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Two Inducible, Functional Cyclooxygenase-2 Genes are Present in the Rainbow Trout Genome

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Abstract The cyclooxygenases (Cox) catalyze the initial reactions in prostanoid biosynthesis, and produce the common prostanoids precursor, PGH_2. Mammalian species have two Cox isoforms; constitutively expressed cyclooxygenase-1 (Cox-1) and inducible cyclooxygenase-2 (Cox-2). Database searches suggest three Cox genes are present in many fish species. In this study, we cloned and characterized a second Cox-2 cDNA, Cox-2b, from the rainbow trout. Rainbow trout Cox-2b protein contains all the functionally important conserved amino acids for Cox enzyme activity. Moreover, the Cox-2b message contains AU-rich elements (AREs) in the 3′ untranslated region (3′UTR) characteristic of inducible Cox-2 mRNAs. We took advantage of the existence of a rainbow trout cell line to demonstrate that expression from both the originally reported Cox-2 (Cox-2a) and Cox-2b genes is inducible. However, differential induction responses to alternative inducers are observed for rainbow trout Cox-2a and Cox-2b. Both Cox-2a and Cox-2b proteins expressed in COS cells are enzymatically active. Thus the rainbow trout has two functional, inducible Cox-2 genes. The zebrafish also contains two Cox-2 genes. However, genome structure analysis suggests diversion of the Cox-2a gene between zebrafish and rainbow trout. J. Cell. Biochem. 102: 1486–1492, 2007.

Key words: Oncorhynchus mykiss; prostaglandins; gene duplication; Cox-2; teleost genome

Prostanoids are fatty acid derivatives that mediate a wide range of physiologic processes. In mammals, prostanoids play important roles in normal functions that include thermal regulation, water balance, parturition, and ovulation. Aberrant prostanoid expression mediates many pathophysiological conditions, including pain, fever, chronic and acute inflammation, neurodegenerative diseases, and a variety of different cancers. Prostanoids are formed following the action of cyclooxygenases (Cox). The Cox catalyze the conversion of arachidonic acid to prostaglandin H_2, the common intermediate for all prostanoids [Herschman, 2003]. Mammalian species have two Cox genes, constitutively expressed cyclooxygenase-1 (Cox-1) and inducible cyclooxygenase-2 (Cox-2) [Kujubu et al., 1991; O'Banion et al., 1991; Xie et al., 1991]. A "Cox-3" enzyme has been reported in dogs; however, Cox-3 is a splice variant, produced from the Cox-1 gene, which retains intron 1 [Chandrasekharan et al., 2002].

Cox-2 expression is regulated by a wide variety of cell-specific inducers, in a number of biological responses. Cox-2 is also overexpressed in many types of tumors, both in humans and in animal models. The signal transduction pathways, the transcription factors, and the *cis*-acting elements of the Cox-2 gene that mediate Cox-2 expression have been analyzed in a number of mammalian physiological and pathophysiological contexts [Herschman, 2004].

Cox genes have also been identified in species other than mammals. Coral and the sea squirt also have two Cox genes. However, these latter Cox-2 isoforms may be the result of a gene duplication event independent of the event that produced vertebrate Cox-1 and Cox-2 [Jarving et al., 2004]. In insects, eicosanoids, including

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prostaglandins, play a role in cellular immunity, fertility and regulation of febrile response [Stanley, 2006]. However, Cox genes have not been reported in this class. Most vertebrate species investigated, including birds and teleosts, have at least two Cox. In fish species, Cox orthologues have been cloned from rainbow trout [Zou et al., 1999], brook trout [Roberts et al., 2000], and zebrafish [Grosser et al., 2002].

