

Evidence for two distinct functional glucocorticoid receptors in teleost fish

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Abstract

Using RT-PCR with degenerated primers followed by screening of a rainbow trout (*Oncorhynchus mykiss*) intestinal cDNA library, we have isolated from the rainbow trout a new corticosteroid receptor which shows high sequence homology with other glucocorticoid receptors (GRs), but is clearly different from the previous trout GR (named rtGR1). Phylogenetic analysis of these two sequences and other GRs known in mammals, amphibians and fishes indicate that the GR duplication is probably common to most teleost fish. The open reading frame of this new trout GR (named rtGR2) encodes a protein of 669 amino acids and *in vitro* translation produces a protein of 80 kDa that appears clearly different from rtGR1 protein (88 kDa). Using rtGR2 cDNA as a probe, a 7.3 kb transcript was observed in various tissues suggesting that this gene would lead to expression of a steroid receptor. *In vitro* studies were used to further characterize this new corticosteroid receptor. Binding studies with recombinant rtGR1 and rtGR2 proteins show that the two receptors have a similar affinity for dexamethasone (GR1 $K_d=5.05\pm 0.45$ nM; GR2 $K_d=3.04\pm 0.79$ nM). Co-transfection of an rtGR1 or rtGR2 expression vector into CHO-K1 or COS-7 cells, along with a reporter plasmid containing multiple consensus glucocorticoid response elements, shows that both clones are able to induce transcriptional activity in the presence of cortisol and dexamethasone. Moreover, at 10^{-6} M 11-deoxycortisol and corticosterone partially induced rtGR2 transactivation activity but were without effect on rtGR1. The other major teleost reproductive hormones, as well as a number of their precursors or breakdown products of these and corticosteroid hormones, were without major effects on either receptor. Interestingly, rtGR2 transactivational activity was induced at far lower concentrations of dexamethasone or cortisol (cortisol $EC_{50}=0.72\pm 0.87$ nM) compared with rtGR1 (cortisol $EC_{50}=46\pm 12$ nM). Similarly, even though RU486 inhibited transactivation activity in both rtGR1 and rtGR2, rtGR1 was more sensitive to this GR antagonist. Altogether, these results indicate that these two GR sequences encode for two functionally distinct GRs acting as ligand-inducible transcription factors in rainbow trout.

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Introduction

In mammals the major hormone involved in regulating mineral balance is aldosterone (Bonvalet 1998, Farman 1999). In teleost fish the interrenal

tissue (akin to the adrenal cortex in mammals) is unable to synthesize this mineralocorticoid. This is demonstrated by the inability of eel cytochrome P450 (11 β) (11 β -hydroxylase) to synthesize aldosterone (Jiang *et al.* 1998), and that the interrenal

tissues of tilapia and salmon are unable to produce aldosterone (Sandor & Medhi 1980, Balm *et al.* 1989, Sangalang & Uthe 1994). Evidence strongly suggests that the major corticosteroid produced by this gland, cortisol, regulates both glucocorticoid and mineralocorticoid activities in fish (Bern & Madsen 1992, Wendelaar Bonga 1997), and that target gene expression is achieved via cortisol binding to corticosteroid receptors which act as ligand-inducible transcription factors (Hollenberg *et al.* 1985, Arriza *et al.* 1987, Gronemeyer & Laudet 1995).

