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Hoxd-4 Expression during Pharyngeal Arch Development in Flounder (Paralichthys olivaceus) Embryos and Effects of Retinoic Acid on Expression

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ABSTRACT—Teleost fish develop seven pharyngeal arches (mandible, hyoid, and five gill arches) which give rise to the jaw and gills, and skeletal cell populations which originate from the cranial neural crest. The anterior border of expression of the Deformed (Dfd) group is located in the hindbrain and pharyngeal region. To investigate pharyngeal skeletal formation in the teleost fish, we cloned the cDNA coding Hoxd-4 from a cDNA library for flounder (Paralichthys olivaceus) embryos, and analyzed gene expression pattern during embryogenesis and the effects of retinoic acid (RA) on this gene expression. Between the 21-somite and prim-5 stages, Hoxd-4 was expressed in the central nervous system from rhombomere 7 to the spinal cord, and in the pharyngeal region posterior from gill arch 2. Its expression then became restricted to cartilage precursor cells of gill arches 2-5. When embryos in the early shield stage were exposed to RA at concentrations above 10⁻⁷ M, the anterior border of Hoxd-4 expression shifted anteriorly in a dose-dependent manner, both in the central nervous system and pharyngeal region. We propose that, during gill skeleton formation, Hoxd-4 functions in the specification of regional identity between gill arches 1 and 2, and that their identity is affected by treatment with RA.

INTRODUCTION
Embryos of teleost fish develop seven pharyngeal arches. During organogenesis, the 1st (mandible) and 2nd (hyoid) arches form the jaw and jaw support structures, respectively, and the 3rd-7th arches differentiate into five sets of gill bars (Schilling and Kimmel, 1994). Mesenchymal populations in the pharyngeal arches, including cartilage precursor cells, bone and connective tissue, originate from cranial neural crest cells which migrate from the hindbrain to the pharyngeal arches during the early somite stage (Sadaghiani and Vielkind, 1990; Kimmel et al., 1995; Schilling and Kimmel, 1994). The crest cells of each arch migrate from certain limited rhombomeric segments of the hindbrain. Based on the migration pathway in mouse and chick embryos (Hunt et al., 1991b, c; Köntges and Lumsden, 1996; Lumsden et al., 1991; Krumlauf, 1993; Osumi-Yamashita et al., 1994; Wilkinson, 1993), it is supposed that in the teleost the crest cells of the mandible arch come from the posterior midbrain and rhombomere (r) 1-3; the hyo-oid arch from r3-5; and the five gill arches from r5-8 and the anterior part of the spinal cord. In the zebrafish embryo, it was ascertained that the mandible crest cells come from r1-3 and the hyoid crest cells from r3-5 (Schilling and Kimmel, 1994), but the correspondence between rhombomeric segments and gill arches is still obscure.

Vertebrate Hox genes, which are located in four clusters, instruct cells to undergo appropriate developmental decisions such as the segmentation of hindbrain and axial skeleton (Manak and Scott, 1994). Pharyngeal cartilage components develop through complex interactions involving rhombomeric segments of hindbrain, neural crest cells and pharyngeal arches (Hall, 1987; Igelzi et al., 1995). Four Hox genes located at the 3' region of each gene cluster are expressed sequentially in a 3' to 5' pattern in rhombomeres and also in the cranial neural crest (Hunt et al., 1991a; Krumlauf, 1993; Wilkinson, 1993). Targeted disruption of mouse Hox genes, including Hoxa-1, a-2, a-3 and d-3, demonstrated that the Hox gene products function to specify regional identity in the pharyngeal skeleton (Chisaka and Capecchi, 1991; Condie and Capecchi, 1994; Gendron-Maguire et al., 1993; McGinnis and Krumlauf, 1992; Lufkin et al., 1991; Rijli et al., 1993). Therefore, the Hox genes are thought to be key molecules that control morphogenesis of the pharyngeal skeleton.

