

## The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes

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### Abstract

Cyclooxygenase is a key enzyme in prostanoid biosynthesis. Mammalian species have two cyclooxygenases, constitutively expressed cyclooxygenase-1 (Cox-1) and inducible cyclooxygenase-2 (Cox-2). Cox-1 and/or Cox-2 have been also identified in other vertebrates, including fish. We identified a second zebrafish Cox-2 gene orthologue, Cox-2b. All of the functionally important amino acids for cyclooxygenase enzymes are conserved in Cox-2b. The 3' untranslated region of the Cox-2b message contains AU rich elements characteristic of regulation at the level of mRNA stability. Constitutive tissue expression patterns for Cox-2a and Cox-2b are distinct, but overlap. Both Cox-2a and Cox-2b expression are inducible in the kidney when fish are exposed to tetradecanoylphorbol acetate. Like Cox-2a, Cox-2b protein, expressed in COS cells is functionally active. Thus, the zebrafish genome contains two functional, inducible Cox-2 genes. Database searching demonstrates that some fish genomes contain multiple Cox-1 or Cox-2 cyclooxygenase genes, suggesting alternate duplication and retention of this gene.

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The cyclooxygenase (Cox) enzymes catalyze the conversion of arachidonic acid to prostaglandin H<sub>2</sub>, the common precursor for the various prostanoids (the prostaglandins, prostacyclins, and thromboxanes). Prostanoids play important roles in a wide range of biological processes that include platelet aggregation, reproduction, thermoregulation, wound healing, water balance, glomerular filtration, and hemostasis. One class of the most commonly used pharmacologic agents, the non-steroidal anti-inflammatory drugs or NSAIDs, exert their pharmacological effects by inhibition of cyclooxygenase enzyme activity. The effects of NSAIDs suggest a role for cyclooxygenase products in a wide range of pathophysiological conditions that include chronic and acute inflammation, neurodegenerative diseases,

cardiovascular disease and a number of different cancers [1].

Cyclooxygenase genes have been identified in all vertebrates investigated, including mammals, birds, teleosts, and cartilaginous fishes. In contrast, cyclooxygenases have not been identified in unicellular organisms, insects or plants. Mammalian species have two cyclooxygenase genes, constitutively expressed cyclooxygenase-1 (Cox-1) and inducible cyclooxygenase-2 (Cox-2). The Cox-1 and Cox-2 proteins share conserved amino acid sequences (approximately 60%), similar three-dimensional structures, and identical reactions in prostanoid synthesis [2–4].

In fish species, cyclooxygenase orthologues have been cloned from rainbow trout [5], brook trout [6], and zebrafish [7]. Knockdown of Cox-1 in zebrafish, using antisense morpholino oligonucleotides, results in gastrulation arrest and defects in vascular tube formation during development. In addition, PGE<sub>2</sub> produced by Cox-1 is essential for gastrulation movement in the zebrafish [8]. In contrast,

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knockdown in the zebrafish of the single Cox-2 described previously does not cause a developmental phenotype [7,9].

Genome sequencing projects have revealed sequences of entire genomes for a number of organisms. Sequence analysis can identify potential orthologous genes in different species, and conserved syntenic regions can help to define chromosomal evolution. The last common ancestor of fish and mammals is likely to be the origin of the vertebrate lineage. Sequence comparisons between such distantly related organisms are informative, and understanding of genome evolution provides leads to understanding the function of genes [10]. In this study, we identified a second zebrafish Cox-2 orthologue, by searching the publicly available genome database. We have characterized this gene, which we refer to as Cox-2b, as a second inducible and functional Cox-2 homologue in the zebrafish, produced from a gene distinct from the zebrafish Cox-2 gene first identified by Grosser et al. [7]. Database search also revealed two potential Cox-1 genes in some fish species and two potential Cox-2 genes in other fish, suggesting that duplication of alternative chromosomal regions during teleost evolution has resulted in differential retention of cyclooxygenase genes.