In vitro cortisol-binding studies have identified the presence of a single high-affinity, low-capacity corticosteroid receptor in a number of different teleost species and tissues (e.g. Sandor *et al.* 1984, Weisbart *et al.* 1984, Maule & Schreck 1990, Pottinger & Moore 1997, Weyts *et al.* 1998, Marsigliante *et al.* 2000), and full-length glucocorticoids receptor (GR) cDNAs (rainbow trout (*Oncorhynchus mykiss*): Ducouret *et al.* (1995), Takeo *et al.* (1996); Japanese flounder, Genbank ABO1344) and a partial tilapia GR cDNA (Tagawa *et al.* 1997) have been cloned. Expression of rainbow trout GR (rtGR) transcript is found in a number of tissues (Ducouret *et al.* 1995, Takeo *et al.* 1996), is widely distributed in the brain (Teitsma *et al.* 1997, 1998) and has been identified in gill chloride cells (Uchida *et al.* 1998), the cells believed to be responsible for branchial ion movement (Perry 1997). Despite the absence of aldosterone in fish, a recent study identified a partial cDNA sequence of a corticosteroid receptor in rainbow trout, whose amino acid sequence showed a high homology with known mineralocorticoid receptors (MRs). This putative trout MR shows a higher ability to bind cortisol than aldosterone (Colombe *et al.* 2000). This is clearly different from the situation in mammals where the MR binds cortisol (or corticosterone) and aldosterone with equal affinity (Funder *et al.* 1988). Thus, it appears that in teleosts, corticosteroid control of physiological processes is more complex than initially thought and involves a multi-receptor system (GR and MR). However, recent data indicated that this picture is probably even more complex. In the present study, we report the isolation of a full-length cDNA whose sequence has similarities, but is distinct from the first rtGR (Ducouret *et al.* 1995, Takeo *et al.* 1996), suggesting the presence of a second GR in rainbow trout. This paper focuses on the functional characteristics of

this new trout GR (named rtGR2), and our data reveal that this receptor appears to be different from the previously described rtGR (named rtGR1: Ducouret *et al.* (1995), Takeo *et al.* (1996)).

Materials and methods

Cloning of rtGR2

The first sequence for the second rtGR was obtained by RT-PCR using the degenerate primers designed based on the conserved regions of the DNA- and steroid-binding domains of known steroid receptors (see Colombe *et al.* 2000), R1: 5'-CGCGGATCCTGTGGVAGCTGCAARGTS TT-3', and R2: 5'-ATAGAATTCAGTTCCTTG ATGTARTTCAT-3'. Poly A+ RNA was isolated from testis using a FastTrack mRNA purification system (Invitrogen) and 1 µg Poly A+ RNA was reverse transcribed using 200 units MoMLV-reverse transcriptase (Gibco BRL) at 37 °C for 1 h in the presence of 500 µM of each dNTP (Boehringer), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 10 ng oligo (dT)₁₂₋₁₈ (Pharmacia) and 10 U RNasin (Promega), in a total volume of 20 µl. PCR was carried out with approximately 10 ng equivalent PolyA+ RNA cDNA, 0.25 U Amplitaq DNA polymerase (Perkin Elmer) in the presence of 200 µM of each dNTP (Boehringer), 2 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 30 pmol of each primer, R1 and R2. The total volume of the reaction was 12.5 µl and cycling conditions were 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min for a total of 30 cycles. The PCR product was subcloned into BamHI and EcoRI linearized Bluescript vector after purification (Geneclean, BIO101, La Jolla, CA, USA). cDNA sequence was determined on both strands by fluorescent automatic sequencing using ABI prism 310 from Perkin Elmer.

Alignment of this sequence with other receptors showed high homology with rtGR (Ducouret *et al.* 1995). From this sequence two specific primers were chosen, R3: 5'-CAGAGGCCAGGTCGCT GTCCCA 3' and R4: 5'-TACTCGTCATGAG ACACCTGC. The resulting PCR product was of 440 bp and was used to generate a probe for screening a rainbow trout intestinal cDNA library. Screening and hybridization protocols followed those previously described (Ducouret *et al.* 1995).

The four positive phages identified were excised by EcoRI restriction enzyme cutting and inserted into EcoRI linearized pGEM4Zf (Promega) plasmid. The cDNA sequences of these clones were determined as described above.

