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

Authors: Suzuki, Tohru, Oohara, Ichiro, and Kurokawa, Tadahide

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

Tohru Suzuki*, Ichiro Oohara and Tadahide Kurokawa

Metabolism Section, National Research Institute of Aquaculture, Nansei, Mie 516-01, Japan

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 hyoid 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; Ignelzi *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

* Corresponding author: Tel.   +81-5996-6-1830;
                                          FAX. +81-5996-6-1962.

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 (Morriss-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)GGGTCG(AGCT)CA(AG)AC(AGCT)TA(CT)A-3'
Hom-2: 5'-C(TG)(AGCT)C(TG)(GA)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)GTGTT(TC)GT(ATGC)AACGG-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 *Eco*RI linkers, and then ligated to *Eco*RI-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 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

Fig. 1. Nucleotide and predicted amino acid sequence of flounder *Hoxd-4* cDNA.

```
flounder Hoxd-4  MAMSSYMVNSKYVDPKFPPCEEY---------------------------SQNNY--IP  30
chick Hoxd-4     MAMSSYMVNS-YVDPKFPPCEEY---------------------------LQSSY--LG  29
human HOXD4      MVMSSYMVNSKYVDPKFPPCEEY---------------------------LQGGY--LG  30
mouse Hoxd-4     MAMSSYMVNSKYVDPKFPPCEEY---------------------------LQGGY--LG  30
mouse Hoxc-4     -------------------------------------------------MIMSSYLMDSN  11
chick Hoxb-4     MAMSSFLINSNYVDPKFPPCEEY--------------------------SHSDYL-P-  30
human HOXB4      MAMSSFLINSNYVDPKFPPCEEY--------------------------SQSDYL-PS  31
chick Hoxa-4     MTMSSFLINSNYIEPKFPPCEEYTQHSGSAGSSASYHPHHPHPHAPPPPPPPPPHLHAA  60
mouse Hoxa-4     MTMSSFLINSNTIEPKFPPFEEFAPHGGPGGGDGAVGGGPGYPR-PQSAPHLPAPNPHAA 59


flounder Hoxd-4  EQG---SDYFS-PSQDTDFQHPG-IYPRPNYTEQ---PF--SCTTVQDSTVQPRGHVQE-  79
chick Hoxd-4     EQG---AEYYG-ASQGSDFQHQG-LYPRSNYSEQ---PF-WLAG-AQGSAVQPRGHGQEQ  79
human HOXD4      EQG---ADYYGGGAQGADFQPPG-LYPRPDFGEQ---PF-GGSGPGPGSALPARGHGQEP  82
mouse Hoxd-4     EQG---ADYYGSGAQGADFQPSG-LYPRPDFGEQ---PF-GGGGPGPGSALPARGHGQEP  82
mouse Hoxc-4     YIDPKFPPCEEYSQNSYIPE--HSPEYYGRTRESG-FQHHHQELYPPPPPRPSYPERQYS  68
chick Hoxb-4     NHSP--EYY-SSQRRESTFQHEAMYQPRSACSEQ----LYPSCQSSG-----HQAAVLSP  78
human HOXB4      DHSP--GYYAGGQRRESSFQPEAGFGRRAACTVQ---RYAACRDPGPPPPPPPPPPPPP  86
chick Hoxa-4     HPGPALPEYFPRPRREPGYQAPAAPPGPPGPPPEALYPAQAPSYPQAPYSYSSAGSAAPG 120
mouse Hoxa-4     RQ-P--PAYYAPRAREPSY------PG--G-----LYP--APA-AACPYACRGASPGRPE 100