## Materials and methods

**Cloning of the zebrafish Cox-2b coding region.** Three zebrafish genomic sequences with high homology to the mouse Cox-2 amino acid sequence were identified by BLAST search (tblastn) of the Ensemble zebrafish genome. Two of these sequences, Cox-1 and Cox-2, have previously been reported by Grosser et al. [7]. Two zebrafish expressed sequence tag (EST) clones (GenBank Accession Nos. CF997612 and BQ450716) with high similarity to the third zebrafish genomic Cox sequence were obtained and sequenced.

**RT-PCR detection of zebrafish COX mRNA.** Total RNA samples from a number of zebrafish tissues were isolated with the Trizol reagent (Invitrogen). cDNA was reverse-transcribed with AMV RT XL (Takara). To amplify each cyclooxygenase, the following primer sets were used; zCOX1F3 (5'-ATCTGAAACCCTACACATCCTTCGC-3') and zCOX1R1 (5'-AGACGTTTTGCTAAAGTTCGCCGTG-3') for Cox-1, zCOX2aF3 (5'-TACTCATCCTTTGAGGAGATGACAG-3') and zCOX2aR1 (5'-GACCTTTTACAGCTCTGAACTCCGC) for Cox-2a, zCOX2bF2 (5'-TTTCAACAGCCCTGAACC-3') and zCOX2bR2 (5'-GTTGAAGGACTCAACCAAGC-3') for Cox-2b, zGAPDH1 (5'-CATTGAGAAGGCCTCAGCTC-3') and zGAPDHR1 (5'-ACGGACACATCAGCGACTGG-3') for zebrafish GAPDH.

**Induction of Cox-2 genes with TPA in adult zebrafish.** TPA (12-*O*-tetradecanoylphorbol-13-acetate, Sigma) was added to the tank water at a final concentration of 50 ng/ml. After 1 h, the fish were dissected, and RNA samples were isolated.

**Cyclooxygenase activity.** The zebrafish cyclooxygenase expression vectors were constructed by cloning open reading frame sequences into pcDNA3.1 (Invitrogen). The open reading frame sequences were amplified using the following primer sets; zCOX1F1 (5'-TGTGTTCAAAA CTACAATGAGAGAG-3') and zCOX1R1 for Cox-1, zCOX2aF1 (5'-A ACATTCTGGAATGAATAAACTGG-3') and zCOX2aR1 for Cox-2a, zCOX2bF1 (5'-CACGCCACTGGAATGAAAAGTTCGG-3') and zCOX2bR1 (5'-TACACTAATCAGAGCTCAGATGTCC-3') for Cox-2b. For PCR templates, the following EST clones were used; AW342787 for Cox-1, BC056736 for Cox-2a, CF997612 for Cox-2b. Each expression vector was transiently transfected with Lipofectamine 2000 (Invitrogen) into COS-1 cells. Cyclooxygenase activity in microsomal fraction was

measured with COX Activity Assay kit (Cayman) according to the manufacturer's instructions.

**Database search.** The genomic sequences for cyclooxygenases of the puffer fish *Takifugu rubripes* and *Tetraodon nigroviridis* were obtained using BLAST search (tblastn) of Ensemble fugu (assembly 4) and tetraodon genome (assembly 7), respectively. The deduced protein sequences of rainbow trout cyclooxygenases were obtained by BLAST search (tblastn) of The Institute of Genomic Research (TIGR) *Oncorhynchus mykiss* gene index. The Cox gene sequences for medaka (*Oryzias latipes*) were obtained from the medaka genome sequencing project. These data have been provided freely by the National Institute of Genetics and the University of Tokyo for use in this publication only.

