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Identification of cDNAs Encoding Two Subtypes of Vitamin D Receptor in Flounder, *Paralichthys olivaceus*[1]

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cDNAs encoding two subtypes of the vitamin D receptor (VDR) are cloned from a teleost (flounder, *Paralichthys olivaceus*). This is the first report of VDR subtypes in fish. Flounder VDR (fVDR) a and b share 86% identity at the amino acid level. With human (h), rat, mouse, quail, and *Xenopus* VDRs, fVDRa shares 72%, 71%, 71%, 69%, and 71% identity, and fVDRb shares 70%, 69%, 69%, 67%, and 68% identity, respectively. The peptide sequences of the DNA-binding domain (DBD) and hormone-binding domain (HBD) of both subtypes have particularly high homology to those of the tetrapods; e.g. 92% identity for DBP and 74% for HBD between fVDRa and hVDR. In an evolutionary tree constructed with peptide sequences of VDRs and related members of the nuclear receptor superfamily, fVDRa and b are more closely related to each other than to other molecules, and situated in the cluster of VDRs at a position which corresponds well with the evolutional position of fish in the vertebrates. Additional independent genome duplication which is thought to have occurred in ray-finned fish phylogeny may explain the existence of two subtypes of VDR in flounder. © 2000 Academic Press

The concentration of calcium in plasma is regulated at a constant level in the vertebrates. Vitamin D is one of the hormones controlling the concentration. It increases the plasma calcium level by stimulating the adsorption of calcium at the intestine (1). In fish, the gill and skin as well as the intestine function in calcium metabolism (2). Thus, the metabolism is characterized by different properties in fish than in the tetrapods, however, the system of molecular control for calcium metabolism in fish is poorly understood.

Abbreviations used: EF-1α, elongation factor-1α; ONR, orphan nuclear receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; THR-β, thyroid hormone receptor-β.

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The action of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D, is mediated by their specific receptor, VDR, which controls the expression of hormone-sensitive genes. VDR belongs to the nuclear receptor superfamily which includes steroid hormones, thyroid hormone, retinoic acid, and unknown ligands (orphan receptors) (3). The absence of VDR in mice caused by gene targeting results in the disease vitamin D-dependent rickets type II, demonstrating that VDR is an essential factor in calcium homeostasis and bone formation (4). VDRs have been cloned from tetrapod animals including human (5), bovine (6), mouse (7), rat (8), chicken and quail (9), and frog (10). At present, no sequential data on VDRs is available in fish. To reveal the vitamin D endocrine system of fish as well as the molecular evolution of VDR, it is important to gain VDR cDNAs from fish. Flounder is now reproduced in tanks at hatcheries, and bone malformations often occur in the larvae. Thus, flounder should serve as a good model with which to analyze the functions of vitamin D and VDR. So, we attempted to clone VDR cDNA from Japanese flounder (*Paralichthys olivaceus*). In contrast to the tetrapods, in which only a single type of VDR gene has been identified, this teleost was found to have two subtypes of VDR. Here, we report the full coding sequence of two subtypes of flounder VDR and their tissue expressions.

MATERIALS AND METHODS

PCR amplification of VDR fragments. cDNA was synthesized from flounder intestine mRNA as described (11). We designed several sets of degenerated PCR primers to amplify VDR fragments, and the following set at conserved sequences of DIGMMKE in DBD and YAKMIQK in LBD gave a clear band of expected length (~900 bp): VDR primer A, 5′GAYATHGGNATGATGAARGA; VDR primer B, 5′TTYTGDATCATYTTNGCRTA. The PCR was conducted for 40 cycles; cycling parameters were 95°C for 0.5 min, 60°C for 1 min, and 72°C for 2 min, followed by a single cycle of 72°C for 2 min. The band was gel purified, ligated to pCRII vector (Invitrogen), and sequenced using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer).

RACE amplification of 5′ and 3′ ends. We obtained 5′ and 3′ ends of VDR cDNAs by RACE. The 5′ RACE was performed, as previously described (12). The 3′ RACE was done by the methods of Frohman (13).