Recently, we identified a second zebrafish Cox-2 orthologue, Cox-2b, and characterized this gene as a second inducible and functional Cox-2 homologue in the zebrafish [Ishikawa et al., 2007]. This was the first report of the expression of two distinct inducible and functional Cox-2 isoforms in any species. Database searching suggests that some fish species have two potential Cox-1 genes, while other fish species have two Cox-2 genes, suggesting that duplication of alternative chromosomal regions during teleost evolution has resulted in differential retention of Cox genes. In this study, we cloned a second rainbow trout Cox-2 orthologue, identified in an EST database, which we term Cox-2b. Biochemical characterization of the product of the rainbow trout Cox-2b gene demonstrates its enzymatic functionality. Because there exists a cell line derived from the rainbow trout, we were able to examine the effect of alternative inducers on the expression of the Cox-2a and Cox-2b genes. We find that, in the RTG-2 cell line, the Cox-2a and Cox-2b genes demonstrate differential inducibility.

MATERIALS AND METHODS

Growth of RTG-2 Rainbow Trout Cells

RTG-2 cells were obtained from ATCC (Manassas, VA). The cells were maintained at room temperature (22°C) in 25 cm^2 flasks in MEM with 10% FBS (Omega Scientific, Tarzana, CA). Cells were passaged by gentle trypsinization with 0.25% trypsin and 0.03% EDTA, and diluted at a ratio of 1:2–1:5 when cultures were nearly confluent.

Cloning of the Rainbow Trout Cox-2b cDNA

Unknown EST sequences with high homology to the mouse Cox-2 amino acid sequence were identified by BLAST search (tblastn) of The TIGR Gene Index Databases (The Institute of Genomic Research, Rockville, MD, URL: http://www.tigr.org/tdb/tgi) *Oncorhynchus mykiss* gene index. Based on these sequences, the presumptive open reading frame sequence was amplified from TPA-treated (50 ng/ml, 6 h) rainbow trout RTG-2 cells using the following primer set; rtCOX2bF1 (5′-GGA ATG AAC AGA ATC ATA TCT GCG C-3′) and rtCOX2bR1 (5′-TTG TCT TAT CAG AGC TCA GTG GTC C-3′). The presumptive 3′UTR sequence was also amplified, using the following primer set; rtCOX2bF3 (5′-TGA GCT CTG ATA ACA CAA TTC AGC-3′) and rtCOX2bR3 (5′-AGA ACA ATA AGG AGT CAG CTC AAG-3′). PCR fragments were subcloned and sequenced.

Identification of Exon/Intron Borders

Genomic sequences spanning a portion of exon 4 to a portion of exon 5 for the rainbow trout Cox-2a and Cox-2b genes were amplified using the following primer sets; rtCOX2aF4 (5′-TCA CAT TTG GTG GAC AGC CCA CCG-3′) and rtCOX2aR4 (5′-ATG GCC CAA GGC TTT GGT GAA AGC-3′) for Cox-2a, rtCOX2bF4 (5′-TCT CAT CTG ATC GAC AGT CCA CCG-3′) and rtCOX2bR4 (5′-AGA ACT GGT GGG TGA AGT GTT GGG-3′) for Cox-2b. Amplified fragments were subcloned and sequenced.

RT-PCR Detection of Rainbow Trout Cox mRNA

RTG-2 cells were plated at 5×10^5 cells/flask in 25 cm^2 flasks in 5 ml MEM with 0.5% serum. After overnight culture, cells were treated with tetradecanoyl phorbol acetate (TPA, 50 ng/ml, Sigma, St. Louis, MO), TPA + dexamethasone (1 μM, Sigma), LPS (from Escherichia coli serotype 0111:B4, 100 ng/ml, Fluka, Ronkonkoma, NY), forskolin (20 nM, Sigma), PGE_2 (50 nM, Cayman, Ann Arbor, MI) or murine IL-1β (10 ng/ml, Cell Biology Products, Gaitherburg, MD) for 6 h without medium change. Control cells are without treatment. The cells were maintained at room temperature (22°C) during induction. Total RNA samples were isolated with the RNeasy kit (QIAGEN, Valencia, CA). cDNA was reverse-transcribed with AMV RT XL (Takara, Otsu, Shiga, Japan). To amplify each Cox, the following primer sets were used; rtCOX1F1 (5′-ATG AGT ATT TCT TCA TGA GTG CAG C-3′) and rtCOX1R2 (5′-TTG TGT TCT CTC AGC CAC AGG GTG G-3′) for Cox-1, rtCOX1F2 (5′-GGC AAT GTC TAC GGA GAT AAT CTG G-3′) and rtCOX1R1 (5′-CAA TGG CTT ACA GTT CAG TGG ACT G-3′) for Cox-1, rtCOX2aF1 (5′-TTT CGG GGA TGA ATA GAG TAA TCT G-3′) and rtCOX1aR1 (5′-TTG TGT