## Results

Blast search of the genome databases of zebrafish (*Danio rerio*) using the mouse Cox-2 sequence identified three highly homologous sequences. Two of the mammalian Cox-related sequences are identical to zebrafish Cox-1 (Chr. 5) and Cox-2 (Chr. 2) cDNA sequences reported previously [7]. The third Cox-related sequence, not previously reported, is present on Chr. 20 (GenBank Accession No. DQ494791). Zebrafish expressed sequence tag (EST) clones related to this third Cox zebrafish genomic sequence were identified in the Washington University Genome Resources EST database.

### Sequence comparisons of the predicted zebrafish cyclooxygenase proteins

Sequence analysis of these previously uncharacterized, Cox-related ESTs reveals a 1818 bp open reading frame. The deduced amino acid sequence (606 aa) was determined from the ORF and is compared in Fig. 1a with zCox-1 (597 aa) and the “zCox-2” cDNA described by Grosser et al. [7], which we now term Cox-2a (601 aa). For optimal alignment of the zebrafish Cox-2 related sequences, it is necessary to insert a seven amino acid deletion that follows amino acid residue 152 in the Cox-2a sequence.

All the important structural and functional domains implicated in cyclooxygenase enzyme function are conserved in the new putative cyclooxygenase enzyme, which we term Cox-2b. Like the mammalian cyclooxygenases and the zebrafish Cox-1 and Cox-2a cyclooxygenases previously described, Cox-2b contains an active site tyrosine (Tyr-385), proximal and distal haem-binding histidines (His-207 and His-388), the aspirin acetylation site (Ser-530), potential *N*-glycosylation sites and a haem-binding domain. The two haem-binding sites are identified by the black boxes in Fig. 1a. The zebrafish Cox-2b sequence is more closely related to mouse Cox-2 (70.5% identity) than to mouse Cox-1 (61.0%), suggesting this gene is a second zebrafish orthologue of the mammalian Cox-2 gene. Zebrafish Cox-2b contains an 18 amino acid C-terminal insertion that is conserved in mammalian Cox-2 proteins (and in zebrafish Cox-2a) and absent in the Cox-1 protein. In contrast, the N-terminal hydrophobic amino acid insertion

Fig. 1. (a) Comparison of the deduced amino acid sequences of the Cox-1, Cox-2a, and Cox-2b cyclooxygenases from the zebrafish. Conserved amino acids of the cyclooxygenases are shaded. Functionally important amino acids are indicated by asterisks; the active site of Cox (tyrosine-385, histidine-388, and serine-530), the substrate-binding site (arginine-120), the *N*-glycosylation site (asparagines-68, -144, and -410), and the sites crucial for peroxidase activity (glutamine-203 and histidine-207). The two domains that define the haem-binding sites are identified by the black boxes. The amino acids which define conformational differences of the substrate-binding channels between Cox-1 and Cox-2 in the mammalian enzymes are indicated by a “+” (arginine-513 and valine-523 in mammalian Cox-2 proteins). (b) The sequences of the AU-rich elements present in the zebrafish Cox-2a and Cox-2b 3' untranslated regions. Shaw-Kamen AUUUA pentamer motifs are underlined.

Amino acids at positions 513 and 523 are postulated to determine the differences in flexibility for the substrate channels of mammalian Cox-1 and Cox-2, and to be responsible for the difference in the specificity of Cox-2 specific inhibitors (reviewed in [12]). Mammalian Cox-1 enzymes have His-513 and Ile-523; while mammalian Cox-2 enzymes have Arg-513 and Val 523 at these residues. Grosser et al. [7] report that both Cox-1 and Cox-2 have Arg and Val in position 513 and 523. The Arg and Val residues at these positions are conserved between Cox-2a and Cox-2b (Fig. 1a).