Phylogenetic analysis

The new GR sequence was compared with known GR sequences from NUREBASE (Duarte *et al.* 2002), including seven mammals: *Homo sapiens* (U01351), *Aotus nancymae* (U87952), *Tupaia belangeri* (Z75079), *Mus musculus* (X04435), *Rattus norvegicus* (M14053), *Cavia porcellus* (L13196), *Saimiri boliviensis* (AAC51133), the amphibian *Xenopus laevis* (X72211), and the teleost fishes *Paralichthys olivaceus* (AB013444) and *Tilapia mossambicus* (D66874), as well as the previously known trout GR (Z54210). In addition, putative GR genes were found by BLAST (Altschul *et al.* 1997) analysis of genome survey sequences from *Tetraodon nigroviridis* (GSS=CNS01UL9) and *Takifugu rubripes* (GSS=T012764 Scaffold 12767; GSS2=T002578 Scaffold 2578). Phylogenies were rooted with MR sequences from NUREBASE (Duarte *et al.* 2002). Protein sequences were aligned by hand using Seaview (Galtier & Gouy 1996). Phylogenetic trees were reconstructed using neighbour-joining (Saitou & Nei 1987), as implemented in Phylo_win (Galtier & Gouy 1996), correcting for multiple substitutions by a Poisson model, using only complete sites. The results were checked with other methods, including rate heterogeneity between sites with a gamma law, estimated by TREE-PUZZLE (Strimmer & von Haesler 1996) with eight rate categories, in neighbour-joining or quartet puzzling maximum likelihood. The solidity of nodes in the tree was evaluated by bootstrap (Felsenstein 1985), with 2000 repetitions, and by the Kishino–Hasegawa likelihood test (Kishino & Hasegawa 1989) implemented in TREE-PUZZLE (Strimmer & von Haesler 1996), between the alternative hypotheses of the two rtGRs grouping together vs a duplication shared by different fishes.

RNA preparation and Northern blot

All experimental animals were housed, handled and used in accordance with internationally recognised guidelines. Tissue samples (gills, intestine, kidney, liver, muscle, heart, spleen, skin, brain)

were taken from five rainbow trout (approximate weight 100 g) acclimated to dechlorinated fresh-water and immediately frozen in liquid nitrogen, pooled together, and stored at -70°C until use. Total RNA was prepared from frozen samples using Trizol (Gibco) by the procedures described by the manufacturers. PolyA⁺ RNA was prepared from total RNA using a Polytract mRNA isolation system (Promega). The purity and quantity of RNA was determined by spectrophotometric methods and gel electrophoresis and 15 μg PolyA⁺ RNA from each tissue were electrophoresed in a 1% agarose gel under denaturing conditions. The RNA was transferred to nylon membranes (Biodyne Pall, Portsmouth, UK) and pre-hybridized in Church buffer (0.5 M phosphate buffer, pH 7.2, 1 mM EDTA, 1% BSA, 7% SDS) containing 50 $\mu\text{g}/\text{ml}$ denatured calf thymus DNA at 65°C for 3 h. The membrane was hybridized in the same buffer containing a probe labelled with [³²P]dCTP as described above and generated by PCR from the 5'-untranslated region and A/B domain from nucleotides 142–720 of the GR2 sequence in Fig. 1. After hybridization for 16 h the membrane was washed twice at room temperature in $2 \times \text{SSC}$, 0.1% SDS and a further two times at 60°C in $0.2 \times \text{SSC}$, 0.1% SDS. Northern blot analysis for rtGR1 was performed using the same membrane, which had been de-hybridized in 5 mM Tris, 2 mM EDTA, 0.5% Denhardt's solution, pH 8 for 30 min at 60°C . Pre-hybridization and hybridization followed the same protocol described above, except the rtGR1 probe was generated by PCR from rtGR1 nucleotides 598–1063, a sequence from the 5'-untranslated region (Ducouret *et al.* 1995). Probe specificity was confirmed by Southern blot (data not shown). The membrane was further de-hybridized and hybridized with an actin probe that was generated by PCR using salmonid-specific primers (Genbank AF012125).

