The Melanocortin System in Fugu: Determination of POMC/AGRP/MCR Gene Repertoire and Synteny, As Well As Pharmacology and Anatomical Distribution of the MCRs

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The G-protein–coupled melanocortin receptors (MCRs) play an important role in a variety of essential functions such as the regulation of pigmentation, energy homeostasis, and steroid production. We performed a comprehensive characterization of the MC system in Fugu (Takifugu rubripes). We show that Fugu has an AGRP gene with high degree of conservation in the C-terminal region in addition to a POMC gene lacking γ-MSH. The Fugu genome contains single copies of four MCRs, whereas the MC3R is missing. The MC2R and MC5R are found in tandem and remarkably contain one and two introns, respectively. We suggest that these introns were inserted through a reverse splicing mechanism into the DRY motif that is widely conserved through GPCRs. We were able to assemble large blocks around the MCRs in Fugu, showing remarkable synteny with human chromosomes 16 and 18. Detailed pharmacological characterization showed that ACTH had surprisingly high affinity for the Fugu MC1R and MC4R, whereas α-MSH had lower affinity. We also showed that the MC2R gene in Fugu codes for an ACTH receptor, which did not respond to α-MSH. All the Fugu receptors were able to couple functionally to cAMP production in line with the mammalian orthologs. The anatomical characterization shows that the MC2R is expressed in the brain in addition to the head-kidney, whereas the MC4R and MC5R are found in both brain regions and peripheral tissues. This is the first comprehensive genomic and functional characterization of a GPCR family within the Fugu genome. The study shows that some parts of the MC system are highly conserved through vertebrate evolution, such as regions in POMC coding for ACTH, α-MSH, and β-MSH, the C-terminal region of AGRP, key binding units within the MC1R, MC2R, MC4R, and MC5R, synteny blocks around the MCRs, pharmacological properties of the MC2R, whereas other parts in the system are either missing, such as the MC3R and γ-MSH, or different as compared to mammals, such as the affinity of ACTH and MSH peptides to MC1R and MC4R and the anatomical expression pattern of the MCRs.

Introduction

The Japanese pufferfish (Takifugu rubripes), or Fugu, has become subject of increasing interest for diversity of biological research during the past few years, as the Fugu was the second vertebrate species whose entire genome was sequenced (Aparicio et al. 2002). Although there are approximately 400 Myr since the lineage leading to teleosts split from the lineage leading to mammals (Carroll 1988), it is clear, that human and fish have large similarities in their genome and proteome organization. Fugu can thus serve as a good model organism for better understanding of the function and formation of the human genome. The genome of Takifugu rubripes is only 365 Mb (one eigth of that of human) and about 33,000 genes (Genscan) have been predicted so far (which is close to that estimated for human). Although rough analysis of the Fugu genome data indicates that 75% of the human proteome have a Fugu counterpart, more precise analysis is needed and information on functional characteristics of the protein families in Fugu is very limited (Aparicio et al. 2002).

The G-protein–coupled receptors (GPCRs) are one of the largest protein families in vertebrate genomes and are the single most pursued group of proteins in drug discovery. There exist over 800 GPCR genes in the human genome and about 40% to 50% of modern drugs are targeted at these receptors. Despite this and the mounting genetic information, only few GPCR families from lower vertebrates have been pharmacologically characterized. The melanocortin receptors (MCRs) are GPCRs and belong to the rhodopsin group (Fredriksson et al. 2003). MCRs respond to the pro-opiomelanocortin (POMC) cleavage products melanocyte-stimulating hormones (α-MSH, β-MSH, and γ-MSH) and adrenocorticotropic hormone (ACTH), all of them possessing agonistic properties on MCRs. The MC system is unique in the sense that it also has two endogenous antagonists named agouti (ASIP) and agouti-related peptide (AGRP). There are five subtypes of MCRs in mammals and aves named MC1R to MC5R (for review see Schioth [2001]) (Gantz and Fong 2003). In mammals, MC1R is expressed in melanocytes and has a main role in determination of skin and hair pigmentation by regulation of the dark eumelanin synthesis, as a response to α-MSH (Rana et al. 1999). Binding of the antagonist ASIP, however, inactivates the signalling pathway and leads to synthesis of yellow phaeomelanin (Lu et al. 1994). Expression of the MC1R is also detected in other cell types of skin and in a number of peripheral tissues and cells (Chhajlani 1996), including leukocytes, where it mediates the broad anti-inflammatory actions, prompting interest from the pharmaceutical industry to find agonist at the MC1R. The MC2R is expressed in adrenal cortex, where it mediates the effects of ACTH on steroid secretion. The mammalian MC2R differs pharmacologically from

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other MCRs in that it is activated only by ACTH and has no affinity for MSH peptides (Schioth et al. 1996). Some expression of MC2R has been found in human adipose tissue, but its role in this tissue is not clear. The MC3R and MC4R are expressed in several brain regions, particularly in the hypothalamus. These receptors have gained great attention during past years because of their involvement in regulation of energy homeostasis. The MC4R is one of the best-characterized monogenic factors of obesity. A number of mutations in this receptor lead to obese phenotypes in humans (Vaisse et al. 1998; Farooqi et al. 2000; Miraglia Del Giudice et al. 2002). Although both receptors are involved in regulation of the energy balance, mice deficient in one of these receptors display different phenotypes (Huszar et al. 1997, Chen et al. 2000). They also differ in pharmacology, as the MC3R has unique preference for γ-MSH among different subtypes of MCRs. MC5R is expressed in a number of human peripheral tissues, including adrenal gland, adipocytes, leukocytes, and others (Chhajlani 1996). The functional properties of MC5R are, however, still not well understood, with the exception of its participation in exocrine function, regulating sebaceous gland secretion in mice (Chen et al. 1997).

The MSH peptides have been intensively studied in lower vertebrates, and it seems that the POMC gene has arisen early in chordate evolution. Two copies of this gene are found in several ray-finned fish species (Okuta et al. 1996; Danielson et al. 1999) and lamprey (Takahashi et al. 1995). It is more likely, however, that the duplications in ray-finned fishes are late evolutionary event and do not represent an ancestry organization of this gene (Danielson et al. 1999). All fish POMC genes contain α-MSH, β-MSH, and ACTH. These sequences, especially that of α-MSH, are very conserved among different vertebrate species. Notable is that the γ-MSH region in ray-finned fishes is quite variable and degenerate, either missing the γ-MSH core motif (Amemiya et al. 1997; Dore et al. 1997) or even the complete γ-MSH sequence (Kitahara et al. 1988; Lee et al. 1999). AGRP has been found in chicken (Takeuchi, Teshigawara, and Takahashi 2000), but the evolutionary origin of AGRP or ASIP is obscure.

Our group has recently cloned the MC4R from zebrafish, goldfish, and dogfish and the MC5R from zebrafish (Ringholm et al. 2002, Cerda-Reverter et al. 2003, Ringholm et al. 2003), indicating high conservation in structure and pharmacology of these two receptors. We also found that MC4R is involved in central regulation of food intake in goldfish (Cerda-Reverter et al. 2003). No functional information is however available about the other MCR subtypes in nonmammalian species. Comparative pharmacological analysis of the entire MCR repertoire has not been previously performed beyond the mammalian lineage.

In this paper we report the identification of the full repertoire of Fugu MCR genes and their ligands. We describe genomic structure, synteny with the human genome, and determine the pharmacological profile and anatomical distribution of the receptors, including the first characterization of MC1R and MC2R from any nonmammalian species and delineate the vertebrate evolution of the MCR system.