ACT TGA ATA GAA CCT AGT G-3′) for Cox-2a, rtCOX2bF1 and rtCOX2bR1 for Cox-2b, rt-GAPDHF1 (5′-GGA ATC AAA GTC GTT GCC AT-3′) and rtGAPDHR1 (5′-GGA TCT CAT GGG GCT TCA TA-3′) for rainbow trout GAPDH. Forward and reverse primers for the various Cox are from distinct putative exons that bridge intervening introns, to distinguish the cDNA products from genomic products.

Cyclooxygenase Activity

The rainbow trout Cox-2a and Cox-2b expression vectors were constructed by cloning open reading frame sequences into pcDNA3.1 (Invitrogen, Carlsbad, CA). Each expression vector was transiently transfected with Superfect (QIAGEN) into COS-1 cells. Cox activity in microsomal fractions was measured with a COX Activity Assay kit (Cayman), according to the manufacturer's instructions. This assay measures the peroxidase activity of the Cox enzyme.

RESULTS

Sequence Comparisons of the Predicted Rainbow Trout Cyclooxygenase Proteins

Blast search of the rainbow trout (*Oncorhynchus mykiss*) EST databases using the mouse *Cox-2* sequence identified highly homologous sequences distinct from the rainbow trout Cox-1 (unpublished work; Genbank accession No. AJ299018) or Cox-2 [Zou et al., 1999] cDNAs reported previously. Based on this sequence, specific primers for this Cox-related sequence were designed. Because the mouse *Cox-2* gene was originally identified as a gene induced by TPA in 3T3 fibroblasts [Kujubu et al., 1991], we used cDNA from TPA-treated RTG-2 cells, a fibroblast like cell line derived from rainbow trout gonad tissue [Wolf and Quimby, 1962], to amplify the unknown Cox-related sequence. Sequence analysis of amplified fragments reveals a 1827 bp open reading frame (Genbank accession No. EF175381). The deduced amino acid sequence (609 a.a.) was determined from the ORF and is compared in Figure 1a with rainbow trout Cox-1 (624 a.a., AJ299018) and the rainbow trout Cox-2 [Zou et al., 1999], which we now term Cox-2a (607 a.a., AJ238307).

The sequence of the new putative Cox has greater similarity to mouse Cox-2 (69.4%) than to mouse Cox-1 (59.3%). This sequence contains a 20 amino acid C-terminal insertion that is conserved in mammalian Cox-2 proteins and is absent in Cox-1 proteins. In contrast, the N-terminal insertion, rich in hydrophobic amino acids, that is characteristic of Cox-1 proteins is absent in the new rainbow trout putative Cox. These sequence comparisons suggest this gene is a second orthologue of the Cox-2 gene in rainbow trout; we term this gene "Cox-2b".

The key amino acid residues known to be essential for structure and function of Cox proteins are well conserved in the rainbow trout Cox-2a and Cox-2b sequences. Both Cox-2 predicted proteins contain an active site tyrosine (Tyr-411), proximal and distal haem-binding histidines (His-233 and His-414), the aspirin acetylation site (Ser-556), potential N-glycosylation sites, and a haem-binding domain (Fig. 1a). The deduced amino acid sequence also revealed the presence of Arg-539 and Val-549. In mammalian Cox-1 the amino acids at these positions are His-539 and Ile-549, while mammalian Cox-2 enzymes have Arg-539 and Val-549. These amino acid differences contribute to the specificity of Cox-2 selective inhibitors [Flower, 2003]. The Arg-539 and Val-549 residues found in mammalian Cox-2 proteins are present in rainbow trout Cox-1, Cox-2a, and Cox-2b.