Members of the Deformed (Dfd) group of Hox genes are expressed in the hindbrain posteriorly from r7 and in neural crest derivatives that have migrated to the pharyngeal region (Hunt et al., 1991a; Krumlauf, 1993; Wilkinson, 1993). Since disruption of mouse Hoxd-4, a member of the Dfd family, induces homeotic transformation of the cervical vertebrae and malformation of the basioccipital bone, this Hox gene is thought to play an important role in the development of the vertebrae.
and pharyngeal skeletons (Horan et al., 1995a, b). Teleost fish retain seven pharyngeal arches, the posterior parts of which (pharyngeal arch 5-7) have degenerated in mammals, and thus the study of teleost fish may provide insight into the original pattern and regulation of vertebrate pharyngeal development.

In human embryonic carcinoma cells, retinoic acid (RA), a putative morphogen, induces the activation of Hox genes in a 3’→5’ sequential pattern (Simeone et al., 1991). RA has the ability to activate RA-receptors that function as transcriptional factors and interact with RA-responsive elements at the 3’ end of the Hox gene clusters (Langston and Gudas, 1992; Ogura and Evans, 1995). In addition, HOXD-4 possesses its own RA-responsive upstream element (Morrison et al., 1996). Thus, RA is a candidate for the regulation of Hox gene expression. Exogenous RA exerts teratogenic effects on the skeleton and central nervous system of vertebrates, from fish to mammals (Holder and Hill, 1991; Herrmann, 1995). The hindbrain and pharyngeal region of the head are particularly sensitive to the effects of RA (Holder and Hill, 1991; Marshall et al., 1992; Alexandre et al., 1996). RA is thought to induce abnormality in vivo by shifting the border of expression of Hox genes, eventually causing a transformation of anterior cell fates to more posterior cell fates (Morris-Kay et al., 1991). Fish embryos are useful for the study of the effects of RA on embryogenesis because the RA concentration in the environment can be controlled.

We previously reported on the morphological features of pharyngeal arch development in flounder (Paralichthys olivaceus) embryos (Suzuki and Kurokawa, 1996). In this study, we cloned the Hoxd-4 gene from a cDNA library for flounder embryos and analyzed its temporal and spatial pattern of expression during the development of the pharyngeal skeleton. Finally, the effects of RA on Hoxd-4 expression were analyzed to test whether RA induces an anterior shift in Hoxd-4 expression in the central nervous system and in the pharyngeal arches of flounder embryos.

MATERIALS AND METHODS

Embryos

Fertilized eggs of flounder, Paralichthys olivaceus, were collected and kept at 17°C as previously described (Suzuki and Kurokawa, 1996). Embryos were staged according to morphological criteria for zebrafish (Kimmel et al., 1995).

cDNA preparation

mRNA was prepared from a mixture of equal volumes of the shield, 14-16 somite and prim-5 embryos of flounder using the QuickPrep mRNA Purification Kit (Pharmacia). First strand cDNA was synthesized using an oligo(dT)12-18 primer, and 2nd strand cDNA was generated by random priming using the QuickPrep cDNA Synthesis Kit (Pharmacia). The cDNA was used as a substrate for the polymerase chain reaction (PCR) and for the preparation of the cDNA library.

PCR amplification and sequencing

The following PCR primers were synthesized to amplify a partial cDNA fragment (152 bp) inside the homeobox domain.

Hom-1: 5′-A(GC)GGTGCG(ACT)CA(AG)AC(ACT)TA(CT)A-3′

Hom-2: 5′-C(TG)(AGT)(CT)G(AG)TT(CT)TG(GA)AACCA(AGT)A-3′

Taq DNA polymerase from Takara was used with the supplier’s buffer. PCR was carried out for 50 cycles; cycling parameters were 95°C for 0.5 min, 50°C for 1 min and 72°C for 2 min, followed by a single cycle of 72°C for 5 min, using a Zymoreactor II thermal cycler (Atto). The PCR product of 152 bp was gel purified and cloned into a TA (pCRII) vector from Invitrogen. Inserts were sequenced using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer).

To obtain the RNA probe of putative Pax-1 or Pax-9 for in situ hybridization to visualize pharyngeal endoderm (Deutsch et al., 1988; Müller et al., 1996), the following PCR primers were synthesized to amplify a 361-bp fragment corresponding to the paired box of the genes.

