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Multiple Corticosteroid Receptors in a Teleost Fish: Distinct Sequences, Expression Patterns, and Transcriptional Activities

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Corticosteroid hormones, including the mineralocorticoids and the glucocorticoids, regulate diverse physiological functions in vertebrates. These hormones act through two classes of corticosteroid receptors (CR) that are ligand-dependent transcription factors: type I or mineralocorticoid receptor (MR) and type II or glucocorticoid receptor (GR). There is substantial overlap in the binding of these two receptor types to hormones and to DNA. In fish, the overlap in processes controlled by CRs may be different from that in other vertebrates, as fish are thought to synthesize only glucocorticoids, whereas they express both GR and MR. Here we describe the characterization of four CRs in a cichlid fish, *Haplochromis burtoni*: a previously undescribed GR (HbGR1), another GR expressed in two splice isoforms (HbGR2a and HbGR2b), and an MR (HbMR). Sequence comparison and phylogenetic analysis showed that these CRs sort naturally into GR and MR groups, and that the GR duplication we describe will probably be common to all teleosts. Quantitative PCR revealed differential patterns of CR tissue expression in organs dependent on corticosteroid action. *Trans*-activation assays demonstrated that the CRs were selective for corticosteroid hormones and showed that the HbMR was similar to mammalian MRs in being more sensitive to both cortisol and aldosterone than the GRs. Additionally, the two HbGR2 isoforms were expressed uniquely in different tissues and were functionally distinct in their actions on classical GR-sensitive promoters. The identification of four CR subtypes in teleosts suggests a more complicated corticosteroid signaling in fish than previously recognized. (*Endocrinology* 144: 4226–4236, 2003)

STEROID HORMONES ARE essential for the regulation of a wide variety of physiological processes. The cortex of the adrenal gland secretes two classes of corticosteroid hormones that modulate functions, such as glucose metabolism, mineral balance, and behavior. These corticosteroid hormones, the glucocorticoids and the mineralocorticoids, were originally named for their distinct bodily functions in mammals. Glucocorticoids, including cortisol and corticosterone, were identified for their role in liver glycogen deposition, whereas the mineralocorticoids, predominantly aldosterone, were recognized to regulate mineral balance principally by controlling sodium retention in the kidney (1). It is now known that glucocorticoids also mediate the stress response, play important roles in cognition, act as immune suppressants and antiinflammatories, and mediate some processes traditionally thought to depend on mineralocorticoids, such as salt balance (2). In addition to its primary role in the kidney, aldosterone is involved in the regulation of blood pressure, perspiration, and salt taste sensitivity (3).

The effects of corticosteroid hormones are mediated through two related intracellular receptors that act as ligand-dependant transcription factors. The mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) are members of the steroid/thyroid/retinoid receptor superfamily and share a canonical structure comprised of distinct functional domains (4). The amino-terminal A/B domain is quite variable among species and is important in modulating transcriptional activity. The highly conserved C domain contains two zinc fingers responsible for DNA binding and receptor dimerization. Conformational change during receptor binding is accommodated by the D domain, and hormones bind to the E domain at the carboxyl terminus. Although the two classes of corticosteroid receptor (CR) are conventionally described as MR or GR, there is substantial overlap in the pharmacology of these two receptor types. Specifically, cortisol binds to and induces the transcriptional activity of both GR and MR. In fact, MR typically has 10-fold higher affinity for cortisol than GR, leading to the original characterization of these receptors as GR type I and type II, respectively (5, 6). MR is also activated by aldosterone. However, as circulating levels of cortisol are much higher than those of aldosterone, MR is thought to be continually occupied by cortisol under basal conditions. Aldosterone specificity can be achieved in certain cells that have mechanisms for limiting cortisol concentrations. For instance, in some cells MR is colocalized with the enzyme 11β-hydroxysteroid dehydrogenase type 2, which selectively inactivates cortisol and corticosterone, but not aldosterone (7). In the tissues in which MR is present without such mechanisms, it is thought to function as a high affinity cortisol receptor.

GR and MR not only share ligand responsiveness, but both also bind to the same glucocorticoid response elements

Abbreviations: CR, Corticosteroid receptor; FBS, fetal bovine serum; G3PDH, glyceraldehyde 3-phosphodehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MR, mineralocorticoid receptor; RACE, rapid amplification of cDNA ends.

(GREs), which are specific sequence motifs within gene promoters (4, 8, 9). Steroid receptors bind to their response elements as dimers, and GR-GR, MR-MR, and GR-MR pairs have been reported (10, 11). Despite this overlap in response element sequence specificity, MR and GR regulate different cellular processes (3, 12). This specificity arises through differential cellular localization and, in a given cell, through the interaction of their distinctive A/B domains with unique sets of transcription factors (13–15).

In fish the overlap in the processes controlled by the two classes of CR may be more complete than that described in other vertebrates. As previous studies have failed to convincingly determine that fish synthesize aldosterone, it was long held that cortisol acted through a single GR to control both glucocorticoid and mineralocorticoid functions in fish (16–20). However, a fragment of a mineralocorticoid-like receptor was recently cloned from rainbow trout (21). This discovery is perhaps not surprising given what is now known about the important role of MR as a high affinity cortisol receptor in many cells. Moreover, it has been suggested that MR and GR may have evolved from duplication of a single CR, and only later, after evolution of the enzyme machinery for aldosterone synthesis and selective cortisol inactivation, did MR gain additional mineralocorticoid-specific functions (22, 23). Thus, in fish both MR and GR may act primarily as cortisol receptors.

Corticosteroid signaling in fish may indeed be more complex than previously thought. Here we identify four CRs in a cichlid fish. We describe two GRs, one of which also exists as a splice variant for which we demonstrate important functional consequences. We also describe the cloning of a MR and demonstrate its exquisite sensitivity to both cortisol and aldosterone.

Materials and Methods

Animal care

Haplochromis (*Astatotilapia*) *burtoni* derived from wild-caught stock were kept in aquaria at pH 8 and 28 C water temperature, with a 12-h light, 12-h dark cycle with 10 additional min of twilight in the morning and evening. Fish were fed every morning with cichlid pellets and flakes (AquaDine, Healdsburg, CA). All work was performed in compliance with the animal care and use guidelines at Stanford University and was approved by the Stanford University Administrative Panel on Laboratory Animal Care committee.

Isolation of cDNA clones

GR. Two fragments of GR, GR-E and GR-A/B, were generated by RT-PCR. GR-E was amplified with the following primers designed to the hormone-binding domain of tilapia GR (24): upper primer, 5′-AGT GCT CCT GGC TGT TYC TNA TG-3′; and lower primer, 5′-TTT CGG TAA TTG GTT GCT GAT GAT-3′. The location of the 5′ end of the upper primer was at amino acid 683 of the resulting *H. burtoni* sequence and at 849 for the lower primer. GR-A/B was amplified with the following degenerate primers designed primarily within the A/B region (consensus degenerate hybrid oligonucleotide primers) (Ref. 25 and http://blocks.fhcrc.org/blocks/codehop.html): upper primer, 5′-GGT GCA CCC CTG GAG TCR WNR ARC ARG A-3′; and lower primer, 5′-CGT CGG ATC TTG TCG ATG ATR CAR TCR TT-3′. The location of the 5′ end of the upper primer was at amino acid 461 of the resulting *H. burtoni* sequence and at 649 for the lower primer. GR-E and GR-A/B fragments were PCR-amplified from whole brain cDNA (Rapidcycler, Idaho Technologies, Idaho Falls, ID) in a 10-μl reaction volume in glass microcapillary tubes using the following parameters: 15-sec denaturation at 94 C, followed by 35 cycles of a 0-sec denaturation at 94 C, 0-sec primer annealing at 60 C for 5 cycles, 58 C for 5 cycles, 56 C for 5 cycles, and 55 C for 20 cycles, and 15-sec extension at 72 C. Note that heat transfer in capillary tubes is extremely rapid, so the temperature does not need to be maintained for any length of time at the denaturation or annealing temperatures, yielding a 0-sec hold. Klentaq1 DNA polymerase (AB Peptides, St. Louis, MO) was used with the supplied buffer, with a final magnesium ion concentration of 3 mM, and the polymerase was prebound with TaqStart Antibody (Clontech, Palo Alto, CA) to reduce nonspecific binding. Primers were used at 0.5 μM each. Resulting products were sequenced and determined to have high similarity to GR sequences from other fish. Full-length sequence was obtained by library screening. Phage (1×10^6) from an adult *H. burtoni* brain cDNA library (Uni-ZAP XR λ vector; Stratagene, La Jolla, CA) were screened with two probes against the different regions of GR. The probes were labeled with [^{32}P]deoxy-CTP by random priming (DECAprimeII kit, Ambion, Inc., Austin, TX). Single phage clones were isolated by subsequent rounds of screening and were excised and independently sequenced on both strands. GR-E probe isolated a clone that we named HbGR1, and GR-A/B isolated two clones, named HbGR2a and HbGR2b.