Mammalian Cox-2 mRNAs contain AU-rich elements (AREs) in their 3' untranslated regions (3'UTR). The presence of AREs is a common characteristic of immediate-early genes and is implicated in post-transcriptional regulation of mRNA stability (reviewed in [13,14]) AREs of mammalian Cox-2 genes contain multiple, often overlapping, copies of the pentameric AUUUA motif originally

Constitutive expression of the three zebrafish Cox genes, Cox-1, Cox-2a, and Cox-2b, was examined by RT-PCR in adult zebrafish organs (Fig. 2a). Cox-2b transcripts are present in many tissues, and are particularly high in the gill, heart and ovary. Intermediate constitutive Cox-2b message levels are present in kidney, gut and testis, with lower transcript levels observed in the brain and eye. Constitutive Cox-2b expression is not detected in liver. The Cox-1 and Cox-2a expression patterns are similar to those previously reported by Grosser et al. [7]. Cox-1 message is present in all tissues, albeit weakly in brain. The most robust constitutive Cox-2a signals are observed in the gills, gut and testis, followed by heart and brain, with

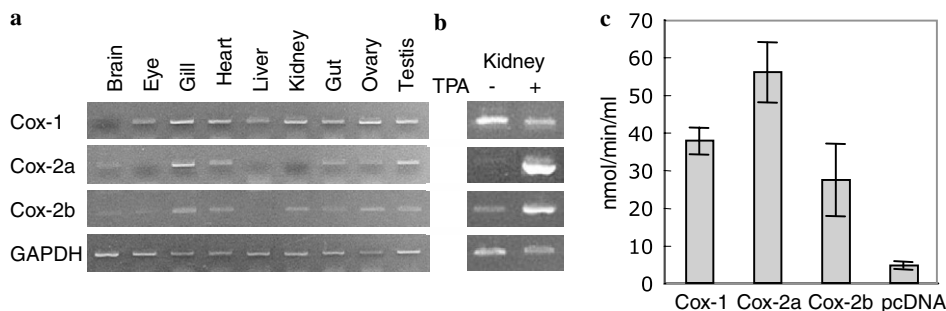


Fig. 2. (a), Constitutive Cox-1, Cox-2a, and Cox-2b mRNA expression in adult zebrafish organs. Total RNA (100 ng) from each adult organ was reverse-transcribed and amplified with specific primer sets for Cox-1, Cox-2a or Cox-2b. GAPDH was also amplified as a control. (b), Induction of cyclooxygenase expression by TPA in zebrafish tissues. Fish were treated with TPA (50 ng/ml) in the tank water for 1 h. Total RNA from kidney was reverse-transcribed and amplified to detect the individual cyclooxygenase isoforms and the GAPDH control. (c) Enzymatic activity of the cyclooxygenases produced from plasmids expressing the zebrafish cyclooxygenase isoforms. COS cells were transfected with Cox-1, Cox-2a, Cox-2b or control pcDNA expression vectors. Two days after transfection, cell lysates were prepared, microsomal fractions were isolated, and peroxidase enzyme activity was measured. Data are the averages  $\pm$  SD from triplicate assays.

relatively low expression levels in the eye, liver and kidney. Thus the constitutive expression patterns of the two Cox-2 related zebrafish genes are different, but overlap in the various organs.

The mouse *Cox-2* gene was originally identified in our laboratory as a gene induced by TPA in 3T3 fibroblasts [2]. To examine whether the Cox-2b gene is inducible, zebrafish Cox transcripts were analyzed after challenge of fish in vivo with TPA (Fig. 2b). Constitutive expression of all three cyclooxygenases is observed in kidney, although Cox-2a transcripts are quite low. When the fish are treated with TPA, both the Cox-2a and Cox-2b message levels increase substantially in this organ. In contrast, the kidney Cox-1 transcript level is, if anything, reduced following TPA treatment.

#### *The zebrafish Cox-2b gene, like the Cox-1 and Cox-2a genes, encodes a functional cyclooxygenase*

Sequence analysis, mRNA expression and inducibility all suggest that Cox-2b, like Cox-2a, is a functional orthologue of mammalian *Cox-2*. To analyze Cox-2b function, we expressed the protein and measured its enzymatic activity. The ORFs of the Cox-1, Cox-2a, and Cox-2b cDNAs were subcloned into a mammalian expression vector and expressed in COS cells. Cyclooxygenase is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activity. Lysates were prepared from transfected cells and assayed for peroxidase activity characteristic of the cyclooxygenases (Fig. 2c). Like Cox-1 and Cox-2a, Cox-2b encodes peroxidase activity, demonstrating that the zebrafish has three, rather than two, functional cyclooxygenase genes.