flounder Hoxd-4  -RS-GHASPFDAQ-TEQGAPVPVAGP--RTCGQQQNTK--SQNG-IQA------------ 120
chick Hoxd-4     SAPPSHFPGQAEHC---P-PPPCPIPSC-GQ-QPAL-K--APHG-SAV------------ 118
human HOXD4      GGPGGHYAAPGEPCPAPPAPPPAPLPGARAYSQ-SDPKQ-PPPG-TAL------------ 128
mouse Hoxd-4     SGPGSHYGAPGEPC---PAPPPAPLPGARACSQPTGPKQ-PPPG-TAL------------ 126
mouse Hoxc-4     CTSLQGPGNSRAHGPAQAG-HHHPEKSQPLCEPAPLSGTSASPSPAPPACSQPAPDHPSS 127
chick Hoxb-4     RG-HVH-P-PAGLQSHLSEPNHPCEP-GTPSPPPSCSQNSLNQSPSNSSC---------- 124
human HOXB4      PGLSPRAPAPPPAGALLPEPGQRCEAVSSSPPPPPCAQNPLHPSPSHSAC---------- 136
chick Hoxa-4     PEQPPPGASPPPPPPAKGHPGPAQPLLPGHALQRRCEAAPAAGAGTGPGCALLPDKSLPG 179
mouse Hoxa-4     -QSPAPGA-HPSPAP---QP-PV-P-L-GHCAPGPTTPAVATGGSAPACPLLLADQGPAG 150


flounder Hoxd-4  ---KQPAVVYPWMKKVHVTTVNPDYTGPEPKRSRTAYTRQQVLELEKEFHFNRYTTRRRR 176
chick Hoxd-4     ---KQPAVVYPWMKKVHVNSVNPNYSGGEPKRSRTAYTRQQVLELEKEFHFNRYLTRRRR 174
human HOXD4      ---KQPAVVYPWMKKVHVNSVNPNYTGGEPKRSRTAYTRQQVLELEKEFHFNRYLTRRRR 184
mouse Hoxd-4     ---KQPAVVYPWMKKVHVNSANPNYTGGEPKRSRTAYTRQQVLELEKEFHFNRYLTRRRR 182
mouse Hoxc-4     AASKQPI-VYPWMKKIHVSTVNPNYNGGEPKRSRTAYTRQQVLELEKEFHYNRYLTRRRR 186
chick Hoxb-4     ---KEP-VVYPWMKKVHVSTVNPNYSGGEPKRSRTAYTRQQVLELEKEFHYNRYLTRRRR 180
human HOXB4      ---KEP-VVYPWMRKVHVSTVNPNYAGGEPKRSRTAYTRQQVLELEKEFHYNRYLTRRRR 192
chick Hoxa-4     LKGKEP-VVYPWMKKIHVSTVNPNYSGGEPKRSRTAYTRQQVLELEKEFHFNRYLTRRRR 239
mouse Hoxa-4     PKGKEP-VVYPWMKKIHVSAVNSSYNGGEPKRSRTAYTRQQVLELEKEFHFNRYLTRRRR 210


flounder Hoxd-4  IEIAHTLCLSERQIKIWFQNRRMKWKKDHKLPNTKGRS----APAS--S------HLQSI 224
chick Hoxd-4     IEIAHTLCLSERQIKIWFQNRRMKWKKDHKLPNTKGRS----SSSS-ASN--P--HLQTV 224
human HOXD4      IEIAHTLCLSERQIKIWFQNRRMKWKKDHKLPNTKGRSSSSSSSSSSCSSSVAPSQHLQPM 244
mouse Hoxd-4     IEIAHTLCLSERQIKIWFQNRRMKWKKDHKLPNTKGRS--SSSSSSCSSSAAPGQHLQPM 239
mouse Hoxc-4     IEIAHSLCLSERQIKIWFQNRRMKWKKDHRLPNTKVRSAPPAGAAPSTLSAATPGTSEDH 246
chick Hoxb-4     VEIAHSLCLSERQIKIWFQNRRMKWKKDHKLPNTKIRS--NPSSSSASLQIPPAA-SQSR 237
human HOXB4      VEIAHALCLSERQIKIWFQNRRMKWKKDHKLPNTKIRS-GGAAGSAG--G-PP---GRP- 244
chick Hoxa-4     IEIAHTLCLSERQVKIWFQNRRMKWKKDHKLPNTKMRSSNQPSL-GQQQAKAQTQG---H 295
mouse Hoxa-4     IEIAHTLCLSERQVKIWFQNRRMKWKKDHKLPNTKMRSSNTASAPAGPPGKAQTHSPHPH 270


flounder Hoxd-4  QKDNQTDITAL            235
chick Hoxd-4     PKDHQTDLTTL            235
human HOXD4      AKDHHTDLTTL            255
mouse Hoxd-4     AKDHHTDLTTL            250
mouse Hoxc-4     SQSATPPEQQRAEDITRL     264
chick Hoxb-4     SSGPASSL               245
human HOXB4      NGGP-RAL               251
chick Hoxa-4     PRPLDGA-APNAAAL        309
mouse Hoxa-4     PHPLPGASTPIPSSI        285
```

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.

