Subfunctionalization of Expression and Peptide Domains Following the Ancient Duplication of the Proopiomelanocortin Gene in Teleost Fishes

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The proopiomelanocortin gene (*POMC*) encodes several bioactive peptides, including adrenocorticotropin hormone, α-, β-, and γ-melanocyte-stimulating hormone, and the opioid peptide β-endorphin, which play key roles in vertebrate physiology. In the human, mouse, and chicken genomes, there is only one *POMC* gene. By searching public genome projects, we have found that *Tetraodon* (*Tetraodon nigroviridis*), Fugu (*Takifugu rubripes*), and zebrafish (*Danio rerio*) possess two *POMC* genes, which we called *POMCα* and *POMCβ*, and we present phylogenetic and mapping evidence that these paralogue genes originated in the whole-genome duplication specific to the teleost lineage over 300 MYA. In addition, we present evidence for two types of subfunction partitioning between the paralogues. First, in situ hybridization experiments indicate that the expression domains of the ancestral *POMC* gene have been subfunctionalized in *Tetraodon*, with *POMCα* expressed in the nucleus lateralis tuberis of the hypothalamus, as well as in the rostral pars distalis and pars intermedia (PI) of the pituitary, whereas *POMCβ* is expressed in the preoptic area of the brain and weakly in the pituitary PI. Second, *POMCβ* genes have a β-endorphin segment that lacks the consensus opioid signal and seems to be under neutral evolution in tetraodontids, whereas *POMCα* genes possess well-conserved peptide regions. Thus, *POMC* paralogues have experienced subfunctionalization of both expression and peptide domains during teleost evolution. The study of regulatory regions of fish *POMC* genes might shed light on the mechanisms of enhancer partitioning between duplicate genes, as well as the roles of POMC-derived peptides in fish physiology.

Introduction

Proopiomelanocortin (POMC) is a prohormone that gives rise to several different bioactive peptides through cell-type–specific posttranslational processing. POMC-derived peptides are produced mainly in the pituitary gland and the brain, being involved in processes as divergent as stress, pain, energy balance, and, in fishes and amphibians, adaptation to background color (reviewed in Hadley and Haskell-Luevano 1999; Volkoff et al. 2005). In the pituitary gland, the *POMC* gene is expressed in two types of cells: corticotrophs in the pars distalis, where POMC is processed into adrenocorticotropin hormone (ACTH) and β-lipotropin and melanotrophs in the pars intermedia (PI), where it gives rise to α-melanocyte-stimulating hormone (α-MSH) and β-endorphin. In the vertebrate brain, *POMC* is mainly expressed in the ventral hypothalamus, where POMC is processed into α-MSH, β-MSH, γ-MSH, and β-endorphin.

Genes and cDNAs encoding POMC have been cloned and sequenced from representatives of all vertebrate classes, including several fishes (Takahashi et al. 2001). Birds and mammals have only one *POMC* gene, but some fishes and amphibians possess more than one. Gene duplicates may arise through a variety of mechanisms, ranging from single-gene to whole-genome duplication. The recent sequencing and assembly of the genome of the teleost *Tetraodon nigroviridis* (family Tetraodontidae), as well as studies on gene duplicates in the genome of another tetraodontid, *Takifugu rubripes* (Fugu), give strong support to the idea that an ancient whole-genome duplication occurred in the teleost lineage around 320 MYA (Taylor et al. 2001; Christoffels et al. 2004; Jaillon et al. 2004; Vandepoele et al. 2004). In addition, some fishes like carp, rainbow trout, and sturgeons have undergone recent polyploidization events (Volff 2005).

After a gene is duplicated, one of the copies usually accumulates nonsense mutations and becomes a pseudogene, although sometimes both paralogue copies are retained in the genome. In the case of tetraodontids, the percentage of duplicate genes retained after the proposed teleost-specific duplication has been calculated to be 3%–7% (Christoffels et al. 2004; Jaillon et al. 2004). The retention of the duplicated copies may be accomplished when one of the paralogues acquires a new function in relation to the original gene (neofunctionalization) or, alternatively, the functions of the original gene are partitioned between the new duplicates, as stated in the duplication-degeneration-complementation (DDC) model (Force et al. 1999; Postlethwait et al. 2004). The latter process, known as subfunctionalization, is most often evidenced by the partitioning of the original expression pattern. For instance, if the original gene was expressed in regions A and B, the differential loss of regulatory regions might cause one duplicate to be expressed in region A and the other in region B so that both paralogues are kept under selective pressure and become fixed in the genome. In teleosts, partitioning of expression domains has been observed in several paralogue genes, including the transcription factors Engrailed1 (Force et al. 1999), Hoxb1 (McClintock, Kheirbek, and Prince 2002), and Mitf (Altschmied et al. 2002), retinol-binding proteins (Liu et al. 2005),

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the neuropeptide somatostatin (Low 2003), among others. In addition, subfunctionalization may also involve the differential partitioning of peptide domains (Force et al. 1999).

Here, we present molecular and phylogenetic evidence that higher teleosts possess two *POMC* paralogue genes, which we named *POMCα* and *POMCβ*, that originated in the ancient teleost whole-genome duplication. In addition, we show that both *Tetraodon POMC* gene duplicates have undergone subfunctionalization, as evidenced by their complementary expression patterns in brain and pituitary. Moreover, the peptide repertoire of *POMC* paralogues has also been subfunctionalized, with the loss of β-endorphin from many teleost *POMCβ* genes.

Materials and Methods

Animals

Adult fish from the genus *Tetraodon* (*T. nigroviridis* and *T. fluviatilis*) and zebrafish (*Danio rerio*) were purchased from a local aquarium and processed immediately for nucleic acid extraction or tissue fixation. Experimental protocols were approved by the local animal care and use committees and were consistent with the Guide for the Care and Use of Laboratory Animals.

Reverse Transcriptase–Polymerase Chain Reactions and Sequencing

Total RNA from *Tetraodon* pituitaries and preoptic area was isolated using TRIzol (Invitrogen, Carlsbad, Calif.). For 5′ and 3′ rapid amplification of cDNA ends (RACE) *POMCα* polymerase chain reactions (PCRs), cDNA was prepared using the MATCHMAKER Library Construction & Screening Kit (Clontech, Palo Alto, Calif.) incorporating a SMART oligo at the 5′ end and using CDS III–modified oligo-dT, according to the instructions of the manufacturer. Nested PCRs were performed with the following primer pairs: for 5′ RACE, 5′-ACGCAGAGTGGCCATTATGGCCGGG-3′ (complementary to the SMART oligo)/5′-CATGCTGCTGGCCTCGGAGT-3′ (complementary to exon 2); and for 3′ RACE, 5′-GAGCGCCCACCTCCAACT-3′ (complementary to exon 3)/5′-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA-3′ (complementary to CDS III oligo). To determine the exon-intron structure of *POMC* genes, PCRs were performed with the following primer pairs: for *POMCα*, 5′-CCATCAGCAGCCAGCACGA-3′ (complementary to exon 1)/5′-CCAACAACCGTCATGAGC-3′ (complementary to exon 3); for *POMCβ*, 5′-ACGGCGGGAGGAAAGCAA-3′ (complementary to exon 1)/5′-CAGTATCCTCCTCTTGTC-3′ (complementary to exon 2); and 5′-GCTGATGACGTACCTGTG-3′ (complementary to exon 2)/5′-TGCCCTCGCTCGTCCCTGT-3′ (complementary to exon 3). Gel-purified PCR bands were subjected to automated DNA sequencing.