Materials and Methods
Identification of MCR Genes

Two closely related pufferfish, Takifugu rubripes and Tetraodon nigroviridis, as well as zebrafish, Danio rerio, MC1R and AGRP genes were identified from the whole-genome shotgun databases found at http://www.ncbi.nlm.nih.gov and other genomic Web pages: http://fugu.hgmp.mrc.ac.uk; http://genome.jgi-psf.org containing Fugu sequences, http://www.genoscope.cns.fr containing Tetraodon sequences, and http://www.sanger.ac.uk/Projects/D_rerio/ for zebrafish sequences. TBLASTN searches were carried out on the databases using a number of MCR, AGRP, and ASIP sequences, including these of human, mouse, chicken, and known receptor sequences from fish species. BioEdit software (Hall 1999) was used for local Blast searches. The following contigs were identified and retrieved from databases. Takifugu rubripes scaffold numbers (only scaffolds from assembly release 3 are shown): MC1R (scaffold_431), MC2R, and MC5R (scaffold_1144), MC4R (scaffold_662), AGRP (scaffold_3097), and AGRP-like motifs A1 and A2 (scaffold_26029 and scaffold_305, respectively). Tetraodon nigroviridis contig numbers (Assembly version 6 at http://www.genoscope.cns.fr): MC1R (1071_4), MC2R (36902_1), MC4R (4178_2), MC5R (6538_1), and AGRP (227_2). Danio rerio contig numbers (Assembly 06/Assembly Zv1 at http://www.sanger.ac.uk/Projects/D_rerio/): MC1R (z06s08391), MC2R and MC5bR (z06s005606), and MC3R (z06s010438). To identify the full-length genes, contigs were then analyzed with Genscan (http://genes.mit.edu/GENSCAN.html) or with SpliceView (http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html) followed by manual correction.

Comparative Synteny Analysis Between Fugu and Human

Sequence from MCR and AGRP containing Fugu scaffolds were used in repeated BlastN search against different releases of genome shotgun sequence data sets. Identified scaffolds were downloaded and assembled to extend the sequence around the genes of interest. Sequences were assembled manually, based on results of pairwise Blast. Four contigs were obtained containing the following scaffolds (scaffolds starting with M represent Assembly release 3; scaffolds starting with S represent Assembly release 2): MC1R (M000431, S000194), MC2R and MC5R (M001144, M003114, M000533, S003468, S001069, S002629), MC4 (M006683, M000622, S000597, S000417, S002052), and AGRP (M 001144, S003468). Assembled contigs were subjected to Genscan analysis. Obtained protein sequences were individually compared with human genome assembly at NCBI using BlastP. Sequence similarity was evaluated based on quality and length of the match, including “score” and “Expect” values. Human genome map positions were identified for selected sequences from matching reads at http://www.ncbi.nlm.nih.gov/mapview/. Identified human sequences were blasted back to Fugu genome databases using TBLASTN to ensure the correctness of similarity and...
Cloning and Sequencing

The MCR sequences were amplified with Pfu Turbo DNA polymerase (Stratagene) from genomic DNA or cDNA. *Fugu* genomic DNA and a whole animal for tissue analysis were provided by Dr. Greg Elgar from *Fugu* Genomics Group (UK). PCR products were purified from 1% agarose gel using Gel Extraction Kit (Qiagen), followed by incubation with Taq polymerase (Invitrogen) in a reaction volume of 20 µl, containing 250 µM dATP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂ and were cloned into pCR1 vector and transformed into TOP10 cells (TOPO TA-Cloning Kit [Invitrogen]). Where possible, the entire coding sequences, including flanking regions, were cloned into TOPO cloning vector for sequencing. Sequencing reactions were performed using the ABI PRIZM Big Dye Terminator cycle sequencing kit according to the manufacturer’s recommendations and analyzed on ABI PRIZM-310 or 3100 Automated Sequencers (Applied Biosystems). Sequences were compiled and aligned using the Seqman program from the DNastar package (Lasergene). Sequences were compared against database assemblies using BlastN and analyzed on ABI PRIZM-310 or 3100 Automated Sequencers (Applied Biosystems). Sequences were compiled and aligned using the Seqman program from the DNastar package (Lasergene). Sequences were compared against database assemblies using BlastN and BlastX. For expression, full-length coding sequences were amplified by means of PCR from receptor gene containing TOPO plasmids with Pfu polymerase using HindIII and XhoI restriction sites containing primers for N-terminus and C-terminus, respectively. Obtained fragments were then digested with both restriction enzymes and gel purified before ligation into modified pCRII expression vector (Lundell et al. 2001). All constructs were sequenced to ensure identity of sequence to the original.

Alignments and Phylogenetic Analysis

Alignment of predicted full-length amino acid sequences for identified genes with other known MCR and ligand sequences were made using ClustalW version 1.8 software (Thompson, Higgins, and Gibson 1994). The following sequences (with their accession codes) were retrieved from GenBank for this analysis: *Homo sapiens* (Hsa) MC1R (NM_002386), MC2R (NM_000529), MC3R (XM_009545), MC4R (NM_005912), MC5R (XM_008685), AGRP (NM_001138), ASIP (NM_001672), *Mus musculus* (Mmu) AGRP (NM_007427), ASIP (NM_001138), AGRP (NM_001138), ASIP (NM_001672), *Gallus gallus* (Gga) MC1R (XM_009545), MC4R (NM_005912), MC5R (XM_008685), AGRP (NM_001138), ASIP (NM_001672), *Takifugu rubripes* (Tru) POMC (AAL11984). The identified genes have the following accession numbers: *Takifugu rubripes* MC1R (AY227791), MC2R (AY227793), MC4R (AY227794), MC5R (AY227796), and AGRP (BK001439); *Tetraodon nigroviridis* (Tni) MC1R (AY332238), MC2R (AY332239), MC4R (AY332240), and MC5R (AY332241). Phylogenetic trees were constructed by MEGA version 2.2. (Kumar et al. 2001) using maximum-parsimony and distance-neighbor-joining methods. Human cannabinoid 2 receptor (hCB2 [S36750]) was used to root the receptor tree. The outgroup sequence that was used for AGRP/ASIP phylogeny was made artificially from alignment consensus sequence randomizing all positions except the fully conserved ones. Bootstrapping was performed with 1,000 random replicates.

Cell Culture and Transfection

HEK 293-EBNA cells were transfected with 2 to 5 µg of the constructs using FuGENE Transfection Reagent (Roche) according to the manufacturer’s instruction. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/ Nut Mix F-12 with 10% fetal calf serum containing 0.2 mM L-glutamine, 250 µg/ml G-418, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Gibco BRL). Semistable cell lines were obtained for every construct expressing *Fugu* MCR by selecting cells for growth on medium containing 100 µg/ml hygromycin B (Invitrogen). Hygromycin was first added to medium 24 h after transfection. The MC2R was expressed in M3 cells. M3 cells were purchased by ATCC and were cultured in Kaighn’s modification of Ham’s F-12 medium (F-12K) supplemented with 15% horse serum (ATCC), 2.5% fetal bovine serum, and 1% Glutamax (Invitrogen). The cells were kept in humidified atmosphere of 95% air and 5% CO₂ at 37°C. One day before transfection, 200 000 cells were plated in 35-mm culture dishes. Transfections were carried out with 1.5 µg DNA using Lipofectamine Reagent PLUS (Invitrogen) according to the manufacturer’s instructions. Transiently transfected cells were used 72 h after transfection.