Table 1. Time course of flounder embryo development

| stage[a]	h[b]
1-cell	0
4-cell	4
Dome	9
Shield	22
5-somite	30
14-somite	33
21-somite	36
Prim-5 (hatching period)	60
Prim-25	80
High-pec	100
Protruding-mouth	130

[a] Stages of zebrafish (Kimmel *et al.*, 1995) were adapted to flounder judged from morphological criteria.
[b] Embryos were incubated at 17°C.

stage. 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)
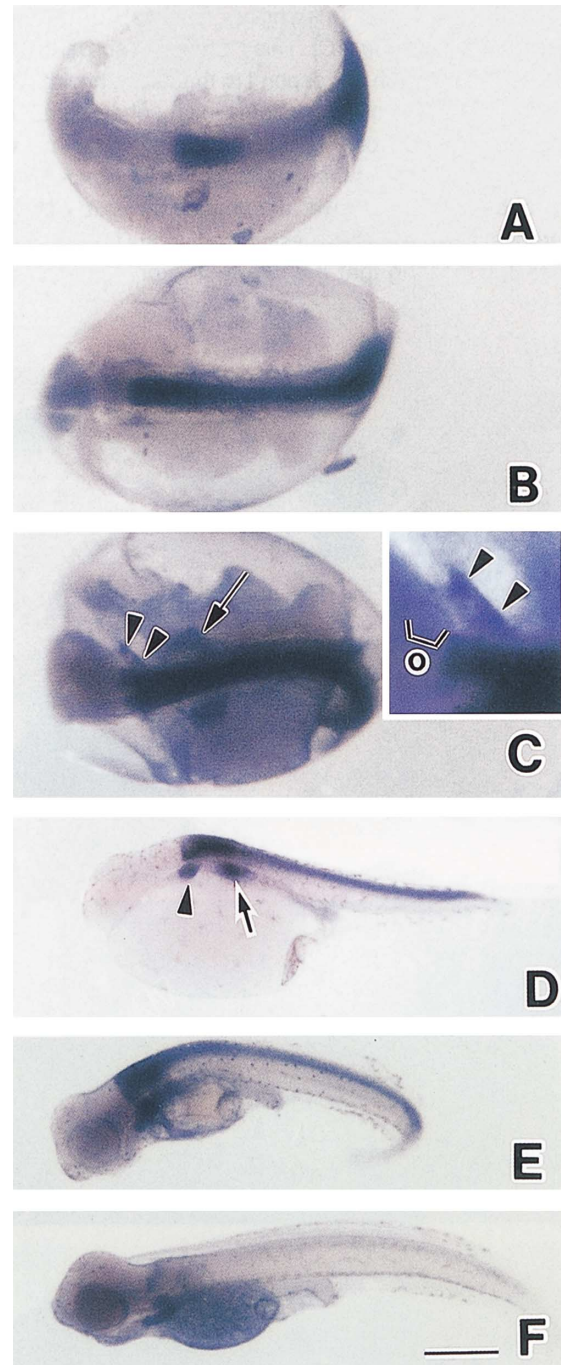
Fig. 3. *Hoxd-4* expression during flounder embryogenesis in whole-mount *in situ* hybridizations. (**A**) 5-somite stage. *Hoxd-4* expression can be detected in part of the central nervous system. The dark color at the tail region is emphasized background due to the curled tail. (**B**) 14-somite stage. *Hoxd-4* is sequentially expressed in the central nervous system from the hindbrain to the posterior end of the spinal cord. (**C**) 21-somite stage. In addition to the central nervous system, two pharyngeal arches (arrowheads) and pectoral fin buds (arrow) express *Hoxd-4* on the lateral sides of the body. Insert is the higher magnification of the pharyngeal region. o; otic vesicle. (**D**) Prim-5 stage. The hybridization signal can be detected in the central nervous system, pharyngeal region (arrowhead) and pectoral fin bud (arrow), as is the case at the 21-somite stage. (**E**) Prim-25 stage. Expression in the spinal cord diminishes in the middle to posterior parts. (**F**) High-pec stage. Expression remains in the pharyngeal region. Bar: 180 µm in **A-C** (80 µm in insert of **C**); 500 µm in **D**, **E** and **F**.