Induced Expression of the Rainbow Trout Cox-2a and Cox-2b Genes

Mammalian Cox-2 mRNAs contain AU-rich elements (AREs) in their 3′ untranslated region (3′UTR). AREs are associated with messages that are unstable and rapidly turning over, and are present in many immediate-early genes [Dean et al., 2004; Espel, 2005]. AREs often contain multiple copies of the pentameric AUUUA Shaw–Kamen motif [Shaw and Kamen, 1986]. Rainbow trout Cox-2a mRNA, which contains seven AUUUA sequences in its 3′UTR, is induced in head kidney leucocytes by bacterial challenge [Zou et al., 1999]. As shown in Figure 1b, the proximal 3′UTR sequences of rainbow trout Cox-2b also possess seven AUUUA sequences. The presence of AREs in their 3′UTR sequences suggests that both Cox-2a and Cox-2b might be inducible genes.

The mouse *Cox-2* gene is induced by a variety of stimuli [Kujubu et al., 1991]. To examine whether the rainbow trout Cox-2b gene is inducible, rainbow trout Cox transcripts were

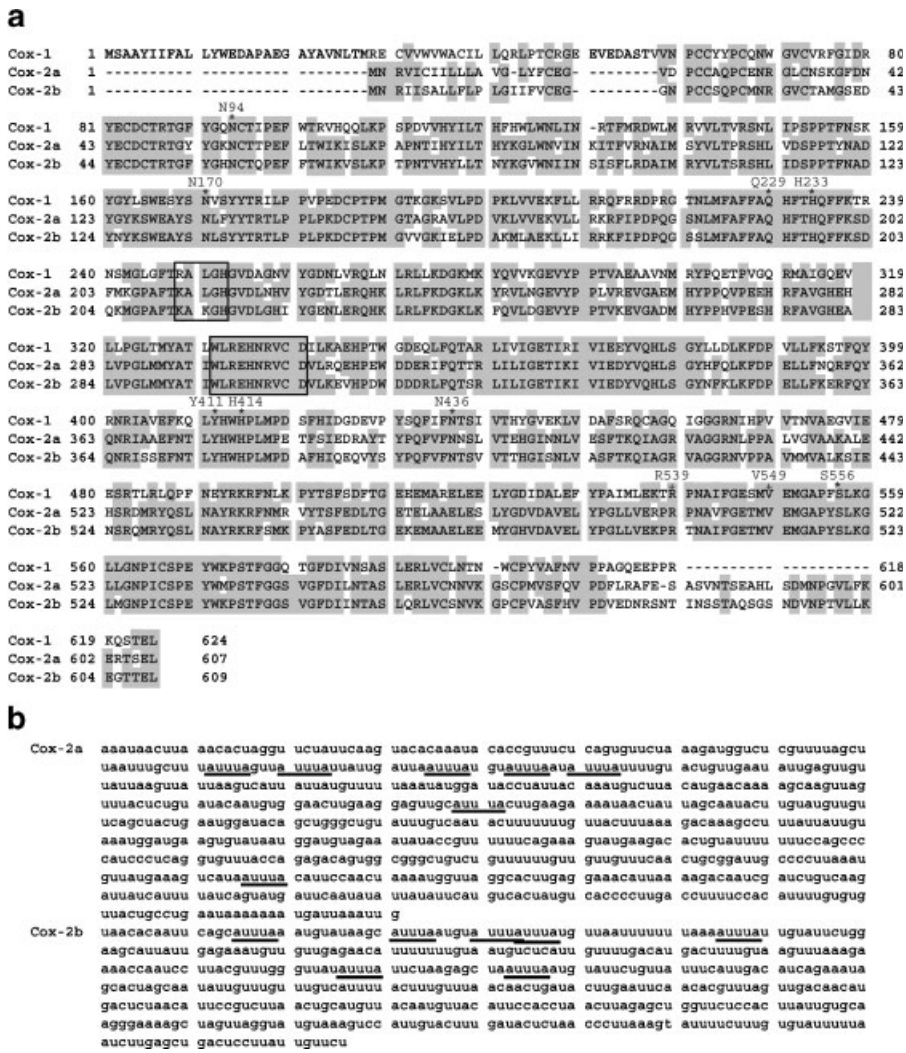

Fig. 1. **a**: Comparison of the deduced amino acid sequences of the Cox-1, Cox-2a, and Cox-2b cyclooxygenases from the rainbow trout. The Cox-1 sequence was obtained from Genbank accession No. AJ299018. Conserved amino acids of the cyclooxygenases are shaded. Functionally important amino acids are indicated by asterisks; these include the cyclooxygenase active site (Tyr-411, His-414, and Ser-556), the N-glycosylation site (Asn-94, -170, and -436), and the sites crucial for peroxidase activity (Gln-229 and His-233). 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 ''+'' (Arg-539 and Val-549 in mammalian Cox-2 proteins). **b**: The sequences of the rainbow trout Cox-2a and Cox-2b 3′ untranslated regions. Shaw–Kamen AUUUA pentamer motifs are underlined.