GR splice variant analysis. To determine whether HbGR1 was expressed as a splice variant, similar to HbGR2, we designed the following primers spanning the DNA-binding domain: upper primer, 5′-GCA CCG CTA CAT CCT CCA CAC AAG-3′; and lower primer, 5′-TGG TGG GTT GCT CTG CTG GAC GGC-3′, which amplified a product of 309 bp. Total RNA from adult *H. burtoni* brain, gill, heart, liver, spleen, and kidney/interrenal gland was isolated, and cDNA was reverse transcribed as described below. Five percent of the cDNA synthesis reaction was amplified using a Rapidcycler (Idaho Technologies) in a 10-μl reaction volume in glass microcapillary tubes using the following cycling program: 15-sec denaturation at 94 C, followed by 35 cycles of a 0-sec denaturation at 94 C, 0-sec primer annealing at 65 C for 5 cycles, 63 C for 5 cycles, 61 C for 5 cycles, and 60 C for 20 cycles, and 15-sec extension at 72 C. Reaction components were as described above. Products were size separated by electrophoresis through a 2% agarose gel and visualized using ethidium bromide staining with UV illumination.

MR. Degenerate primers were designed based on an alignment of mammalian and fish androgen receptor sequences and were as follows: upper primer, 5′-AYG ARG CNG GGA TGA CDY TDG GAG-3′; and lower primer, 5′-TRT GCA TXC KRT AYT CRT T-3′. The location of the 5′ end of the upper primer was at amino acid 724 of the resulting *H. burtoni* sequence and at 897 for the lower primer. A cDNA fragment was amplified from whole brain cDNA using a Rapidcycler and the following cycling conditions: 15-sec denaturation at 94 C, followed by 40 cycles of a 0-sec denaturation at 94 C, 0-sec primer annealing at 55 C for 5 cycles, 53 C for 5 cycles, 51 C for 5 cycles, and 50 C for 25 cycles, and 15-sec extension at 72 C. The resulting product was determined to have high similarity to mammalian MR sequences. Specific primers were then designed for rapid amplification of cDNA ends (RACE): 5′ RACE primer, 5′-CCT CCA GCG CCA CCT GTA GCA GTT TGG-3′; and 3′-RACE primer, 5′-CCT CAG CTG GCG CTC CTA CAA ACA CA-3′. 5′- and 3′-RACE reactions were performed using MARATHON RACE technology (Clontech) according to the manufacturer's directions. 5′ RACE products were PCR-amplified (PerkinElmer GeneAmp 9600, PE Applied Biosystems, Foster City, CA) using the following cycling conditions: 10 cycles at 94 C for 5 sec and 72 C for 4 min, 5 cycles at 94 C for 5 sec and 72 C for 4 min, and 25 cycles at 94 C for 5 sec and 69 C for 4 min. 3′ RACE product was generated with 5 cycles at 94 C for 5 sec and 72 C for 3 min, 5 cycles at 94 C for 5 sec and 70 C for 3 min, and 20 cycles at 94 C for 5 sec and 68 C for 3 min.

Genomic Southern blot

H. burtoni genomic DNA was isolated from a whole juvenile fish according to Sambrook *et al.* (25a). Twenty micrograms of DNA were digested with *Bam*HI and *Eco*RI and size separated in two lanes of 10 μg each by electrophoresis through a 0.8% agarose gel. The DNA was transferred to an uncharged nylon membrane (Schleicher & Schuell, Keene, NH), and UV cross-linked to the membrane (Stratalinker, Stratagene). The two lanes were separated and prehybridized in Church-Gilbert buffer at 55 C for 30 min. Each lane was hybridized overnight with specific probes to either HbGR1 or HbGR2. Templates specific to

each transcript were generated by cDNA amplification of 35 cycles using the following cycling conditions: 15-sec denaturation at 94 C, followed by 40 cycles of a 0-sec denaturation at 94 C, 0-sec primer annealing at 65 C for 5 cycles, 63 C for 5 cycles, 61 C for 5 cycles, and 60 C for 25 cycles, and 15-sec extension at 72 C. Each reaction contained approximately 5 pg of either HbGR1 or HbGR2 clone cDNA and 0.5 μM of each gene-specific primer. Primer sequences were as follows: HbGR1: upper primer, 5′-GCG CGC AAT TAA CCC TCA CTA AAG GGG CCG AAG CTG GGT GTG TTG TAT C-3′ (contains T3 promoter sequence); and lower primer, 5′-CGC GCG TAA TAC GAC TCA CTA TAG GGC TGG GCT CAA AAG CAT TAT CAA GG-3′ (contains T7 promoter sequence), yielding a 711-bp product; HbGR2: upper primer, 5′-GCG CGC AAT TAA CCC TCA CTA AAG GGG TTA GAT ATA TTG CGG AGA ATG-3′ (contains T3 promoter sequence); and lower primer, 5′-CGC GCG TAA TAC GAC TCA CTA TAG GGC CAC CGG TGC TCT CTT GTA GAA TG-3′ (contains T7 promoter sequence), yielding a 918-bp product. Templates were random prime labeled with [^{32}P]deoxy-CTP using a DECAprimeII kit (Ambion, Inc.).

Phylogenetic tree

Sequences from HbGR1, HbGR2, and HbMR were aligned with GR and MR sequences from other fish [flounder GR (GenBank accession no. O7363), trout GR (P49843), and trout MR (AAF61206)], and tetrapods [human GR (NP_000167), human MR (NP_000892), rat MR (NP_037263), mouse GR (NP_032199), and *Xenopus* GR (P49844)] using ClustalX (26). Note that the trout MR sequence is a fragment including part of the C domain and the entire D and E domains. PAUP software (Sinauer Associates, Sunderland, MD) was used to generate phylogenetic trees using neighbor-joining and bootstrap-jackknife algorithms. Trees were generated either using all functional domains or using only the C, D, and E domains. Rat androgen receptor sequence (GenBank accession no. P15207) was used as an out-group.