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
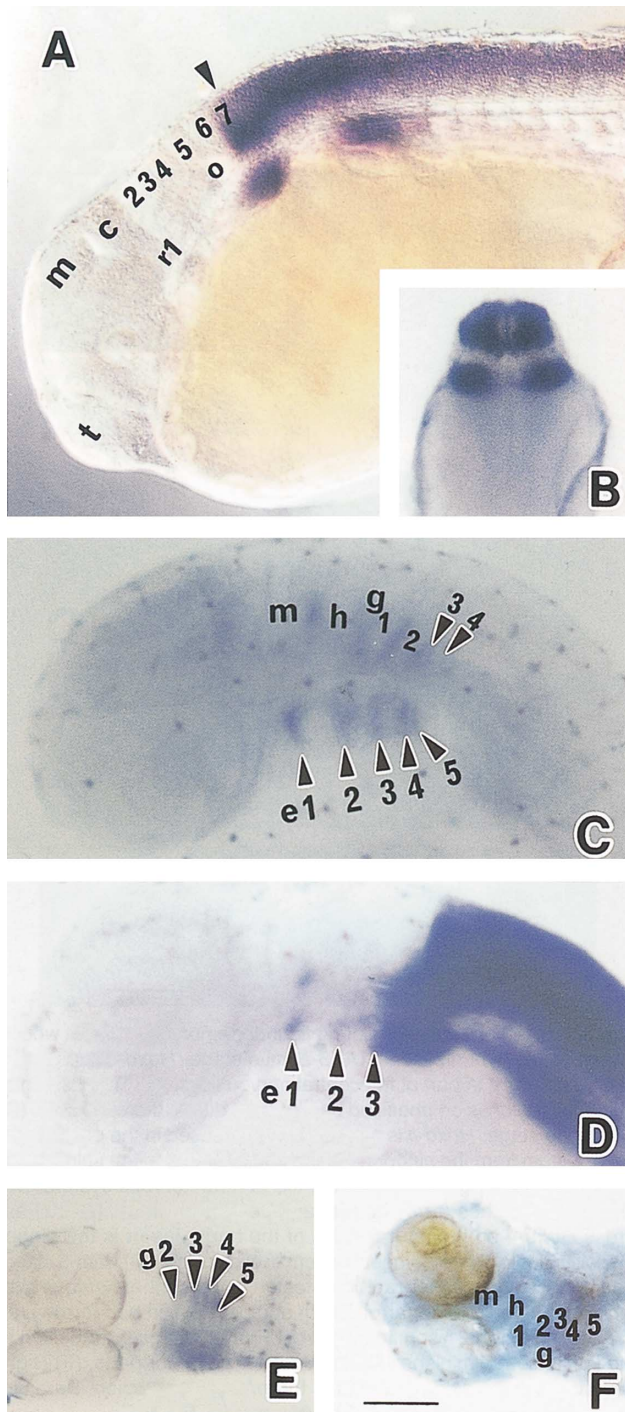
Fig. 4. *Hoxd-4* expression in the anterior part of flounder embryos at the prim-5 and high-pec stages. (**A-D**) Prim-5 stage. **A**; Nomarski microscopy. Rhombomere segments of hindbrain can be seen. The number of rhombomeres (r) was determined with reference to zebrafish embryos in which r5 locates 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**.

```
flounder PCR   CAGGCCGCTCCCCAACGCCATCCGGCTCCGGATCGTGGAGCTGGCCCAGCTCGGGATTCG   60
chick Pax-1    GCGTCCCCTGCCCAACGCCATCCGCCTGCGGATCGTGGAGCTGGCCCAGCTCGGCATTCG   188
cbick Pax-9    GAGGCCCCTGCCCAACGCCATCCGGCTCCGGATTGTGGAACTGGCACAGCTGGGGATCAG   107

flounder PCR   ACCCTGTGACATTAGCCGGCAGCTGCGCGTCTCCCACGGCTGTGTCAGCAAGATCCTGGC   120
chick Pax-1    CCCGTGCGACATCAGCCGCCAGCTGCGGGTCTCGCACGGCTGCGTCAGCAAGATCCTGGC   248
cbick Pax-9    GCCCTGCGACATCAGCCGCCAGCTCCGCGTTTCCCACGGCTGTGTGAGCAAAATCCTGGC   167

flounder PCR   CCGCTACAACGAGACCGGCTCCATCCTCCCGGGGGCCATCGGGGGCAGCAAGCCACGGGT   180
chick Pax-1    CCGCTACAACGAGACCGGCTCCATCCTGCCCGGAGCCATCGGGAGGCAGCAAGCCGCGGGT   308
chick Pax-9    TCGCTACAACGAGACCGGCTCCATCCTACCGGGGGGCTATCGGAGGCAGCAAGCCGCGGGT   227

flounder PCR   CACCACACCCACCGTGGTCAAGCACATACGGACATACAAGCAGAGGGACCCGGGGATTTT   240
chick Pax-1    CACCACCCCCAACGTGGTCAAGCACATCCGCGACTACAAGCAGGGAGACCCCGGCATCTT   368
chick Pax-9    CACCACCCCCACGGTGGTTAAACATATCCGGACCTACAAACAAAGGGATCCGGGCATCTT   287

flounder PCR   TGCCTGGGAGATCCGGGACAGGCTACTCGCCGACGGGGTTTGCGATAAGTTCAACCTCCC   300
chick Pax-1    CGCCTGGGAGATCCGCGACCGTCTGCTGGCCGACGGTGTGTCTGTGACAAGTACAACGTGCC   428
chick Pax-9    CGCCTGGGAGATCCGGGATCGCCTGCTGGCCGACGGCGTGTGCGACAAGTACAACGTGCC   347

flounder PCR   CTCCGTCAGCTCCATCAGTCGG   322
chick Pax-1    CTCGGTCAGCTCCATCAGCAGG   450
chick Pax-9    GTCGGTCAGCTCTATCAGCCGC   369
```

