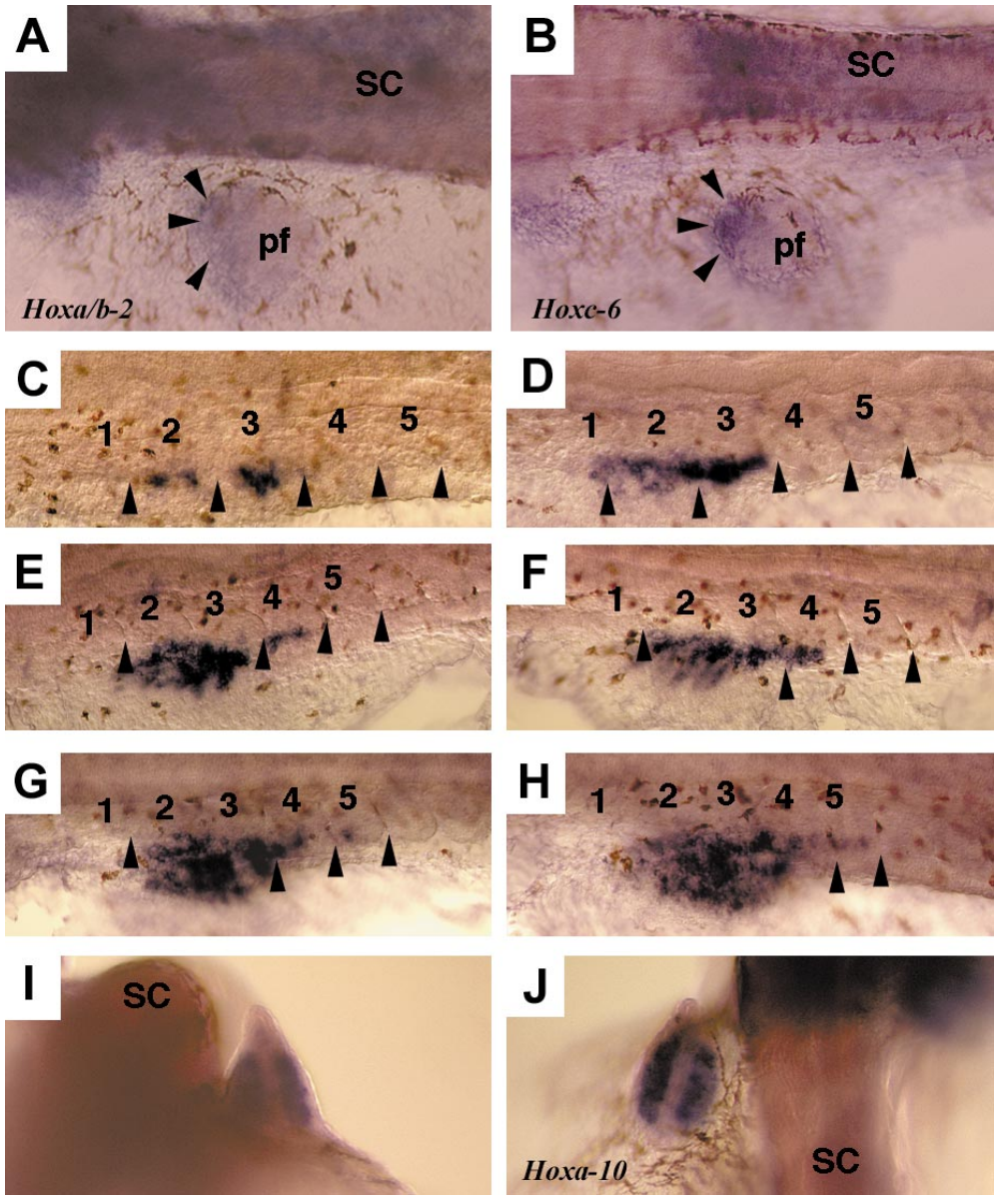

Fig. 6A–J Expression of stickleback *Hox* genes in the pectoral fin bud. **A**, **B** dorsal view with anterior to the left. *SC* Spinal cord. **A** *Hoxa/b-2*; "vitelline vein below the level of the heart" stage. Expression is limited to the anterior of the pectoral fin bud (*pf*). **B** *Hoxc-6*; at "vitelline vein at heart level" stage. Expression forms an anteroposterior gradient. **C**–**J** Expression of *Hoxa-10* during pectoral fin morphogenesis. **C**–**H** Dorsolateral view with anterior to the *left*. Somite boundaries are marked with *arrowheads*. Somites are marked by *numbers*. Embryos range from 18- to 24-somite stages. **I**,**J** *Hoxa-10* expression at "subocular branch" stage. **I** Anterior view. **J** Dorsal view