Comparison of expression levels using quantitative real-time PCR

Total RNA from brain, gill, heart, and kidney/interrenal gland (these tissues are intermeshed in euteleosts) of five female *H. burtoni* and liver and spleen from four females was isolated using the Ultraspec-II RNA isolation protocol (Biotecx Laboratories, Houston, TX). cDNA was reverse transcribed from 10 μg total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and an anchored poly T primer, VdT_{18}. Controls performed in parallel omitted reverse transcriptase from the reaction to identify possible genomic contamination. Primers for real-time PCR were designed using Oligo 6 software (Molecular Biology Insights, Cascade, CO) according to the iCycler iQ Multi-Color Real Time PCR Detection System manual (Bio-Rad Laboratories, Hercules, CA). Specifically, primers that amplified products between 75–250 bp were chosen using high stringency search parameters in Oligo. Templates were analyzed for secondary structure using a web-based program (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). Primers had a GC content of 50–60%, and upper and lower primers had similar melting temperatures, which were higher than any template secondary structure. Primers were then screened for high efficiency using standard curve analysis (see below) and were analyzed for primer dimer formation using melt curve analysis. Primers specific to each gene were as follows: HbGR1: upper primer, 5′-TGC CTC TGT CAC TGC CAC CGT AG-3′; and lower primer, 5′-AGT CGT CTG CGT CTG AAG TAA CTG-3′, generating a 109-bp product; HbGR2: upper primer, 5′-CAT CAG AGC CCA CCA TTA GCA ACA-3′l and lower primer, 5′-GGT TCT ATG GCC TTC AGT ATG GA-3′, generating a 103-bp product; and HbMR: upper primer, 5′-GAT GAG GCA TCG GGT TGT C-3′; and lower primer, 5′-TCC CAG CGC ACA GGT AGT TA-3′, generating a 102-bp product. Additionally, to isolate the expression of the splice variant form of HbGR2, one primer was designed with the 3′ end anchored within the 27-bp insertion of HbGR2b: upper primer, 5′-TGT TGG CTT CTC CGG TTC ATC AC-3′; and lower primer, 5′-GTT GTG CTG GCC ATC TGT GTT T-3′, generating a 223-bp fragment (see Fig. 5).

Expression levels of the housekeeping gene glyceraldehyde 3-phosphodehydrogenase (G3PDH; GenBank accession no. AF123727) were used to control for differences in loading and cDNA synthesis efficiency between samples. Primers for G3PDH generating a 78-bp product were as follows: upper primer, 5′-CAC ACA AGC CCA ACC CAT AGT CAT-3′; and lower primer, 5′-AAA CAC ACT GCT GCT GCC TAC ATA-3′.

Real-time PCRs were performed using an iCycler (Bio-Rad Laboratories). Reactions were 30 or 40 μl and included 1× IQ SuperMix (Bio-Rad Laboratories), 0.1× SYBRgreen1 (Molecular Probes, Eugene, OR), 10 nM fluorescein (Bio-Rad Laboratories), 0.5 μM of each primer, and 10 ng cDNA (RNA equivalent). Cycling conditions were as follows: 1 min at 95 C, then 40 cycles of 30 sec at 95 C, 30 sec at 60 C, and 30 sec at 72 C, followed by melt curve analysis. Fluorescence at 490 nm was determined during the annealing step. All reactions were run in triplicate. The mean coefficient of variation for all samples was 0.8%. Reactions that included template from cDNA synthesis reactions with omitted reverse transcriptase did not amplify above background levels or above levels from control reactions that contained no DNA template.

Standard curves were created using serially diluted cDNA samples of 50, 5, 0.5, and 0.05 ng cDNA (RNA equivalent). The efficiency of the primer pairs was calculated using the formula: $E = 10^{[-1/\text{slope}]}$, and was as follows: $E_{HbGR1} = 2.05$, $E_{HbGR2a+b} = 1.96$, $E_{HbGR2b} = 1.95$, $E_{HbMR} = 1.97$, and $E_{G3PDH} = 2.04$. Product melt curves were plotted using iCycler software, and each product was determined to have one peak. PCR reactions were baseline subtracted, and the threshold cycle (CT), the cycle when the fluorescence level rose above background, was determined using iCycler software (Bio-Rad Laboratories, Hercules, CA). Expression levels for each sample relative to G3PDH were calculated with the equation: $E(G3PDH)^{[CT(G3PDH)]}/E(gene)^{[CT(gene)]} \times 100$ (27). Resulting values are the relative gene expression as a percentage of G3PDH expression levels. As G3PDH levels can vary significantly across tissues (27, 28), we have restricted our discussion to within-tissue comparisons.

Trans-activation assays

Subcloning. HbGR1, HbGR2a, HbGR2b, and HbMR were inserted into pcDNA3.1 vector (Invitrogen) for use in expression studies. The HbGR1 library clone in pBSIISK$^-$ (Stratagene) was digested using *Xba*I and *Apa*I, and the insert was isolated using gel electrophoresis and was ligated into pcDNA3.1 Hygro$^+$ in a standard reaction using T4 ligase (Invitrogen). HbGR2a was subcloned using a T/A cloning strategy. The HbGR2a insert was generated by cDNA amplification using the following cycling parameters: 30 sec at 94 C, 25 cycles of a 5-sec denaturation at 94 C and a 5-min annealing/extension at 72 C, followed by a final 5-min hold at 72 C to extend deoxy-ATP nucleotide overhangs. The following primers were designed outside of the coding region and generated a 2821-bp cDNA fragment: upper primer, 5′-CTT CAC CTT GTG GAC GTT GCG GCA TTT-3′; and lower primer, 5′-CAC AGG GCT ATG GGT GTA GAG TAG GTG-3′. Advantage cDNA Polymerase Mix (Clontech) was used with the supplied buffer. Vector (pcDNA3.1 Hygro$^-$) was digested using *Eco*RV to generate blunt ends. Overhanging thymidine nucleotides were added to digested vector in a reaction containing 2 mM magnesium, 2 mM deoxy-TTP, and 1 U Klentaq1 polymerase (AB Peptides, St. Louis, MO) and incubated for 2 h at 72 C. The HbGR2a amplification product was combined with this vector in a standard ligation reaction. To generate a pcDNA3.1 clone containing the splice insertion, the HbGR2b library clone in pBSIISK$^-$ was digested using *Sgr*AI and *Hpa*I and ligated into *Sgr*AI- and *Hpa*I-digested HbGR2a$^+$pcDNA3.1 Hygro$^-$. Full-length HbMR cDNA was generated using as upper primer, 5′-TGC CCA AGC AAC ACA GCC ACC TGA CAA C-3′, and as lower primer, 5′-TGC CGC TTC TGT CCC ATC TGT GTA AAG-3′, in a reaction using 25 cycles of 94 C for 5 sec and 72 C for 5 min. Full-length HbMR product was subcloned into pCRII-TOPO vector (Invitrogen). HbMR in the pCRII TOPO vector was digested with *Bam*HI and *Xho*I and was ligated into pcDNA3.1Hygro$^-$.