Immunohistochemistry

Adult fishes were anaesthetized by immersion in a neutral buffered solution of 0.1% (w/v) tricaine methane sulfonate (Sigma, St. Louis, Mo.), and heads were fixed overnight in Bouin's fixative. Brains with attached pituitaries and part of the skull (cranium base) were dissected and washed with 70% (v/v) ethanol for 24 h, cryoprotected with 30% (w/v) sucrose, and frozen in embedding medium (Tissue Tek, Sakura Tek, Torrance, Calif.). Serial transversal and sagital sections (20 μm) were cut using a cryostat microtome (IEC Microtome, Walldorf, Germany) at −20°C. Sections were thaw mounted on Vectabond-coated (Vector Laboratories, Burlingame, Calif.) slides and stored at −70°C until use. Alternatively, some brains were embedded into paraffin and 4- or 10-μm slices were cut in a microtome (SM2000R, Leica, Nussloch, Germany). Immunohistochemistry proceeded as described previously (de Souza et al. 2005) with a polyclonal antihuman ACTH IC-1 antibody (1:300; National Hormone and Pituitary Program, National Institutes of Health).

Radioactive In Situ Hybridization

Cryosections obtained as described above were treated with 10 μg/ml proteinase K in 10 × TE buffer (100 mM Tris, 10 mM ethylenediaminetetraacetic acid [EDTA]) at room temperature (RT) for 15 min and then washed twice with the same buffer. Slides were then treated with 0.1 M triethanolamine (TEA) pH 8.0 for 3 min, incubated in 0.0025% (v/v) acetic anhydride in 0.1 M TEA for 10 min, washed twice in 2 × standard saline citrate (SSC) (NaCl, sodium citrate), and finally dehydrated quickly in ascending ethanol concentrations. For prehybridization, slides were incubated under parafilm coverslips with the hybridization buffer (66% [v/v] formamide, 260 mM NaCl, 1.3 × Denhardt's, 1.3 mM EDTA, 13 mM Tris-HCl pH 8.0, 13% [w/v] dextran sulfate) for 1 h/57°C. [^{35}S]-labeled riboprobes were heated to 65°C/5 min (with 0.5 mg/ml tRNA, 10 mM dithiothreitol [DTT] in water) and added to the hybridization buffer in a concentration of 5 × 10^6–10^7 cpm/ml. Hybridization followed overnight at 57°C. Next day, slices were washed four times in 4 × SSC and subjected to ribonuclease (RNase) A digestion (20 μg/ml RNase A, 0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) at 37°C/30 min, desalted in 2 ×, 1 ×, 0.5 × SSC/1 mM DTT, and subjected to a final wash in 0.1 × SSC/1 mM DTT at 30 min/65°C. After a quick rinse in 0.1 × SSC/1 mM DTT RT, slides were dehydrated through 5-min steps in ethanol at 70%, 95%, and 100% (v/v), dried at room temperature, dipped in Kodak N-TB2 emulsion (diluted 1:1 in distilled water), and exposed for 3–6 days. Slides were developed, counterstained with cresyl violet (Sigma), dehydrated with ethanol, and coverslipped under Permount (Fisher Scientific, Pittsburgh, Pa.). To prepare riboprobes, PCR fragments of exon 2 of *Tetraodon POMCα* (123-bp product; TE2SE: 5′-CCAGAATGAGTCCTGCGTGGCT-3′ and TE2AN: 5′-CATGCTGCTGGCCTCGGAGT-3′) and exon 3 of *POMCβ* (302-bp product; 2TPE3SE4: 5′ CGGAGCAGCAACAGGGAC 3′ and 2TPE3AN2: 5′ AACGCCTGCGGGCACGGA-3′) were cloned into pGEMT-Easy vector (Promega, Madison, Wisc.). Antisense RNA probes were synthesized by incubating 1 μg of linearized plasmid with transcription buffer (40 mM Tris-HCl, pH 8.0, 15 mM MgCl2, 5 mM DTT, 0.05 mg/ml bovine serum albumin), 10 mM DTT, 60 U RNase out (Invitrogen), 0.5 mM of adenosine triphosphate, cytidine triphosphate, and guanosine

triphosphate, 1,000 μCi α[^{35}S]UTP (1,250 Ci/mmol; Perkin-Elmer, Wellesley, Mass.), and 16 U of T7 polymerase (Amersham Biosciences, Piscataway, N.J.). After 70 min of incubation at 37°C, plasmid was digested with DNAse I (Invitrogen). Riboprobes were precipitated with 10 μg tRNA, 0.1 vol 5 M NH_4 acetate, and 2.5 vol 100% (v/v) ethanol for 1 h at −70°C, washed with 70% (v/v) ethanol, and resuspended in RNAse-free water.

Sequences and Databases

The genomic regions around *POMC* loci from Fugu and *Tetraodon* were located via Blast searches (Altschul et al. 1990) in the Ensembl database Web site (http://www.ensembl.org/index.html) using *POMC* sequences from other species. *POMCα* loci are present in the scaffolds 970 in Fugu and 10884 in *Tetraodon*, while *POMCβ* loci are located in scaffold 130 in Fugu and chromosome 14 in *Tetraodon*. A complete cDNA sequence for Fugu *POMCα* was assembled from expressed sequence tag (EST) clones EFRz001apsG3, EFRa090apsE11, and EFRz002apsD6 retrieved from the Fugu Home Page of the Rosalind Franklin Centre for Genomic Research (http://www.hgmp.mrc.ac.uk). A cDNA sequence for *Tetraodon POMCβ* (clone name FD0ADA50CB12AAP1) was retrieved from the Genoscope Home Page (http://www.genoscope.cns.fr/externe/tetranew). Data on the *POMC* neighborhood in chicken (*Gallus gallus*) and humans were obtained from the Ensembl and Mapviewer Web sites (http://www.ncbi.nih.gov/mapview/), respectively. Zebrafish *POMCα* and *POMCβ* were located, respectively, in sequences AL590149 (from linkage group [LG] 17) and AL845420 (from LG 20) deposited in GenBank (http://www.ncbi.nih.gov). We also retrieved from GenBank the POMC peptide and cDNA sequences from the following species and accession numbers: *Verasper moseri* (barfin flounder) POMC-A (AB051424); POMC-B (AB051425) and POMC-C (AB051426); *Oncorhynchus mykiss* (rainbow trout) POMC-A (X69808) and POMC-B (X69809); *Oncorhynchus keta* (chum salmon) (X01122); *Polypterus senegalus* (bichir) (AF465781); *Acipenser trasmontanus* (sturgeon) POMC-A (AF092937) and POMC-B (AF092936); *Polyodon spathula* (paddlefish) POMC-A (AF117302) and POMC-B (AF117303); *Cyprinus carpio* (common carp) POMC-I (Y14618) and POMC-II (Y14617); *D. rerio* (zebrafish) (NM_181438); *Homo sapiens* (man) (NM_000939); *Carassius auratus* (goldfish) (AJ431209); *Ictalurus puntactus* (channel catfish) (AY174050); *Paralichthys olivaceus* (bastard halibut) POMC-I (AF184066) and POMC-II (AF191593); *Anguilla japonica* (Japanese eel) (AY158010); and *Anguilla rostrata* (American eel) (AF194969).

