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Short Contribution

Hoxb-5 is expressed in gill arch 5 during pharyngeal arch development of flounder *Paralichthys olivaceus* embryos

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ABSTRACT ***Hox*** **genes are expressed in domains with clear anterior borders exhibiting 3'→5' hierarchy in hindbrain and in the pharyngeal area commonly in vertebrate embryos. Teleost embryos form seven pharyngeal arches, the mandibular arch, hyoid arch and the gill arches 1-5. We previously reported that, in Japanese flounder** ***(Paralichthys olivaceus)*** **embryos,** ***Hoxd-4*** **is expressed from rhombomere 7 to the spinal cord in the central nervous system and at gill arches 2-5. At present, the hierarchy of** ***Hox*** **genes at gill arches 3-5 of teleost fish is unclear. Here, we investigated the expression domains of** ***Hoxb-5*** **in the flounder embryo by whole-mount** ***in situ*** **hybridization to gain insight into the** ***Hox*** **code at gill arches. The initial signal indicating** ***Hoxb-5*** **expression was identified in the spinal cord at hatching, corresponding with the prim-5 stage of zebrafish. Then, intense signals were detected from the anterior part of the spinal cord and from the posterior part of the pharyngeal area at 36 h after hatching. By serially sectioning the hybridized embryos, it was found that signal in the pharyngeal area came from the most posterior gill arch 5. Therefore, it is speculated that** ***Hoxb-5*** **functions in regional identification of gill arch 5 in this teleost.**

KEY WORDS: *pharyngeal arch, neural crest, Hoxb-5, flounder, teleost*

Teleost fish embryos form seven pharyngeal arches (P1-7), the lining mandibular arch, hyoid arch and five sets of gill arches. In mouse embryos which develop five pharyngeal arches (P1-4, and 6), *Hox* genes of the paralogous group 2-4 are expressed in domains with clear anterior borders exhibiting 3'→ 5' hierarchy at the hindbrain and pharyngeal arches, collectively controlling the patterning of their A-P axis (reviews in McGinnis and Krumlauf, 1992; Krumlauf 1994; Manak and Scott, 1994; Boncinelli, 1997). The mesenchymal populations in the pharyngeal arches, including precursor cells of cartilage, differentiate from cranial neural crest cells emigrating from the hindbrain (Schilling and Kimmel, 1994). Cranial neural crest cells migrate from certain rhombomeres into specific pharyngeal arches, where they again express identical *Hox* genes as in hindbrain, under the influence of the surrounding tissues (Saldivar *et al*., 1996). Therefore, a corresponding hierarchy exists in the *Hox* code from the hindbrain and pharyngeal area. It has been recently demonstrated in zebrafish that the expression domains of paralogous groups 1-4 at the hindbrain are the same as those of the mammalian counterparts with some modifications (Prince *et al.,* 1998b). We reported that the anterior border of *Hoxd-4* expression is at r6/7 and gill arches 1/2, corresponding to mouse P3/4, in Japanese flounder, *Paralichthys olivaceus*, indicating a conservation of the expression domain of this gene between flounder and mouse (Suzuki *et al.,* 1998). From these results, it is supposed that the mandibular arch, hyoid arch and gill arches 1-2 of teleost embryos, corresponding to mouse 1-4, respectively, are coded by a hierarchy of *Hox* genes similar to mouse embryos. At present, the hierarchy of *Hox* code at caudal gill arches 3-5 in teleosts is unclear. Because the most caudal pharyngeal arch (P6) of mouse embryos, is coded by *Hoxb-5* (Kuratani and Wall, 1992), members of paralogous group 5 are the candidates for coding the posterior gill arches in teleosts. *Hox b-5* and *c-5* are expressed in the central nervous system in zebrafish (Ericson *et al.,* 1993; Njølstad and Fjose 1988; Prince *et al.,* 1998a), but their expression domains in the pharyngeal area have not been described.

Since a cDNA fragment of *Hoxb-5*, a member of paralogous group 5, was cloned from flounder embryos (Suzuki *et al.,* 1998), this report aimed to reveal the expression domain of this gene in the embryos to gain insight into which *Hox* genes are expressed in gill arches. The expression of *Hoxd-4,* which

Abbreviations used in this paper: A-P axis, anterior-posterior axis.

