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Identification of four FGF receptor genes in Medaka fish (*Oryzias latipes*)

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Four types of cDNA clones encoding tyrosine kinases highly homologous to mammalian fibroblast growth factor receptors (FGF-R) were isolated from Medaka fish (*Oryzias latipes*) by the reverse transcription-polymerase chain reaction. Comparison of the four deduced amino acid sequences with four known mammalian FGF-Rs indicated that four FGF-R species corresponding to mammalian FGF-Rs exist universally in vertebrates including fishes, although FGF-R4 might have diverged sequences between fishes and mammals. Each of four FGF-R genes is transcribed to various extents as multiple mRNAs possibly by alternative splicing in adult fishes.

Medaka; *Oryzias latipes*, Fibroblast growth factor; Fibroblast growth factor receptor, RT-PCR

1. INTRODUCTION

Fibroblast growth factors (FGFs) constituting a family of seven members show a variety of physiological activities in mesodermal and mesectodermal cells (reviewed in [1]). The signals of FGFs are transduced via their specific receptors (FGF-Rs) at the cell membrane into intracellular signalling pathways by means of kinase activity associated with the receptors (reviewed in [2]). Molecular multiplicity of FGF-Rs are shown for mammals, and four genes generating at least five distinct proteins with different physiological activities have been cloned, encoding similar transmembrane tyrosine kinases, although alternative splicing events might bring about more complicated aspects on the polymorphic nature of FGF-R molecules [2]. Similar FGF-R-related genes are also identified in *Drosophila melanogaster* to have important developmental functions [3,4].

Here, we report four FGF-R-related genes in Medaka fish (*Oryzias latipes*), each of which should correspond to four known mammalian FGF-Rs, indicating that four FGF-R genes generally exist in all vertebrates including fishes.

2. MATERIALS AND METHODS

Two oligonucleotide mixed primers were used for the reverse transcription-polymerase chain reaction (RT-PCR). They had the sequences, 5′-GG(A/C/T)GA(G/A)GG(A/C/T)TG(T/C)TT(T/C)GG(A/C/T)CA(G/A)GT-3′ (sense) and 5′-TC(A/G/T)GGNGCCATCC-A(C/T)TTNAC(T/G/A)GG-3′ (anti-sense, where N denotes all four nucleotides) corresponding to the amino acid sequences GEGCFGQV and PVKWMAPE, respectively. Single-stranded cDNA template for RT-PCR was synthesized by reverse transcriptase using poly (A)$^+$-RNA of adult Medaka fishes (*O. latipes*) according to a standard method [5]. One ng of the cDNA template was conducted for RT-PCR in a solution containing 20 pmol each of the primers at a condition that annealing temperature was 55°C and cycle number was 30. After the reaction, DNA products were blunt-ended by Klenow fragment of *Escherichia coli*, 5′-phosphorylated by T4 polynucleotide kinase, and inserted into the *Sma*I site of pUC18 vector. Clones positive to the DNA probe of *Drosophila* FGF-R [3] were sequenced by the dideoxy chain termination method [6].

A genomic Southern experiment was performed by a standard procedure using 10 μg DNA per lane digested with appropriate restriction enzymes [5]. Northern hybridization was also carried out using 1 μg poly (A)$^+$-RNA of adult Medaka fishes per lane [5]. In both experiments, the final wash of the filters was usually carried out in 0.1 × SSC containing 0.1% SDS at 65°C.

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3. RESULTS

3.1. *Molecular cloning of FGF-R-related cDNA clones of Medaka fish*

We sequenced 24 RT-PCR clones which were positive to the *Drosophila* FGF-R probe [3]. They all coded for tyrosine kinases highly related to mammalian FGF-Rs [2], and were divided by mutual comparison into four distinct groups (MFR1 to 4) each of which contained 4 to 8 clones and had essentially the same sequence. The deduced amino acid sequences of four Medaka FGF-Rs (MFR1 to 4) were highly homologous to each other (76.2 to 86.6% homology, Fig. 1) and to other known FGF-R sequences [2] (around 80% homology), but the sequences were distinct around kinase subdomains I to II around kinase inserts among MFRs (Fig. 1), and between Medaka and human FGF-Rs (see below, Fig. 2).