Evolutionary Analyses

POMC peptide sequences were aligned with the ClustalW program (http://www.ebi.ac.uk/clustalw) (Thompson, Higgins, and Gibson 1994). The alignment used for phylogenetic inference using the PHYLIP 3.62 Program Package (Felsenstein 2004) is shown in Supplementary Figure 1 (Supplementary Material online). For maximum-likelihood (ML) phylogenetic inference, the PROTML program was used with the amino acid substitution model of Jones, Taylor, and Thornton (1992) using 100 replicates generated with the SEQBOOT program. A consensus tree was created with CONSENSE. Ka/Ks ratios were calculated using the DnaSP 4.0 program (J. Rozas and R. Rozas 1999) downloaded from http://www.ub.es/dnasp.

Results

Identification of Duplicate *POMC* Genes in Teleost Fishes

We searched the public genome projects of *Tetraodon* (Jaillon et al. 2004) and Fugu (Aparicio et al. 2002) using the Ensembl database Web site. In each tetraodontid genome, we identified two *POMC* genes which we named *POMCα* and *POMCβ*, according to their identity to mammalian POMC at the peptide level (45% and 35%, respectively). In the *Tetraodon* genome project, *POMCβ* has been assigned to chromosome 14, while *POMCα* is found in scaffold 10884 of unknown location. In the Fugu genomic database, where scaffolds have not yet been assigned to chromosomes, *POMCα* and *POMCβ* are located in scaffolds 970 and 130, respectively.

The complete cDNA from Fugu *POMCα* was assembled from ESTs retrieved from the Fugu database of the Rosalind Franklin Centre for Genome Research, and this sequence was used to deduce the exon-intron structure of both Fugu and *Tetraodon POMCα* genes. Reverse transcriptase (RT)–PCR and 5′/3′-RACE reactions using *Tetraodon* pituitary cDNA were done to confirm exon-intron boundaries and the transcriptional start site. The structure of tetraodontid *POMCβ* genes was defined using a *Tetraodon POMCβ* cDNA clone from the Genoscope Web site, and exon-intron boundaries were confirmed by sequencing RT-PCR fragments. Both tetraodontid *POMC* genes have three exons: exon 1 is noncoding, exon 2 contains the starting ATG, and most of the coding region lies in exon 3 (fig. 1*A*). This organization is remarkably similar to that of *POMC* genes of all tetrapods studied to date.

A gene and a cDNA encoding zebrafish POMC have been described previously (Hansen et al. 2003; Liu et al. 2003). In addition, González-Nuñez, González-Sarmiento, and Rodríguez (2003) have reported a second, incomplete cDNA corresponding to a more divergent POMC sequence, isolated from a brain cDNA library. Based on the phylogeny of the genes (see below), we propose to call these zebrafish genes *POMCα* and *POMCβ*, respectively. Searching the GenBank database, we identified zebrafish genomic scaffolds of LGs 17 and 20 that contain *POMCα* and *POMCβ*, respectively. Comparing the reported partial *POMCβ* cDNA (González-Nuñez, González-Sarmiento, and Rodríguez 2003) with genomic sequence, we identified two exons which, as judged from sequence alignments, contain the whole coding region of zebrafish *POMCβ*. These exons correspond probably to exons 2 and 3 because all *POMC* genes studied to date contain a noncoding exon 1. A putative exon 1 could not be identified from sequence comparisons.

Characterization of Duplicate POMC Peptides

Figure 1*B* shows an alignment of deduced *Tetraodon*, Fugu, and zebrafish POMCα and POMCβ amino acid

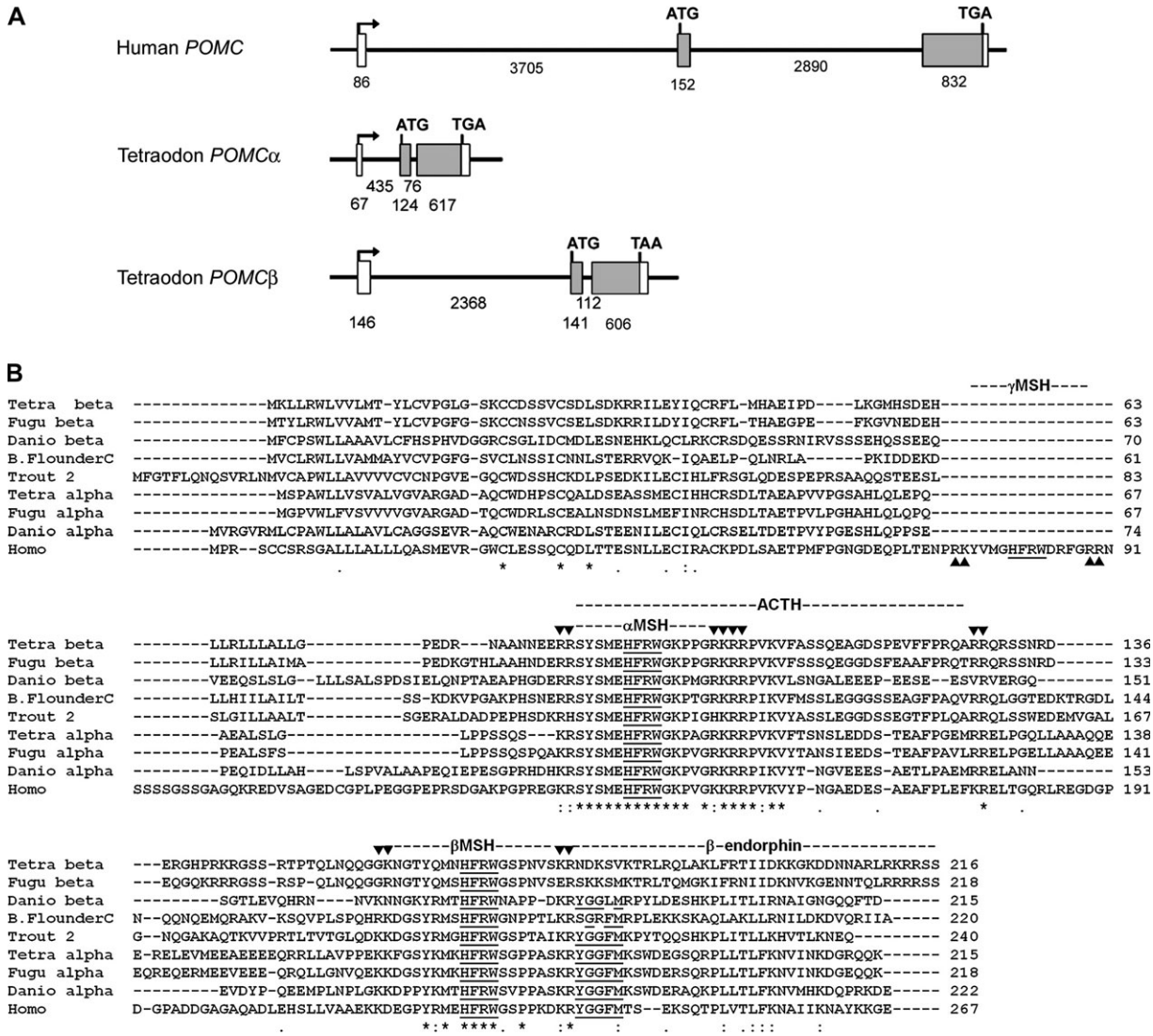

FIG. 1.—(*A*) Organization of *Tetraodon POMC* paralogues compared to the human *POMC* gene. Note the shorter size of introns in *Tetraodon*. (*B*) Alignment of *Tetraodon* POMC amino acid sequences with those of different vertebrates. Peptides derived from POMC are shown on top of the sequences. Putative dibasic cleavage sites are indicated by arrowheads. Asterisks and dots below the sequences indicate identical and conservative residues, respectively. Melanocortin and opioid core sequences are underlined. Note that both teleost POMCα and POMCβ lack γ-MSH and that *Tetraodon* and Fugu POMCβ lack the opioid signal.