Radioligand Binding

HEK 293-EBNA cells expressing *Fugu* receptors were harvested from plate and resuspended in binding buffer composed of 25 mM HEPES (pH 7.4) containing 2.5 mM CaCl₂, 1 mM MgCl₂, and 0.2 % bacitracin. To obtain the membrane, preparation cells were homogenized using UltraTurrax. The cell suspension was centrifuged for 3 min at 1,300 g and membranes were collected from the supernatant by centrifugation for 15 min at 14,000 g. The pellet was resuspended in binding buffer. The binding was performed in a final volume of 100 µl for 3 h at room temperature. Saturation experiments were carried out with serial dilutions of [¹²⁵I]-NDP-MSH. Nonspecific binding was determined in presence of 1 µM unlabeled NDP-MSH. Competition experiments were performed with constant 0.6 nM concentration of [¹²⁵I]-NDP-MSH and serial dilutions of competing unlabeled ligands: NDP-MSH, α-MSH, β-MSH, γ-MSH.
ACTH(1-24), MTII, and HS024 (Neosystem). The membranes were collected by filtration on glass fibre filters, Filtermat A (Wallac), using a TOMTEC Mach III cell harvester (Orange, Conn.). The filters were washed with 5 ml per well of 50 mM TrisHCl (pH 7.4) and dried. MeltiLex A scintillation sheets (Wallac) were melted on dried filters and radioactivity was counted with automatic Microbeta counter 1450 (Wallac). Binding assay was performed in duplicates from at least three independent experiments. Nontransfected cells did not show any specific binding with [125I]NDP-MSH. The results were analysed with the Prism version 3.0 software package (Graphpad).

cAMP Assay in HEK 293 EBNA Cells

Cyclic adenosine 3′,5′-cyclic monophosphate production was determined on semistable HEK 293 EBNA cells expressing appropriate the MCR. A confluent layer of semistable cells was incubated for 3 h with 2.5 µCi of [3H]ATP (specific activity 29 Ci/mmol [Amersham]) per ml of medium. Cells were collected, washed with PBS, and incubated for 10 min in PBS containing 0.5 mM isobutylmethylxanthine (IBMX) (Sigma). Stimulation reaction was performed for 10 min in a final volume of 150 µl of PBS supplemented with 1 mM IBMX containing approximately 2 × 10⁶ cells and various concentrations of α-MSH peptide. After incubation, cells were centrifuged and 200 µl of 0.33 M perchloric acid (PCA) was added to pellets to lyse the cells. After centrifugation, 200 µl of lysate was added to Dowex 50W-X4 resin (Bio-Rad) column (Bio-Rad, poly-prep columns), previously washed with 2 × 10 ml H₂O. As an internal standard, 750 µl 0.33 M PCA containing 0.5 nCi/ml [14C]cAMP (Amersham) was added to column. Columns were washed with 2 ml H₂O to remove ATP, which was collected in scintillation vials to estimate the amount of unconverted [3H]ATP. Four milliliters of Ready Safe scintillation cocktail (PerkinElmer) was added to the vials before counting. Dowex columns were then placed over alumina (Sigma) columns (preshashed with 8 ml 0.1 M imidazole), and the cAMP was transferred onto the alumina column using 10 ml H₂O. cAMP was eluted from alumina column using 4 ml 0.1 M imidazole and collected into scintillation vials to which 7 ml of scintillation fluid was added. ¹H and ³⁵C were counted on Tri-carb liquid scintillation beta counter. The amount of obtained [14C]cAMP was expressed as a fraction of total [14C]cAMP ([14C]cAMP/total [14C]cAMP) and was used to standardize [³H]cAMP to column efficiency. Results were calculated as the percent of total [³H]ATP (obtained as a sum of [³H]ATP from first column and [³H]cAMP from second column) to [³H]cAMP and used to determine EC₅₀ values by nonlinear regression using the Prism version 3.0 software. All experiments were made in duplicates and repeated three times.

cAMP Assay in M3 Cells

Intracellular cAMP production induced by MC2R expressed in M3 cells was measured by sequential chromatography on Dowex and alumina columns as described for HEK 293 EBNA cells with following modifications (Gallo-Payet and Payet 1989). Seventy-two hours after transfection, MC2R transiently transfected M3 cells plated on 35-mm culture dishes were incubated 1 h at 37°C with complete culture medium containing 2 µCi/ml [³H]-adenine (NEN). The cells were then washed twice with Hank’s buffer saline and equilibrated in the same buffer containing 1 mM isobutyl methylxanthine (IBMX) for 15 min at 37°C. ACTH was added to the incubation medium for further 15 min at 37°C. The reaction was ended by aspiration and adding 1 ml ice-cold 5% (w/v) trichloroacetic acid. Cells were harvested, and 100 µM ice-cold 5 mM ATP plus 5 mM cAMP solution was added to the mixture. Cellular membranes were pelleted at 5,000 g for 15 min at 4°C and the supernatants sequentially chromatographed on Dowex and alumina columns according to the method of Salomon, Londos, and Rodebell (1974), allowing the elution of [¹H]-ATP and [¹H]-cAMP, respectively. cAMP formation was calculated as percent conversion = [¹H]-cAMP/ ([³H]-cAMP + [³H]-ATP) × 100 and expressed as fold stimulation over basal cAMP level.

RT-PCR and Southern Analysis

The total RNA was isolated from number of peripheral tissues (head-kidney, eye, skin, muscle, intestine, and gut) and several brain regions (telencephalon, optic tectum, hypothalamus, cerebellum, and brain stem). Tissues were dissected from frozen animal and kept in RNAlater (Ambion) before RNA extraction. Tissues were homogenized and total RNA was isolated using Rnasy Mini Kit (Qiagen), including processing with DNA shredder and DNase treatment (Qiagen) as recommended by manufacture’s protocol. Because some of the samples retained genomic DNA, total RNA was exposed for another treatment with 1U/µl RNase-free Dnasel (Roche) for 10 min, followed by heat inactivation of DNase for 5 min at 70°C. Absence of genomic DNA in RNA preparations was confirmed in PCR reactions with primers specific to β-actin gene using 10 to 100 ng of total RNA as a template and genomic DNA as a positive control. Messenger RNA was reverse transcribed using the First Strand cDNA Synthesis kit (Amersham). The produced cDNA was used as a template for PCR, with the specific primers for the receptor genes (see below). The quantity of cDNA was roughly estimated with PCR reactions on β-actin gene by ethidium bromide-stained agarose gel inspection of PCR product taken after cycles 20, 25, 30, 35, and 40 of PCR reaction. The conditions for the PCR on receptor genes were 1 min initial denaturation, 20 s at 95°C, 30 s at appropriate annealing temperature, 60 s at 72°C for 30 or 40 cycles, and finished by 5 min at 72°C, using Taq polymerase (Invitrogen). The following primers were used: 5′-TCT CCT TGT GCA TCC GGG CG-3′ and 5′-CAT CAA GGA GAA GAT GTG CT-3′ for β-actin gene (expected size 317 bp); 5′-TCA GGG GCC AAA GCA CCA GG-3′ and 5′-TCA GGG GCC AAA GCA CCA GG-3′ for MC1R gene (expected size 430 bp); 5′-TTA GCG CCA CCT CCA GTT TA-3′ and 5′-ACG TGG TGG ACT CGC TGC-3′ for MC2R gene (expected size...
for cDNA 626 bp); 5'-TCA CAT GCA CAC CAG CAT TT-3' and 5'-TGC TGG CGC GCC TGC ACA TG-3' for MC4R gene (expected size 332 bp); 5'-TCA GTA CTT ACC TGG AAG AG-3' and 5'-ACC TCC TTA ACA AGC AGC-3' for MC5R gene (expected size of cDNA 709 bp). The PCR products were analyzed on 1% agarose gel. DNA from the gel was transferred onto nylon filters overnight, using 0.4 M NaOH. The filters were hybridized with a random-primed 32P-labeled, receptor-specific probe. Probes were generated by Megaprime kit (Amersham Biosciences) using sequence-verified PCR products amplified from plasmids containing Fugu MCR genes. Hybridization was carried out at 65°C in 25% formamide, 6× SSC, 10% dextran sulfate, 5× Denhardt's solution, and 0.1% SDS overnight. The filters were washed three times in 0.2× SSC, 0.1% SDS for 1 h at 65°C and exposed to autoradiography film (Amersham). As positive controls in the Southern blots, the PCR products obtained from genomic DNA was used. A number of MC2R and MC5R RT-PCR bands were cut out from agarose gel, purified, and sequenced to ensure their identity and correct splicing, because these RT-PCR products were of different size as compared with genomic DNA as a result of the presence of introns in these genes. The RT-PCR reactions with 30 and 40 cycles and respective Southern blots were performed at least two times each.