#### *Structure of the zebrafish Cox-2 genes*

The genomic structure of the zebrafish cyclooxygenase genes was determined by comparing their cDNA sequences with genomic database sequences. Maps of the murine and

zebrafish cyclooxygenase gene structures are shown in Fig. 3. The exon/intron structures of zebrafish cyclooxygenases are similar to those of the mouse cyclooxygenase genes, although intron lengths vary substantially. The exon/intron structures of zebrafish Cox-1 and mouse *Cox-1* are identical; 11 exons and 10 introns. Like murine *Cox-2*, zebrafish Cox-2b has one less intron and one less exon than the *Cox-1* genes; both murine *Cox-2* and zebrafish Cox-2b have 10 exons and nine introns. In contrast, in zebrafish Cox-2a, the intron separating exons 4 and 5 is missing; exons 4 and 5 form a continuous coding exon sequence in zCox-2a. Thus, in comparison to murine *Cox-2* and zebrafish Cox-2b, Cox-2a has one less exon and one less intron. The “fused” exon 4 of the Cox-2a gene, encoding sequences homologous to exons 4 and 5 of murine *Cox-2* and zebrafish Cox-2b, is 21 nucleotides shorter in length than the sum of the nucleotides present in the murine *Cox-2* or zebrafish Cox-2b exons 4 and 5 (305 nucleotides versus 326 nucleotides). This difference in exon length accounts for the seven amino acid deletion observed in the zebrafish Cox-2a protein, when compared to murine *Cox-1*, *Cox-2*, and the zebrafish Cox-1 or Cox-2b proteins (Fig. 1a).

The mouse and human *Cox-1* genes are approximately 22–25 kb in length, whereas *Cox-2* genes are 8 kb (reviewed in [11]). In zebrafish, the Cox-1 gene is also greater than 20 kb in length. In contrast, both zebrafish Cox-2 genes, Cox-2a and Cox-2b, are relatively small; 7.3 and 5.5 kb in length, respectively.

#### *Evolutionary relationships among fish Cox genes*

Jarving et al. [16] report that database searches suggest the puffer fish fugu (*Takifugu rubripes*) genome sequence contains three cyclooxygenase related sequences. In contrast to zebrafish, two of the fugu cyclooxygenase genes resemble mammalian *Cox-1*, while the third fugu gene more closely resembles *Cox-2*. Our identification of a second Cox-2 gene in the zebrafish prompted us to examine