In vitro expression of proteins and ligand-binding studies

In vitro translation of proteins from a 500 ng rtGR1 or rtGR2 plasmid template was performed using a TNT-coupled reticulocyte lysate *in vitro* translation system (Promega). The pGEM4Zf-rtGR2 template was used for rtGR1, and for rtGR1 expression the full-length rtGR1 cDNA was excised from pUC H1/P4 by EcoRI and SpHI digestion (Ducouret

1 gcgccgctcttactttcatttttggctgggtgaacgacacagctaattccggtgattttcgggtgcacttttatacctaa
 80 cacattgggttggatgatccgattaatgacttgccttattcgtgtcctgctacgattcgcaaggagaacgcaatcagatg
 159 ttttcgtgtttctggcccattttgcgagcaaaataacaccaaacaagaacaacaatgctcagacgagtgcatggagtcaca
 238 tccagctaccaaacgcctgaggggattgtaagatgggtcaaggaggagtgagagcaggagtaacaaaacaacagcaga
 317 gttagggcgcgtgtgatagaaaaccctcagagggaaacccttctcaactacgctcccaacatgtccggtgcttggaaa
 396 ccaccatcgcgccttgttccctcgatccagccaactgatgcaaccaggtgacgtgccagcgggaaccagtaacagacct
 475 cacctcagccaccgctcctggtggcctctactctggaataccagagcctcccgcacaaaggtttgtacaaggaccaga
 554 gagccagaccagcagacctacatgttctctgagaatctgactcttcatctcctcggggagacctactcctgcttggaaaa
 633 cagctgacttccaccgctccacacacagcatggaactccctaatcggggactcagacccaacctgttccccatgtccat
 1 M D S L I G D S D P N L F P M S M
 712 gaggacagagacagccttctcgcgggaccagaaccctatggatgaccaggaagggtacattggaaaagaccagaaa
 18 R T E T A F S R D Q N P M D M D Q E G Y I G K D Q K
 791 ctgttcagcgacaactctggatctcctccagacttttgagttgacaggtccccctcagacttttacgtagggagc
 44 L F S D N T L D L L Q D F E L T G S P S D F Y V G D
 870 acgcttctctcctcctcctgggtgatgacactctcctgggggatgagagccaggatcgtgggggtctccaactccacctc
 70 D A F L S S L A D D T L L G D E S Q D R G V S N S T S
 949 caagccagctgactatgaccaatagtggtagtttttagcggttccaacaccaccacatgaaatggaaggtagcagctacta
 97 K P A A T M T N S G S F S G S N T T L N G S L L
 1028 gcatgtcaggatgagttccaactccagcaactctatgacaaccaccgcaactttccccatggtgaagatggagaaggaat
 123 A C Q D E S N S S T S M T T T A T F P M V K M E K E
 1107 ctggcttccatccagctgtgtactccaggggtgatcaaacaggagaacacgtctgcatgagcagctcctcctgccagat
 149 S G F I Q L C T P G V I K Q E N T S A M R S S S C Q M
 1186 gagtggtagtaccggcggtccacttctcttctcctccgagctgtccagctccagccccagccccatcctcctgt
 176 S G S T S G T S T S S P S E L S S S S P I S I C
 1265 ggggtgagcacctctggaggacagagctaccactttggaggcaacagcagcattaacaccacctggcttccactacca
 202 G V S T S G G Q S Y H F G G N S S I N T T L A S T T
 1344 gtggagcttctcagcagaaggatcagaagccctcgggtgttcagcctgtatcctccgctgggtgacagtaggagaggcctg