Results

Gene Identification and Sequence Analysis

Melanocortin Receptors

The shotgun sequence databases of two closely related pufferfishes, Fugu (Takifugu rubripes) and Tetraodon (Tetraodon nigroviridis), as well as zebrafish (Danio rerio) were screened using Blast search to identify orthologs of the MCR gene family. This search revealed MC1R, MC2R, MC4R, and MC5R gene sequences from Takifugu rubripes databases and corresponding sequences from Tetraodon nigroviridis database (see Materials and Methods). In addition to previously known MC4R, MC5aR, and MC5bR genes obtained by our group (Ringholm et al. 2002), MC1R, MC2R, and MC3R gene sequences were found in zebrafish databases. Apparently, Fugu lacks a gene for the MC3R. The new genes were designated according to their sequence similarity to known MCRs from human and other species and phylogeny (see accession numbers in Materials and Methods).
resulted in the same topology of the trees. It is interesting to note that the MC5R in Fugu is an ortholog of the MC5a in zebrafish. The sequences of identified Fugu MCR genes and flanking regions were confirmed by sequencing of genomic PCR products. Some minor differences were found in MC1R receptor gene sequence as compared with the released genome sequence data. When analyzing the gene structure of the Fugu MCR, we surprisingly found that Fugu contains one intron in MC2R (position 553 from CDS start) gene and three introns in MC5R gene (positions 156, 263, and 452). None of the previous available sequences of MCRs contains introns (with an exception of human MC1R, where extension of coding sequence has been detected in mRNA because of an alternative splicing [Tan et al. 1999]). Tetraodon MC2R and MC5R genes had the same genomic structure, indicating that introns arose before the split of Fugu and Tetraodon in the pufferfish lineage, which is roughly estimated to be 20 MYA (Cmogorac-Jurcevic et al. 1997). The coding sequences of the ortholog MCR genes are highly conserved between Fugu and Tetraodon (84% to 93% similarity). Moreover, there is remarkable similarity in both length and sequence of the introns in the MC2R gene (60% similarity). The introns of MC5R gene have, however, diverged considerably in length, but still retain sequence similarity. To test the correctness of predicted splice products for Fugu MC2R and MC5R genes, they were verified by sequencing of RT-PCR products obtained using total RNA from Fugu brain. We were able to obtain and sequence full-length gene cDNA sequence for MC5R, whereas in case of the MC2R, RT-PCR products lacked 200 bp from 5′ end. In both cases, the splicing occurred at the predicted positions in these brain cDNAs.

**POMC**

We also searched for the ligand peptides to obtain a better picture on evolution of the MC system. Figure 3A displays the alignment of POMC genes from several relevant species. It should be noted that the overall similarity for POMC gene sequence is quite low or about 40%, but the parts coding for specific active peptides have remarkable high homology. Comparing MSH peptides from human and Fugu, we can see that α-MSH differs only in one position (98% similarity), ACTH peptides have 72% similarity, being almost identical (differs in one position) for ACTH(1-24), and β-MSH has 81% similarity. As observed for other teleosts (Takahashi et al. 2001), the γ-MSH region is not present in Fugu POMC.

**AGRP**

We have also searched for AGRP/ASIP-related genes. Although we were not able to identify such genes in zebrafish, the Fugu search revealed three cysteine-rich motifs with high similarity to the C-terminal part of AGRP/ASIP family. Figure 3B shows amino acid alignment of these sequences compared with peptides from other vertebrate species. We were able to identify and confirm the full-length structure of one such gene. Phylogenetic analysis placed this gene with the AGRP peptides, and the gene was accordingly named Fugu AGRP. For the other two motifs (A1 and A2), full-length genes were not identified because of incompleteness of sequence data in one case and unconfomable Genscan predictions in the other case. The difficulties in analysing the N-terminal part are related to the lack of sequence homology for this part of the peptide. Figure 3C represents the results of phylogenetic analysis using maximum-parsimony method. We also found that Fugu AGRP gene structure resembles that of other vertebrates as seen in figure 3B and D, indicating conservative intron positions, even though the length of the introns is variable. The Fugu, however, has one additional 5′ intron in the AGRP sequence.

**Comparative Synteny Analysis**

To gain more information about organization and synteny of the genome regions in Fugu containing MCR genes, we assembled from 170 kb to 400 kb large contigs using sequence data from different releases of Fugu genome consortium. First we found that MC2R and MC5R are located in close proximity in both Fugu (separated by 2.6 kb) and zebrafish (6.1 kb) (see fig. 4B). In both cases, the MC2R and MC5R genes face each other in sense of direction of transcription. This localization and orientation of the two genes appears to be very conserved among vertebrates. Thus, the human MC2R and MC5R orthologs located on chromosome 18 (18p11.2) are separated by 57.8 kb, whereas the mouse has 67.7 kb “inserted” between these genes. The MC2R and MC5R loci have also been mapped on the same region of chicken chromosome 2 (Schiöth et al. 2003).
To estimate the level of synteny shared between the human and Fugu genomes, we compared the content and order of genes from assembled Fugu contigs with available human genome maps at NCBI map search. Figure 4A shows the result of this analysis. Genes identified on Fugu contigs were named according to the orthologous human gene names. Where official names for human genes did not exist, we used interim names from UniGene cluster or gene model names from LocusLink. In total 20 genes were identified to share synteny with corresponding human genome regions. Thus nine genes from "MC1R contig" have their orthologs on two close loci of HSA16q with the same gene order. The segment of three genes including MC1R, however has been inverted either in humans or Fugu. "MC2R and MC5R contig" share eight genes with the HSA18p in locus containing both the MC2R and MC5R genes. The order and colinearity of these genes seems not to be disrupted between the two species. Finally three genes from "MC4R contig," including MC4R, have orthologs on HSA18q. This contig in contrast to the previous two has 10 genes mapping to other human chromosomes. These genes, with one exception, are organized in three clusters, which correspond to HSA5p, HSA14q11.2, and HSA3q, sharing the order of genes with their human counterparts. Apparently there have been more translocations in this region, and genes were either inserted into this locus in Fugu lineage or moved from this region in human. Because it is believed that Fugu has undergone additional genome duplication early in teleosts lineage, we searched for copies of the identified genes elsewhere in Fugu genome. However, this search did not produce any positive results, with exception of genes from families with many copies and high sequence similarity (e.g., cadherin [CDH] gene family).