sequences and those of other representative vertebrates. We have included in the alignment the sequences of the reported POMC-B from rainbow trout (*O. mykiss*—Salbert et al. 1992) and the POMC-C from barfin flounder (*Verasper moseri*—Takahashi et al. 2005), which we have assigned to the POMCβ group. In general, conservation of typical POMC features is high between teleost POMCα sequences but lower in POMCβ sequences. The amino acid sequence of α-MSH is highly conserved (93%–100%) in POMCα and POMCβ in all species (fig. 1*B*). β-MSH is also conserved, but zebrafish POMCβ lacks a dibasic cleavage site at the prospective amino-terminal of the peptide (fig. 1*B*). The hormone ACTH, which encompasses α-MSH and CLIP, is potentially derived from POMCα and POMCβ in all species except zebrafish POMCβ, which lacks a dibasic cleavage site at the C-terminal of the prospective peptide (fig. 1*B*; González-Nuñez, González-Sarmiento, and Rodríguez 2003). Amino acid identity between human and teleost ACTH is 74%–77% for POMCα and 59% for POMCβ sequences. Both teleost POMCα and POMCβ sequences lack the region corresponding to γ-MSH (fig. 1*B*), a third melanocortin which is present in other vertebrate POMC sequences (Dores and Lecaude 2005).

The region corresponding to the β-endorphin peptide is more conserved in teleost POMCα than POMCβ sequences (fig. 1*B*). Amino acid identity between human and teleost β-endorphins ranges from 59% to 67% for POMCα and only 20%–44% for POMCβ. The N-terminal met-enkephalin sequence of β-endorphin, which contains the opioid motif YGGFM, presents several degrees of degeneration in teleost POMCβ sequences. The motif is changed to YGGLM in zebrafish POMCβ (González-Nuñez, González-Sarmiento, and Rodríguez 2003) and to SGRFM in barfin flounder POMC-C (Takahashi et al.

2005; fig. 1*B*). Strikingly, the opioid motif is completely absent from POMCβ β-endorphin sequences in *Tetraodon* and Fugu (fig. 1*B*). Because the opioid signal is necessary for stimulation of the opioid receptors (Reinscheid et al. 1995; Waldhoer, Bartlett, and Whistler 2004), it is likely that *POMCβ* from tetraodontids and, possibly, barfin flounder and zebrafish are incapable of generating a β-endorphin with opioid activity. Nevertheless, β-endorphin peptides containing the canonical opioid signal are present in the POMC-B sequences of trout (fig. 1*B*) and salmon, indicating that the peptide repertoire of POMCβ sequences varies among teleosts.

The absence of opioid activity in β-endorphin of *Tetraodon* and Fugu POMCβ indicates that the C-terminal of POMCβ might be nonfunctional and, consequently, might be accumulating a high number of nonsynonymous mutations. To check this possibility, we calculated the ratio of the number of nonsynonymous substitutions per nonsynonymous sites (Ka) to the number of synonymous substitutions per synonymous sites (Ks) (Hurst 2002) of the β-endorphin regions of tetraodontid POMCα and POMCβ genes. β-endorphins from POMCα showed a Ka/Ks much lower than 1 (Ka/Ks = 0.12), indicating that this region is under purifying selection in POMCα genes. In contrast, β-endorphins of POMCβ have a Ka/Ks slightly higher than 1 (Ka/Ks = 1.26), suggesting that the β-endorphin region of POMCβ came under relaxed selection in the tetraodontid lineage. ACTH regions, in contrast, seem to be under purifying selection in both tetraodontid POMCα (Ka/Ks = 0.36) and POMCβ (Ka/Ks = 0.17) genes. The unique *POMC* gene of distantly related sarcopterygians like humans and Australian lungfish (*Neoceratodus forsteri*, Dores et al. 1999) is under strong purifying selection in both the β-endorphin (Ka/Ks = 0.15) and ACTH (Ka/Ks = 0.07) regions. We found no strong evidence for relaxed or positive selection in ACTH or β-endorphin regions in other fish lineages.

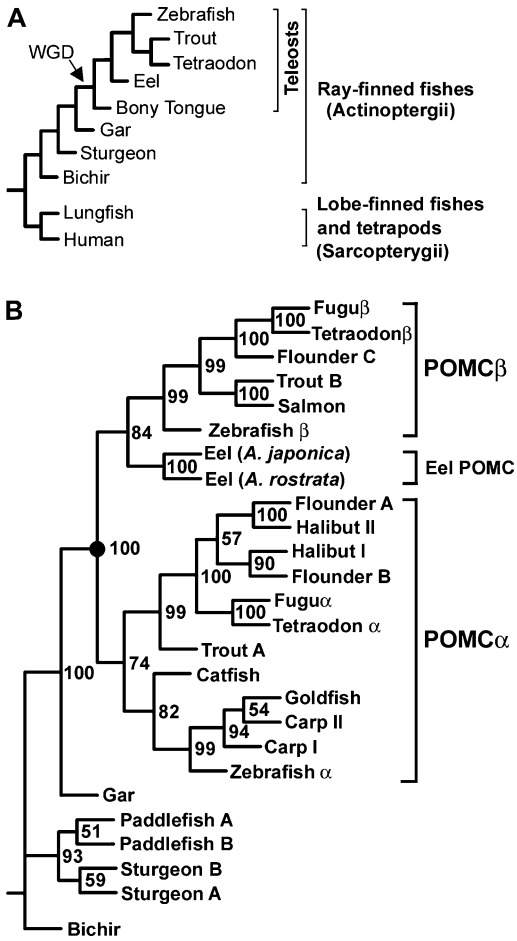

FIG. 2.—Phylogenetic relationships between POMC sequences. (*A*) Putative phylogenetic relationships between relevant vertebrate groups adapted from Kikugawa et al. (2004) and Inoue et al. (2004). Putative timing of teleost whole-genome duplication (WGD) (Hoegg et al. 2004) is indicated by an arrow. (*B*) ML phylogenetic tree of POMC amino acid sequences of ray-finned fishes. Percentage bootstrap values (100 replicates) are indicated. The node grouping teleost POMC sequences is highlighted with a black circle, and POMCα and POMCβ clades are indicated. See *Materials and Methods* for details on the species.