 228 S G A S Q Q K D Q K P S V F S L Y P P L V T V G E A W
 1423 gaacaacattagctatgggagctcaggaatgcaaggcctctccagccctcctccagccctcttctccagcagctat
 255 N N I S Y G D G A S G M Q G L S S P T S A F S S S Y
 1502 gccagttctacctccaagcttgggtgggtggcgtcgtcatgtacaaccagggaaggccgggaccaccataaggtct
 281 A S S T S K L G G G A A S C T T Q G K A G T T H K V
 1581 gtctgggtgttcagatgagggcgttggctgtcactcaggggtgtcactgcggaagctgcaaggtgttcttcaagag
 307 C L V C S D E A S G C H Y G V L T C G S C K V F F K R
 1660 agccgtggaaggtacaggggcaaggggcaacacaactacctgtgtgctgggagggaacgactgcatcatcgataagatca
 334 A V E G T G A R G Q H N Y L C A G R N D C I I D K I
 1739 gaaggaagaactgtccagcctgccgcttccgcaagtgtctcctggcagggatgaacctggaagctcgtaaaaccaagaa
 360 R R K N C P A C R F R K C L L A G M N L E A R K T K K
 1818 gctgaaccgtttaaagggggtgcagcagccaccactgcccagctgacccccctccagagggcaggtcgtcgt
 387 L N R L K G V Q Q P T T A E E L T P R P L P E A R S L
 1897 gtccccaggtccatgccccagctcaccaccacctatgctgtcctactaaaggccatcgagccagacaccatctactctg
 413 V P K S M P Q L T P T M L S L L K A I E P D T I Y S
 2976 gctacgacggcacctgccagacacctccaccgcatcatgaccacctcaacaggtggggggagcagaggtggatc
 439 G Y D G T L P D T S T R I M T T L N R L G G R Q V V S
 2055 agccgtcaagtgggccaagccctgccaggggttccggaaccttccactggagcaccagatgactctgctgaggttcc
 466 A V K W A K A L P G F R N L H L D D Q M T L L Q C S
 2134 tggttgttctcctatgtccttccgctgggctggaggtcctaccaacagtgtagcgggaatagctctgcttccgctccag
 492 W L F L M S F G L G W R S Y Q Q C D G N M L C F A P
 2213 acctcgtcatcaaccaggaccgtatgaagctgcctacatggcagaccagtgtagaacagatggtgaagatctccagtg
 518 D L V I N Q D R M L K P Y M A D Q C E Q M L K I S S E
 2292 gtttgtcagactgaggtgtctcatgacgagtatctgtgtatgaaggttctgctgctcagcaccgtcgaaaagat
 545 F V R L Q V S H D E Y L C M K V L L L L S T V P K D
 2371 ggtctgaaagatcaggcaggtttgatgagatcaggatgtcctacatcaaggagttggggaaggccatcgtaagagag
 571 G L K S C Q A V F D E I R M S Y I K E L G K A I V K R
 2450 aggaaaactccagcaactggcaactcttaccagcttccaccaagcttcttgactccatgacagagatggttgggtg
 597 E E N S S Q N W Q R F Y Q L T K L L D S M H E M V G G
 2529 gctgctggatttctgcttctacagttcgtcaacaagctcactgctcgggtggagttccagaaatgtagcagagatcatc
 624 L L D F C F Y T F V N K S L S V E F P E M L A E I I
 2608 agcaaccagttacaaaaattcaaagccgggagcgtcaagccctcctcttccaccagaaatgactgtgtgtgtgtgt
 650 S N Q L P K F K A G S V K P L L F H Q K
 2687 ggtgctaccgcaactggcctgtccaccaccgagacaaatgccttaaaatccctcacttccccctgacagagggaga
 2766 cagggagcggccgcaattc