Pax-A: 5′-G(TC)GG(ATGC)GTGG(TC)GT(ATGC)ACGG-3′
Pax-B: 5′-CC(AGT)ATCTT(AG)TT(ATGC)CGCAGGAT-3′

The PCR product of expected length was sequenced as described for Hoxd-4.

Cloning of flounder Hoxd-4 genes

The flounder embryo cDNA was terminated with EcoRI linkers, and then ligated to EcoRI-digested λZAP II arms (Stratagene). The ligated cDNA was packaged using Gigapak Gold extracts (Stratagene) and the phage were plated using XL1-Blue MRF’ as the host bacteria strain. DIG-labeled probe was prepared by PCR amplification in which the 152-bp homeobox PCR product was reamplified from one of the TA-cloned plasmids using Boehringer Mannheim digoxigenin (DIG)-labeling mix and Hom-1 and Hom-2 primers. Phage plaques were transferred to Hybond-N+ nylon filters (Amersham) and hybridized to the DIG-labeled probe. Hybridization was performed overnight in 500 mM sodium phosphate (Na-Pi) buffer, pH 7.2, containing 7% SDS and 1 mM EDTA at 65°C. The filters were washed in 40 mM Na-Pi buffer containing 1% SDS three times each for 20 min at 65°C. Adsorbed DIG-labeled probe on the filter was detected using a DIG Luminescent Detection Kit (Boehringer Mannheim) according to the manufacturer’s instructions.

DNA sequence analysis

A single positive plaque in the 2nd screening was picked, suspended in 20 mM Tris-HCl buffer, pH 9.3 containing 2 mM EDTA and 1% Triton X-100, and heated at 95°C for 15 min. The heated sample was subjected to PCR (40 cycles; 95°C for 0.5 min, 55°C for 1 min and 72°C for 2 min, followed by a single cycle of 72°C for 5 min) using M13 forward and reverse primers. The PCR product was about 3,000 bp in length. The product was gel purified and sequenced using an automated DNA sequencer (Applied Biosystems 377A).

Whole-mount in situ hybridization

The template for RNA transcription containing the full length Hoxd-4 (about 3,000 bp) was prepared by further PCR amplification (same conditions as above) using T3 and T7 primers from the already mentioned Hoxd-4 PCR product for sequence analysis. As for Pax-1/9, the PCR product including the paired box which was amplified from the TA (pCRII) clone described above, was used as the template for transcription. Antisense and sense RNA probes were prepared using the Boehringer Mannheim RNA Labeling kit with DIG-UTP. In situ hybridization was performed as described by Jowett and Lettice (1994). Since it was difficult to remove the chorionic membrane from living embryos due to the hardness of the membrane, the embryos before hatching period were fixed and immersed in methanol, and then the ventral half of the membrane was removed using forceps. In all cases, only the antisense RNA probe revealed specific signals.

After coloration, embryos were fixed in 4% paraformaldehyde overnight, dehydrated through graded methanol, and finally soaked in 80% glycerol in Tris-buffered saline containing 0.1% Tween-20.
Cartilage staining
Fixed embryos were stained with alcian-blue as previously described (Suzuki and Kurokawa, 1996). For counter staining after in situ hybridization, cartilage staining was performed after paraformaldehyde fixation.

Retinoic acid treatment
All-trans retinoic acid (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions (10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ M). Aliquots of each stock solution (10 µl) were added to 10 ml of 0.2 µm-filtered seawater containing 100 embryos at the shield stage. Embryos were exposed to retinoic acid for 1 hr. Control embryos were incubated in seawater containing 0.1% DMSO without RA. The embryos were then rinsed in seawater three times, and incubated at 17°C. Once the control embryos had developed to the Prim-5 stage, all embryos were fixed for in situ hybridization as described by Jowett and Lettice (1994).