Fig. 5. Nucleotide sequence of the PCR product obtained from flounder embryo, corresponding to a 322 bp fragment inside the paired-box domain of putative flounder *Pax-1* or *Pax-9*. The homology with chick *Pax-1* and *Pax-9* is shown.

a dose of 10^{-7} M RA, the distance between the rostral end and otic vesicle was shortened as compared to that in control embryos (Fig. 6C). With regard to *Hoxd-4* expression, it was found that a part of the hindbrain, judged to be r5 because of the lateral presence of otic vesicles, newly expressed the gene, skipping the r6 region (Fig. 6D, E). In the pharyngeal region, the *Hoxd-4* expression domain shifted anteriorly below the part of hindbrain where new *Hoxd-4* expression was induced. In the embryos treated with 10^{-6} M RA, the development of the eye was severely affected, the otic vesicle was absent, and the development of the pharyngeal region was poor (Fig. 6F). *Hoxd-4* expression in the hindbrain further expanded anteriorly from the position of r5. Noticeably, *Hoxd-4* expression newly appeared where the dorsal mesencephalon normally develops (compare Figs. 4A and 6F, G). Its expression in the pharyngeal region diminished, but expanded to the posterior end of the eye where the mandible arch normally develops. Treatment with 10^{-5} M RA disturbed brain segmentation and eye formation (Fig. 6H). *Hoxd-4* expression in the central nervous system expanded anteriorly approaching the rostral end of the central nervous system (Fig. 6I). The gene expression became diffusely expanded anteriorly at the pharyngeal region and newly appeared at the rostral part of the cranial region.