et al. 1995), and the resulting fragment inserted into the vector pBluescribe. All subsequent procedures followed the protocol suggested by the manufacturer using the T7 and SP6 RNA polymerase for rtGR1 and rtGR2 translation respectively. A luciferase template provided by the manufacturer acted as an internal control. The translation was performed in the presence of 20 μ Ci [35 S]methionine (1000 Ci/mmol), and the radiolabelled products were run on SDS-PAGE electrophoresis, and then visualized after the gel had dried by autoradiography for 8 h.

For steroid-binding studies the lysate containing either the recombinant rtGR1 or rtGR2 protein was diluted 2-fold with ice-cold TEGWD buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 20 mM sodium tungstate and 10% glycerol) and incubated for 4 h at increasing concentrations (0.1 nM–1 μ M) of [3 H]dexamethasone. Bound and unbound steroids were separated via the dextran-charcoal method. Bound steroids was measured by counting the radioactivity of the supernatant. Bound as a function of unbound was analysed using the methods of Claire *et al.* (1978) and the dissociation constant at equilibrium, K_d , calculated.

Transactivation assays

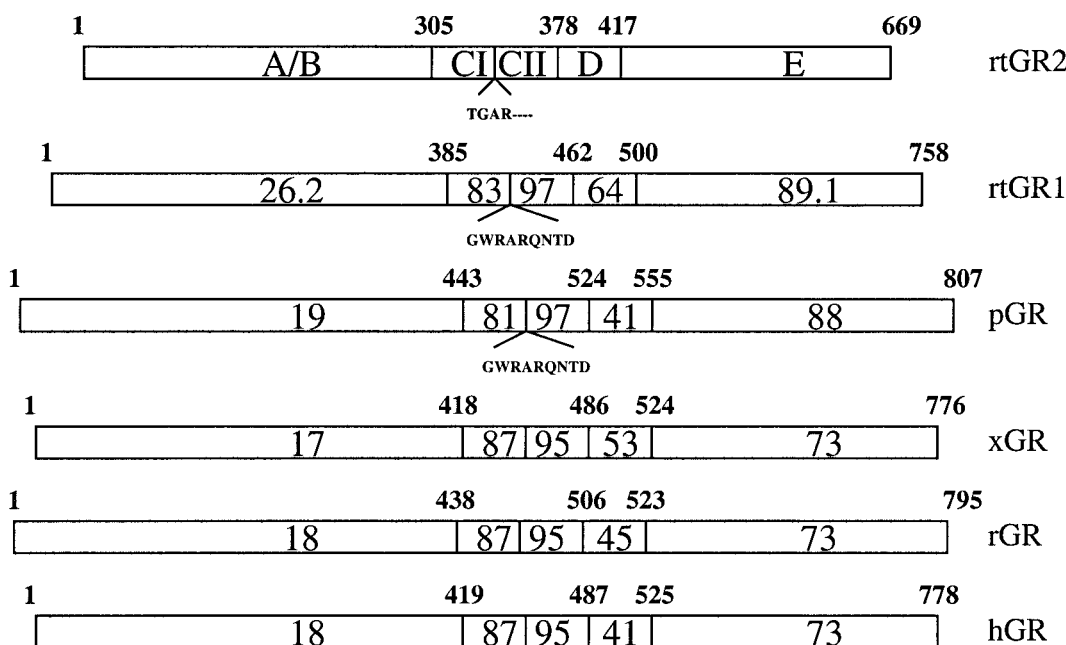
The full clone encoding part of the 5'-untranslated region, the entire coding region and part of the 3'-untranslated region (Fig. 1) was excised from pGEM4Zf by EcoR1 and ligated into Eco-R1 cut dephosphorylated expression vector pCMV5 (pCMrtGR2). Orientation of the insert was confirmed by restriction enzyme profile. The expression vector containing GR1 (pCMrtGR1) was the same as that previously used (Ducouret *et al.* 1995).

Two cell lines were used to assess the transactivational properties of rtGR1 and rtGR2, the Chinese hamster ovary (CHO)-K1 and COS-7 (derived from the African green monkey kidney) cell lines. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 110 mg/l pyruvate (41966; Invitrogen) supplemented with 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 5% (CHO)