analyzed in RTG-2 cells treated with various stimulants (Fig. 2a). Neither Cox-2a nor Cox-2b message is detectable in untreated RTG-2 cells. When treated with TPA, both Cox-2a and Cox-2b mRNA are induced. Induction of both Cox-2s was also observed with forskolin and murine IL-1β. In contrast, LPS induces only Cox-2a expression. Despite using two distinct primer sets, rainbow trout Cox-1 expression is not detected, with or without any stimulant, in RTG-2 cells (data not shown). However, because no cDNA corresponding to the Cox-1 rainbow trout sequence reported in Genbank has been described in the literature, we had no cloned DNA to use as a positive control in these experiments.

Mammalian Cox-2 transcriptional induction in cell lines and tissues is inhibited by glucocorticoids [Kujubu and Herschman, 1992]. Both Cox-2a and Cox-2b expressions induced with TPA are inhibited by dexamethasone. All ligand induction and inhibition experiments were performed twice, with similar qualitative results.

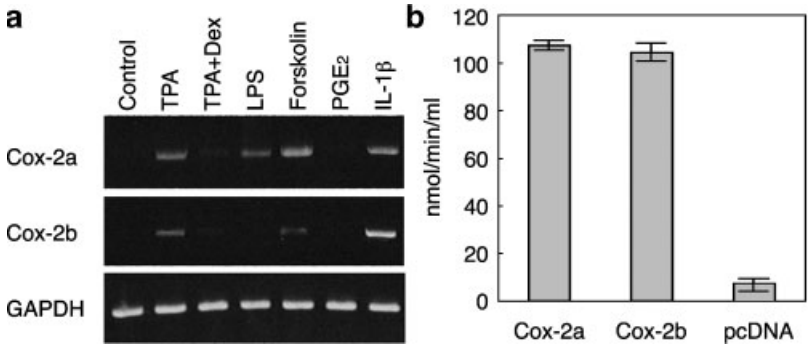

Fig. 2. **a**: Induction of Cox-2a and Cox-2b expression by various stimulants in rainbow trout RTG-2 cells. Cells were treated with TPA [TPA + dexamethasone], LPS, forskolin, PGE_2, or IL-1β for 6 h. mRNA levels for the cyclooxygenase isoforms and for GAPDH were detected by RT-PCR. **b**: Enzymatic activity of the Cox-2a and Cox-2b produced from plasmids expressing the rainbow trout cyclooxygenase isoforms. COS cells were transfected with Cox-2a, Cox-2b, or control pcDNA expression vectors. Two days after transfection, peroxidase enzyme activity in the microsomal fractions was measured. Data are the averages ± S.D. from triplicate assays.

The Rainbow Trout Cox-2a and Cox-2b Genes Both Encode Functional Cyclooxygenases

The rainbow trout Cox-2a gene has not been shown previously to encode an enzymatically active Cox protein. To analyze their enzymatic activity, the ORFs of the rainbow trout Cox-2a and Cox-2b cDNAs were amplified by PCR, subcloned into a mammalian expression vector, and transfected into COS cells. Lysates were prepared from transfected cells and assayed for peroxidase activity characteristic of the Cox (Fig. 2b). Both Cox-2a and Cox-2b-transfected cells contain elevated peroxidase enzymatic activity, demonstrating that the rainbow trout has two functional Cox-2 genes.