DISCUSSION

The *Hox* genes are located in four clusters and the *Dfd* gene family is composed of four members in zebrafish (Misof *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 prevertebrae (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* hybridiza-
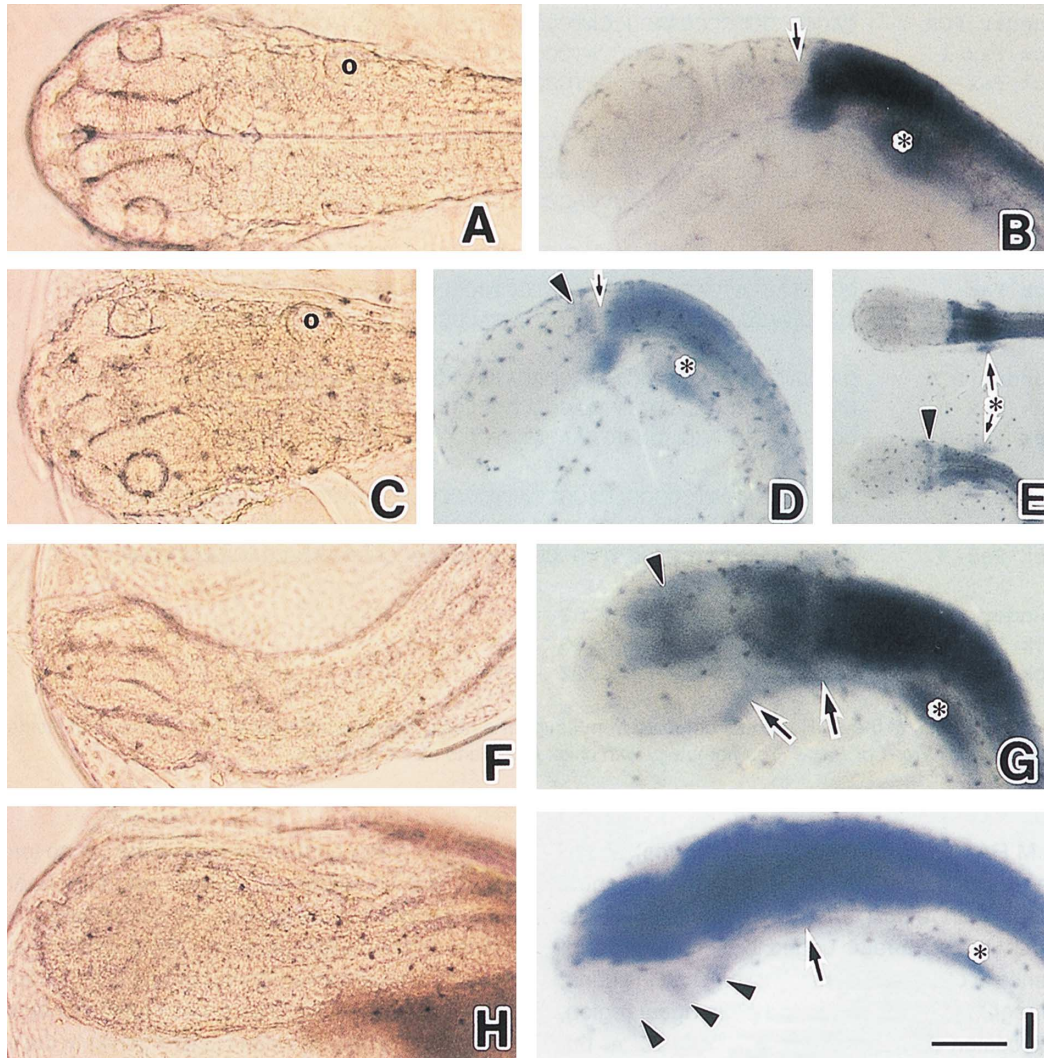
Fig. 6. Effects of RA on the *Hoxd-4* expression in the flounder embryo. Embryos were incubated with 10^{-7}–10^{-5} M RA at the shield stage and allowed to develop until control embryos treated with 0.1% DMSO grew to the prim-5 stage. **A**, **C**, **F** and **H** are ventral views under a phase-contrast microscope. **B**, **D**, **E**, **G** and **I** show *Hoxd-4 in situ* hybridization. **B**, **D**, **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**.

tion using a cDNA fragment corresponding to the *Pax* domain of putative flounder *Pax-1* or *Pax-9*, signal was detected from the ventral side of the trunk (data not shown) as well as pharyngeal endoderm at the prim-5 stage, indicating the presence of sclerotome at this stage. The *Hoxd-4* hybridization signal could not be detected from the ventral region, including sclerotome. The vertebral skeletons of flounder appear at about 20 days after fertilization, long after pharyngeal cartilage formation (data not shown), unlike the simultaneous development of two skeletal systems in mouse. So, regarding

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-

ryngeal arch 3 and 4, respectively), in the pharyngeal region. This result ascertains that the expression domain of *Hoxd-4* at central nervous system and the pharyngeal region is conserved between fish and mammals (Featherstone *et al.*, 1988; Mavilio *et al.*, 1986; Hunt *et al.*, 1991a, b). *Hox* genes are thought to function in specification of regional identity at the anterior border of their 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 *Emx1*, *Emx* and *Dlxl* 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.

REFERENCES

Alexandre D, Clarke JDW, Oxtoby E, Yan Y-L, Jowett T, Holder N (1996) Ectopic expression of *Hoxa-1* in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. Development 122: 735–746

Aparicio S, Hawker K, Cottage A, Mikawa Y, Zuo L, Venkatesh B, Chen E, Krumlauf R, Brenner S (1997) Organization of the *Fugu rubripes Hox* clusters: evidence for continuing evolution of vertebrate complexes. Nat Genet 16: 79–83

Chisaka O, Capecchi MR (1991) Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene *Hox-1.5*. Nature 350: 473–479

Condie BG, Capecchi MR (1994) Mice with targeted disruptions in the paralogous genes *hoxa-3* and *hoxd-3* reveal synergistic interactions. Nature 370: 304–307

Dencker L, Annerwall E, Busch C, Eriksson U (1990) Localization of specific retinoid-binding sites and expression of cellular retinoic acid-binding protein (CRABP) in the early mouse embryo. Development 110: 343–352