RESULTS
Isolation and characterization of flounder Hoxd-4 cDNA
PCR using the pair of primers Hom-1 and Hom-2, designed to amplify a 152-bp fragment inside the homeotic domain, yielded a product of expected length from flounder embryo cDNA. The nucleotide sequence of the PCR product was mostly homologous to that of the homeotic domain of the Antennapedia gene of the grasshopper, Schistocerca americana (Hayward et al., 1995). The λZAP II cDNA library prepared from mixed stages of flounder embryos was screened using the PCR product as a probe. Two clones, H1 with a strong signal and H2 with a weak signal, were isolated from 500,000 phages. The partial sequence data suggested that H1 is a homologue of Hoxb-5, and H2 of Hoxd-4. H2 included an insert of approximately 2,600 bp, which was sequenced.

Figure 1 shows the nucleotide and deduced amino acid sequences of the flounder Hox gene obtained from the clone H2. The predicted Hox protein was composed of 235 amino acids. The homeobox sequence and successive downstream pentapeptide (LPNTKGRS) of flounder Hoxd-4 were identical to those of Hoxd-4 of higher vertebrates (Fig. 2). There was a 15 amino acid sequence around the YPWM box identical to those of the other Hoxd-4 gene family members. The N-terminal region is highly conserved among the Hoxd-4 family. Thus, a comparison of the putative flounder protein with those coded for by human, mouse and chick Hox genes shows that it corresponds to the flounder Hoxd-4.

Expression of flounder Hoxd-4 during embryogenesis
An abbreviated schedule of flounder embryogenesis is shown in Table 1. Whole mount in situ hybridization using the antisense cRNA probe of Hoxd-4 revealed no signal at the shield stage, but then gave a signal with a clear anterior border in the central nervous system at the 5-somite stage (Fig. 3A). During zebrafish embryogenesis (Kimmel et al., 1995), the central nervous system exists as a neural keel which is yet to be segmented at this somite stage and segmentation of the central nervous system is completed at the 18-somite stage.

Fig. 1. Nucleotide and predicted amino acid sequence of flounder Hoxd-4 cDNA.
Fig. 2. Alignment of the protein sequences of Hoxd-4, Hoxc-4, Hoxb-4 and Hoxa-4 homologues. Areas of amino acid identity are represented by shaded boxes.
Expression of *Hoxd-4* in Flounder Embryos

The domain of *Hoxd-4* expression at the later developmental stages (Fig. 3 B-E) indicates that the initial hybridization signal comes from a part of the presumptive spinal cord.

At the 14-somite stage, *Hoxd-4* expression in the central nervous system had expanded both anteriorly and posteriorly to include the whole spinal chord (Fig. 3B). *Hoxd-4* expression in the pharyngeal region could be detected at the 21-somite stage in two sets of pharyngeal arches just ventral to the anterior border of its expression in the central nervous system (Fig. 3C), indicating cranial neural crest cells migrating into the pharyngeal arches. *Hoxd-4* expression was also detected in the mesenchyme at paired locations where the pectoral fin buds form, though the latter could not be identified by Nomarski microscopy until the prim-15 stage.

At the prim-5 stage, *Hoxd-4* expression was maintained at the same locations as observed at the 21-somite stage, including the central nervous system, pharyngeal region and pectoral fin buds (Fig. 3D). In zebrafish at the corresponding stage, the first seven rhombomeres are distinguishable in the hindbrain, with rhombomere (r) 5 located just above the otic vesicle, serving as a marker for rhombomeres identities (Holder and Hill, 1991; Kimmel et al., 1995). Correspondingly, seven rhombomeric segments and the otic vesicle could be observed in the flounder embryos (Fig. 4A). Using embryos at this stage, we tried to locate the anterior border of *Hoxd-4* expression in the central nervous system and pharyngeal region. Similar to its mouse homologue (Hunt, 1991a), the anterior border of *Hoxd-4* expression in flounder embryos was found at r6/7 boundary (Fig. 4A). It was difficult to discern the number of pharyngeal arches in the flounder embryo with Nomarski microscopy (Suzuki and Kurokawa, 1996). The endoderm of the pharyngeal arches expresses the *Pax-1* and *Pax-9* genes (Deutsch et al., 1988; Müller et al., 1996). Since a PCR fragment highly homologous to the paired box region of both chick *Pax-1* and *Pax-9* was obtained from the flounder embryo cDNA (Fig. 5), we used the putative *Pax-1/9* RNA probe prepared from the PCR product as a marker to border the pharyngeal arches and reveal the anterior border of *Hoxd-4* expression in the pharyngeal region. Signals were visible as five sets (1-5).