Phylogenetic Analysis of Teleost *POMC* Genes

POMC cDNAs have been sequenced from several phylogenetically diverse species of fishes, and POMC duplicates have been identified in some of them. To understand the origin of POMC paralogues in teleost genomes, we constructed phylogenetic trees of POMC amino acid sequences using ML analyses. Figure 2*B* shows an ML phylogenetic tree of POMC peptides of ray-finned fishes (Actinopterygii). Supplementary Figure 1 (Supplementary Material online) shows the alignment used to build this tree. To serve as reference, figure 2*A* shows the phylogenetic relationships between selected fishes adapted from Kikugawa et al. (2004) and Inoue et al. (2004). Recent molecular phylogenetic studies agree on the relationships of most groups shown, but there is controversy over the relationship between Semionotiformes (gar) and Acipenseriformes (sturgeons and paddlefishes). For a similar but slightly different molecular phylogeny, see Inoue et al. (2003). The POMC phylogenetic tree shown in figure 2*B* corresponds roughly to the phylogenetic relationships between the fish species. POMCα and POMCβ sequences from euteleosts (higher teleosts), as well as POMC sequences from two eel species (which are basal teleosts), are grouped in a branch with 100% bootstrap support (fig. 2*B*). Importantly, the analysis supports (99% bootstrap) a branch with the POMCβ sequences of zebrafish, *Tetraodon*, and Fugu, together with rainbow trout POMC-B, barfin flounder POMC-C, and a POMC sequence from chum salmon. POMCα sequences from euteleosts are grouped in another branch, being monophyletic in the tree, although with relatively low statistical support (74%). The available eel POMC sequences cluster with the POMCβ branch with moderate statistical support (84% bootstrap).

Our phylogenetic results indicate that the duplication event that originated the *POMCα* and *POMCβ* paralogues happened in the teleost lineage, after the branching of the longnose gar lineage. Within euteleosts, the duplication seems to have happened before the separation of the superorders Ostariophysii (zebrafish, goldfish, and carp), Protacanthopterygii (salmon and trout), and Acanthopterygii (*Tetraodon*, Fugu, barfin flounder, and halibut). POMC sequences from eels, which are not considered euteleosts,

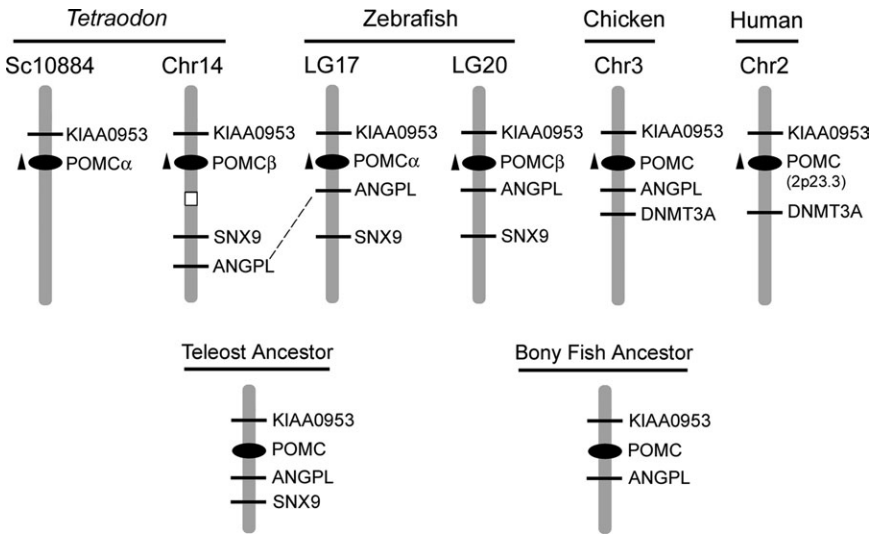

FIG. 3.—Analysis of *POMC* neighborhood in different vertebrate genomes. Genomic scaffolds and chromosomes where the loci are located in each species are indicated. Gene neighborhoods around Fugu *POMC* loci (not shown) are identical to that of *Tetraodon*. Genes were named according to the human orthologs. Only genes showing homology to others are shown (black bars). White square denotes omitted intervening genes. The dotted line denotes change in gene order. Arrowheads show the direction of transcription of *POMC* genes. Hypothetical reconstructions of *POMC* gene neighborhood in the ancestor of *Tetraodon* and zebrafish (teleost ancestor) and teleosts and tetrapods (bony fish ancestor) are shown.

are grouped in the same branch as euteleost POMCα and POMCβ, suggesting that the *POMC* gene duplication happened before the branching of elopomorphs (eels and relatives) from the lineage that led to euteleosts (see *Discussion*).

Apart from the duplication that originated POMCα and POMCβ, there have been other duplications involving the *POMC* gene in the history of teleosts. As evidenced in fig. 2*B*, there are duplicates of POMCα (POMC-I and -II) in the halibut and carp. In the case of barfin flounder, there are three POMC sequences reported: two belonging to the POMCα group (POMC-A and -B) and another to the POMCβ group (POMC-C). In addition, there are two similar POMC duplicates in the primitive chondrostean fishes sturgeons and paddlefish (POMC-A and -B). These very similar pairs of *POMC* genes in teleosts and chondrosteans may have arisen in recent, independent genome duplications in these lineages (see *Discussion*).

Neighborhood of *POMC* Genes

To determine whether *POMCα* and *POMCβ* originated in a single-gene duplication or in a higher order event (segmental, chromosomal, or whole-genome duplication), we compared the gene content around *POMC* loci from several species. *POMCα* and *POMCβ* genes from fish, as well as the *POMC* genes from mammals, chicken, and the frog *Xenopus tropicalis* are all flanked at the 3′ end by a 21-exon gene of unknown function, dubbed *KIAA0953* (fig. 3). *KIAA0953* encodes a protein similar to a *Drosophila* transmembrane lipase (Huang et al. 2004). Phylogenetic analyses show that the *KIAA0953* genes downstream of all *POMC* genes are homologues (not shown). At the 5′ side, both zebrafish *POMC* genes and the unique chicken *POMC* gene in chromosome 3 are flanked by a gene encoding an angiopoietin-like factor (*ANGPL*, fig. 3). *Tetraodon* and Fugu *POMCβ* genes also have an *ANGPL* gene located at the 5′ side, although separated from *POMCβ* by a few intervening genes. Phylogenetic analyses show that the angiopoietin-like factors near the *POMC* genes from zebrafish, tetraodontids, and chicken are true homologues (data not shown). In addition, the gene encoding *sortin nexin 9* (*SNX9*) is conserved at the 5′ side of teleost *POMC* genes (fig. 3).

We conclude that the gene order *ANGPL-POMC-KIAA0953* was likely found in the last common ancestor of ray-finned and lobe-finned fishes (fig. 3), which lived 400–450 MYA (Kumar and Hedges 1998). In addition, the gene order *SNX9-ANGPL-POMC-KIAA0953* was probably present in the ancestor of higher teleosts. The conservation of gene content around duplicated *POMC* loci in teleosts indicates that these paralogues are not the result of a single-gene duplication, but rather of a higher order duplication event. The possibility of a chromosomal or genome duplication is further supported by the fact that the zebrafish *POMC* loci are located in different chromosomes.