Deutsch U, Dressler GR, Gruss P (1988) *Pax 1*, a member of a paired box homologous murine gene family, is expressed in segmented structures during development. Cell 53: 617–625

Durston AJ, Timmermans JPM, Hage WJ, Hendriks HFJ, de Vries NJ, Heideveld M, Nieuwkoop PD (1989) Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 304: 140–144

Featherstone MS, Baron A, Gaunt SJ, Mattei M-G, Duboule D (1988) *Hox-5.1* defines a homeobox-containing gene locus on mouse chromosome 2. Proc Natl Acad Sci USA 85: 4760–4764

Gendron-Maguire M, Mallo M, Zhang M, Gridley T (1993) *Hoxa-2* mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. Cell 75: 1317–1331

Hall BK (1987) Tissue interactions in the development and evolution. In "Development and Evolutionary of the Aspects of the Neural Crest" Ed by R Fleischmajer, RE Billingham, Williams & Wilkins, Baltimore, pp 1–30

Hayward DC, Patel NH, Rehm EJ, Goodman CS, Ball EE (1995) Sequence and expression of grasshopper antennapedia: comparison to *Drosophila*. Develop Biol 172: 452–465

Herrmann K (1995) Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (*Brachydanio rerio*) as assessed by a novel scoring system. Toxic in Vitro 9: 267–283

Holder N, Hill J (1991) Retinoic acid modifies development of the midbrain-hindbrain border and affects cranial ganglion formation in zebrafish embryos. Development 113: 1159–1170

Horan GSB, Ramirez-Solis R, Featherstone MS, Wolgemuth DJ, Bradley A, Behringer RR (1995a) Compound mutants for the paralogous *hoxa-4*, *hoxb-4*, and *hoxd-4* genes show more complete homeotic transformations and a dose-dependent increase in the number of vertebrae transformed. Genes Develop 9: 1667–1677

Horan GSB, Nagy Kovàcs E, Behringer RR, Featherstone MS (1995b) Mutations in paralogous *Hox* genes result in overlapping homeotic transformations of the axial skeleton: evidence for unique and redundant function. Develop Biol 169: 359–372

Hunt P, Gulisano M, Cook M, Sham M-H, Faiella A, Wilkinson D, Boncinelli E, Krumlauf R (1991a) A distinct *Hox* code for the branchial region of the vertebrate head. Nature 353: 861–864

Hunt P, Wilkinson D, Krumlauf R (1991b) Pattering the vertebrate head: murine Hox 2 genes mark distinct subpopulations of premigratory and migrating cranial neural crest. Development 112: 43–50

Hunt P, Whiting J, Nonchev S, Sham M-H, Marshall H, Graham A, Cook M, Allemann R, Rigby PWJ, Gulisano M, Faiella A, Boncinelli E, Krumlauf R (1991c) The branchial *Hox* code and its implications for gene regulation, patterning of the nervous system and head evolution. Development 1991 Supplement 2: 63–77

Ignelzi Jr MA, Liu Y-H, Maxson Jr RE, Snead ML (1995) Genetically engineered mice: tools to understand craniofacial development. Crit Rev Oral Biol Med 6: 181–201

Jowett T, Lettice L (1994) Whole mount *in situ* hybridizations on zebrafish embryos using a mixture of digoxigenin and fluorescein labelled probes. Trends Genet 10: 73

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Develop Dyn 203: 253–310

Köntges G, Lumsden A (1996) Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122: 3229–3242

Krumlauf R (1993) *Hox* genes and pattern formation in the branchial region of the vertebrate head. Trends Genet 9: 106–112

Langston AW, Gudas LJ (1992) Identification of a retinoic acid responsive enhancer 3' of the murine homeobox gene *Hox-1.6*. Mech Develop 38: 217–228

Lee YM, Osumi-Yamashita N, Ninomiya Y, Moon CK, Eriksson U, Eto K (1995) Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells. Development 121: 825–837

Lufkin T, Dierich A, LeMeur M, Mark M, Chambon P (1991) Disruption of the *Hox1.6* homeobox gene results in defects in a region corresponding to its rostral domain of expression. Cell 66: 1105–1119

Lumsden A, Sprawson N, Graham A (1991) Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. Development 113: 1281–1291

Manak JR, Scott MP (1994) A class act: conservation of homeodomain protein functions. Development 1994 Supplement: 61–71