Expression analysis. First strand cDNAs from total RNA of flounder tissues prepared previously (12) were used for RT-PCR. The sites

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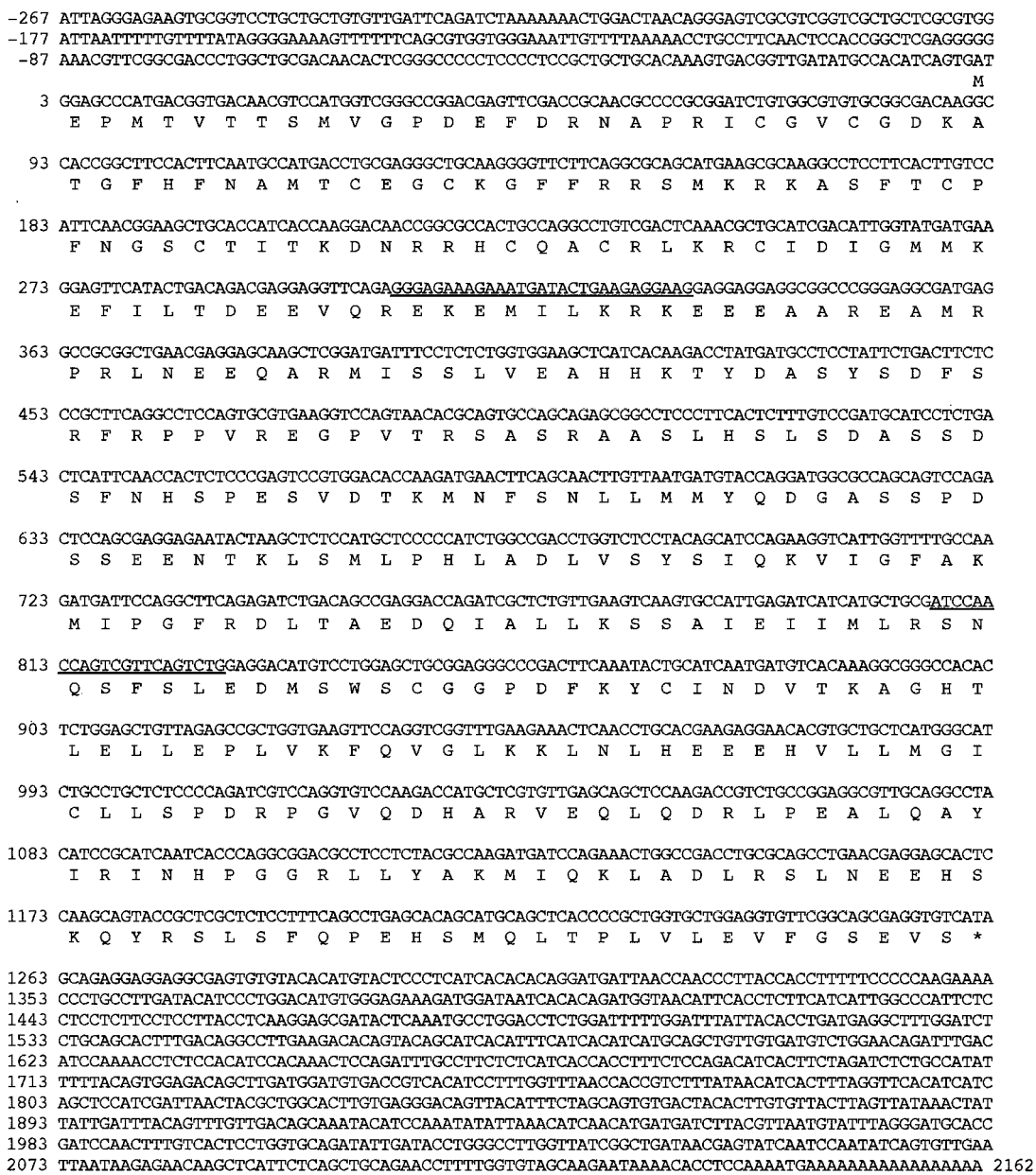

FIG. 1. cDNA sequence and deduced amino acid sequence of flounder VDRa. The sites of paired primers used for RT-PCR in Fig. 6 are underlined.

of the gene specific primers which amplify 524 bp and 729 bp fragments of VDRa and b, respectively, are indicated in Figs. 1 and 2. The primers gave a single PCR product of expected length only from the corresponding plasmid. The PCR was carried out for 20 cycles; cycling parameters were 95°C for 0.5 min, 60°C for 1 min, and 72°C for 2 min. The conditions for PCR amplification of the flounder EF-1α fragment (308 bp) were described previously (14).