Cell culture and transfection. CR-deficient primate renal CV-1b cells were maintained in 5% CO_2 at 37 C in DMEM (Invitrogen) supplemented with 5% stripped (charcoal treated to remove endogenous steroids) fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml). Cells were plated at 2×10^5/well in six-well plates in DMEM containing 5% stripped FBS, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) 18 h before transfection. For cotransfection assays the resulting subconfluent cells (60–80% confluent) were trans-

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HbGR2   MDQGGLKRNGNRDDGL...........TFAEIEGTGDTPGSLFQTAMH..LPGSPPPATVAPNRQGGTNG..........   57
HbGR1   MDKGGVKKIAYRRDDH...........LSKLVYTESPEEGGLLRVAPHSAMSVTSPASVVLPSSSLMQPG..........   59
HbMR    METKRYQSFFDGKDTENRWPQVPGTMEYCCSTEDSSLTGSDILMDIVNVNCPAGSPATDCKDNNTKKQEQPMLQLSQNQP   80
        *+ +++++ + +++*                    ++ ++ + + ++  + +   +  +*+  + ++    + +

HbGR2   ......QGELGGLFESHQHHVLGEGTDMKEGKMIRMQK.....QQQQQQQQ.......................DIGIF  102
HbGR1   ......QVPNGLNNSTLPEELTSASVTATVGSLIDSPQPRGLTKDQRPQHQ.......................QLLQT  109
HbMR    FVLPHFNNSLLGHKQEMDSKELSKTVAESMGLYMNAAREADFAFNQQGANTSPGKLYPACGRPLEENQCGPTKSPKLKPF  160
              +  +++          ++  +     *  +             *+ + +                       +  +

HbGR2   GMGDSLPLFNQCISDTPTSVINTSDTSVLGNLPLPDLFSHPIKTESILSLDKDLGTYSG...HTGTGPCDLDGNSGRLIE  179
HbGR1   QTTFGHQTLSENLSQLDASMADITQSSMDSLIGGSDPNFFPLKTED.FSLDKGE...........QDPIDLDNAFEPIGK  177
HbMR    GFQQPSATPRECTAGTPVSSASMLASSLSCSPQTSSAISSPGGSNNMVSSTTSPPTCFAPLCSSVSSPVSQTSCAATLAN  240
        +     +  ++ + ++ * +      +*         ++  + * +++    ****    +   +          * +++       +

HbGR2   DTEIWQDLDLPSSLPEISAFELD...SEVAHLDNILQESTGGGCPVGGLP.KEIKPLMGNGEN................C  239
HbGR1   DMDVNQKLFSDNTLDLLQDFDLSGSPSDFYVGDDAFLSSLADDSLLGVTSERDIKPAVVDSSN................T  241
HbMR    IKRRNSVTCSPVESSTVGSPPLTSPLNVMRSPMSSPQSMSSVRSPPSCSTTCNIRSSVSSPTAGSCSSKTTNNCNTAMPS  320
        +   ++ + ++   +      + *     +      +   +++     ++ +       *++ +     +

HbGR2   TSVNGTKQQHHALHPHQQQQQHHQLIHHQQHQPQHHQPQALLSTIIIKEEKDPDESFIQICTPG.............VIK  306
HbGR1   TGAVPVALNGSSVTSP.......DLSSPTISTTTSLSPTTTLFAMVKKEK...DADFIQLCTPG.............VVK  298
HbMR    ISSPATAGNMTASSPQNPSSGGFPVSSPADGLGLVQNDTSSPEAGLTRETDFKNFEFPKVEMVDGEVFNVGLDQMGMVKY  400
        ++   ++ +  +  +          ++++          ++  + +   +*      +  *++ ++++                * +

HbGR2   QEKQDNGFCQPQCLQSGISSLHGG.......................................................G  331
HbGR1   QEKTSGG..QSYCQISGTASRDMA.......................................................G  321
HbMR    IKNEPGTDFRSMCLGSSKCNASNTPFITQIKSEPNKSEGCMNQQPYGEQSPSIGLFSASETTYLSLRNNIDEYSLSGILG  480
        +++  ++  ++  + *+  +   +                                                       *

HbGR2   PRPMSSP..............VSVGAVPGYHYTAN.LSSTMDIQ.DQKPFD.MYSNMPLMGDGWARGKRYGETS..GIQS  392
HbGR1   TNAISVCG.............VSTSGGQTYHFGVNTLSSDTPLQNEQKPVSSLFLPVTTIGGIWNRGQGIGNNS..LVQR  386
HbMR    PPVSSVNGNYESDVFSNLSKGVKQEATDGSYYQENNSMSTSAIVGVNSGGHSFHYQIGAQGTMSFTRHDVRDQTNPLLNL  560
        +   *+ +              *+  +  ++++  * +++*  ++  +++  +           *  + ++   + +   + +

                                                            DNA Binding Domain
HbGR2   SDDGPTPVASLAPFSVGFSGSSPREGE.......ISSSVVPAQSKTSGQTHKICLVCSDEASGCHYGVVTCGSCKVFFKR  465
HbGR1   AGEG...FSSSPSYPTSFTR...QEG.........STATSSTQGKSG..THKICLVCSDEASGCHYGVLTCGSCKVFFKR  449
HbMR    ISPVTALMESWKSHPGISQGSLAARGEGYPGQNCISDGMSSSPLRQPSSTAKVCLVCGDEASGCHYGVVTCGSCKVFFKR  640
            +      *  + +  ++ +   ++*      +*   ++ + +    *+*******+**************+**********

HbGR2   AVEGwrarqntdgQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGMNLEARKNKKLIKMKVHR..............  530
HbGR1   AVEG.........QHNYLCAGRNDCIIDKIRRKNCPACRFRKCLMAGMNLEARKLKKNRLKGVQQ..............  505
HbMR    AVEG.........QHNYLCAGRNDCIIDKIRRKNCPACRVRKCLQAGMNLGARKSKKLKLKGVSEDLQGSKDGQTATGGA  711
        ****         *******************************+*****+********* *+*+****

                                            Hormone Binding Domain
HbGR2   ....AGASEPTISN..MPVPVVP.RSMPQLVPTMLSILKAIEPEIIYSGYDSTLPDTSSRLMSTLNRLGGQQVVSAVKWA  603
HbGR1   ....SNPPEVTPSPPVETRSLVP.KCMPQLVPTMLSLLKAIEPDTIYAGYDSTLPDNFTRLMTTLNRLGGRQVISAVKWA  580
HbMR    GGYLSSEKELNASAANALVPHRPGVVTPFLPPSICSVLELIEPEEVYSGYDNSQPDTTDHLLSSLNRLAGKQMVRMVKWA  791
             +   *++ *      +++++* + +*+***+**+*******+ +*******+****+*******+********+*+****

HbGR2   KSLPGFRNLHLDDQMTLLQCSWLFLMSFSLGWRSYEQCNGSMLCFAPDLVINKDRMKLPFMTDQCEQMLKICNEFVRLQV  683
HbGR1   KALPGFRNLHLDDQMTLLQYSWLFLMTFSLGWRSYQQCNGNMLCFAPDLVINEERMKLPYMTDQFEQMLKICSEFVRLQV  660
HbMR    KVLPGFRSLPIEDQITLIQYSWMCLSSFCLSWRSYKHTNGQMLYFAPDLIFNEERMQQSAMYDLCLGMRQVSQEFVRLQL  871
        * *******+++++*+***+*+*+******+*******  ++*+*****+*****+***+****+*++*******+*******+

HbGR2   SYEEYLCMKVLLLLSTVPKDGLKSQAVFDEIRMTYIKELGKAIVKREENPSQNWQRFYQLTKLLDSMQEMVEGLLQICFY  763
HbGR1   SHDEYLCMKVLLLLSTVPKDGLKSQAVFDEIRMSYIKELGKAIVKREENSSQNWQRFYQLTKLLDSMHEMVGGLLSFCFY  740
HbMR    TYDEFLSMKVLLLLSTVPKEGLKNQAAFEEMRVNYIKELRRSVGKATNNSGQTWQRFFQLTKLLDAMHDLVGNLLDFCFY  951
        ++++*+++*******************+****+****+*+++*+++++*+++*+*+***+*+*+*+**********+*+++ ****

HbGR2   TFVN.KTLSVEFPEMLAEIISNQIPKFKDGNVKALLFHQK  802
HbGR1   TFVN.KSLSVEFPKMLAEIISNQLPKFKAGSVKPLLFHQR  779
HbMR    TFRESQALKVEFPEMLVEIISDQIPKVESGLTHTIYFHKK  991
        **++ ++++*****+*+*****+**** * ++ ++***+
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FIG. 1. Alignment of predicted CR amino acid sequences. ClustalX was used to generate an alignment of HbGR1, HbGR2, and HbMR. The nine-amino acid splice insertion found in HbGR2b (residues 470–479) is shown in *lowercase*. The predicted DNA- and hormone-binding domains are indicated by *solid* and *dashed lines*, respectively. Consensus between the sequences is indicated for two or three identical residues by a *plus sign* or an *asterisk*, respectively.