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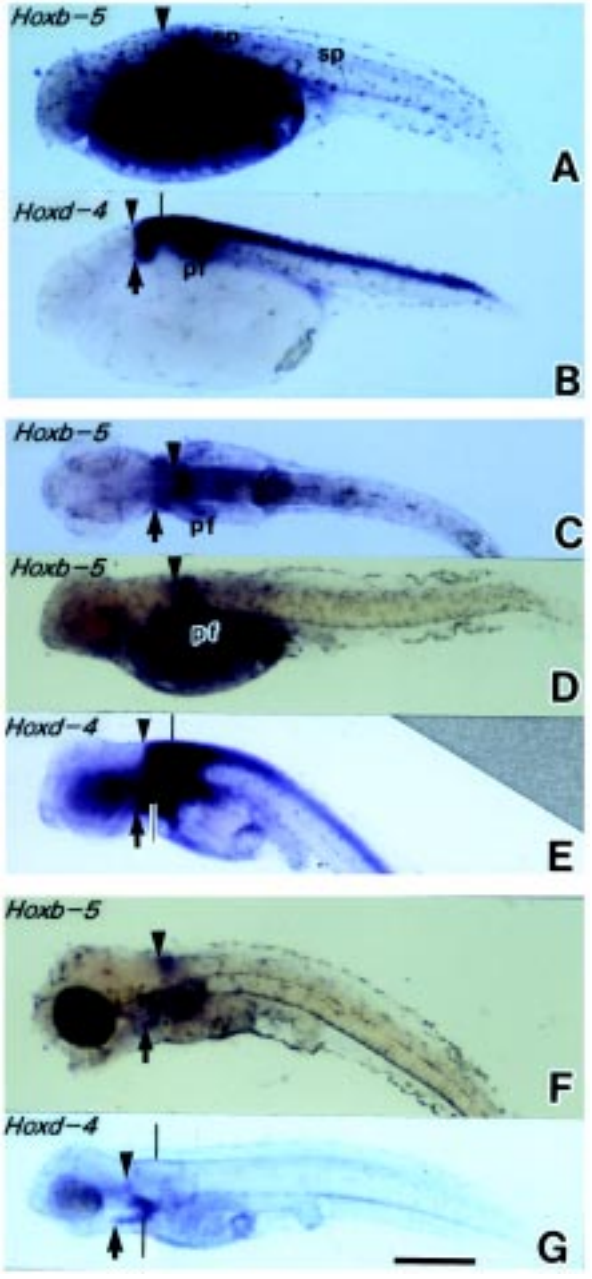

Fig. 1. Whole-mount *in situ* hybridization of flounder embryos using *Hoxb-5* and *Hoxd-4* riboprobes. (A,C,D *and* **F)** *Hoxb-5 expression.* **(B,E** *and* **G)** *Hoxd-4 expression.* **(C)** *Dorsal view, the others are lateral views. The anterior border of the Hox expression domains in the central nervous system and pharyngeal area is marked by an arrowhead and arrow, respectively. For comparison, the position of the Hoxb-5 expression borders observed in A,D and F is marked by bars in B,E and G, respectively.* **(A,B)** *Hatching period. The spinal cord (sp) gave weak Hoxb-5 expression. The Hoxd-4 expression domains in the central nervous and pharyngeal area show a clear anterior border located at the position of rhombomere 6/7 and gill arches 1/2, respectively (refer to Suzuki et al., 1998). Hoxd-4 is also expressed at the pectoral fin bud (pf).* **(C,D,E)** *Thirty six hours after hatching. Intense expression of Hoxb-5 can be seen in part of the spinal cord. Hoxb-5 expression is also seen at the posterior part of the pharyngeal area. Hoxd-4 expression is maintained at the same level as at hatching.* **(F,G)** *Ninety six and 72 h after hatching, respectively. The expression of both Hoxb-5 and Hoxd-4 is maintained in the pharyngeal area.*

shows a clear anterior border both in the central nervous system and pharyngeal area (Suzuki *et al.,* 1998), was also observed to describe the borders of *Hoxb-5* expression domains.

On hatching, corresponding to the prim-5 stage of zebrafish development (Kimmel *et al.,* 1995), the hindbrain has formed seven prominent rhombomere segments, and paired primordia of each mandible, hyoid and gill arches locate on the ventral side of the body, between the eye and pectoral fin (Suzuki and Kurokawa, 1996; Suzuki *et al.,* 1998). At this stage, a weak hybridization signal to *Hoxb-5* riboprobe could be detected from the spinal cord, but not from the pharyngeal area (Fig. 1A). At this stage, *Hoxd-4* had established expression domains both in the central nervous system and pharyngeal area, showing clear anterior borders at r 6/7 and gill arches 1/2, respectively (Fig. 1B; refer to Suzuki *et al.,* 1998). The *Hoxb-5* expression level was found to be relatively low, and the anterior boundary of expression in the central nervous system was not clear when compared with the *Hoxd-4* expression at any stage.

At 36 h after hatching (the high-pec stage of zebrafish), the paired primordia of mandibular and hyoid arches have become fused at the medial line. The anterior border of the *Hoxd-4* expression domain in the central nervous system was still clear (Fig. 1E). The *Hoxb-5* expression in the central nervous system had become restricted to the anterior part of spinal cord, where the expression level had increased (Fig. 1C,D). The border of anterior expression was located posterior to that of *Hoxd-4* (compare Fig. 1D and E). *Hoxb-5* expression was first detected from the pharyngeal area at this stage (Fig. 1C,D), and was maintained through 70 h after hatching (Fig. 1F). The expression domain of *Hoxb-5* in the pharyngeal area was more posterior than that of *Hoxd-4* (compare Fig 1D and E; Fig. 1F and G). The initiation of *Hoxb-5* expression at the pharyngeal area was about 30 h later than *Hoxd-4,* which occurs at the 21-somite stage (Suzuki *et al.,* 1998).