RESULTS AND DISCUSSION

Cloning and Analysis of Flounder VDR cDNAs

We sequenced ten plasmid clones containing PCR products from intestinal cDNA, which included two kinds of fragments with high homology to tetrapod VDRs. Since the PCR fragments contained approximately 70% of the coding sequence of VDR, the remaining cDNA was sequenced by 5′ and 3′ RACE. Figures 1 and 2 show the nucleotide and deduced amino acid sequences of the two cDNAs. The two cDNAs were 2429 and 2659 nucleotides long, respectively. These cDNAs had highest homology with mouse and human VDR cDNAs, respectively, in a FastA computer homology search. So, we named the peptides coded by flounder VDR (fVDR) a and b, respectively. A possible initiation codon (ATG) was found at a position to give an

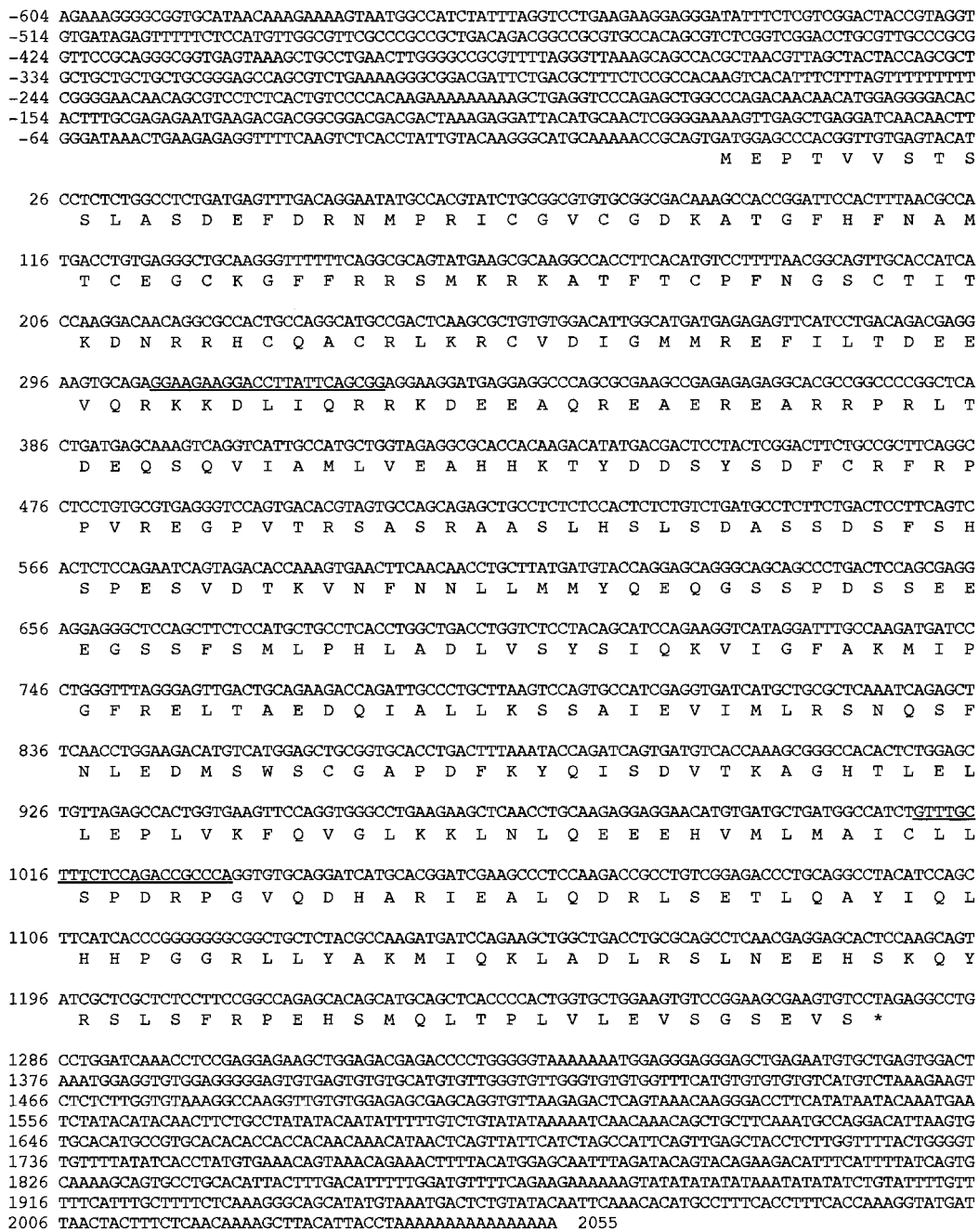

FIG. 2. cDNA sequence and deduced amino acid sequence of fVDRb. The sites of the paired primers used for RT-PCR in Fig. 6 are underlined.