fected using Opti-MEM reduced serum medium containing the Lipofectamine-DNA complex (Invitrogen). Cells were transfected with the following plasmids: 50 ng TAT3-LUC reporter plasmid, 25 ng β-galactosidase expression vector, 900 ng Bluescript KS^-vector plasmid as carrier DNA, and 25 ng HbGR1, HbGR2a, HbGR2b, or HbMR pcDNA3.1 receptor expression plasmid. TAT3-LUC contains a trimerized GRE with the unit sequence TGTACAGGATGTTCT fused to the *Drosophila* alcohol dehydrogenase minimal promoter (−33/+4) driving luciferase expression (11). Additionally, in parallel experiments we cotransfected either rat GR or rat MR plasmid with TAT3-LUC plasmid as described above to directly compare the responsiveness of the cichlid receptors to mammalian receptors. Five hours after transfection, 1 ml fresh DMEM containing 10% stripped FBS was added to each well without removing the transfection mixture, and cells were incubated for an additional 16–18 h. In control experiments performed in parallel, cells were transfected as described above with all plasmids except the steroid receptor plasmid. Steroid hormones (Sigma-Aldrich, St. Louis, MO) were added as described below, cells were harvested 24 h later, and cell extracts were prepared as previously described (14). Cell extracts were quantified by a modified Bradford protein assay (Bio-Rad Laboratories), and 4 mg

protein were subjected to further analysis. Luciferase activity was measured (Luciferase Assay System, Promega, Madison, WI) and normalized to β-galactosidase activity to control for differences in transfection efficiency (14). Receptors were tested with 10-fold titrations of cortisol and/or aldosterone and with no hormone added. Additionally, the following noncorticosteroid hormones were tested at a maximal concentration of 100 nM: estradiol, progesterone, testosterone, and 11-ketotestosterone. Data are reported as fold activation relative to baseline, which is taken as luciferase activity in the absence of exogenous hormone. Values represent the mean and SE of at least four separate transfections. EC_{50} values were determined from a plot of the luciferase expression for each hormone concentration normalized by the maximum response for a given receptor-hormone combination.

Results

Isolation and sequencing of CR cDNAs from H. burtoni

GR. We amplified two GR fragments, one within the hormone-binding domain and the other primarily within the A/B domain. Library screening using these two fragments and subsequent sequencing yielded two transcripts that shared high similarity with GRs of other species, but were substantially different from each other. The first transcript, HbGR1 (GenBank accession no. AF263738), obtained using a probe within the hormone-binding domain (see *Materials and Methods*) was 2620 bp long and did not contain a substantial 3′-untranslated region. Predicted translation yielded a protein of 779 amino acids. The second transcript, HbGR2 (GenBank accession no. AF263739), was obtained using a probe within the A/B and DNA-binding domains and was 3894 bp, corresponding to a protein of 793 amino acids.

To determine whether the two GR clones we isolated indeed corresponded to different genes, we performed genomic Southern analysis. The HbGR1 probe recognized three bands (10.1, 7.8, and 6.9 kb), and the HbGR2 probe recognized one band that was larger than 12 kb (data not shown). Taken together with identification of many sequence differences between the cDNAs (Figs. 1 and 2), this suggests that HbGR1 and HbGR2 are coded by two separate genes and are not merely splice isoforms.

A third clone, named HbGR2b (GenBank accession no. AF263740), was identical to HbGR2, except for the addition of 27 bp, corresponding to nine amino acids, between the two zinc finger domains. This splice insertion has been previously reported in both rainbow trout and flounder GR cDNAs (19, 29, 30). PCR amplification of the HbGR1 DNA-binding domain revealed only one product from all tissues we tested (data not shown), suggesting that the expression of a DNA-binding domain splice insertion was unique to HbGR2.

MR. Degenerate primers designed against androgen receptor sequences amplified a fragment with high similarity to mammalian MRs. The full-length MR sequence was obtained using RACE. The full-length HbMR transcript was 3285 bp, corresponding to 993 amino acids (GenBank accession no. AF263741).

Sequence comparison and phylogenetic analysis of CR

An alignment of HbMR, HbGR1, and HbGR2 revealed that the GRs were more similar to each other than to HbMR (see Fig. 1). A comparison of amino acid identity as a function of

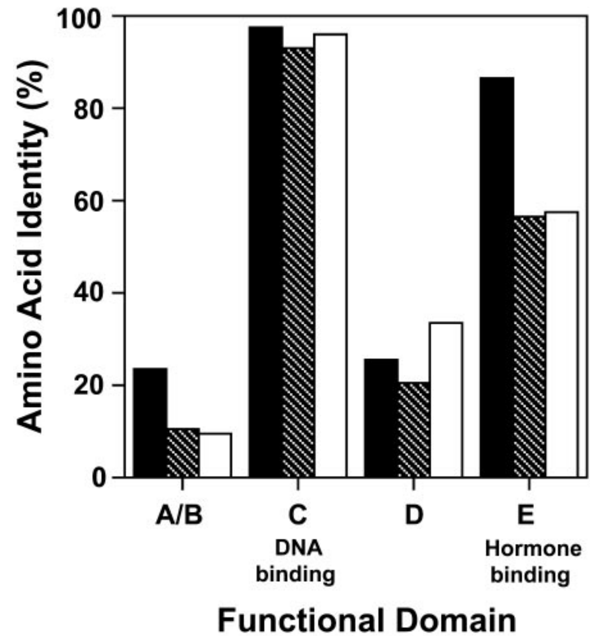

FIG. 2. The percent amino acid identity as a function of receptor domain. Sequences were aligned (ClustalX), and identity matrexes were generated (BoxShade). ■, Comparison of HbGR1 and HbGR2; ▨, comparison of HbGR1 and HbMR; □, comparison of HbGR2 and HbMR.

domain (see Fig. 2) showed that all three receptors shared greater than 90% identity in the DNA-binding C domain. Within the E domain, which is responsible for ligand binding, HbGR1 and HbGR2 were more similar (86% identical) than HbMR and either GR (~57%). Additionally, the GRs were more similar to each other than to HbMR within the A/B domain, which contains subregions important in *trans*-activation (23% between GR, 10% highest identity between MR and one GR). Interestingly, within the D domain, or hinge, GR2 and MR shared the most identity (33%).

A phylogenetic tree comparing the C, D, and E domains of MRs and GRs was generated using the neighbor-joining algorithm (Fig. 3). Identical branching patterns were obtained regardless of which method was used to produce the tree or which functional domains were included (data not shown). As expected, MRs and GRs group on separate branches, and HbMR clusters within the MR branch. Within the GR line, teleost and nonteleost GRs form distinct branches, with the second GR duplication clustering within the teleost lineage.

Comparison of mRNA expression patterns in organs

Receptor mRNA expression levels determined using quantitative PCR were quite different in various body tissues (Fig. 4). In the brain, HbMR expression levels were 2-fold higher than either HbGR1 or HbGR2. In the heart, liver, and spleen, the expression of HbGR2 was higher than that of HbGR1 or HbMR. In the kidney and spleen, HbMR levels were much lower than those of either GR. The distribution of all three receptors was overlapping in the gill.

To examine the distribution of the HbGR2 spice variants, we used PCR to isolate the expression of HbGR2b (see *Ma-*

terials and Methods and Fig. 5). The expression of HbGR2b relative to total HbGR2 was different as a function of tissue type (Fig. 5). Specifically, in most animals HbGR2 was preferentially expressed in the gill and liver, whereas HbGR2a was primarily expressed in the kidney and spleen. In the heart and the brain, a combination of both variant forms was expressed, and the ratio differed among animals.