**Pharmacological Properties of Fugu MCRs**

For pharmacological testing of the Fugu MCR repertoire, the coding regions of the receptors were cloned into an expression vector containing cytomegalovirus (CMV) promoter. All receptors were expressed in HEK-293 EBNA cells except for the MC2R that was tested separately in M3 cells (see below). Semistable cell lines were used for radioligand binding and cAMP assays. The endogenous peptides we used were of the human origin. α-MSH is fully conserved between the species, but there are minor differences in the sequences of β-MSH and ACTH. These differences are not within the predicted core-binding region of the peptides, but it is not known if these may affect the pharmacology.

**Radioligand Binding**

We performed saturation analysis using high-affinity ligand [125I] NDP-αMSH and competition-binding analysis using NDP-MSH, α-MSH, β-MSH, γ-MSH, and [125I] aMSH.
ACTH(1–24), MTII, and HS024 as competitors. MTII and HS024 are synthetic ligands widely used for physiological studies. The results of binding experiments are shown in figure 5. Table 1 shows Kd and Ki values obtained from saturation and competition experiments, respectively, for the MSH-binding Fugu receptors. Table 1 also includes previously published results for the human MCRs for comparison, tested with the same methodological approach.

Fig. 4.—(A) The Fugu, HSA16, and HSA18 comparative gene maps. The genes were named according to the human orthologs. The syntenic genes are displayed as black boxes and linked by lines. The Fugu genes with orthologs elsewhere in the human genome are denoted by gray boxes. The gray boxes with no name indicate the genes whose human ortholog could not be identified. The human genes that do not share synteny with the Fugu contigs are indicated with white boxes. (B) The gene linkage for MC2R and MC5R in several vertebrate species. The distances between the stop codons of the MC2R and MC5R are obtained from the genome assembly data. The direction of the transcripts is shown with arrows. The chicken MC2R and MC5R genes were mapped on the same locus of chromosome 2 (Schroth et al. 2003). Other genes that share synteny between the species are listed in parenthesis below the species name. The abbreviations used are human (HSA), zebrafish (DRE), Fugu (Tru), mouse (MMU), and chicken (GGA).
It should be mentioned that these human MCR binding values have been tested repeatedly for over a decade with very consistent results. The results show that NDP-MSH binds to a single saturable site for all *Fugu* receptors, except for the MC2R that was tested separately in M3 cells (see below). The affinity for labeled NDP-MSH was high, indicating that this radioligand is appropriate for characterization of the *Fugu* MCRs, except the MC2R. The affinity of NDP-MSH was similar for both the labeled (saturation) and cold (competition) experiments and also similar between the *Fugu* and human orthologs. The endogenous ligands α-MSH, β-MSH, and γ-MSH had clearly lower affinity (64-fold, 7-fold, and 94-fold, respectively) for the *Fugu* MC1R than the human MC1R. The ACTH(1–24), which is generally considered to be equipotent to the full-length ACTH(1–39), had however only 4.7-fold higher affinity for the *Fugu* MC1R as compared with the human MC1R. The synthetic ligand MTII had about 10-fold lower affinity for the *Fugu* MC1R, whereas HS024 had similar affinity for the *Fugu* MC4R as compared with the human MC4R. In contrast to the *Fugu* MC1R, the *Fugu* MC4R had 23-fold, sevenfold, and 277-fold higher affinity for α-MSH, β-MSH, and γ-MSH, respectively, as compared with the human MC4R. MTII had higher affinity, whereas HS024 had lower affinity for the *Fugu* MC4R as compared with the human MC4R. In similar
ACTH (1–24)

Cyclic AMP Assay

We also tested cells expressing the *Fugu* receptors in a cAMP assay to determine the ability of these receptors to couple to G-proteins and induce accumulation of cAMP upon exposure to natural ligands. The results are shown in figure 6. All the MCRs, except the MC2R, reached maximal levels of response, when stimulated with α-MSH. As α-MSH had very low potency to the *Fugu* MC1R, we also tested ACTH(1–24) at this receptor. The *Fugu* MC1R showed clearly higher potency for ACTH(1–24) α-MSH than in agreement with the binding data (table 1). In contrast to the MC1R, the MC4R and MC5R responded with high potency to α-MSH.

There are general difficulties of expressing MC2R in common cell lines as observed by many researchers because of failed transport of MC2R to the membrane (Noon et al. 2002). Expression of the MC2R has only been achieved in some adrenocortical and melanoma cell lines (Schimmer et al. 1995; Penhoat et al. 2000). We therefore used M3 melanoma cell line to functionally express *Fugu* MC2R and tested it in cAMP assay. These cells possess some basal activity in response to ACTH because of endogenous MC1R. However, expression of human MC2R has proved to be successful, showing a response to ACTH at much lower doses and with higher maximum response than the endogenous MC1R response. Our results in figure 7 show the response of ACTH in M3 cells transfected with the human MC2R, which is in good agreement with previous data (unpublished data). Moreover, we also received reproducible ACTH dose-dependent stimulation of at least twofold higher cAMP production in M3 cells transiently transfected with the *Fugu* MC2R. The results from the *Fugu* MC2R, although it seems to have slightly lower affinity (EC50 = 2.0 × 10⁻⁹ M), No activity was observed in the same cells stimulated with NDP-MSH.

**Table 1**

<table>
<thead>
<tr>
<th>Ligand</th>
<th><em>Tru</em> MC1</th>
<th><em>Hsa</em> MC1</th>
<th><em>Tru</em> MC3</th>
<th><em>Tru</em> MC4</th>
<th><em>Hsa</em> MC4</th>
<th><em>Tru</em> MC5</th>
<th><em>Hsa</em> MC5</th>
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</thead>
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<tr>
<td>[125I] NDP-MSH (K_i)</td>
<td>0.116 ± 0.003</td>
<td>0.109 ± 0.062</td>
<td>0.412 ± 0.121</td>
<td>1.52 ± 0.01</td>
<td>1.78 ± 0.36</td>
<td>1.47 ± 0.45</td>
<td>5.05 ± 1.00</td>
</tr>
<tr>
<td>NDP-MSH (K_i)</td>
<td>0.266 ± 0.097</td>
<td>0.046 ± 0.011</td>
<td>0.319 ± 0.064</td>
<td>1.24 ± 0.02</td>
<td>1.96 ± 0.39</td>
<td>1.65 ± 0.42</td>
<td>2.39 ± 0.10</td>
</tr>
<tr>
<td>α-MSH (K_i)</td>
<td>13.4 ± 2.4</td>
<td>0.210 ± 0.089</td>
<td>21.2 ± 5.3</td>
<td>22.8 ± 0.3</td>
<td>522 ± 122</td>
<td>120 ± 36</td>
<td>8240 ± 1670</td>
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<tr>
<td>β-MSH (K_i)</td>
<td>17.1 ± 0.8</td>
<td>2.53 ± 0.93</td>
<td>15.1 ± 3.4</td>
<td>52.7 ± 0.6</td>
<td>387 ± 208</td>
<td>1790 ± 470</td>
<td>14400 ± 1670</td>
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<tr>
<td>γ-MSH (K_i)</td>
<td>253 ± 14</td>
<td>2.68 ± 0.35</td>
<td>7.45 ± 2.55</td>
<td>187 ± 45</td>
<td>51800 ± 12000</td>
<td>3440 ± 670</td>
<td>42600 ± 6600</td>
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<tr>
<td>ACTH (1–24) (K_i)</td>
<td>0.983 ± 0.194</td>
<td>0.209 ± 0.052</td>
<td>32.8 ± 6.7</td>
<td>1.14 ± 0.15</td>
<td>755 ± 151</td>
<td>ND</td>
<td>2760 ± 780</td>
</tr>
<tr>
<td>MTII (K_i)</td>
<td>7.62 ± 1.12</td>
<td>0.686 ± 0.109</td>
<td>52.6 ± 7.9</td>
<td>1.00 ± 0.14</td>
<td>6.60 ± 0.32</td>
<td>24.4 ± 7.4</td>
<td>46.1 ± 7.9</td>
</tr>
<tr>
<td>HS024 (K_i)</td>
<td>22.4 ± 1.1</td>
<td>18.6 ± 3.3</td>
<td>15.1 ± 3.0</td>
<td>5.36 ± 0.64</td>
<td>0.341 ± 0.089</td>
<td>27.4 ± 8.1</td>
<td>3.29 ± 1.15</td>
</tr>
</tbody>
</table>