open reading frame of 1263 bp for fVDRa and 1278 bp for fVDRb. The deduced sequences of fVDRa and b, 420 and 425 amino acids long, respectively, were aligned with tetrapod VDRs (Fig. 3). With human, mouse, rat (r), quail, and *Xenopus* VDRs, fVDRa shares 72%, 71%, 71%, 69%, and 71% identity, and fVDRb shares 70%, 69%, 69%, 67%, and 68% identity, respectively. fVDRa and b had 92% and 88% identity with hVDR at the DBD, 74% and 73% identity at the LBD, and 49% and 48% at the hinge region (HR), respectively. Thus, the identity between fVDRs and hVDR is particularly high at the DBD and LBD. The eight cysteine residues essential for Zn finger formation in the DBD are conserved in fVDRa and b. It is indicated that R274, C288, C337, E395, H397, and K399 in the LBD of hVDR are important for high affinity binding of $1,25(OH)_2D_3$ (15–17). VDR forms a heterodimer with RXR, and R391, F244, K246, L254, Q259, and L262 are essential for the

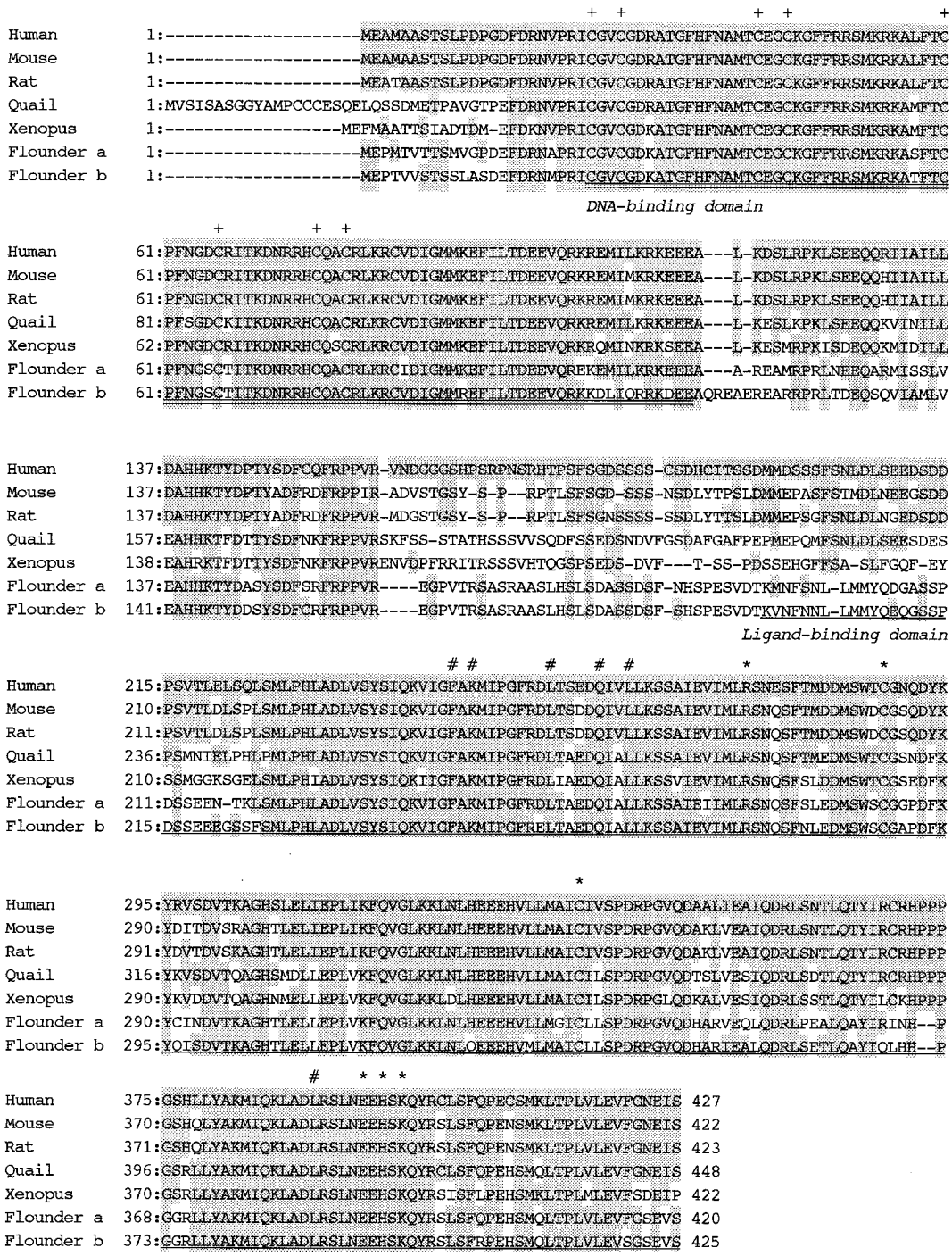

FIG. 3. Alignment of flounder VDRs with human, mouse, rat, quail, and *Xenopus* VDRs. The amino acid residues conserved with human VDR are shaded. +; cysteine residues critical for Zn finger formation. *; amino acids important for high affinity binding of $1,25(OH)_2D_3$. #; amino acids important for heterodimalization with RXR.

heterodimerization in hVDR (18, 19). These residues essential for ligand specificity and heterodimerization are conserved in both fVDRa and b. The sequential data support that both peptides coded by cloned cDNAs are VDRs of this teleost. The peptide sequences of fVDRa and b were compared (Fig. 4). The overall identity between fVDRa and b was 86%. The identity at each domain was 91% at the DBD, 88% at the LBD, and 80% at the HR. The identity of the nucleotide sequence was 81% at the ORF. Multiple isoforms of rVDR are formed by alternative splicing (20). Because the sequences of the 5′ and 3′ untranslated regions of