anteroposterior gradient (Fig. 6B), similar to the transcription of *Hoxc-6* in the forelimb buds of chicken (Nelson et al. 1996) and zebrafish (Molven et al. 1990). Unexpectedly, *Hoxa/b-2* is found to be expressed in a subset of anterior pectoral fin bud cells as well (Fig. 6A). *Hoxa-10*, by contrast, is expressed uniformly and at high levels in the mesenchyme cells that appear to originate from cells lying adjacent to somites at the same axial level as the presumptive fin bud and later migrate into it. Transcription is first detected at the 15-somite stage as a small cluster of cells lying adjacent to the third somite. These are soon joined by a second cluster adjacent to the second somite (Fig. 6C), with fusion of these two clusters of cells resulting in a single large patch of *Hoxa-10* expression (Fig. 6D). As somitogenesis proceeds, further clusters of cells appear opposite the fourth (20-somite stage; Fig. 6E) and fifth (23-somite stage; Fig. 6G) somites, such that the anteroposterior extension of the mesenchyme of the fin bud eventually includes cells lying from the s1/2 boundary to the s5/6 boundary (Fig. 6H).

This cluster of fin-bud precursor cells enlarges and becomes more coherent as a single cluster up until the stage at which the vitelline vein reaches the level of the heart, at which point it closely resembles reported patterns of expression of the zebrafish *Hoxa-10* gene (Sordino et al. 1996). Soon the entire fin bud becomes partitioned with the appearance of nonexpressing cells between groups of *Hoxa-10* expressing mesenchymas that may later give rise to the muscles of the pectoral fins (Fig. 6I, J). Expression of *Hox* genes in prospective muscle cells has been reported previously for *Hoxa-11* and *Hoxa-13* during development of chicken limb buds (Yamamoto et al. 1998), suggesting that *Hoxa-10* performs similar functions in stickleback fin buds. *Hoxa-10* expression persists during later phases of fin morphogenesis, until it decays to a small patch in the center of the

fin toward the end of embryogenesis. Interestingly, at no point is a second domain of expression akin to that which patterns the proximodistal axis of tetrapod limbs (Haack and Gruss 1993) observed.

Discussion

Hox genes and the evolution of teleost and tetrapod vertebral morphology

Stickleback *Hox* genes appear to be expressed, and are hence most likely used in the patterning of the main body axis, in a similar manner to that of other vertebrates. In the absence of linkage information it is not possible to confirm that there is colinearity of order of expression domains with gene order along the chromosome in *G. aculeatus*, but all indications are that this is likely to be the case. Using sequence similarity as a guide, homeobox sequences belonging to more 3' paralogous groups are expressed with more anterior limits (Fig. 7) and are activated earlier in development. In general, expression extends more rostrally in the CNS than in the mesoderm, and there is some degree of overlap in expression domains, satisfying necessary conditions for the existence of a combinatorial *Hox* code (Hunt and Krumlauf 1992).

In several respects the stickleback *Hox* expression patterns resemble those of zebrafish more closely than those of mammals and birds and thus appear to reflect a teleostean type of usage. Most notably, the anterior limits of expression of genes in groups 5–10 are located in the somites of the trunk region where ribs are present in all but the last few vertebrae and thus are not obviously coincident with morphological transitions between vertebral types as they are in amniote tetrapods (Burke et al. 1995). The finding that two highly divergent species of teleosts share this condition suggests that this is not a property unique to zebrafish or sticklebacks and hence is likely to represent a general feature among modern-day fishes. Although it may be surprising to find that the pos-