Functional analysis of transcriptional activity

We compared the ability of the cichlid CRs to induce transcriptional activity on a trio of canonical GREs (responsive to both MR and GR) using cotransfection assays. In the absence of cotransfected CR, the TAT3-LUC reporter plasmid directed the expression of low levels of luciferase that did not increase with any hormone application (data not shown). Noncorticosteroid hormones did not cause activation above baseline, whereas cortisol and aldosterone induced all four receptors (see Fig. 6).

Comparisons of the sensitivity of the receptors to titrations of cortisol and aldosterone revealed differences between HbMR and GRs. Table 1 shows the EC_{50} values and maximal fold induction of luciferase for each receptor, and data are plotted in Fig. 7. HbMR appeared equally sensitive to cortisol and aldosterone; the EC_{50} values were 0.02 and 0.05 nM, respectively. In parallel experiments we compared the activity of cichlid MR to that of rat MR (Fig. 7). As is typical of rat MR, we found that it was sensitive to aldosterone at a concentration 10-fold lower than for cortisol (see Table 1). Compared with rat MR, HbMR was equally sensitive to aldosterone and was nearly 10-fold more sensitive to cortisol. EC_{50} values of rat MR for aldosterone were lower than those reported in the literature for reasons that remain uncertain at this time. Importantly, the sensitivity of HbMR to aldosterone was similar to that of its well characterized rat counterpart. These data suggest that HbMR functions like a classical MR *in vitro*.

The sensitivities of the cichlid GRs to cortisol were very similar to each other, with EC_{50} values in the range of 2–5 nM, comparable to the value of 3 nM obtained with the rat GR (Fig. 7 and Table 1). All three cichlid GRs were approximately 100-fold less sensitive to cortisol than HbMR. Additionally, the cichlid GRs were unresponsive to aldosterone concentrations less than 10^{-9} M. The EC_{50} of GRs to aldosterone was not determined because the responses did not reach a plateau at 10^{-6} M, which was the maximum concentration used in these experiments (data not shown). Thus, HbMR and HbGR1/2 can be functionally separated into two different groups, aligning with the classifications for tetrapod CR; HbMR is more sensitive to both cortisol and aldosterone than either GR.

A comparison of the maximal luciferase expression revealed additional differences among the cichlid corticosteroid receptors (Table 1). Among the GRs, HbGR1 elicited the highest luciferase expression, followed by HbGR2a. HbGR2b, the isoform with a nine-amino acid insertion be-

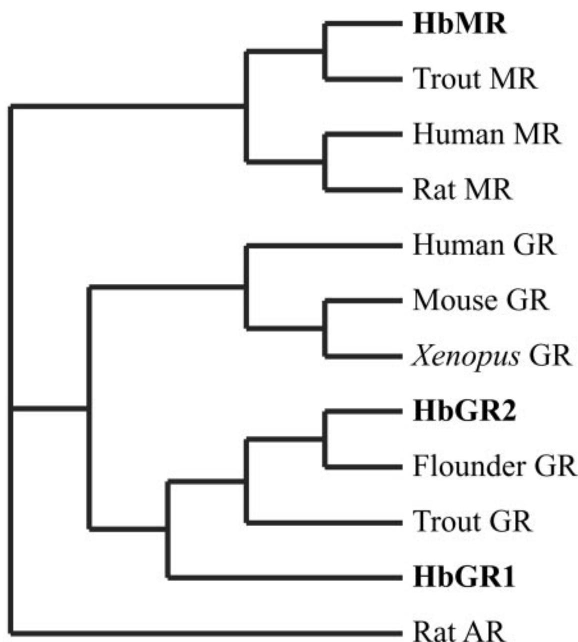

FIG. 3. Phylogenetic tree comparing the sequences of GR and MR C, D, and E domains. PAUP software was used to generate a phylogenetic tree using the neighbor-joining method. Sequences from *H. burtoni* are shown in **bold**. Rat androgen receptor (AR) is included as an out-group.

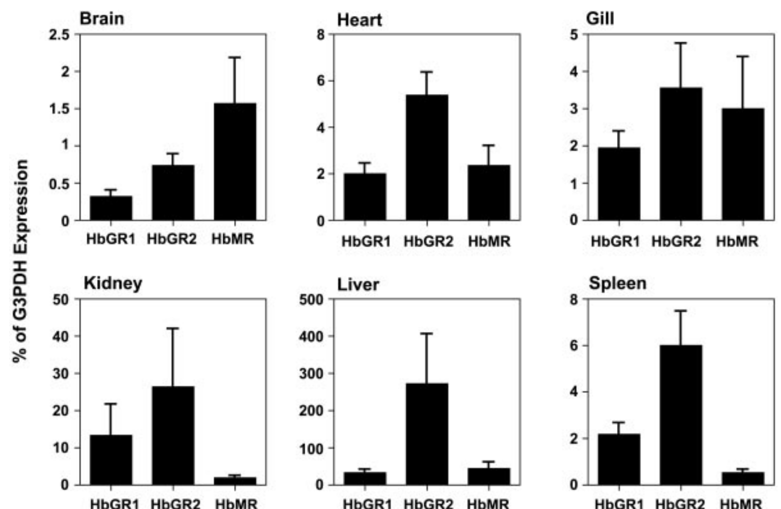

FIG. 4. Comparison of gene expression patterns within body tissues. Real-time PCR was performed on tissues from five adult *H. burtoni* females (n = 4 for liver and spleen). Relative expression of HbGR1, HbGR2, and HbMR is shown as a percentage of the G3PDH expression level for the various tissues. Data are plotted as means, with SE designated by *error bars*.

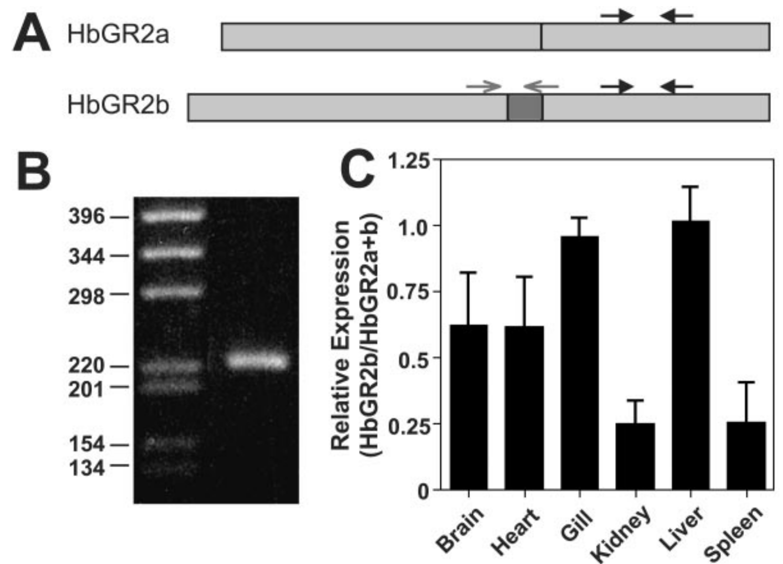

FIG. 5. HbGR2 splice variant expression patterns. A, A diagram of the two splice variants shows the locations of the primers used to specifically amplify HbGR2b (*gray arrows*). Primers designed to detect both variants are shown with *black arrows*. B, Ethidium bromide-stained agarose gel showing a real-time PCR of brain cDNA using HbGR2b-specific primers. A single band was seen at 223 bp. False priming of HbGR2a would produce a band at 198 bp, which was not present. C, Relative expression of the splice variant HbGR2b as a ratio of total HbGR2 expression levels determined by real-time PCR. Data are shown as the mean and SE for tissue from five fish, except for liver and spleen, where n = 4.