Marshall H, Nonchev S, Sham M-H, Muchamore I, Lumsden A, Krumlauf R (1992) Retinoic acid alters hindbrain *Hox* code and induces transformation of rhombomeres 2/3 into a 4/5 identity. Nature 360: 737–741

Mavilio F, Simeone A, Giampaolo A, Faiella A, Zappavigna V, Acampora D, Poiana G, Russo G, Peschle C, Boncinelli E (1986) Differential and stage-related expression in embryonic tissues of a new human homeobox gene. Nature 324: 664–668

McGinnis W, Krumlauf R (1992) Homeobox genes and axial patterning. Cell 68: 283–302

Misof BY, Blanco MJ, Wagner GP (1996) PCR-survey of Hox-genes of the zebrafish: new sequence information and evolutionary implications. J Exp Zool 274: 193–206

Morrison A, Moroni MC, Ariza-McNaughton L, Krumlauf R, Mavilio F (1996) *In vitro* transgenic analysis of a human *HOXD4* retinoid-responsive enhancer. Development 122: 1895–1907

Morriss-Kay GM, Murphy P, Hill RE, Davidson DR (1991) Effects of retinoic acid excess on expression of *Hox-2.9* and *Krox-20* and on morphological segmentation in the hindbrain of mouse embryos. EMBO J 10: 2985–2995

Müller TS, Ebensperger C, Neubüser A, Koseki H, Balling R, Christ B, Wilting J (1996) Expression of avian *Pax1* and *Pax9* is intrinsically regulated in the pharyngeal endoderm, but depends on environmental influences in the paraxial mesoderm. Develop Biol

178: 403–417

Ogura T, Evans RM (1995) A retinoic acid-triggered cascade of *HOXB1* gene activation. Proc Natl Acad Sci USA 92: 387–391

Osumi-Yamashita N, Ninomiya N, Doi Y, Eto K (1994) The contribution of both forebrain and midbrain crest cells to the mesenchyme in the frontonasal mass of mouse embryos. Develop Biol 164: 409–419

Oxtoby E, Jowett T (1993) Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. Nucleic Acids Res 21: 1087–1095

Rijli FM, Mark M, Lakkaraju S, Dierich A, Dollé P, Chambon P (1993) A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. Cell 75: 1333–1349

Sadaghiani B, Vielkind JR (1990) Distribution and migration pathways of HNK-1-immunoreactive neural crest cells in teleost fish embryos. Development 110: 197–209

Sasaki H, Yokoyama E, Kuroiwa A (1990) Specific DNA binding of two chicken *Deformed* family homeodomain proteins, *Chox-1.4* and *Chox-a*. Nucleic Acids Res 18: 1739–1747

Schilling TF, Kimmel CB (1994) Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. Development 120: 483–494

Simeone A, Acampora D, Arcioni L, Andrews PW, Boncinelli E, Mavilio F (1990) Sequential activation of *HOX 2* homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature 346: 763–766

Simeone A, Acampora D, Nigro V, Faiella A, D'Esposito M, Stornainolo A, Mavilio F, Boncinelli E (1991) Differential regulation by retinoic acid of the homeobox genes of the four *Hox* loci in human embryonal carcinoma cells. Mech Develop 33: 215–227

Simeone A, Avantaggiato V, Moroni MC, Mavilio F, Arra C, Cotelli F, Nigro V, Acampora D (1995) Retinoic acid induces stage-specific antero-posterior transformation of rostral central nervous system. Mech Develop 51: 83–98

Sordino P, van der Hoeven F, Duboule D (1995) *Hox* gene expression in teleost fins and the origin of vertebrate digits. Nature 375: 678–681

Stoner CM, Gudas LJ (1989) Mouse cellular retinoic acid binding protein: cloning, complementary DNA sequence, and messenger RNA expression during the retinoic acid-induced differentiation of F9 wild type and RA-3-10 mutant teratocarcinoma cells. Cancer Res 49: 1497–1504

Suzuki T, Kurokawa T (1996) Functional analyses of FGF during pharyngeal cartilage development in flounder (*Paralichthys olivaceus*) embryo. Zool Sci 13: 883–891

Wilkinson DG (1993) Molecular mechanisms of segmental patterning in the vertebrate hindbrain and neural crest. BioEssays 15: 499–505

Zhang Z, Balmer JE, Lovlie A, Fromm SH, Blomhoff R (1966) Specific teratogenic effects of different retinoic acid isomers and analogs in the developing anterior central nervous system of zebrafish. Develop Dyn 206: 73–86