3.2. *Similarity between Medaka and mammalian FGF-Rs*

To know the precise relationship between four Medaka FGF-Rs (MFR1 to 4) and four mammalian

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MFR1   VMMGEVLGLDKEKPNRVTNVAVKMLKSDATEKDLSDLISEMEMMKIIGKHKNIINLLGACTQDGPLYVIVEYASKGNLREYLR
MFR2   VVMAEALGIDKDKPKEAATVAVKMLKDDATEKDLSDLVSEMEMMKMMGKHKNIINLLGACTQDGPLYVIVEYASKGDLREYLR
MFR3   VVMAEAIGIDKEKPNKPLTVAAKMLKDDATDKDLSDLVSEMEMMKMIGKHKNIINLLGACTQDGPLYVLVEFASKGNLREYLR
MFR4   VVRAEAYGINKDGPEQATTVAVKMLKDDATDKDLADLISEMELMKVMDKHKNIINLLGVCTQDGPLYVLVEYASKGSLREYLR
       *---*- *--* -*     -**-****-*** ***-** ****-**   -**********-********* **-**** ******
       I                  II                III          IV                         V

 ARRPPGMEYCYNPDQVSVETMSIKDLVSCAYQVARGMEYLASKKCIHRDLAARNVLVTEDNVMKIADFGLARDIHHIDYYKKTTNGRLP
 ARRPPGMEYSYDIARVSDEQLTFKDLVSCTYQVARGMEYLASKKCIHRDLAARNVLVTESNVMKIADFGLARDVHNIDYYKKTTNGRLP
 ARRPPGMDYSFDTCKIPDEQLTFKDLVSCAYQVARGMEYLASQKCIHRDLAARNVLVTDDNVMKIADFGLARDVHNIDYYKKTTNGRLP
 ARRPPGMDYTFDVTKVPEEQLTFKDLLSCAYQVARGMEYLASKRCIHRDLAARNVLVTEDNVMKIADFGLARGVHQIDYYKKTTNGRLP
 ******* *   -   -   *----***-**-**************--**************--************--* *************
       -     K I     -              VIa         VIb                 VII                    VIII
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Fig. 1. Alignment of four deduced amino acid sequences of Medaka FGF-Rs (MFR1 to 4). The amino acid sequences deduced from the cDNA sequences of four groups from 24 clones are aligned. Identical residues to four and three sequences are marked with asterisks and hyphens, respectively. Regions of subdomains (I to VIII) and kinase inserts (KI) are shown below the marks.

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                   SUBDOMAINS I-II          KINASE INSERTS

Human flg          LAEAIGLDKDKPNRVTKVAV     EYCYNPSHNPEEQLSS
MFR1               MGEVLGLDKEKPNRVTNVAV     EYCYNPDQVSVETMSI
                     /  /*// //**** ///     //***/      /  *

Human bek          MAEAVGIDKDKPKEAVTVAV     EYSYDINRVPEEQMTF
MFR2               MAEALGIDKDKPKEAATVAV     EYSYDIARVSDEQLTF
                   //// ///////*** ////     /////* **   // //

Human FGFR3        MAEAIGIDKDRAAKPVTVAV     DYSFDTCKPPEEQLTF
MFR3               MAEAIGIDKEKPNKPLTVAA     DYSFDTCKIPDEQLTF
                   /////////      ** ///    ///*/*** / /////

Human FGFR4        RAEAFGMDPARPDQASTVAV     DLSPDGPRSSEGPLSF
MFR4               RAEAYGINKDGPEQATTVAV     DYTFDVTKVPEEQLTF
                   */// /      / ** ////    /   /      /  /
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Fig. 2. Comparison of amino acid sequences between Medaka and human FGF-Rs around kinase subdomains I to II and kinase inserts. Identical and characteristic residues for each type are marked by asterisks, and other identical residues are marked by slashes.