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Table 1. Time course of flounder embryo development

<table>
<thead>
<tr>
<th>stage*</th>
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<tr>
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<td>4-cell</td>
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<td>30</td>
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<tr>
<td>14-somite</td>
<td>33</td>
</tr>
<tr>
<td>21-somite</td>
<td>36</td>
</tr>
<tr>
<td>Prim-5 (hatching period)</td>
<td>60</td>
</tr>
<tr>
<td>Prim-25</td>
<td>80</td>
</tr>
<tr>
<td>High-pec</td>
<td>100</td>
</tr>
<tr>
<td>Protruding-mouth</td>
<td>130</td>
</tr>
</tbody>
</table>

* Stages of zebrafish (Kimmel et al., 1995) were adapted to flounder judged from morphological criteria.

* Embryos were incubated at 17°C.

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![Fig. 3. *Hoxd-4* expression during flounder embryogenesis in whole-mount in situ hybridizations.](https://bioone.org/journals/Zoological-Science)
of endodermal borders (pharyngeal pouches) delineating the pharyngeal arches (Fig. 4C). This means that six pairs of pharyngeal arches, which correspond to mandible, hyoid, and gill arches (g) 1, 2, 3 and 4 were bordered by pharyngeal endoderm. The g 5 was probably not yet segmented by endoderm at this stage. To assess the anterior border of Hoxd-4 expression, double hybridization was performed with Hoxd-4 and Pax-1/9 probes. Hoxd-4 was expressed posteriorly from endoderm border 3, indicating that g 2, 3 and 4 expressed this gene (Fig. 4D). Thus, the anterior border of Hoxd-4 in the pharyngeal region was between g 1 and 2.

At the prim-5 stage, cartilage precursor cells in the pharyngeal arches are prechondroblasts and have formed compact aggregates composed of about 60 cells in each arch which are well segregated from surrounding mesenchymal tissue (Suzuki and Kurokawa, 1996). Hoxd-4 was expressed evenly in the pharyngeal arches, showing no sign of restriction by cell lineage (Fig. 4B).

Cartilage matrix appears in the mandible and hyoid arches at the high-pec stage, then in the gill arches at the protruding-mouth stage (Suzuki and Kurokawa, 1996). Hoxd-4 expression in the central nervous system diminished from the middle to posterior region of the spinal cord after the prim-5 stage (Fig. 3E), and finally disappeared from both hindbrain and spinal cord at the high-pec stage (Fig. 3F). At the high-pec stage, a strong Hoxd-4 hybridization signal appeared only in the pharyngeal region. The signal was restricted to the cartilage precursor cells of g 2, 3, 4 and 5 (Fig. 4E, F). Alcian blue staining after hybridization showed that Hoxd-4 expression was restricted to gill components located posteriorly to cartilage components of the mandible and hyoid arches (Fig. 4F). By the protruding-mouth stage, Hoxd-4 expression in the gill arches had become very weak.

**Effects of RA on Hoxd-4 expression**

Flounder embryos were exposed to RA in the range of \(10^{-8} - 10^{-5}\) M for 1 hr at the early shield stage, then hybridized with Hoxd-4 probe when control embryos treated with RA vehicle (0.1% DMSO) had developed to the prim-5 stage. In control embryos and those treated with \(10^{-8}\) M RA (Fig. 6A, B), the pattern of Hoxd-4 expression did not differ from that of untreated embryos. Above a dose of \(10^{-7}\) M RA, the anterior border of Hoxd-4 expression in the central nervous system and pharyngeal region shifted anteriorly in a dose-dependent manner (Fig. 6B, D, E, G and I). In contrast, Hoxd-4 expression at the pectoral fin bud remained constant, serving as a marker for the degree of anterior shift in the above regions. At