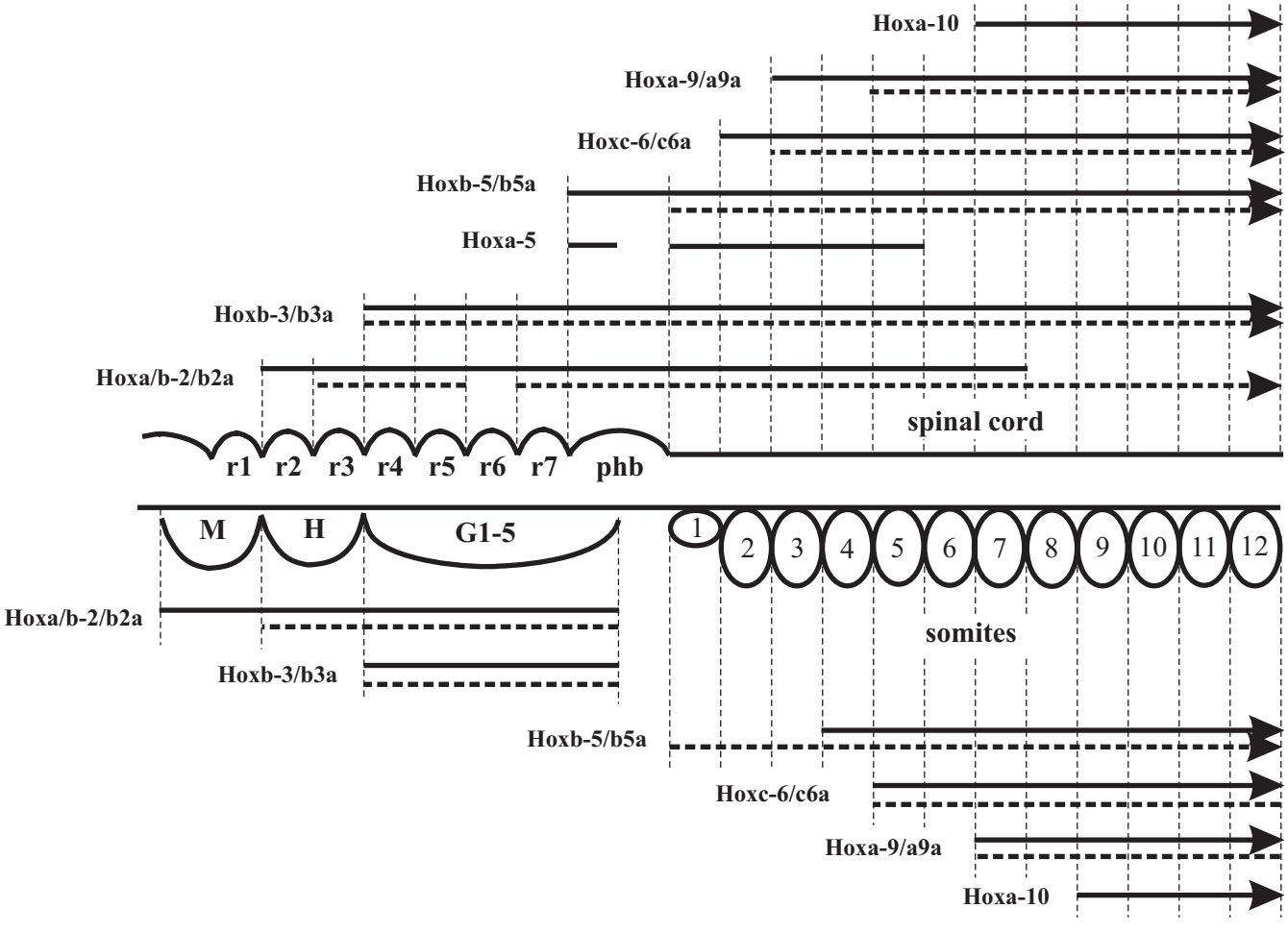

Fig. 7 Comparison of expression domains of *Hox* genes between threespine stickleback and zebrafish embryos at the 20-somite stage. *M* Mandibular arch; *H* hyoid arch; *G1–G5* gill arches 1–5; *r1–r7* rhombomeres 1–7; *phb* posterior hindbrain. *Top* Expression in the CNS; *bottom* expression in the pharyngeal arches and in somites. Expression domains of stickleback (this study) and zebrafish (Prince et al. 1998a, 1998b; Yan et al. 1999) *Hox* genes are represented by *lines* and *dashed lines*, respectively. *Arrows* indicate expression in the remainder of CNS or somites. For zebrafish genes, nomenclature of Amores et al. (1998) is used (see the supplementary material for Amores et al. 1998 at the *Science* web site for the revision of zebrafish *Hox* gene nomenclature)

terior limit of the dorsal rib-bearing vertebrae is set by different genes in teleosts and tetrapods, this result is actually quite consistent with the fossil and anatomical evidence relating to the evolution of the vertebrate body axis.