To elucidate which pharyngeal arch expresses *Hoxb-5*, we examined serially sectioned embryos which had been stained by whole-mount *in situ* hybridization for hybridization signal. In a lateral section, in which mandibular, hyoid and gill arches 1-5 were visible, hybridization signal was detected from the mesenchyme in gill arch 5 (Fig. 2A). In a horizontal section, hybridization signals appeared as a pair of foci in gill arch 5 just in front of the cleithrum and pectoral fin cartilages (Fig. 2B), confirming that the signal came from the most posterior gill arch. It is thought that *Hox* genes function at their anterior limit of expression in the regional specification along the A-P axis of the body (see review; McGinnis and Krumlauf, 1992). So, it is speculated that in flounder embryos, *Hoxd-4* and *Hoxb-5* function in distinction between gill arch 1 and 2, and between gill arch 4 and 5, respectively. Alternatively, it is also possible that *Hoxb-5* expression defines not any specific arch but the caudalmost arch, such as pharyngeal arch 6 in mice (Kuratani and Wall, 1992). In most teleosts including flounder, gill arch 5 has lost a respiratory function and gained a masticatory function with developed pharyngeal teeth (Harder, 1975). Hence, this arch is morphologically distinct from gill arches 1-4. Such morphological modification may relate to the fact that gill arch 5 has a different *Hox* code from the anterior arches.

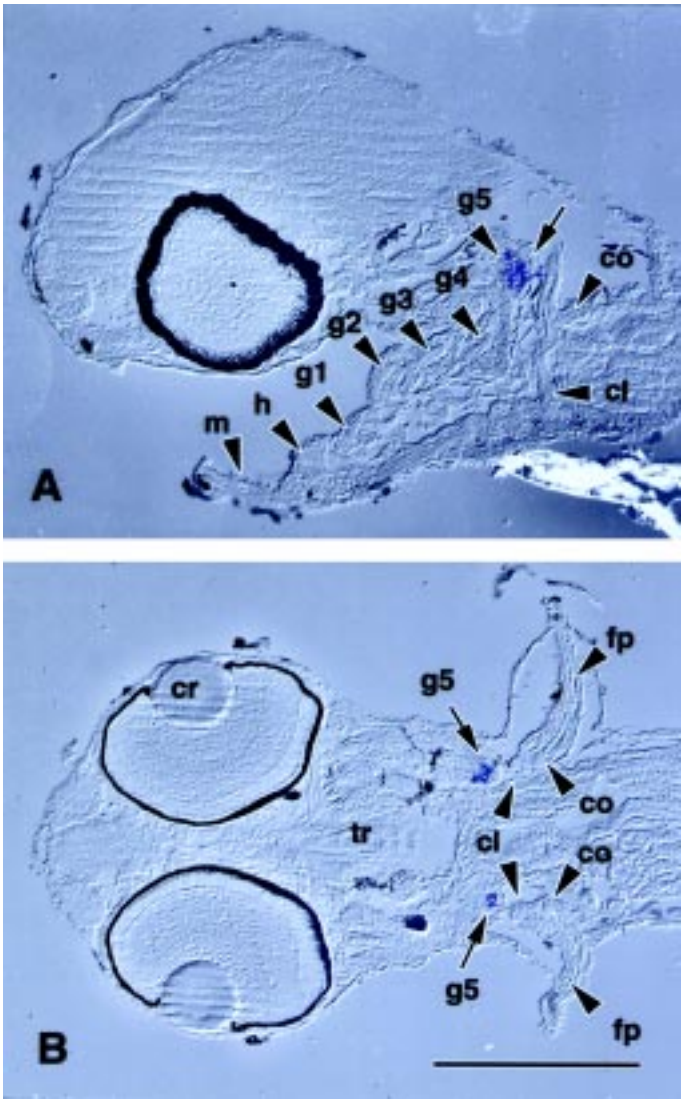

Fig. 2. Sections of flounder embryos hybridized with *Hoxb-5* riboprobe at 96 h after hatching. (A) *Lateral section through the mandibular arch (m), hyoid arch (h) and five gill arches (g1-g5). The hybridization signal (arrow) came from the gill arch, g5, located just in front of cleithrum (cl). co; coracoid-scapula cartilage.* **(B)** *Horizontal section through crystalline lens (cr), trabeculae cartilage (tr), dorsal part of g5, cleithrum, and coracoid-scapula and fin plate cartilage (fp) in the pectoral fins. Paired hybridization signals in g5 (arrows) are just anterior to the cleithrum.*

Experimental Procedures

Embryos

Japanese flounder *(Paralichthys olivaceus)* embryos at the 2-4 cell stage were collected from a hatching tank and reared further in 50 l tanks, as previously described (Suzuki and Kurokawa, 1996). They were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, overnight, washed in PBS, and then kept in methanol at -20°C.

In situ hybridization

Digoxigenin (DIG)-labeled riboprobes of *Hoxd-4* and *Hoxb-5* were prepared from λ ZAP II cDNA clones which possessed inserts of the respective genes, as described previously (Suzuki *et al.,* 1998). Whole-mount *in situ* hybridization was performed on fixed embryos kept in methanol at -20°C, according to Jowett and Lettice (1994).

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