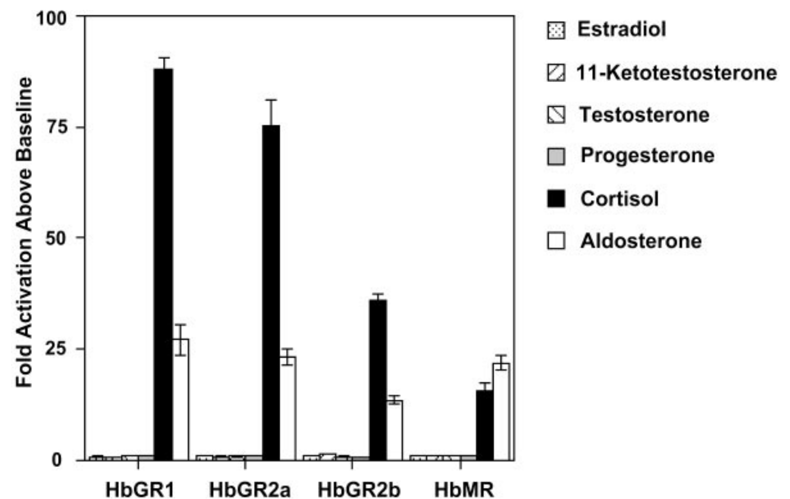

FIG. 6. Selectivity of CR to various steroid hormones. HbGR1, HbGR2a, HbGR2b, or HbMR was cotransfected with TAT3-LUC reporter plasmid into CV-1b cells. Induction of luciferase expression above baseline (no hormone) by each CR with the following steroid hormones is shown: estradiol, 11-ketotestosterone, testosterone, progesterone, cortisol, and aldosterone. Hormones were applied at a concentration of 100 nM. Values represent the mean and SE of at least four separate transfections.

TABLE 1. Summary of *trans*-activation data

	Hormone	EC_{50}	Maximum activation
HbGR1	Cortisol	3.6	88
HbGR2a	Cortisol	5.4	75
HbGR2b	Cortisol	2.1	36
RatGR	Cortisol	3.0	23
HbMR	Cortisol	0.02	16
HbMR	Aldosterone	0.05	25
RatMR	Cortisol	0.3	13
RatMR	Aldosterone	0.04	21

EC_{50} is the concentration in nanomoles at which the response is half-maximal. Maximum activation is the highest amount of fold induction of luciferase expression over baseline (no hormone) levels.

tween the zinc fingers, caused the lowest maximal expression of all GR on this reporter, which is driven by a trio of canonical GREs. The *trans*-activation induced by HbMR in response to cortisol was lower than that of any GR. This finding is similar to our comparison of rat GR and MR, and with what has previously been described for mammalian receptors (13, 31). These effects may reflect the lower capacity for transcriptional synergy on GRE multimers in MR relative to GR (15, 31, 32) and perhaps in HbGR2b relative to HbGR2a. Interestingly, the cichlid GR all induced higher luciferase expression than the rat GR.

Discussion

We have described the expression of four distinct receptors for corticosteroid hormones in one species of cichlid fish. Two of these genes are similar to tetrapod GR genes, and one of these GRs is expressed in two variant isoforms. We identified another CR whose sequence is orthologous to mammalian MRs. These four receptors are differentially responsive to cortisol and are expressed in distinct patterns throughout the body, suggesting the possibility of an even more intricate system regulating corticosteroid effects in fish than that described for tetrapods.

Tissue expression of CR

Analysis of gene expression levels using quantitative PCR revealed differential tissue distribution of the four receptors. HbMR was the dominant CR in the brain and was expressed at low levels in the kidney/interrenal and spleen compared

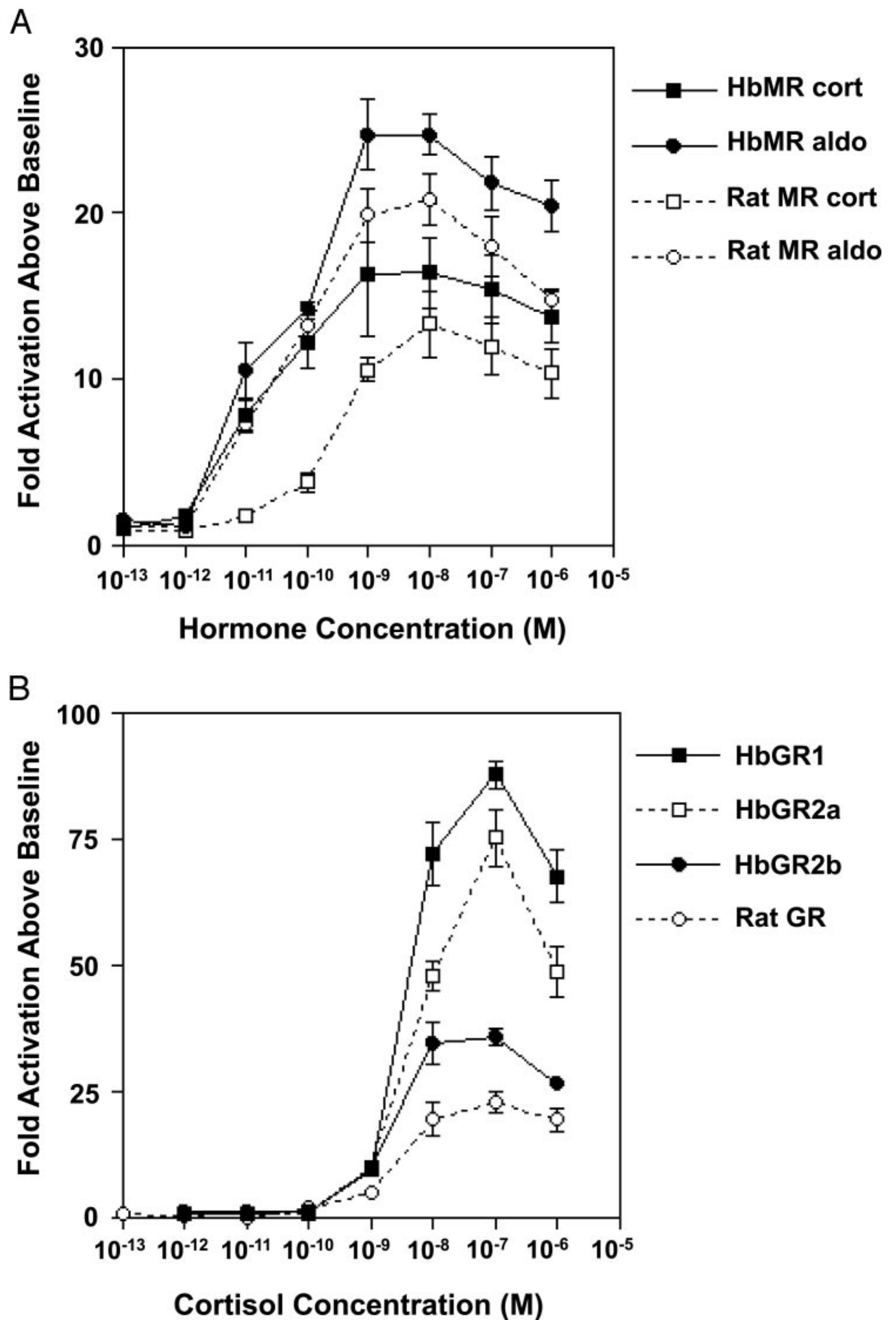

FIG. 7. CR induced *trans*-activation in response to titrations of hormone. Each CR was transfected into CV-1b cells along with a luciferase reporter plasmid containing a three GRE promoter (TAT3-LUC). A, HbMR and RatMR were presented with titrations of cortisol and aldosterone. B, HbGR1, HbGR2a, HbGR2b, and RatGR were presented with titrations of cortisol. Data are shown as the fold induction over the no hormone condition and represent the mean and SE of at least four separate transfections.