Partitioning of Expression Domains of *POMC* in *Tetraodon*

The persistence of two ancient *POMC* duplicates in teleosts suggests that each of them performs a unique function. Because it has been observed that gene duplicates have frequently partitioned their expression domains, we decided to check this possibility for *POMC* paralogues. At first, to verify which regions express the POMC protein, we performed immunohistochemistry on slices of brain and pituitary of adult *Tetraodon* using an anti-ACTH antibody that also recognizes the whole POMC prohormone. Because the ACTH region is very similar in both *Tetraodon POMC* paralogues (fig. 1*B*), it is expected that the antibody will recognize the products of both genes.

As shown in figure 4, ACTH-positive perykaria were found in three regions: (1) the pituitary gland, (2) the nucleus

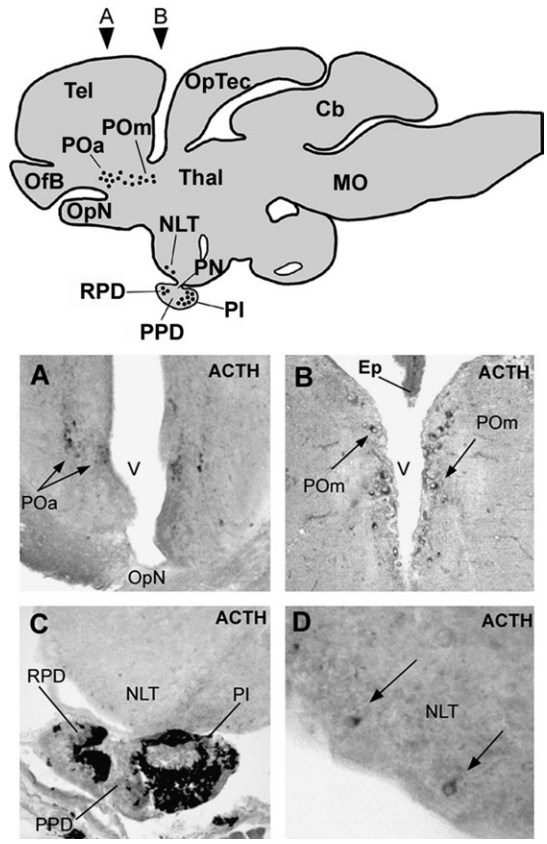

FIG. 4.—ACTH immunoreactivity in *Tetraodon* brain and pituitary. The ACTH antibody recognizes the whole POMC prohormone. Top drawing illustrates the location of POMC-expressing cell groups. Anterior is to the left in the drawing and panels (*C*) and (*D*). Arrowheads indicate the planes of the transversal cuts in panels (*A*) and (*B*). (*A*) Transversal cut at the level of the anterior preoptic area (POa) showing ACTH-immunoreactivity cell groups (arrows) around the diencephalic ventricle (V). (*B*) Transversal cut showing ACTH-positive cells in the magnocellular part of the preoptic area (POm, arrows). (*C*) Sagital cut showing ACTH immunoreactivity in the rostral pars distalis (RPD) and pars intermedia (PI) of the pituitary. (*D*) ACTH-positive cells (arrows) in a sagital cut of the nucleus lateralis tuberis (NLT). Other abbreviations: Cb, cerebellum; Ep, epiphysis; OfB, olfactory bulb; OpN, optic nerve; OpTec, optic tectum; MO, medulla oblongata; PPD, posterior pars distalis; PN, pars nervosa; Tel, telencephalon; and Thal, thalamus.

lateralis tuberis (NLT) in the ventral hypothalamus, and (3) the preoptic nucleus area (POA). In the pituitary, POMC is found in the rostral pars distalis (RPD), presumably in corticotrophs, as well as in melanotrophs of the PI. No staining was observed in the pars nervosa (PN) or in the posterior pars distalis (PPD). In the ventral hypothalamus, we detected ACTH-like immuoreactivity in a few cells of the NLT. This pattern of expression in the pituitary and NLT of *Tetraodon* is similar to that of other fish like the barfin flounder (Amano et al. 2005), zebrafish (Herzog et al. 2003; Liu et al. 2003), and the cichlid *Cichlastoma dimerus* (Pandolfi et al. 2003). In addition to expression in these two areas, we observed ACTH immunoreactivity in the preoptic area, both in the anterior periventricular area as well as in the more dorsoposterior magnocellular region (POm). ACTH immunoreactivity in the preoptic area has already been observed in the carp (Metz et al. 2004) and other nonteleost vertebrates (see *Discussion*).

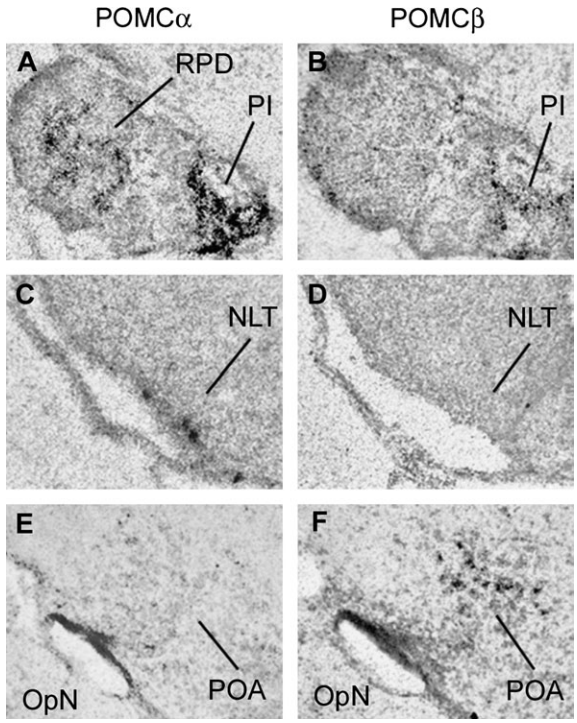

FIG. 5.—Radioactive in situ hybridization of *POMC* paralogues in the brain and pituitary of *Tetraodon*. Riboprobes are complementary to the exon 2 of *POMCα* (left panels) and the exon 3 of *POMCβ* (right panels). All panels show sagital cuts, anterior to the left, dorsal is up. (*A*, *B*) Pituitary cuts. RPD, rostral pars distalis; PI, pars intermedia. (*C*, *D*) Nucleus lateralis tuberis (NLT) cuts. (*E*, *F*) Preoptic area (POA) cuts; OpN, optic nerve. Note that *POMCα* is expressed in the RPD, PI, and NLT, while *POMCβ* is expressed in the POA and weakly in the PI.

To check whether the expression patterns of *POMC* might have been partitioned between the paralogues, we perfomed radioactive in situ hybridization with specific *POMCα* and *POMCβ* antisense riboprobes on slices of adult *Tetraodon* brains and pituitaries. As shown in figure 5, *POMCα* is strongly expressed in pituitary RPD and PI, as well as in a few cells of the NLT. No expression was observed in preoptic nuclei or in other brain areas. *POMCβ*, on the other hand, is expressed in the POA and in the pituitary PI, while no expression was detected in the pituitary RPD, NLT, or other brain area. In the pituitary PI, expression of *POMCα* is much higher than that of *POMCβ* (compare fig. 5*A* and *B*). The combined expression patterns of *POMCα* and *POMCβ* correspond to the distribution of ACTH-positive perikarya in *Tetraodon* pituitary and brain. Because there is strong evidence that *POMC* was expressed in the POA, NLT, and pituitary RPD and PI of ancestral ray-finned fishes (see *Discussion*), these results indicate that the expression domains of the *POMC* gene were partitioned between *POMCα* and *POMCβ* during the evolution of *Tetraodon*.