Birds and mammals appear to have highly derived vertebral columns (Romer and Parsons 1986). In many fishes, reptiles, and early amphibians, dorsal ribs extend from the neck to the base of the tail without interruption or obvious morphological differentiation. Caudal vertebrae are better distinguished from trunk vertebrae by the presence of hemal arches, since in some species "caudal" ribs can also be found on the dorsal side of several vertebrae in the tail (Carroll 1988). Fossil paleoniscoids that represent primitive ray-finned fish and crossopterygian lobe-finned fish that are thought to represent the basal tetrapod condition both have structurally rather homogeneous vertebral columns, with the only clear distinction being between "trunk" and "caudal" vertebrae (Romer 1966). By the assumption of parsimony, this distinction defines the ancestral state for both ray-finned and lobe-finned fishes, which indicates that the relationship between expression domains of *Hox* genes and the vertebral morphology in the common ancestor of the two groups probably more closely resembled that seen in teleosts. In the lineage leading to modern-day amniote tetrapods morphological differentiation of trunk vertebrae evolved gradually during the transition to land, beginning with modification of sacral ribs to support the pelvis, followed by modification and loss of ribs in the cervical and lumbar regions to promote flexibility (Radinsky 1987). In ray-finned fishes, including teleosts, various changes have been superimposed on the trunk region in different lineages, including modification of the ventral ribs and hemal arches, of the neural arches and spines dorsally, and of ossification and the shape of vertebral centra (Ford 1937).

Combining the morphological and gene expression data, it can be inferred that the staggering of *Hox* expression boundaries in the trunk region of primitive gnathostomes provided a "precode" that has been used to derive diverse axial plans. Although we have not analyzed group 11–13 clones in sticklebacks, the data presented here are consistent with the hypothesis that *Hox* genes collectively pattern only the precaudal region of the vertebral column in this species, as postulated for all vertebrates by van der Hoeven et al. (1996). These authors implicated *Hoxd-13* in determination of the location of the proctodaeum anlage, which also typically marks the transition point from trunk to caudal vertebrae. Within the trunk region, *Hoxc-6* consistently marks the axial level adjacent to the posterior-most spinal nerve that innervates the pectoral appendages, which appears to be the case in sticklebacks as well. The anterior limits of *Hoxb-9*, *Hoxc-9*, and *Hoxd-9* in the lateral plate mesoderm are staggered over the posterior region of the forelimb bud in the chick (Cohn et al. 1997), and in stickleback *Hoxa-9* is expressed in the lateral plate mesoderm one or two somites posterior to the pectoral fin bud. Each of these features may thus have been present in the common ancestor of tetrapods and teleosts, implying that the *Hox* code was first used to determine the location of the limbs and trunk-tail boundary.

By contrast, the coincidence of *Hox* expression boundaries with transitions between cervical, thoracic, lumbar, and sacral types of vertebrae that is seen in birds and mammals appears to be a recent innovation. While transposition of the locations of each of these boundaries may have evolved through alteration in *Hox* expression domains, the genesis of different vertebral types must have required modification of Hox protein function. In this sense there are clear parallels between the suppression of appendage development in the evolution of insects, which was brought about in part by modulation of target gene specificity of more posterior *Hox* genes (Averof and Akam 1995; Warren and Carroll 1995), and suppression or modulation of rib development in the evolution of terrestrial vertebrates.

Differences between stickleback and zebrafish *Hox* expression patterns

Although the exact identities of our stickleback *Hox* clones are uncertain in some cases, particularly for middle group genes (Fig. 2), there nevertheless appear to be many similarities as well as differences to the expression profiles of presumed homologs in zebrafish. These two species have fairly similar axial morphologies, with of the order of 15 trunk vertebrae, and therefore no gross differences in the expression patterns within the paraxial mesoderm were expected. Indeed, comparison of anterior limits of expression between homologous genes of these two species seems to indicate that expression domains of many of the *Hox* genes are more or less identical in the paraxial mesoderm although somewhat different expression domains are observed in the spinal cord (Fig. 7). At present it is not clear whether such gross similarity in *Hox* expression domains is the result of convergent evolution due to having a similar number of body segments or to the retention of ancestral features. Analysis of *Hox* expression in more divergent fish species with longer body axes would be necessary to resolve this issue.

What might the anterior limits of teleost *Hox* genes be marking in the paraxial mesoderm? One possibility is that *Hox* genes in fish affect the axial positioning of median skeletal elements such as the pterygiophores and predorsals (for a discussion see Ahn and Gibson 1999). The strong expression and sharpness of the anterior limits observed in the CNS are more consistent with a fundamental role for *Hox* genes in patterning of the nervous system. It is also possible that the genes are not involved in setting the locations of skeletal elements at all, and rather that there is a general requirement for Hox proteins in the mesoderm with the differences in axial level of expression being driven by shared CNS enhancers. In any case, the differences between fish and tetrapod ex-

pression patterns highlight the need for more experimental and genetic studies of *Hox* gene function in fish species.