Structure of the Zebrafish and Rainbow Trout Cox-2a and Cox-2b Genes

The murine and human Cox-2 genes have 10 exons and 9 introns [Herschman et al., 2003]. The zebrafish Cox-2b gene also has 10 exons and 9 introns. In contrast, the zebrafish Cox-2a gene has one less exon and one less intron; the intron separating exons 4 and 5 is missing and seven amino acids are deleted from the predicted Cox-2a protein sequence [Ishikawa et al., 2007]. Because genomic sequences of rainbow trout Cox-2 genes are not available in existing databases, the exon boundaries for exons 4 and 5 of the rainbow trout Cox-2a and Cox-2b genes were determined by using mammalian Cox-2 genomic structure/sequence as guide sequences. Based on the mammalian Cox-2 gene exon/intron structure, primer sets were designed to amplify across putative exons 4 and 5, to include intron sequences from genomic DNA, for both the rainbow trout Cox-2a gene and the rainbow trout Cox-2b gene. Sequence data for these Cox-2a and Cox-2b genomic amplification products revealed that both the Cox-2a and Cox-2b genes of the rainbow trout contain an intron between putative exons 4 and 5 (Fig. 3). These data suggest that the deletion of the intron separating exons 4 and 5, and 21 nucleotides (a seven amino acid sequence)

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                          Exon 4                Intron 4                Exon 5

zebrafish          T   P   D   -   -   -   -                          -   -   -   L   P
Cox-2a             ACA CCT GAT                                                   CTC CCA

zebrafish          T   P   M   G   V   A   G                          K   K   E   L   P
Cox-2b             ACG CCA ATG GGA GTC GCA G gtgagcct.....tttgatag GT AAG AAA GAG CTG CCA

rainbow trout      T   P   M   G   T   A   G                          R   A   V   L   P
Cox-2a             ACA CCT ATG GGA ACC GCA G gtaagtga.....ccttatag GA AGA GCA GTG CTC CCG

rainbow trout      T   P   M   G   V   V   G                          K   I   E   L   P
Cox-2b             ACC CCT ATG GGA GTC GTA G gtaatcca.....ccatccag GT AAG ATA GAG CTA CCT
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Fig. 3. The sequences of the exon 4/intron 4 and the intron 4/exon 5 borders of the zebrafish and rainbow trout Cox-2b isoforms. Uppercase letters in the DNA sequences represent nucleotides present in exons. Lowercase letters represent nucleotides present in the introns. Amino acids are indicated by single capital letters present above the first nucleotide of each codon.

encoded by exons 4 and 5 in zebrafish Cox-2a, occurred after zebrafish and rainbow trout diverged in evolution.

Using data from our own studies on zebrafish and rainbow trout, published studies from other laboratories, and unpublished data from several gene databases, we propose a sequential scheme (shown in Fig. 4) for gene duplication in an ancestral species, followed by loss of either one of the two Cox-1 genes or one of the two Cox-2 genes for the species shown in the figure. Based on the differences observed between the genomic sequences for the Cox-2a genes of zebrafish and rainbow trout, we also indicate in Figure 4 this distinction between these two species.

DISCUSSION

In this study, we identified a third Cox gene, Cox-2b, in the genome of the rainbow trout *Oncorhynchus mykiss*. Expression of mammalian Cox-2 genes is induced by wide variety of signals, including growth factors, cytokines, endotoxin, and neuronal depolarization, as well as under many pathophysiological conditions such as neuronal degeneration, inflammation, and cancer [Herschman, 2004]. Zebrafish contains two Cox-2 genes that are inducible in response to alternative stimuli [Ishikawa et al., 2007]. In rainbow trout, Cox-2a expression is not constitutive but can be induced in head kidney leucocytes in vivo following challenge with a bacterial pathogen [Zou et al., 1999]. The availability of culture cell lines from the rainbow trout offers a simple, tractable system to more thoroughly study the similarities and