Discussion

In this work, we show that: (1) *Tetraodon* and Fugu have two *POMC* genes, *POMCα* and *POMCβ*; (2) these two genes are the product of an ancient duplication and are present in all higher teleosts; (3) the expression domains

of the primitive *POMC* gene have been partitioned, with *POMCα* being expressed in the NLT and pituitary RPD and PI, and *POMCβ* in the POA and pituitary PI; and (4) the peptide repertoire of *POMC* has also been subfunctionalised because a bona fide β-endorphin is lacking in POMCβ in tetraodontids and other lineages.

Phylogeny of *POMC* Duplicates in Teleosts

Molecular phylogenetic studies indicate that, among higher teleosts, one of the first groups to diverge from the lineage leading to the Acanthopterygii (which includes *Tetraodon*, Fugu, halibut, and barfin flounder) was the Ostariophysii (zebrafish, carps, catfishes, and their relatives), followed by the Protacanthopterygii (salmons and trouts) (fig. 2*A*). In our phylogenetic analyses, POMCβ sequences from fishes as divergent as zebrafish, salmon, and *Tetraodon* are grouped together in a branch clearly separated from their α paralogues, which also tend to group together. Thus, the duplication that originated the two *POMC* genes likely occurred in a common ancestor to all higher teleosts and, therefore, *POMCα* and *POMCβ* genes will probably be present in the genomes of all 22,000 higher teleost species. A recent inventory of the melanocortin system in Fugu identified only one *POMC* gene (Klovins et al. 2004), which corresponds to *POMCα*. The low level of nucleotide identity of Fugu *POMCβ* to other *POMCs* has probably prevented the identification of this gene in that study.

Recently, the hypothesis of an ancient, teleost-specific tetraploidization followed by rediploidization has gained much support (reviewed in Postlethwait et al. 2004; Volff 2005). In Fugu and zebrafish, phylogenetic analyses of duplicate paralogues suggest that an extensive gene duplication event, most likely a tetraploidization, happened around 320 MYA (Taylor et al. 2001; Christoffels et al. 2004; Vandepoele et al. 2004). In addition, analyses of *Tetraodon* and zebrafish genomes show that many syntenic segments (paralogons) occurring once in mammalian genomes are found duplicated in teleost genomes (Woods et al. 2000; Jaillon et al. 2004). Because *POMCα* and *POMCβ* seem to be common to all higher teleosts, it is likely that these genes are derived from the ancient teleost tetraploidization. This possibility is supported by the conservation of gene content around *POMC* paralogues in tetraodontids and zebrafish, which rules out the possibility of a single-gene or retrovirus-mediated duplication of *POMC*. In addition, the fact that the zebrafish *POMC* loci are found in different LGs, i.e., in chromosomes 17 and 20, is also compatible with a whole-genome duplication. Importantly, zebrafish LGs 17 and 20 have other genetic markers in common (*snap25*, *bmp2*, *sox11*), consistent with their being, to a great extent, sister chromosomes originated in the teleost genome duplication (fig. 4 in Woods et al. 2000; Taylor et al. 2003).

The phylogenetic timing of the genome duplication in the evolution of teleosts is still not known. Recently, Hoegg et al. (2004) analyzed duplicated genes from basal ray-finned fishes and basal teleosts (bony tongues and eels) and concluded that the genome duplication happened after the separation of the bichir, sturgeon, and gar, but before the separation of bony tongues (Osteoglossomorpha) and eels (Elopomorpha) from the higher teleost lineage (see fig. 2*A*), even though this hypothesis and the timing of 320 MYA for the genome duplication do not agree with the available actinopterygian fossil record (Benton 2000; see Discussion in Christoffels et al. 2004 and Hoegg et al. 2004). Our results are compatible with the phylogenetic dating proposed by Hoegg et al. (2004) because the POMC of eels (*Anguilla*) are grouped tightly within the branch of POMCα and POMCβ from higher teleosts (fig. 2*B*). In addition, there were duplications of the *POMC* gene which occurred independently from the duplication that originated *POMCα* and *POMCβ*. It is likely that many of these extra *POMC* genes are the result of more recent whole-genome duplications, which are known to have happened in sturgeons (Ludwig et al. 2001), salmonids (Palti et al. 2004), and some cyprinids like carp and goldfish (David et al. 2003).

Subfunctionalization of *POMC* Duplicates

The *POMC* gene plays several roles in vertebrate physiology. Among its functions are the regulation of serum glucocorticoid levels (mediated by pituitary ACTH), pain sensitivity (mediated by brain β-endorphin), food intake (mediated by brain melanocortin peptides α-MSH, β-MSH, and γ-MSH), and the control of skin pigmentation (mediated by pituitary α-MSH). This diversity of functions is, to a great extent, a consequence of the modular structure of the POMC prohormone, which can give rise to several bioactive peptides in different cell types. The POMC peptide repertoire has undergone different specializations in some vertebrate groups. For example, cartilaginous fishes have an extra melanocortin, δ-MSH, not present in other lineages (Dores and Lecaude 2005). In another case, the segment encoding γ-MSH, which is found in all vertebrate groups, has been lost from teleost POMC before the divergence of eels (Takahashi et al. 2001; Dores and Lecaude 2005). Both POMCα and POMCβ lack γ-MSH, suggesting that the degeneration of this melanocortin took place before the whole-genome duplication in the teleost lineage. Interestingly, mammalian γ-MSH binds preferentially to the MC3R, a receptor that is absent from the genome of tetraodontids (Klovins et al. 2004).

More importantly, we observed a high degree of degeneration of the β-endorphin–coding sequence in teleost POMCβ prohormones (fig 1*B*). β-endorphin is characterized by the N-terminal sequence YGGFM (or close variants), which is necessary for opioid receptor binding (Reinscheid et al. 1995; Waldhoer, Bartlett, and Whistler 2004). The absence of this canonical motif suggests that tetraodontid POMCβ peptides cannot stimulate opioid receptors. The missing opioid signal and the fact that these regions seem to be evolving neutrally (Ka/Ks around 1) suggest that the β-endorphin regions of tetraodontid POMCβ sequences are presently nonfunctional, in contrast to the α-MSH/ACTH region, which has strong sequence conservation and low evolving rate (Ka/Ks $\ll$ 1). In other teleosts, β-endorphin peptides from POMCβ show varying degrees of degeneracy: opioid signatures of POMCβ are mutated in zebrafish and barfin flounder but are conserved in salmonids. POMCα, on the other hand, has retained a conserved β-endorphin peptide in all teleosts. Given that salmonids occupy an intermediate phylogenetic position

among euteleosts (fig. 2*A*), it seems that the β-endorphin region of *POMCβ* started degenerating independently in several lineages, probably as a consequence of compensation provided by *POMCα* and/or the partitioning of *POMC* expression domains between the two paralogues. The partitioning of the peptide repertoire between ancient POMC paralogues is one of the few examples of subfunctionalization of peptide functional domains, as suggested by Force et al. (1999). The unusual modular structure of the POMC prohormone, with its distinct functional peptides, makes it an ideal target for this sort of subfunctionalization. The evolution of POMCβ β-endorphin is reminiscent to that of γ-MSH, which also shows distinct levels of degeneration in different lineages of ray-finned fishes: sturgeons and gar have a recognizable but degenerating γ-MSH sequence, while teleosts lack this melanocortin completely (Dores and Lecaude 2005).