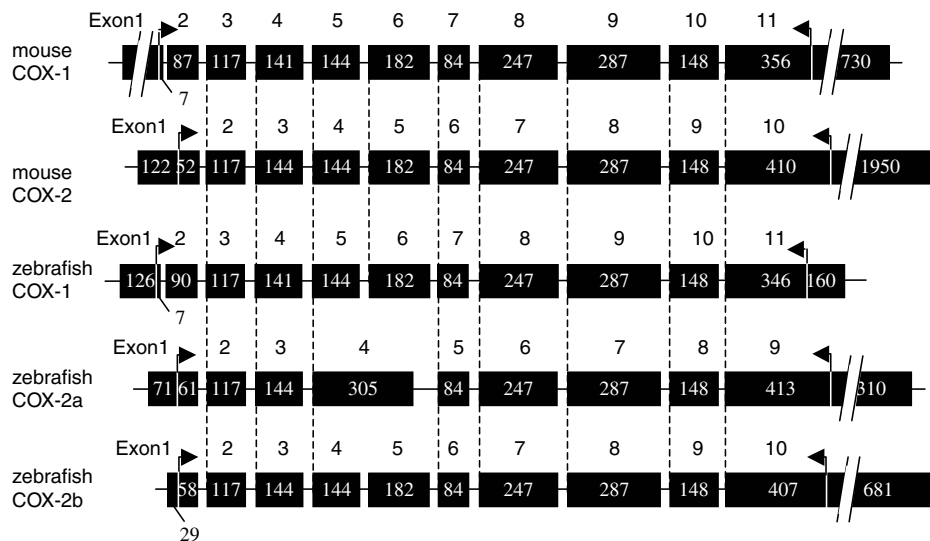


Fig. 3. Structures of the mouse and zebrafish Cox genes. The number and length of the exons of the zebrafish Cox-1, Cox-2a, and Cox-2b genes are compared with exons of the mouse *Cox-1* and *Cox-2* genes. The numbers in the boxes indicate the numbers of nucleotides in each exon. The arrows above the genes indicate the coding regions.

the evolution of the cyclooxygenase genes in the teleost lineage, using existing sequence data. Homology searches were performed on publicly available fish genome databases. Blast searches of the genome database for the fresh water puffer fish *Tetraodon nigroviridis*, using known Cox sequences, also yields three cyclooxygenase-like sequences. Although these genomic sequences have some gaps, sequence similarities and the distribution of 3'UTR AUUUA sequences indicate that *Tetraodon* has two Cox-1 genes and one Cox-2 gene (the Cox-1 genes are on chromosomes 4 and 12; Cox-2 is on chromosome 1). Genome database searches for the Japanese killifish, medaka (*Oryzias latipes*), reveal that this genome also contains two Cox-1 and one Cox-2 like sequences (the Cox-1 sequences are on scaffolds 84 and 283; the Cox-2 gene is on scaffold 230). In contrast, we found a second Cox-2 like gene in the rainbow trout *Oncorhynchus mykiss*. Individual Cox-1 and Cox-2 orthologues have been reported and cloned previously in this species [5]. Searches of EST databases revealed that rainbow trout also have a third cyclooxygenase sequence. Because the predicted amino acid sequence for this third Cox species in the rainbow trout shows a 59.3% identity with mouse *Cox-1* and 69.4% with *Cox-2*, and because the mRNA of this previously undescribed Cox-related sequence also contains 3'UTR AUUUA sequences, we suggest that this gene is a putative second Cox-2 in this genome. These predicted sequences and the known cyclooxygenases from fish species and ancestral corals have been used, in Fig. 4, to construct a phylogenetic tree. The results indicate that teleosts often possess three cyclooxygenase genes; some species have two Cox-1 genes and a single Cox-2 gene, while other species have a single Cox-1 gene and two Cox-2 genes. However, to date only this study in the zebrafish has demonstrated expression and function for three cyclooxygenases, encoded by three distinct genes, in a single species.

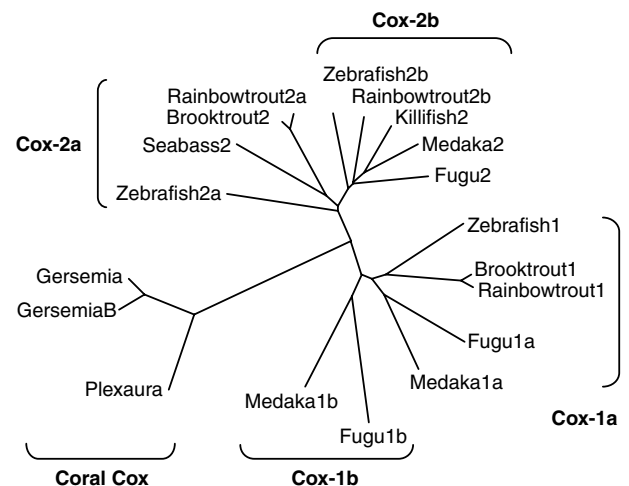


Fig. 4. Unrooted phylogenetic distance dendrogram of fish and ancestral coral cyclooxygenases. The tree was constructed by the neighbor-joining method, using the CLUSTAL program.