Note.—Values are nmol x L⁻¹. ND = not determined.

* Schioth et al. 1997c.
* Schioth et al. 1995.
* Kask et al. 1998.
* Schioth et al. 2002.
* Schioth et al. 1997b.

The tissue distribution of the four *Fugu* MCRs was determined by RT-PCR. Total RNA from a number of tissues, including different brain regions, was isolated from an adult animal and cDNA was obtained. We tested the cDNA by PCR using β-actin primers and confirmed the integrity of the mRNA. We determined the approximate amounts of mRNA in preparations by estimating the kinetics of the PCR reaction. Results of these experiments on actin gene are shown in the figure in Supplementary Material online. Even though the expression levels of β-actin varied slightly among the different types of tissues, this experiment gave a rough estimation of quality and amounts of RNA and helped to interpret the results of receptor-specific RT-PCR in more quantitative terms. For example, because of elastic structure of the *Fugu* skin, we were not able to extract RNA of sufficient amount and quality. Even though β-actin RT-PCR product is observed on actin mRNA in brain is highly expressed compared with other tissues. This fact must be considered when analyzing the expression of the MCRs, because presence of transcripts in tissues with highly expressed mRNA may be caused by unspecific transcription events and can be detectable in RT-PCR after high number of cycles. We therefore used different cycle numbers for RT-PCR to better estimate the quantity and specificity of the expression.

The results of the RT-PCR for each of the receptor gene are shown in figure 8. Each pair of PCR primers was designed to be specific for one receptor subtype. Specificity of the PCR reactions was estimated by Southern blot with the *Fugu* MCR subtype-specific probes. No cross-hybridization was detected in our samples (data not shown). To get an impression of the expression levels for *Fugu* MCRs, we
analyzed the PCR products taken from cycles 30 and 40 of the PCR. Average results from at least three independent experiments are displayed in table 2. The MC1R showed only weak signals and could only be seen in three brain parts on the Southern blot, indicating very low levels of expression. Expression of MC2R (seen with EtBr staining) was found in telencephalon and hypothalamus, but could be detected with hybridization assay in head-kidney (corresponding to the adrenal gland) at the cycle 40. RT-PCR of MC4R was detected in high levels (seen on EtBr gel after cycle 30) in all brain parts except the brain stem and slightly lower levels in head-kidney and gut. A signal for the MC5R was found in brain, head-kidney, and eye.

Discussion

There are two remarkable genomic features of the MCRs in Fugu. Firstly, there exist introns in two of the subtypes that are not found in any MCR in any other species (see below). Secondly, it seems almost certain that the Fugu does not have any MC3R subtype and that we have identified the full set of the MCR genes in Fugu, based on Blast search results of the nearly completed genomes, Takifugu rubripes with 95% genome coverage and Tetraodon nigroviridis with 83% coverage. It is unlikely (probability of 0.008) that MC3R gene is not found in these fishes because of an incomplete data set. Considering the other subtypes, the Fugu, Tetraodon, and zebrafish MCRs share high amino acid identity with the respective human orthologs (53% to 69% identity), which is higher than the identity of MCRs between the subtypes (38% to 60%). The phylogenetic analysis indicates clearly that newly identified receptors are orthologs of human MCR family (fig. 2). The TM2, TM3, TM6, and TM7 in the Fugu MCRs display the highest degree of conservation with MCRs from mammalian species, whereas they show almost no similarity in the N-terminal and C-terminal regions. This is in line with results that suggest involvement of TM2, TM3, TM6, and TM7 in the recognition and binding of MSH peptides (Schioth 2001).

We showed that there exist functionally spliced introns in two of the MCR genes in Fugu. This is intriguing, considering that none of the MCRs cloned so far, including the MCRs in zebrafish, have introns in their coding sequences. Moreover, our group has cloned additional MCR genes from the teleost lineage, but we have not found any introns in these genes (unpublished results). In general, positions and the number of introns are very conserved between different species of vertebrates. Nevertheless, several reports exist about recent gain or differences in intron composition in vertebrates (Logsdon, Stoltzfus, and Doolittle 1998; Stoltzfus et al. 1997; O’Neill et al. 1998) and fish species in particular (Venkatesh, Ning, and Brenner 1999; Figueroa et al. 1995; Sandford et al. 1997). It is intriguing that the MC2R intron is located at the same position as one of the MCR1 introns. Moreover, introns in this very same amino acid sequence (DRY) can be found in many GPCRs. This is one of the most important motifs that distinguishes the large rhodopsin group of GPCRs and is believed to be crucial in keeping the receptors in an inactivated form in absence of a ligand. The question arises as to whether this is an ancient intron that has been lost in all other members of MCR family but remained intact in two Fugu genes, as suggested by Logan et al. (2003), or whether these introns have been inserted into the genome later in the evolution of Fugu. We tend to believe that the introns found in MCR genes in Fugu are the result of recent insertions. Firstly, MC2R and MC5R are the most divergent members in the MCR family and may therefore have split from a common ancestor earlier than other MCR genes, where no traces of introns have been found. It seems unlikely that two genes could retain introns in Fugu, whereas many other MCR genes from related teleost and more ancestral species are intronless (unpublished results). Secondly, the genetic code of the DRY motif can form a protosplice site C/A, A, G, R, which is believed to be target site for insertion of spliceosomal introns by a process called reverse splicing (Dibb and Newman 1989; Dibb 1991, 1993). Although this theory is still debated, recent gene findings in “lower” animals and plants confirm the preferable insertion of spliceosomal introns into protosplice sites (Di Maro et al., 1997).
We thus believe that many examples of intron position in DRY motif of GPCR can rather be explained by intron insertion that, in some cases, may have occurred late in the vertebrate evolution such as in the MCRs in Fugu, whereas others, such as the ones in galanine or neurokinin (NK) receptors, have occurred much earlier, giving rise to conserved introns in mammals and "lower" vertebrates (unpublished data). Moreover, the fact that rhodopsin GPCRs that have the DRY intron are not phylogenetically clustered, as far as we can determine, also indicates that the suggestion of common DRY intron in the GPCRs may be wrong. For example, the MC, NK, and galanine receptors all belong to different clusters of rhodopsin GPCRs or the α, β, and γ subgroups of the rhodopsin family, respectively (Fredriksson et al. 2003). It is possible that intron insertion process in pufferfish is more frequent taking advantage of the protosplice site compared with other species, and it is possible that it has played some role in large genomic rearrangements, resulting in the compactness of Fugu genome.