In addition to the subfunctionalization of peptide domains, the expression patterns of *POMCα* and *POMCβ* in the brain and pituitary of *Tetraodon* also indicate subfunctionalization. *POMCα* mRNA is detected in the PI and rostral pars distalis in the pituitary and in the NLT in the hypothalamus. *POMCβ* mRNA, on the other hand, is detected in the preoptic area (POA) in the brain and, weakly, in the PI of the pituitary. What about other teleosts? In the case of zebrafish, in situ hybridization using a *POMCα* riboprobe detected expression in the pituitary and ventral hypothalamus but not in the POA (Herzog et al. 2003; Liu et al. 2003). However, ACTH- and β-endorphin–like immunoreactivity is detected in the POA of a close species, the common carp (Metz et al. 2004). Thus, the partitioning of *POMC* expression domains might be a feature shared by other teleosts.

In mammals, *POMC* mRNA is mainly detected in the pituitary and in the arcuate nucleus of the hypothalamus. In teleosts, the NLT is considered to be the topological and functional equivalent to the arcuate nucleus. In contrast, the teleost POA is considered to be the functional and topological equivalent to the paraventricular and supraoptic nuclei of the hypothalamus, which do not express *POMC* in mammals. Is *POMC* expression in the POA a teleost novelty? It seems not. In the lungfish *Protopterus annectens*, the pituitary, NLT, and POA show α-MSH immunoreactivity (Vallarino, Tranchand Bunel, and Vaudry 1992). Similarly, studies on the frog *Xenopus laevis* show *POMC* mRNA expression in the same regions (Tuinhof et al. 1998). These data suggest that *POMC* was expressed in the pituitary, NLT, and POA of the last common ancestor of lobe- and ray-finned fishes. After the genome duplication in teleosts, the expression domains of the original *POMC* gene were partitioned between the two newly arisen paralogues so that each of them performs an essential subfunction and, therefore, is kept under selective pressure. In addition, perhaps after *POMCβ* expression was restricted to the POA, its β-endorphin module started degenerating in different lineages. Thus, it is likely that β-endorphin does not play an essential role in POA neurons of tetraodontids and, possibly, other teleosts as well.

The subfunctionalization of *POMC* paralogues in teleosts has some similarities to what happened in a lower vertebrate, the marine lamprey *Petromyzon marinus* (a jawless fish). This lamprey has two *POMC* genes with an unique organization regarding their peptide content: proopiomelanotropin (*POM*) gives rise to two different MSHs and β-endorphin but no ACTH, whereas proopiocortin (*POC*) produces ACTH, MSH, and a different β-endorphin (Takahashi et al. 2001; Dores and Lecaude 2005). *POM* is expressed in the pituitary PI, while *POC* is expressed in the RPD and weakly in the PPD, with none of the paralogues being expressed in the lamprey brain (Ficele et al. 1998). Thus, there has also been expression and peptide subfunctionalization between the lamprey paralogues, underlining the tendency of the modular POMC prohormone for subfunction partitioning. The duplication that originated these paralogues is very ancient, being probably specific to the lamprey lineage (Dores and Lecaude 2005). In any event, the *POMC* duplication in lamprey occurred independently of the duplication we observed in teleosts.

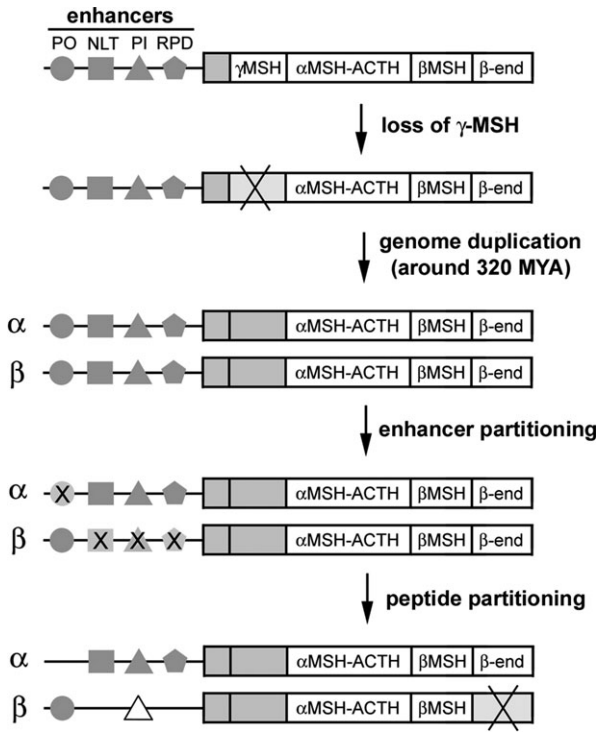

FIG. 6.—Hypothetical scheme showing the evolution of the *POMC* gene in teleosts. The genes are shown with the peptide-coding domains and putative enhancers for expression in the nucleus lateralis tuberis (NLT), preoptic area (PO), and pituitary rostral pars distalis (RPD) and pars intermedia (PI). After γ-MSH is lost from the *POMC* gene, a whole-genome duplication gives rise to two identical *POMC* copies, which subsequently undergo enhancer partitioning and, in some teleost lineages including *Tetraodon*, peptide partitioning. The PI enhancer maintains residual activity in *Tetraodon POMCβ* (denoted by white triangle). Enhancer and peptide partitioning did not occur necessarily in the order presented in the scheme.

What could be the mechanism for the partitioning of POMC expression domains? As stated in the DDC model (Force et al. 1999), differential degenerative mutations in the regulatory regions of each duplicate gene might have led to the loss of expression of the *POMCα* paralogue in the POA and loss of *POMCβ* expression from the pituitary RPD and NLT, provided that *POMC* regulatory regions are modular in nature. Figure 6 summarizes the most likely

scenario for *POMC* evolution in the *Tetraodon* lineage, starting with the loss of γ-MSH and the origin of two *POMC* paralogues in the whole-genome duplication until the differential loss of enhancers and β-endorphin. *POMC* enhancers are shown as modular blocks that work independently of each other. We have previously shown that the expression of the mammalian *POMC* gene is controlled by two independent regulatory regions: one is located near the promoter and directs expression to the pituitary (Rubinstein et al. 1993) and the other, composed of two enhancers located around 10 kb upstream of the gene, directs expression to the arcuate nucleus (de Souza et al. 2005). Further studies on the regulatory regions of tetraodontid *POMC* paralogues, taking advantage of the reduced amount of intergenic DNA in this species, might shed light on the mechanisms of subfunction partitioning of duplicate genes, a crucial process in the evolution of vertebrates.

Supplementary Material

Supplementary Figure 1 is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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