## Discussion

In this study, we identified a third functional cyclooxygenase gene, Cox-2b, in the zebrafish. To our knowledge, expression of two distinct inducible, enzymatically active Cox-2 isoforms has not previously been described in any species.

Both Cox-2a and Cox-2b messenger RNAs contains ARE sequences in their 3'UTRs. In contrast, like the mammalian Cox-1 genes, zebrafish Cox-1 has no ARE sequences in its mRNA. AREs are divided generally into three categories; the ARE region of mammalian Cox-2 is classified as class II, in which mRNAs contain multiple AUUUA pentamers, often with some overlap [13,14]. AREs act as potent mRNA destabilizing sequences. The

mouse *Cox-2* ARE has three overlapping and four separated AUUUA motifs in close proximity. *Cox-2b* has six overlapping and one separated ARE motifs, while *Cox-2a* has two overlapping and three separated ARE motifs. It will be instructive to compare the mRNA destabilizing effects of the zebrafish *Cox-2a* and *Cox-2b* 3'UTRs with that of human and/or mouse *Cox-2* 3'UTRs. Such a comparison might help to elucidate which of the two zebrafish *Cox-2* genes is more closely related functionally to the single mammalian *Cox-2* gene.

Exon–intron junctions of cyclooxygenases are highly conserved in all mammalian species studied. *Cox-1* genes contain an extra intron (intron 1) compared with *Cox-2* genes. Comparison of the zebrafish *Cox-2b* cDNA sequence with genomic database sequences indicates that the exon–intron structure of the zebrafish *Cox-2b* gene is identical to mammalian *Cox-2*, with 10 exons and nine introns. In contrast, zebrafish *Cox-2a* lacks one intron when compared both to mammalian *Cox-2* genes and to zebrafish *Cox-2b*. Thus comparison of gene structures also suggests that zebrafish *Cox-2a* is more divergent than is zebrafish *Cox-2b* from mammalian *Cox-2* genes.

By database search of genomic and EST sequences, we also detected three cyclooxygenase genes in other teleosts; the puffer fishes *Takifugu rubripes* and *Tetraodon nigroviridis*, the Japanese killifish *Oryzias latipes*, and the rainbow trout *Oncorhynchus mykiss*. Among these species, both puffer fish species and killifish have two putative *Cox-1* genes and one putative *Cox-2* gene. In contrast, like the zebrafish, the rainbow trout has two *Cox-2* genes. For many gene families, fish have more members than do tetrapods. The frequency of two paralogous genes in fish species corresponding to one ortholog in tetrapods has suggested that a whole-genome duplication occurred in the teleost lineage after its divergence from the tetrapod lineage [17–20]. Recently, Woods et al. [21] showed that different duplicated genes have been lost in zebrafish and *Tetraodon*, although similar overall numbers of duplicated genes may have been retained in these two species. The differences in retention of the *Cox-2* and *Cox-1* gene pairs respectively in zebrafish and *Tetraodon* are consistent with the conclusions of Woods et al. [21]. The *Cox-1* and *Cox-2* genes are likely to have been duplicated after the divergence of teleosts from tetrapods; subsequently puffer fishes and killifish retained two *Cox-1* genes, while zebrafish and rainbow trout retained two *Cox-2* genes.

Expression of the single *Cox-2* genes in mammals is induced by a wide variety of signals, including growth factors, cytokines, and neuronal depolarization, as well as in many pathophysiological conditions, such as neuronal degeneration, inflammation, and cancer [22]. It will be of great interest to compare the regulation of the two *Cox-2* genes present in zebrafish and rainbow trout, to determine whether induction of the two genes shows substantial overlap in response to alternative ligands, or demonstrates differential induction responses to alternative stimuli.

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