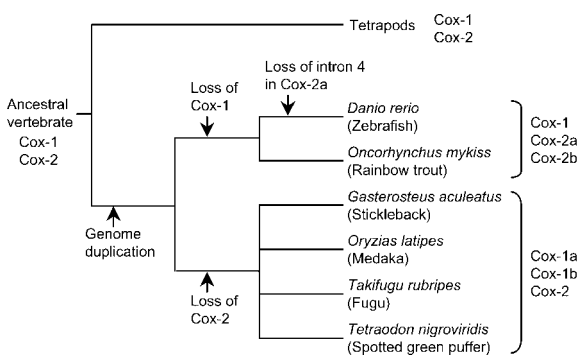

Fig. 4. Schematic view of cyclooxygenase gene isoform variations during the evolution of teleosts. Data obtained from cloning and sequencing experiments in our laboratory, other published reports, and unpublished database sequences were used to construct this hypothetical proposal for cyclooxygenase gene alterations during teleost evolution.

differences in the regulation of the Cox-2a and Cox-2b genes. Cox-2a expression is also induced in RTS11 cells, a macrophage-like cell line from rainbow trout spleen, by LPS [Brubacher et al., 2000], IL-1β [Hong et al., 2001] and antimicrobial peptides [Chiou et al., 2006]. In the RTG-2 cell line, a fibroblast-like cell line from rainbow trout gonad tissue, we find that both Cox-2a and Cox-2b expressions are induced by TPA, forskolin, and IL-1β, demonstrating that rainbow trout expresses two distinct, inducible Cox-2 genes. In contrast, LPS induces Cox-2a, but not Cox-2b, in RTG-2 cells. The different responses of the two Cox-2 genes to this stimulant suggest the two genes may have distinct physiological functions; Cox-2a and Cox-2b may have adapted for different roles during fish evolution, after gene duplication and retention resulted in two functional orthologues. The interpretations of studies that have investigated the role of "Cox-2" in fish development and biology [Cha et al., 2005] may need to be reconsidered in the light of the discovery of two functional Cox-2 genes in the zebrafish and the rainbow trout.

Analyzing publicly available databases suggest that two putative Cox-1 genes and one putative Cox-2 gene exist in fugu (*Takifugu rubripes*), spotted green puffer (*Tetraodon nigroviridis*), and Japanese killifish (*Oryzias latipes*) [Ishikawa et al., 2007]. More recently, genomic database data for the stickleback (*Gasterosteus aculeatus*) have become available. Blast search of this database suggests two putative Cox-1 genes and one putative Cox-2 gene in the stickleback. Identification of each orthologue was determined by sequence similarity and by the presence of ARE sequences in the presumptive 3′UTRs. The presence of alternative Cox-2 and Cox-1 genes in these species suggests that zebrafish and rainbow trout diverged early from the ancestor of puffer fish, killifish, and stickleback (Fig. 4). This divergence in teleost lineage is consistent with the phylogenetic hypotheses based on morphology and molecular analysis [Crollius and Wessenbach, 2005]. The deletion observed in the Cox-2a gene of zebrafish, absent in the rainbow trout Cox-2a gene, will provide a simple, easily analyzable genomic characteristic to map an interesting genetic event in teleost evolution.

The genome sequencing data suggest that a Cox gene probably occurred in ancestral multicellular invertebrates and that Cox(s) play a

role in prostanoid generation in all protostomate and deuterostomate animals. It is currently not known whether primitive vertebrates such as jawed cartilaginous fish (sharks and rays) and the jawless lampreys and hagfishes express one or multiple Cox genes. The Cox cDNA cloned from the shark *Squalus acanthias* is constitutively expressed in a wide range of tissues, and has sequence homology essentially equivalent for mammalian Cox-1 and Cox-2 [Yang et al., 2002]. Although Val-523, present in the shark Cox protein, has been considered characteristic of the mammalian Cox-2 gene and is thought to confer isoform sensitivity to Cox-2 selective inhibitors, valine is present at this position in Cox-1, as well as in Cox-2a and Cox-2b in zebrafish [Grosser et al., 2002; Ishikawa et al., 2007] and rainbow trout ([Zou et al., 1999] and Fig. 1 of our Results). More complete characterization of the genomes of these primitive vertebrates may clarify whether an early vertebrate may have a single Cox gene.

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