It seems evident that not only the four MCRs are conserved in Fugu but also their ligands show a high degree of conservation. It is well established that there exist a high level of similarity between the α-MSH peptide sequence in POMC among vertebrate species. As seen from figure 3, the MSH peptides contain amino acid sequence HFRW known to be the core sequence in ligand-receptor recognition. It has also been known that a number

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**FIG. 8.—Expression of Fugu MC1R (A), MC2R (B), MC4R (C), and MC5R (D) mRNA as determined by RT-PCR.** The tissues, controls, and expected sizes of the PCR products are denoted at the top of each figure. Ethidium bromide–stained agarose gels are presented on top and autoradiographs of Southern blots, hybridized with gene-specific probes are shown below. The names of the brain parts are abbreviated as follows: telencephalon (Tel), optic tectum (OT), hypothalamus (HT), cerebellum (CB), and brain stem (BS).
of teleost fishes lost the region in the POMC gene coding for γ-MSH. This fact becomes particularly interesting in *Fugu*, which is missing MC3R, because MC3R in mammals has selectivity for γ-MSH. The role and importance of MC3R in teleost fish species remains, however, obscure, considering that the zebrafish has a MC3R.

Fairly recently, it was established that MCRs have not only POMC products as ligands but also AGRP and ASIP. AGRP has previously been shown to exist in chicken, and our data show that there exists an Agrp peptide in fish as well. It is thus conceivable that the dual ligand system consisting of both MCR agonist and antagonist arose early in vertebrate evolution. The Cys rich part in the C-terminal of the *Fugu* AGRP seems to be very well conserved, whereas the other part is much less conserved. This is in agreement with data showing that this part of the peptide is important for the interaction with MCRs (Tota et al. 1999). We also found partial genes that may account for additional genes that are likely to represent additional members of the AGRP/ASIP group of peptides. The low similarity in the N-terminal region and inconsistency of several gene prediction programs made it impossible with certainty to predict the full length of these genes, but blasts and phylogeny experiments (data not shown) indicate that these are more similar to AGRP than the agouti peptides.

The availability of the complete repertoire of MCR clones from a nonmammalian vertebrate species made it in particular interesting to investigate the functionality of these receptors, and we performed a thorough characterization of the pharmacological properties. One of the important characteristics of the mammalian MC1R is that it has very high affinity to α-MSH and similar or slightly lower affinity for ACTH. Surprisingly, we found that the *Fugu* MC1R had much lower affinity for α-MSH as compared with the human MC1R. In similar fashion, the *Fugu* had also lower affinity for the β-MSH and γ-MSH, whereas the potency order of these endogenous peptides was the same as for the mammalian MC1Rs. Remarkably, we also found that ACTH had more than 10-fold higher affinity than α-MSH to the *Fugu* MC1R. Our new data may suggest that the early vertebrate MCR had preference to ACTH peptides, whereas the sensitivity for the shorter POMC products such as α-MSH, β-MSH, and γ-MSH has appeared as the MCR subtypes gained more specified functions. This could also fit to the notion that the short MSH peptide sequences are copies of the original ACTH peptide sequence in the POMC gene. Previous studies have also indicated the mouse MC1R has much lower affinity for ACTH than α-MSH (Mountjoy 1994), suggesting that the mouse MC1R (that is perhaps evolving faster than the human one, considering that the mouse genome evolves about twice as fast as the human one) is losing its affinity for ACTH peptides. Taken together, this may support the hypothesis that the MC1R has evolved from being an ACTH-preferring receptor to being a specific α-MSH receptor in mammals.

One of the remarkable properties of the MCR family in mammalian species is that one of the MCRs does not bind MSH peptides at all. The MC2R is not able to bind MSH peptides, only recognizing ACTH peptides. It is not known how this unique property has evolved, and the MC2R in *Fugu* is the most “distant” receptor of this type that has been characterized. The pharmacological data show that the *Fugu* MC2R responds to ACTH, but it is not activated by α-MSH, showing similar characteristics as the mammalian MACTH receptors. This clearly indicates that the specific ACTH selectivity and perhaps functionality in mediating steroidoneogenesis arose early in evolution of vertebrates and that this property is likely to be crucial for its function in most vertebrates.

The MC4R is one of the most pursued GPCR for drug development, yet it is surprising how low affinity it has for the natural MSH peptides. The *Fugu* MC4R shows about 10-fold higher affinity for the α-MSH and β-MSH as compared with the human MC4R, whereas, in similar fashion as the MC1R, the affinity is much higher for ACTH. The high potency of ACTH at the MC1R, MC2R, and MC4R in *Fugu* provides further support to the possibility that it was indeed the ACTH that was the "original" ligand for the early MCRs, which apparently was created very early during vertebrate evolution. One of the most distinct pharmacological characteristics of the MC4R in mammals is a particularly low affinity for γ-MSH. The MC3R has preference to γ-MSH and, together with the fact that these two MCRs are the most abundantly

### Table 2
Anatomical Localization of the *Fugu* MCR mRNA Expression As Estimated by RT-PCR with Cycle Numbers 30 and 40

<table>
<thead>
<tr>
<th><em>Fugu</em> Gene</th>
<th>Receptor</th>
<th>Tel</th>
<th>OT</th>
<th>HT</th>
<th>CB</th>
<th>BS</th>
<th>Head-Kidney</th>
<th>Eye</th>
<th>Skin</th>
<th>Liver</th>
<th>Muscle</th>
<th>Intestine</th>
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Note.—Plus signs indicate specific PCR fragments visible on ethidium bromide–stained gels. Specific PCR bands observed in Southern blots are marked with asterisks. The bottom row represents the results of kinetics of β-actin–specific PCR. The number of PCR cycles shows when the PCR product can be detected on an EtBr-stained gel.
expressed in brain of the MCR repertoire in mammals, has made this property very valuable in physiological studies aiming to delineate the specific central effects of the MCRs. Remarkable, \( \gamma \)-MSH has several hundred–fold higher affinity for the \textit{Fugu} MC4R than it has for the human MC4R. This is very interesting, as it is conceivable that the MC4R has evolved in such manner that it loses its binding ability to \( \gamma \)-MSH. The role of \( \gamma \)-MSH is still quite obscure, but these data clearly indicate the importance of \( \gamma \)-MSH to not interfere with the MC4R and suggest that “this property” has become specifically important in “higher” vertebrates. It is also possible that this inability to bind \( \gamma \)-MSH has been lost in the \textit{Fugu} lineage, as it does not have the \( \gamma \)-MSH sequence in POMC. The inability of the human MC4R to bind \( \gamma \)-MSH has been linked to Tyr268 on the top of the TM6 (Oosterom et al. 2001). It is interesting to note that the \textit{Fugu} MC4R is lacking the corresponding Tyr, as can be seen in figure 1. It is also interesting that we do not observe preference of the \textit{Fugu} MC4R to \( \beta \)-MSH in a similar way as we have previously shown for both the rat and human MC4R (Schioth et al. 2002). There is increasing evidence that \( \beta \)-MSH may have a specific role for the feeding response through MC4R in mammals (Harrold, Widdowson, and Williams 2003). The lack of specificity for the \( \beta \)-MSH at the \textit{Fugu} MC4R may suggest that this is a property that has evolved at later stages of vertebrate evolution. The MC5R in \textit{Fugu} has in general higher affinity for the natural MSH peptides as compared with the human ortholog. This is particularly evident for \( \alpha \)-MSH, which has about 70-fold higher affinity for the \textit{Fugu} receptor, indicating that the mammalian MC5Rs may have lost their affinity to the \( \alpha \)-MSH, \( \beta \)-MSH, and \( \gamma \)-MSH, but so little is known about the functional role of this receptor that it is difficult to speculate about the importance of this. We have also shown that all the MCRs can functionally couple to the Gs-linked signalling pathway in response to \( \alpha \)-MSH and/or ACTH in line with previous results on the mammalian MCRs.