(human) FGF-Rs [7–13], the deduced amino acid sequences (Fig. 1) were compared with four human sequences in diverged regions (sequences between subdomains I and II, and those of kinase inserts). The results are summarized in Fig. 2, which strongly suggests that MFRs 1, 2, 3 and 4 correspond to human *flg* (FGF-R1), *bek* (FGF-R2), FGF-R3, and FGF-R4, respectively. The similarity between MFR4 and human FGF-R4 is relatively low, but several characteristic residues in subdomains IV and XI were conserved in both MFR4 and FGF-R4.

3.3. *Identification of Medaka FGF-R genes*

To identify FGF-R genes in Medaka genome, genomic Southern hybridization was carried out using the four cDNAs. As shown in Fig. 3, distinct and simple profiles were obtained with four respective probes at a

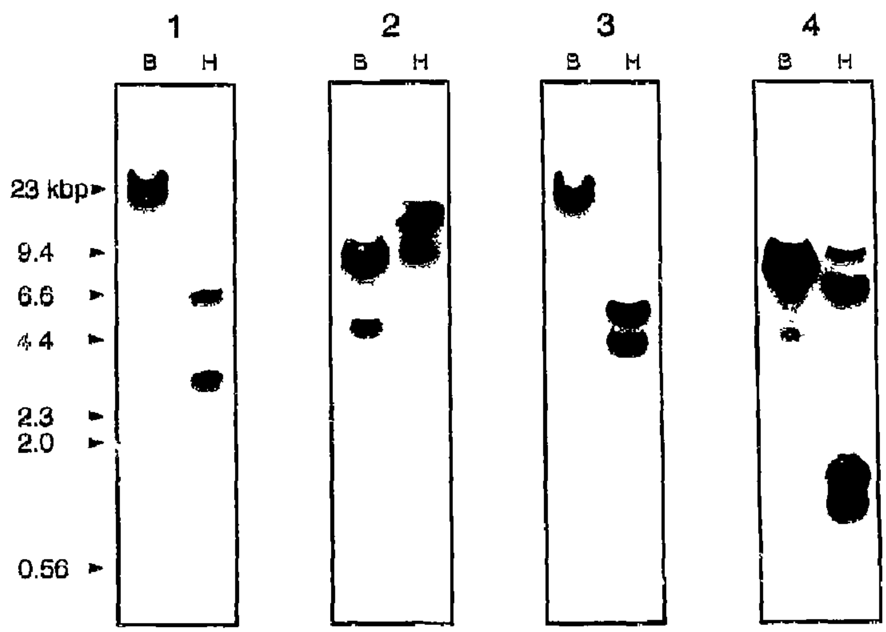

Fig. 3. Identification of four Medaka FGF-R genes by genomic Southern experiment. Panels 1 to 4 represent the profiles of MFR1 to 4, respectively. Hybridization conditions are described in the text. Letters B and H, denote the restriction enzymes used; B, *Bam*HI, and H, *Hin*dIII. Exposure periods are equally 40 h.