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**Fig. 4.** Hoxd-4 expression in the anterior part of flounder embryos at the prim-5 and high-pec stages. (A-D) Prim-5 stage. A: Nomarski microscopy. Rhombomere segments of hindbrain can be seen. The number of rhombomeres (r) was determined with reference to zebrafish embryos in which r5 locates close to the otic vesicle (o) (Holder and Hill, 1991; Kimmel et al., 1995). Arrowhead indicates the position where the embryo was fractured in B. c, cerebellum; m, mesencephalon; t, telencephalon. B: Fractured surface through paired pharyngeal arches expressing Hoxd-4. C) Embryos were hybridized with Pax-1/9 probe (see Fig. 5) and viewed ventrally. Five sets of endoderm borders (e1-5) appeared in the pharyngeal region, segmenting mandible (m), hyoid (h) and gill arches (g) 1, 2, 3 and 4. (D) Double hybridization with Pax-1/9 and Hoxd-4 probes. Hoxd-4 is expressed posteriorly from e 3, indicating that g 2, 3 and 4 express Hoxd-4. (E, F) High-pec stage. E; Hoxd-4 expression in gill arches. Hoxd-4 is expressed in the cartilage precursor cells in four sets of gill bars (g 2-5). F; Alcian blue staining after hybridization. Mandible (m), hyoid (h) and g1 arches have started deposition of cartilage matrix. Bar: 106 µm in A; 166 µm in B; 80 µm in C and D; 125 µm in E and F.
Expression of *Hoxd-4* in Flounder Embryos

![Image](https://bioone.org/journals/Zoological-Science)

**Introduction**

Hox genes are located in four clusters and the *Dfd* gene family is composed of four members in zebrafish (Mii et al., 1996). The peptide sequence coded by the *Dfd* gene family is highly conserved around the amino terminus, YPWM box, and homeodomain. These regions of each member of the respective gene clusters are almost identical among mammals and birds (Sasaki et al., 1990). We cloned a cDNA coding the homeodomain-containing gene from flounder embryos. Since its peptide sequence was nearly identical to that coded by mammalian and avian *Hoxd-4* genes in the above regions, we concluded that the cDNA codes for flounder *Hoxd-4*. Recently, it was demonstrated that, in the *Fugu* (=Takifugu) rubripes, the region coding for *Hoxd-1*, *Hoxd-3*, *Hoxd-4* and *Hoxd-8* has been lost from the *Hoxd* cluster. So, it is hypothesized that teleost fish developed four *Dfd* members after duplications of the *Hox* clusters as is the case in tetrapods and that a large part of *Hoxd* cluster including *Hoxd-4* was deleted in some groups of teleost fish.

In mouse embryos, *Hoxd-4* is expressed in the central nervous system, pharyngeal region, limb bud and vertebral column (Featherstone et al., 1988). During flounder embryogenesis, expression of *Hoxd-4* was detected in the central nervous system, pharyngeal region, and pectoral fin bud. Considering that the pectoral fin is homologous to the fore-limb (Sordino et al., 1995), the expression domain of *Hoxd-4* in flounder embryos corresponds well with that of mouse embryos, except in the vertebral skeletal components. The high degree of conservation of the peptide sequence and expression domain in embryos, between fish and mammals, suggest that *Hoxd-4* has common functions in regulating morphogenesis of these organs. During vertebral development in mouse, *Hoxd-4* functions in the specification of the region identity of the cervical vertebrae (Horan et al., 1995a, b). With the *in situ* hybridization-
Fig. 6. Effects of RA on the Hoxd-4 expression in the flounder embryo. Embryos were incubated with $10^{-7} - 10^{-5}$ M RA at the shield stage and allowed to develop until control embryos treated with 0.1% DMSO grew to the prim-5 stage. A, C, F and H are ventral views under a phase-contrast microscope. B, D, E, G and I are lateral views. E is dorsal view. o; otic vesicle. (A, B) Control embryos treated with 0.1% DMSO. Expression pattern of Hoxd-4 coincides with that of non-treated embryos shown in Figs. 3D and 4A. The arrow in B marks the center of otic vesicle. (C, D, E) Embryos treated with $10^{-7}$ M RA. In E, control embryo (upper) was set with RA-treated embryo (lower). In RA-treated embryo, the hybridization signal newly appeared at the position of r5 (arrowhead), skipping r6. The arrow in D indicates the center of otic vesicle. (F, G) Embryos treated with $10^{-6}$ M RA. Hoxd-4 expression can be detected from the region dorsal to the eye where the dorsal mesencephalon normally develops (arrowhead). The expression in the pharyngeal region diminished, and expanded anteriorly to the posterior end of the eye (arrow). (H, I) Embryos treated with $10^{-5}$ M RA. Eye and segmentation of brain can not be seen. Hoxd-4 is continuously expressed to the rostral part of the central nervous system. New Hoxd-4 expression laterally appeared in the cranial region (arrowheads) as well as the pharyngeal region (arrow). Note that Hoxd-4 expression in pectoral fin bud (·) remained at the same location in the body in B, D, E, G and I. Bar: 110 µm in A-C, and F-I; 167 µm in D; 25 µm in E.