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fVDRa  MEPMTVTTSMVGPDEFDRNAPRICGVCGDKATGFHFNAMTCEGCKGFFRRSMKRKASFTCPFNGSCTITKDNRRHCQACR 80
fVDRb  MEPTVVSTSSLASDEFDRNMPRICGVCGDKATGFHFNAMTCEGCKGFFRRSMKRKATFTCPFNGSCTITKDNRRHCQACR 80
                                   DNA-binding domain
fVDRa  LKRCIDIGMMKEFILTDEEVQREKEMILKRKEEEA---A-REAMRPRLNEEQARMISSLVEAHHKTYDASYSDFSRFRPP 156
fVDRb  LKRCVDIGMMREFILTDEEVQRKKDLIQRRKDEEAQREAEREARRPRLTDEQSQVIAMLVEAHHKTYDDSYSDFCRFRPP 160

fVDRa  VREGPVTRSASRAASLHSLSDASSDSFNHSPESVDTKMNFSNLLMMYQDGASSPDSSEEN-TKLSMLPHLADLVSYSIQK 235
fVDRb  VREGPVTRSASRAASLHSLSDASSDSFSHSPESVDTKVNFNNLLMMYQEQGSSPDSSEEEGSSFSMLPHLADLVSYSIQK 240
                                            ligand-binding domain
fVDRa  VIGFAKMIPGFRDLTAEDQIALLKSSAIEIIMLRSNQSFSLEDMSWSCGGPDFKYCINDVTKAGHTLELLEPLVKFQVGL 315
fVDRb  VIGFAKMIPGFRELTAEDQIALLKSSAIEVIMLRSNQSFNLEDMSWSCGAPDFKYQISDVTKAGHTLELLEPLVKFQVGL 320

fVDRa  KKLNLHEEEHVLLMGICLLSPDRPGVQDHARVEQLQDRLPEALQAYIRINHPGGRLLYAKMIQKLADLRSLNEEHSKQYR 395
fVDRb  KKLNLQEEEHVMLMAICLLSPDRPGVQDHARIEALQDRLSETLQAYIQLHHPGGRLLYAKMIQKLADLRSLNEEHSKQYR 400

fVDRa  SLSFQPEHSMQLTPLVLEVFGSEVS  420
fVDRb  SLSFRPEHSMQLTPLVLEVSGSEVS  425
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FIG. 4. Comparison of amino acid sequence between fVDRa and b. The amino acid residues conserved between two proteins are shaded.

fVDRa and b are different, it is apparent that fVDRa and b are not products of alternative splicing from a single gene.