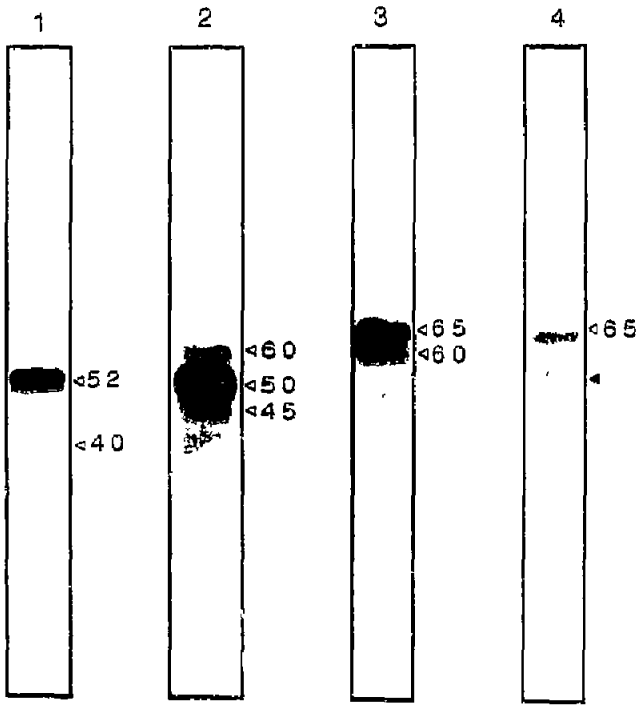

Fig. 4. Identification of MFR mRNAs. Lanes 1 to 4 represent the profiles of MFR1 to 4, respectively. Hybridization conditions are described in the text. Exposure periods are 15 h for lanes 1 and 2 (MFR1 and 2), and 72 h for lanes 3 and 4 (MFR3 and 4). A faint band shown in lane 4 at 5–5.2 kb in length (marked by a closed triangle) might be a cross-hybridization signal with two abundant MFR mRNAs (MFR1 and 2).

stringent condition. The profiles indicate that each MFR gene should be a single copy gene, although some lanes contain more than one band caused possibly by internal restriction site(s) in the introns. At the stringent condition, cross-hybridization signals were scarcely detected in spite of high nucleotide sequence homology (72.7 to 79.2%).

3.4. *Expression of four Medaka FGF-R mRNAs*

The length and abundance of MFR mRNAs were estimated by Northern hybridization using the same cDNA probes as the genomic Southern experiment. As shown in Fig. 4, all four probes detected mRNA band(s) from 4 to 6.5 kb in length; 5.2 kb (major) and 4.0 kb (minor) for MFR1, 5.0 kb (major), 6.0 kb (minor), and 4.5 kb (minor) for MFR2, 6.5 kb and 6.0 kb for MFR3, and 6.5 kb for MFR4. These multiple mRNA species transcribed from single copy genes were probably synthesized by alternative splicing as described for mammalian FGF-Rs [2]. The hybridization signals were significantly different among the four genes; mRNAs for MFR1 and 2 were abundant, that for MFR3 was less abundant, and that for MFR4 was very rare.

4. DISCUSSION

The present study first identified four FGF-Rs (MFR1 to 4) in the non-mammalian vertebrate, Medaka fish (*O. latipes*). The four Medaka FGF-Rs (MFR1 to 4) probably correspond to four mammalian FGF-Rs (*flg*, *bek*, FGF-R3 and FGF-R4) in terms of the deduced amino acid sequences around kinase subdomains I to II and kinase inserts, although MFR4 and FGF-R4 are distantly related (Fig. 2). Then, in fishes, multiple FGF and FGF-R systems should exist and might function in various developmental processes like mammals [1,2]. In other words, it is suggested that all vertebrates have at least four FGF-R genes, which should be under different transcriptional control (Fig. 4) to elicit different functions [2].

On the other hand, in an invertebrate, *D. melanogaster*, two FGF-Rs having sequences distantly related to those of vertebrate FGF-Rs are known to have distinct characteristics and functions for processes such as cell movement [3,4]. Then, it is probable that FGFs and FGF-Rs universally exist at least in the animal kingdom. To further perform comparative studies, the RT-PCR method described here will be very useful, because our primers are applicable to both invertebrates (*Drosophila*) and vertebrates (fish and mammal), and the RT-PCR method overcomes difficulties in cloning for low abundant mRNAs such as MFR4 mRNA (Fig. 4).

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