Some intriguing differences were detected in the rhombomeres, where the expression domains of most of the stickleback *Hox* genes differ from those of the putative zebrafish and mouse homologs. For example, the strongest expression of *Hoxb-3* is found in r7 in stickleback, compared with r5 in the mouse and r5 and r6 in the zebrafish. In mouse and zebrafish this difference is known to be correlated with a change in rhombomeric location of the abducens nuclei (Prince et al. 1998a). We have not examined the organization of the branchiomotor nuclei in the hindbrain of stickleback embryos, but our results suggest the possibility that there is variation among taxa in the precise organization of neuronal identity and function in rhombomeres.

Hox genes and segmentation in teleosts

Variation in the number and location of the various types of vertebrae is common in both aquatic and terrestrial vertebrates. Analysis of patterns of covariation along the anteroposterior axis suggests that meristic effects, namely the variation in number of segments, account for much of the variation in morphology of the vertebral column in sticklebacks (Ahn 1998) and in salamanders (Jockusch 1997). In addition to variation in total number of vertebrae, we have found evidence of homeotic shifts that could account for variation in the axial location of particular types of median skeletal elements in sticklebacks, such as predorsals and fin pterygiophores (Ahn and Gibson, in preparation). A mechanistic understanding of such phenomena requires better understanding of the relationship between the generation of mesodermal segments and the assignment of positional identity to them.

Assuming that *Hox* genes are involved in establishing segmental identity in fish, the expression data presented here suggest that segmentation (or somitogenesis) and identity determination are independent developmental processes. It is difficult to establish the precise relationship between the initiation of *Hox* transcription and segmentation since *Hox* expression during epiboly and early gastrulation is relatively weak, and there appears to be considerable variation in the timing of appearance of distinct somites in different embryos. Nevertheless, it is clearly not the case that the onset of stable *Hox* expression accompanies the birth of each somite (Ahn and Gibson 1999), as occurs in *Drosophila* where the same transcription factors activate expression of *Hox* genes and segmentation genes (Kornberg and Tabata 1993). Furthermore, the lack of expression of *Hox* genes in domain 3 (Fig. 5) during tail morphogenesis (which is also the case in zebrafish; see Prince et al. 1998b) strongly suggests that *Hox* genes do not respond directly to genes such as *her1* and *deltaD* that are expressed in somite-sized blocks of cells in this domain and are likely to be involved in the segmental configuration of presomitic mesoderm (Dornseifer et al. 1997; Müller et al. 1996). An immediate consequence of the uncoupling of the formation and determination of identity of somites would be greater opportunity for the generation of diversity in axial morphology (Ahn and Gibson 1999).

It also seems likely that patterning in the tail region of vertebrates involves different developmental genetic mechanisms than in the trunk. This observation is perhaps not surprising given that the mesodermal precursor cells are generated in the trunk region by the ingression of epiblast cells during gastrulation while they are generated de novo by mitosis in the tailbud (Catala et al. 1995; Kanki and Ho 1997). Refinement of the structures of derivatives of caudal somites may be regulated by activities of homeotic genes other than the *Hox* genes that have not yet been identified. Transient expression of *Hox* genes in the five domains associated with tail bud formation and elongation could also conceivably contribute some positional information. Cell lineage tracing has shown that subsets of cells in the tail region are fated to become axial or paraxial mesoderm during the formation of the tailbud (Kanki and Ho 1997), but it is not known when or how they receive anteroposterior identity.

Alternatively, *Hox* expression in the tail domains may play some direct role(s) in cellular morphogenesis, migration, and proliferation as the tail bud extends along the body axis. *Hox* genes have been proposed to regulate cell division during limb development in the chick and mouse (Duboule 1995) and have been shown to affect formation of constrictions in the midgut of *Drosophila* embryos (Panganiban et al. 1990). The latter process is mediated in part by the secreted growth factor Wingless (Yu et al. 1996), and several members of the homologous Wnt family are known to be expressed in dynamic patterns associated with zebrafish tail morphogenesis (Blader et al. 1996). It is also intriguing that *Hox* expression in domain 4 is reinitiated immediately posterior to newly formed somitogenetic furrows and is restricted to only the anterior halves of each somite (Fig. 5), suggesting that it contributes to defining their polarity. Whatever the functions of *Hox* expression in the tail, they should be considered as another example of the reuse of *Hox* genes for regulation of development other than patterning of the CNS and paraxial mesoderm of the trunk. Given the diversity of tail morphology in vertebrates, experimental and comparative analysis of the role of *Hox* genes in tail formation should be an interesting system for the study of the evolution of development.

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