with the GR forms. In most tissues HbGR2 was more highly expressed than HbGR1, and HbGR2 levels were higher than those of both HbGR1 and HbMR in heart, liver, and spleen. Previous work has localized GR in fish using Northern analysis; however, as of this writing, MR expression patterns in fish have not been previously reported. In the rainbow trout, GR2 mRNA expression levels are highest in the intestine and lower in the gill, muscle, kidney, and liver (19). In another cichlid fish, *Oreochromis mossambicus*, a GR1-like receptor is expressed highly in the gill and spleen; moderately in the brain, heart, and muscle; and at low levels in the liver (24). Thus, similar to mammals, GR is present in fish tissues that are involved in osmoregulation, glucose metabolism, and cognition.

Role of MR in fish

We found that the cichlid MR was highly sensitive to both aldosterone and cortisol. The results from our *trans*-activa-

tion studies suggest that MR serves at least partially as a high affinity cortisol receptor in fish, but whether MR is responsive to aldosterone *in vivo* remains uncertain. Many teleost fish have no measurable aldosterone and do not produce this steroid *in vitro* (33–35), including *O. mossambicus,* the closest relative to *H. burtoni* that was studied (36–38). Although some teleosts do appear to synthesize this mineralocorticoid (39–42), it has been suggested that the minute amounts of aldosterone reported in some of these fish are not biologically relevant (20). However, we showed that HbMR was sensitive to extremely low levels of aldosterone *in vitro*, suggesting that these low circulating levels may indeed be sufficient to activate MR. Additionally, most fish have circulating levels of the cortisol breakdown product cortisone (34, 36–39, 42), which suggests that the activity of 11β-hydroxysteroid dehydrogenase type 2. Thus, MR could be specifically activated by aldosterone in certain cells. Alternatively, it is possible that fish MR retained such high sensitivity for aldosterone in its absence over millions of years of evolution simply as a by-product of being highly sensitive to cortisol.

GR splice variants

Our comparison of expression patterns and *trans*-activation activity revealed that the two HbGR2 splice variants differ in function and tissue distribution. HbGR2b has a nine-amino acid insertion between the two zinc fingers within the DNA-binding domain, which caused a substantially lower maximum response on a three GRE promoter in response to cortisol. This insertion was first identified in another teleost fish, the rainbow trout (19), was later described to be one of two expressed splice variants (29), and has been subsequently described in the flounder (30). In contrast to our results, previous comparisons of the two GR2 splice variants from rainbow trout revealed no obvious differences in transcriptional activities with the application of the synthetic glucocorticoid, dexamethasone (29), with a one or two GRE promoter (43). However, Lethmonier *et al.* (43) reported differences in the affinities of the two isoforms for GRE sequences in a gel-shift assay. Specifically, the trout isoform with the insertion had higher affinity for a single GRE sequence, whereas the noninsert form had higher affinity for two GREs in tandem. Although there was no difference in *trans*-activation of the receptors with the application of dexamethasone, there was a difference in basal transcription levels in the presence of no exogenous hormone. In this case the insert form induced more reporter transcription on a single GRE under hormone-independent conditions. In contrast to these results, we found substantially greater activation by the cichlid noninsert isoform using a promoter with three GREs in response to cortisol, and no difference under hormone-independent conditions. These divergent results may reflect the synergism that can occur at multimeric GREs (15, 44, 45), which may have enhanced differences in the activity of the receptor variants in our experiments.

We found that the GR2 isoforms were differentially expressed in body tissues responsive to corticosteroids. Quantitative PCR analysis showed that in the kidney and spleen, the receptor without the nine-amino acid insert (HbGR2a) was expressed at higher levels, whereas HbGR2b was the dominant form in the gill and liver. In the brain and heart, the ratio of isoform expression varied in different animals. In trout, using conventional RT-PCR, the testis was the only organ to express the noninsert isoform, and there was no expression in the spleen [kidney levels were not reported (29)]. These differences may be accounted for by differences in methodology or by species differences, as rainbow trout and cichlids are in different taxonomic orders.

The nine-amino acid insertion in the DNA-binding domain changes the protein structure between the two α-helical zinc fingers, which could affect the distance between the two receptor dimers and their interaction with DNA (46). Classical GREs are composed of two palindromic half-sites separated by three nucleotides; however, it has been suggested that there may be GREs with alternate spacing or even comprised of multiple independent half-sites in tandem with other regulatory regions (47–50). It is possible that this insertion may alter the spacing between the two zinc fingers so as to improve binding to these nonclassical elements. It is interesting to speculate that the differential expression of the two HbGR2 splice variants that we describe could reflect the regulation of tissue-specific genes by different types of glucocorticoid-sensitive promoters. For instance, tissues such as the gill and liver, which preferentially contain HbGR2b, may express genes whose promoters include these nonclassical GREs.

CR duplications

Our phylogenetic analysis suggests that the identification of two GR paralogues will be unique to fish. This is consistent with previous hypotheses that teleost fish appear to have undergone additional gene duplications compared with the rest of the vertebrate line. This duplication may have occurred in the whole genome (51) or in selected genes (52). In either case, bony fish may be expected to have an increased number of steroid receptors compared with tetrapods (53). Accordingly, a second form of the androgen receptor has been identified in the eel (54) and *H. burtoni* (55), and a third estrogen receptor has been identified in the Atlantic croaker (56). Cichlids, including *H. burtoni*, are diploid (57). Thus, we expect that the presence of an additional GR gene is not merely an effect of increased ploidy and will likely be found in other teleosts. Accordingly, evidence from GR binding studies in salmon and eel that environmental or hormonal manipulations can alter synthetic glucocorticoid binding affinity suggests that other teleosts may indeed have multiple cortisol receptors (58–60).

What has been the fate of duplicated CR? Gene duplication can have many potential consequences over the course of evolution. In general, as redundancy reduces the selective pressure on one gene copy, both genes are not expected to retain similar functions. Most random mutations are predicted to be deleterious, so the parsimonious outcome of gene duplication is for one gene to become a nonexpressed pseudogene (61). Alternatively, the new gene may assume entirely new functions, or the two genes may divide the work of the gene product (62). Clearly the two GR genes are not pseudogenes, as their expression is detected by RT-PCR from

mRNA. Moreover, they have retained the capacity to selectively respond to cortisol, and both share high sequence identity with GR of other species. Given that this duplication event probably occurred many millions of years ago, it appears that selective pressure has maintained the basic functions of a GR in both HbGR1 and HbGR2. Furthermore, as they are expressed in cortisol-responsive tissues, both may contribute to glucocorticoid signaling processes. Their *trans*-activation properties are distinct when tested on a promoter with three classical GREs in tandem, with HbGR1 eliciting a higher level of reporter gene expression. This suggests that their functional responses have diverged. Additionally, evidence from *in situ* hybridization suggests that they are expressed in different patterns in the brain (Greenwood, A. K., and R. D. Fernald, unpublished observations). Thus, each may have embarked on different regulatory paths and are not merely redundant. Whether the GRs have also developed novel functions or simply divided the functions of an ancestral GR remains to be determined.

In summary, the discovery of four CR in a single species of cichlid reveals the possibility of unique regulation of corticosteroid effects in fish. Future work will aim to discover the distinct role that each receptor plays in regulating these effects.

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