Hoxd-4 expression during vertebral development of flounder, it is necessary to examine if Hoxd-4 is expressed temporally at sclerotome induction, or later at vertebral skeletal formation.

Seven pharyngeal arches exist in the teleost fish, in contrast to the degeneration of pharyngeal arches 5, 6 and 7 in mammals. Hoxd-4 expression in flounder embryos showed a clear anterior border both in the hindbrain and pharyngeal region. The border existed between r 6 and 7 in the hindbrain, and between g 1 and 2 (which corresponds with mouse pha-
Expression of Hoxd-4 in Flounder Embryos

In gill arches and cell growth is inactivated (Suzuki and protruding-mouth stage when cartilage matrix production starts 5 to high-pec stages), cell growth is greater in cartilage precursor cells than in surrounding connective tissue cells and

In whole cranial neural crest cells, which arrived at the gill arches (g 3-5) are likely to be identified by Hoxa-1 expression, given the phenotype of mutant mice in which Hox genes were target-disrupted (Chisaka and Capecchi, 1991; Condie and Capecchi, 1994; Gendron-Maguire et al., 1993; Horan et al., 1995a, b; McGinnis and Krumlauf, 1992; Lufkin et al., 1991; Rijli et al., 1993). Taking this into account, we speculate that Hoxd-4 functions in the discrimination between gill arches 1 and 2 of flounder embryos. Recently, by analyzing the phenotype of zebrafish larva in which Hoxa-1 was overexpressed, it has been demonstrated that Hoxa-1 functions in identification of mandible and hyoid cartilages (Alexandre et al., 1996). The posterior gill arches (g 3-5) are likely to be identified by Hox genes, such as Sex combs reduced (Scr) and Antennapedia (Antp), located in the 5' region following the Dfd family members. Since this relationship is unclear, we intend to analyze expression pattern of flounder Hoxb-5 (Scr class) obtained here.

In flounder embryos, it was found that Hoxd-4 is expressed in whole cranial neural crest cells, which arrived at the gill arches, and then became restricted to the cartilage precursor cells up to the high-pec stage. At this stage, as Hoxd-4 expression ceases at the central nervous system and pectoral fin, the cartilage precursor cells of g 2-5 are the only cells maintaining the Hoxd-4 expression. Since hyoid and thyroid cartilages, which develop from pharyngeal arch 4, are smaller in double mutant mice of hoxa-3 and hoxd-3, Hox genes have been suggested to function in the growth regulation of the cartilage precursor cells as well as in the specification of cell identity (Condie and Capecchi, 1994). During the stage when Hoxd-4 expression is restricted in flounder embryos (the prim-5 to high-pec stages), cell growth is greater in cartilage precursor cells than in surrounding connective tissue cells and central nervous system (Suzuki and Kurokawa, 1996). At the protruding-mouth stage when cartilage matrix production starts in gill arches and cell growth is inactivated (Suzuki and Kurokawa, 1996), Hoxd-4 expression in the gill arches ended. Thus, Hoxd-4 expression is likely to be maintained in the cartilage precursor cells during the proliferation stage. The restriction of Hoxd-4 expression to cartilage precursor cells may be related to the function of Hox genes in cell growth regulation.