or 10% (COS-7) denatured fetal calf serum (FCS) at 37 °C in a water-saturated atmosphere with 5% CO₂. Transfection experiments were carried out using the calcium precipitation method (Sambrook & Russell 2001) on cells being in the logarithmic growth phase and having reached 30–50% of confluency in plastic culture dishes (24-well plates for CHO; 6-well plates for COS-7). Four hours prior to transfection, the medium was removed, cells washed twice with PBS, and the medium replaced with DMEM nutrient mixture F-12 Ham (D-2906; Sigma) supplemented with 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 3.7 g/l NaHCO₃ containing 2.5% FCS that had previously been desteroided by dextran/charcoal treatment. In a standard experiment, cells were co-transfected with the following plasmids: a plasmid containing the appropriate hormone receptor cDNA, pCMrtGR2 or pCMrtGR1 (1.2 μ g/24-well plate with CHO or 5 μ g/6-well plate with COS-7), the reporter plasmid pFC31 Luc, which contains the mouse mammary tumour virus promoter upstream of the luciferase gene (MMTV-LUC) (20.4 μ g/24-well plate with CHO or 10 μ g/6-well plate with COS-7), and pSV β (Clontech), a second reporter plasmid containing the β -galactosidase gene under the control of the SV40 promoter and serving as a control for transfection efficiency (2.4 μ g/24-well plate with CHO or 2 μ g/6-well plate with COS-7). Twelve hours after transfection, the cells were washed with PBS and the medium was replaced with medium containing the steroids to be tested. The cells were incubated for a further 36 h, and then harvested using reporter lysis buffer (Promega) following the manufacturer's instructions. Luciferase activities were determined using 10 μ l cell lysate and 50 μ l luciferase assay substrate (Promega) in an ML3000 luminometer. For the measurement of β -galactosidase activity, 200 μ l substrate solution (final concentration: 3 mM *o*-nitrophenyl β -D-galactopyranoside, 0.56 M potassium phosphate, 1 mM MgCl₂, 44 mM β -mercaptoethanol) were added to 55 μ l cell lysate in a 96-well microplate and incubated for 30 min at 37 °C before absorbance at 405 nm was read. All treatments

Figure 1 Nucleotide sequence of the second rtGR (rtGR2), and predicted amino acid sequence of the receptor protein. The longest open reading frame begins at nucleotide 667 (atg=M) and ends at nucleotide 2674 (tga), which corresponds to a protein of 669 amino acids. Nucleotides underlined indicate the A/B domain (transactivation region), those in bold the C domain (DNA-binding region), and those in italics the E domain (ligand-binding domain).

rtGR2	KVCLVCSDEASGCHYGLVTCGSCVKVFFKRAVEGTGAR	GQHNYLCAGRNDCCIIDKIRRNKCPACRFRKC	372	
rtGR1	-I-----N---I-----Q--- -PN-P- -DE-ACA-I-----V-----EA-----S	-----WR--QNTD-----	460	
pGR	-I-----V-----	-----WR--QNTD-----	514	
xGR	-L-----	-----Y---	474	
rgr	-L-----	-----Y---	500	
hgr	-L-----	-----Y---	482	
rtGR2	LLAGMNLEARKTKKLNRLKGVQOQPTTA	ELTPRPLPEARS	LVPKSMPLQTPMTLSLLKAIEPDTIYSGYDG	442
rtGR1	-Q-----N---I-----Q--- -PN-P- -DE-ACA-I-----V-----EA-----S	-----WR--QNTD-----	525	
pGR	-Q-----N---IKM- -HR--GS	AEPISNM-V	PVI-R-----V-----EII-----	580
xgr	-Q-----KI--I--S---TARESPETSMT-	T--A-VA-----LI---EV---EVL---S	549	
rgr	-Q-----KI--I--A-AGVSQDTSEN-	NK	TI--AAL-----LV---EV---EVL-A---A	568
hgr	-Q-----KI--I--A-GVSSQETSEN-GNK	TI- ATL-----LV---EV---EVL-A---S	550	
rtGR2	TLPDTSTRIMTTLNRLGGRRQVVS	SAVKWAKALPGFRNLHLLDDQMTLLQCSWLF	FLMSFGLGWRYSQQCDGNMLCF	515
rtGR1	-I-----L-----Q-----S-----	-----N-G---	598	
pgr	-----L-----Q--I-----S-----	-----S-----E-N---	653	
xgr	SI--TR--SS--M-----R---I-----	-----Y--M--V-A---K-TN-SI-Y-	622	
rgr	SV--SAW-----M-----IA-R---IL-L-----	-----Y--M--A-A---R-SS-L---	641	
hgr	SV- STW-----M-----IA-R---I-----	-----Y--M--A-A---R-SSA-L---	623	
rtGR2	APDLVINQDRMKLPYMAHQCEQMLKISSEFVRLQVSHDEYLCMKVLLLLSTVPKDGLK	SQAVFDEIRMSYIKE	588	
rtGR1	-----DE--T-----T-----T-----Y-----T---	-----T---	671	
pgr	-----KE--F-T-----CN-----Y-----	-----T---	726	
xgr	-----TE--H--F-QER-QE---AG-MSS--I-Y-----	-----MCC-I-E---H-L-E---T---	695	
rgr	-----I--EQ--S--C-Y---KH-FV---LQ---Y-----	-----T-----S---E---EL---T---	714	
hgr	-----I--EQ--T--C-Y---KH--YV---LH---Y-----	-----T-----S---EL---T---	696	
rtGR2	LGKAIVKREENSQNWQRFYQLTKLLDSMHMVGGLLDFCFYTFVNKSLSVFPEMLAEI	IISNQLPKFKAGSV	661	
rtGR1	-----Q-----QI-----	-----D---	744	
pgr	-----A-----Q--E---QII-----T-----T--I---D---	799		
xgr	-----G-----VAEN--A--L---T-----T--I--YSS-NL	768		
rgr	-----G-----V-EN--TY-Q--LD-TM-I-----T--I--YSN-NI	787		
hgr	-----G-----V-EN--NY-Q--LD-TM-I-----T--I--YSN-NL	769		
rtGR2	KPLLFHQK	669		
rtGR1	-----ALNHD'TMP	758		
pgr	-----	807		
xgr	-K-----	776		
rgr	-K-----	795		
hGR	-K-----K	778		