We located fVDRa and b in an evolutionary tree of VDRs and some closely related members of the nuclear receptor superfamily (Fig. 5). fVDRa and b are related more closely to each other than other molecules, and included in a cluster composed of VDRs. Their positions among the VDRs well correspond with the evolutional position of fish in the vertebrates. Thus, in terms of molecular evolution, it is also reasonable to conclude that both fVDRa and b belong to the VDR family. Recently, cluster analyses of *Hox* genes of zebrafish and pufferfish revealed additional independent whole genome duplication in the ray-finned fish phylogeny (21). As for the nuclear receptor superfamily, additional duplication can be seen in RAR and RXR of zebrafish (22). The genome duplication unique to ray-finned fish may explain the existence of two subtypes of VDR in flounder. The evolutionary tree also supports the independent duplication of VDR in flounder.

Tissue Expression of fVDRa and b

The tissue expression of two fVDRs was examined by RT-PCR (Fig. 6). Equal levels of mRNA expression of fVDRb were detected in the kidney, intestine, heart, testis, ovary, brain, gill, vertebra, and fin. Weaker signals of expression were detected also in the liver and muscle. Thus, fVDRa mRNA expression was detected in all the tissues examined.

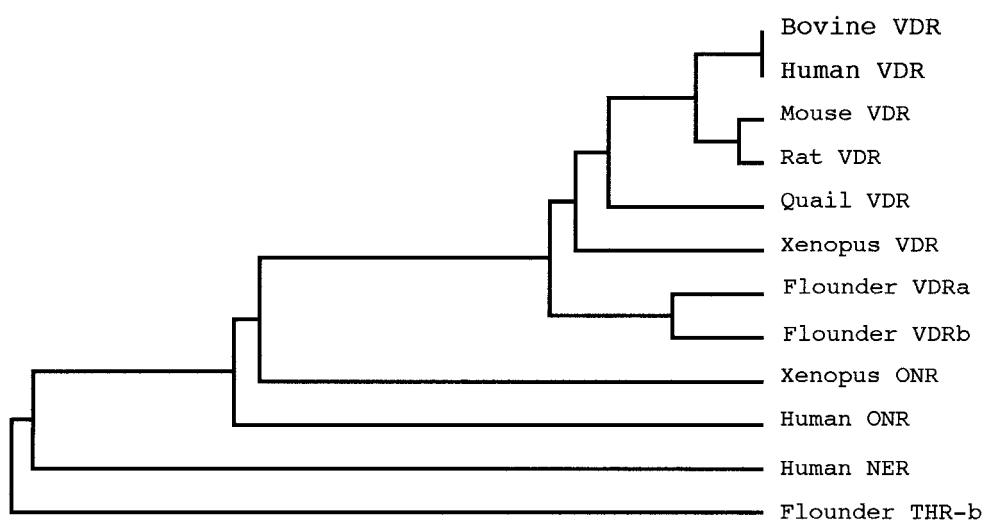

FIG. 5. Evolutionary tree of VDRs, some members of ONR and THR-β. This evolutionary tree was made by the UPGMA method using Genetic Information Processing Software (Software Development, Tokyo). VDRs; human (J03258), bovine (U50200), mouse (D31969), rat (J04147), quail (U12641), *Xenopus* (U91846). ONR; human (Z30425), *Xenopus* (X75163). Flounder THR-β (D45245). NER; human (U07132).

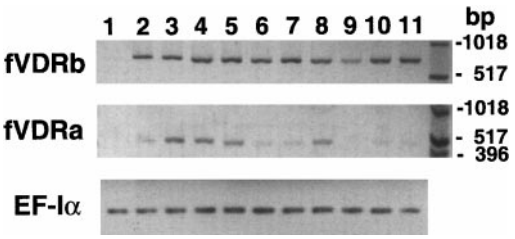

FIG. 6. RT-PCR analysis of tissue expression of fVDRa and b. Lane 1, liver; 2, kidney; 3, intestine; 4, heart; 5, testis; 6, ovary; 7, brain; 8, gill; 9, muscle; 10, vertebra; 11, fin. EF-1α serves as an internal control for the amount of RNA used.

The expression of fVDRa mRNA was relatively strong in the intestine, heart, testis, and gill. Weaker expression could be detected in the kidney, ovary, brain, vertebra, and fin. Unlike fVDRa, fVDRb mRNA could not be detected in liver and muscle. Thus, both subtypes of fVDR are expressed in various tissues, as demonstrated in *Xenopus* VDR (Li *et al.*, 1997). However, because some tissues, such as muscle, vertebra and fin, have lower expression levels of fVDRa, it is possible that the expressions of fVDRa and b are controlled by different transcriptional systems. To discriminate the function of fVDRa and b, one has to reveal their genome structure and mRNA expression pattern, particularly in the early life stage of flounder.

ACKNOWLEDGMENTS

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