Although the RT-PCR is not well suited for quantitative analysis of gene expression, this approach is very effective in detecting expression of genes sets in a wide range of tissues. The expression of the MC2R in the brain is intriguing. The MC2R was found in four of the five brain regions analyzed (see fig. 8 and table 2), and sequencing of the RT-PCR products confirmed the predicted splicing of this gene in the brain. After the first cloning of the MC2R and the subsequent demonstration that this was indeed an ACTH receptor in mammals, several groups performed an extensive search for this receptor in the brain (Xia and Wikberg 1996). This was because of the physiological textbook perception that ACTH played a role in a central negative feedback (Motta, Mangili, and Martini 1965). The MC2R has not been found in brain of any mammal, and the central ACTH-binding sites that were found early on can probably be attributed to the MC3R (Schioth et al. 1997a). It is possible that the presence of the MC2R in the \textit{Fugu} brain may suggest that there exists a negative feedback loop involving this receptor that may have been later taken over by, for example, the MC3R in mammals. Expression of mammalian MC4Rs has only been found in brain tissue. The fact that we have detected MC4R transcripts in telencephalon, optic tectum, hypothalamus, cerebellum, head-kidney, and gut of \textit{Fugu} is in agreement with previously found observations from zebrafish (Ringholm et al. 2002), where MC4R was in addition to brain found to be expressed in the eye, GI tract, and ovaries. In chicken, the MC4R is expressed in a wide variety of peripheral tissues, including the heart, adrenal glands, ovaries, testes, spleen, adipose tissue, and eye, as well as the brain (Takeuchi and Takahashi. 1998). It is therefore possible that restriction of MC4R expression to the brain is a feature of mammalian species and has developed because of needs for more specific CNS-driven regulation of metabolic and behavioral processes. It has recently been demonstrated that MC4R participates in central regulation of food intake in teleost fish (Cerda-Reverter et al. 2003), but the presence of this receptor in peripheral tissues may suggest that it may also have additional functions in fishes. The MC5R is expressed in a wide range of tissues not only in mammals but also in chicken (Takeuchi and Takahashi 1998) and zebrafish. Our results show that also the MC5R in mammals but also in chicken (Takeuchi and Takahashi 1998) and zebrafish. This new found observation from zebrafish (Ringholm et al. 2003). The functional relevance of this linkage of MC2R and MC5R genes is found in all representatives of different vertebrate classes (see figure 4) (Schioth et al. 2003). The functional relevance of this tandem remains a mystery. This conserved linkage suggests that these receptors arose from a common ancestor by a local gene duplication event. It is intriguing that the MC2R and MC5R subtypes are evolutionary the most distant among the MCRs as seen from the phylogenetic analysis (fig. 2). This indicates that a local duplication responsible for this linkage occurred very early, most probably before the two putative tetraploidization, which, according to the 2R hypothesis, took place before the origin of gnathostomes (Holland et al. 1994;
The phylogenetic analysis clearly shows that MC4R and MC3R are more closely related to each other than to other MCRs. This suggests that the MC2R and MC5R linkage have arisen from common ancestor through a local duplication, the MC3R might have arisen later from the common MC5R-related ancestor gene, perhaps through tetraploidization event. MC4R is, however, most likely the result of local duplication from MC5R that occurred after the last common tetraploidization in vertebrate lineage. Alternatively, MC1R and MC2R might have common ancestor gene and appeared through the genome duplication events. Although there is less similarity between these two receptors than in MC3/4/5R group, this scenario might be explained by different evolution rates, perhaps as MC2R has the most divergent pharmacological/physiological characteristics. An important question is when these duplications took place. According to our findings of MCR genes in lamprey (preliminary unpublished results), the appearance of MC1/2R and MC3/4/5R ancestor gene linkage can be dated before the appearance of gnathostomes (jawed vertebrates) but after the first tetraploidization. Taking into account the high similarity between MC3R, MC4R, and MC5R and presence of all these receptors in zebrafish, MC4R or MC3R might appeared through local duplication of MC5R soon after the last genome duplication after the split of gnathostomes. Figure 9 summarizes above-mentioned observations and provides a putative scheme for evolution of MCR genes through the duplication events. The order of appearance for MC3R and MC4R remains unclear. However, MC4R is located on the same chromosome in human, mouse, and chicken, thus indicating that MC4R might be a duplicate of a common ancestor to the MC5R branch. It is intriguing that the Fugu MC5R gene, which is linked with the MC2R gene, is, according to phylogenetic analysis, an ortholog of the zebrafish MC5aR gene. In zebrafish, however, it is the other one, or the MC5bR gene, that is linked with the MC2R gene. This would mean that different copies of MC2R gene have been lost in Fugu and zebrafish, providing further strong evidence for the ancient origin of the remarkable MC2R and MC5R gene linkage. It also indicates that two copies of MC2R gene existed in the teleost lineage before the split of Fugu and zebrafish. This is in agreement with our previous molecular clock investigation of the two zebrafish MC5Rs, dating them to the predicted teleost-specific genome duplication 250 MYA.

In summary, we have characterized the gene repertoire of the MC system in Fugu. We show that there exists an AGRP peptide gene in addition to a POMC gene. The receptor gene repertoire does not include an MC3R gene, but after the first tetraploidization. The MC4R is, however, most likely the result of local duplication from MC5R that occurred after the last common tetraploidization in vertebrate lineage. Alternatively, MC1R and MC2R might have common ancestor gene and appeared through the genome duplication events. Although there is less similarity between these two receptors than in MC3/4/5R group, this scenario might be explained by different evolution rates, perhaps as MC2R has the most divergent pharmacological/physiological characteristics. An important question is when these duplications took place. According to our findings of MCR genes in lamprey (preliminary unpublished results), the appearance of MC1/2R and MC3/4/5R ancestor gene linkage can be dated before the appearance of gnathostomes (jawed vertebrates) but after the first tetraploidization. Taking into account the high similarity between MC3R, MC4R, and MC5R and presence of all these receptors in zebrafish, MC4R or MC3R might appeared through local duplication of MC5R soon after the last genome duplication after the split of gnathostomes. Figure 9 summarizes above-mentioned observations and provides a putative scheme for evolution of MCR genes through the duplication events. The order of appearance for MC3R and MC4R remains unclear. However, MC4R is located on the same chromosome in human, mouse, and chicken, thus indicating that MC4R might be a duplicate of a common ancestor to the MC5R branch. It is intriguing that the Fugu MC5R gene, which is linked with the MC2R gene, is, according to phylogenetic analysis, an ortholog of the zebrafish MC5aR gene. In zebrafish, however, it is the other one, or the MC5bR gene, that is linked with the MC2R gene. This would mean that different copies of MC2R gene have been lost in Fugu and zebrafish, providing further strong evidence for the ancient origin of the remarkable MC2R and MC5R gene linkage. It also indicates that two copies of MC2R gene existed in the teleost lineage before the split of Fugu and zebrafish. This is in agreement with our previous molecular clock investigation of the two zebrafish MC5Rs, dating them to the predicted teleost-specific genome duplication 250 MYA.

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