Positional shift of the otic vesicle occurred at a dose of 10^{-7} M RA administered by pulse-treatment. Also, otic vesicle, eye and brain development was seriously affected at doses of 10^{-6} to 10^{-5} M RA. Thus, RA exerts teratogenic effects on the central nervous system of flounder embryos, as reported for zebrafish (Herrmann, 1995; Holder and Hill, 1991; Zhang et al., 1996) and Xenopus (Durston et al., 1989). In mouse, it has been demonstrated that the anterior expression border of Hox genes in the central nervous system is shifted anteriorly by RA, and that the teratogenic effects of RA on the brain is mediated by re-patterning of Hox gene expression (Marshall et al., 1992; Morriss-Kay et al., 1991; Simeone et al., 1995).

In the human EC cell line, NT2/D1, Hoxd-4 gene expression is induced by exposure to RA above 5×10^{-8} M (Simeone et al., 1990). Using flounder embryos, this study showed that the anterior border of Hoxd-4 expression in the central nervous system shifts anteriorly at the concentration of RA reported to induce HOXD-4 expression in the EC cells, and that the degree of anterior shift depends on the dose of RA.

In the flounder embryos, different regional susceptibility to the effects of RA was noticeable; e.g. the region corresponding to r5 newly expressed Hoxd-4 at 10^{-7} M RA, skipping the r6 region. This difference in regional sensitivity to RA may relate to the distribution of cellular retinoic acid binding protein I (CRABP I), which binds RA with high specificity (Stoner and Gudas, 1989). Hypothesizing that CRABP I exists at particularly high levels in r6 of flounder embryos as observed in mouse embryos (Dencker et al., 1990; Lee et al., 1995), it is possible that RA at this concentration is largely absorbed by CRABP I. To discuss this anterior shift pattern of Hoxd-4 precisely, it is necessary to analyze the expression domain of CRABP I and krox-20, which marks r3 and r5 (Oxtoby and Jowett, 1993), in RA-treated flounder embryos. At 10^{-5} M RA, the anterior border of Hoxd-4 was shifted up to the rostral part of the central nervous system where the forebrain and midbrain form. The morphological features of flounder embryos given this dose of RA are reminiscent of those seen in Xenopus and mice, termed as phenotype B embryos, e.g. segmentation of brain and absence of eyes (Durston et al., 1989). In this type of mouse embryo, the identity of the forebrain region is lost together with the normal pattern of Ems1, Emsx and Dlx1 gene expression (Simeone et al., 1995). Hoxb-1 expression, the normal border of which is between r3 and 4, occurred up to the midbrain region (Morriss-Kay et al., 1991; Simeone et al., 1995). From the present results, it is speculated that the capability of Hox gene expression exists up to the rostral part of the central nervous system in teleost fish. It is also speculated that at a high dose of RA, the region fated to form forebrain and midbrain acquires the identity of posterior hindbrain and spinal cord due to an anterior shift of the Hoxd-4 expression domain, causing loss of otic vesicle and eye.

The domain of Hoxd-4 expression at the pharyngeal region of flounder embryos was also demonstrated to be expanded anteriorly by RA. At the pharyngeal region of embryos treated with 10^{-7} M RA, the anterior border of Hoxd-4 expression shifted below r5 in which new Hoxd-4 expression was induced. r5 gives rise to cranial neural crest cells which enter into hyoid arches in zebrafish (Schilling and Kimmel, 1994). So, it is possible that Hoxd-4 expression, induced in rhombomeres by exogenous RA, is transferred into the pharyngeal arches via cranial neural crest cells. At a higher concentration (10^{-5} M) of RA, Hoxd-4 expression was detected from a more anterior position where eyes normally develop, accompanied by anterior expansion of Hoxd-4 expression in the central nervous system. This may indicate that neural crest cells, destined to form maxilla, are induced to express Hoxd-
4 at this high RA concentration.

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