were performed in triplicate (i.e. three separate wells) and repeated. Luciferase activity was corrected for well-specific transfection efficiency (as determined by β -galactosidase activity) and then expressed as a percentage of the luciferase activity observed in cells transfected with pFC31 Luc and pSV β and incubated with 10^{-6} M of the appropriate steroid.

Initial studies assessed the ability of various steroids at a range of concentrations (10^{-10} – 10^{-6} M) to induce rtGR1 or rtGR2 transactivation in COS-7 cells. The steroids tested included the corticosteroids, cortisol and aldosterone, the synthetic glucocorticoid agonist dexamethasone, as well as a number of their precursors or breakdown products of cortisol production such as 11-deoxycortisol, cortisone, corticosterone, 11-deoxycorticosterone, and 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one. In addition, the effect of the GR antagonist RU486 at 10^{-5} , 10^{-6} and 10^{-7} M on rtGR1 and rtGR2 transactivational activity at 10^{-7} M dexamethasone was also assessed. Transactivation experiments performed with CHO cells included a far more in-depth analysis of the concentration-dependent (10^{-12} – 10^{-6} M) induction of transactivation activity by cortisol and dexamethasone, from which the EC₅₀ for cortisol and dexamethasone-induced luciferase activity were determined by generation of a Hill plot using SigmaPlot 5.0 computer package.

Results

The initial PCR reaction using the degenerate primers yielded numerous cDNA products whose sequence spanned part of the DNA-binding (C) region, the hinge (D) region and the ligand-binding (E) region of the steroid receptor. One of the products had high sequence homology with MR (see Colombe *et al.* 2000) and the other to GR. The latter consisted of 726 bp, whose nucleotide sequence was 79% similar to the known rtGR1 (Ducouret *et al.* 1995). Based on the differences in the nucleotide sequence, gene-specific primers were

designed and the resulting PCR product for the new GR used to screen a rainbow trout cDNA intestinal library. This screening yielded four positive clones; of these clones, two gave an identical sequence with the rtGR1 identified by Ducouret *et al.* (1995). The other two clones were identical in sequence except one was of 2.8 kb in length and the other of 1.1 kb in length. The largest open reading frame of the second clone spanned 2007 nucleotides, corresponding to a predicted protein length of 669 amino acids. The flanking region of the start codon differs from that of the Kozak (1991) sequence but possesses an A and a G at position -3 and $+4$ respectively (Fig. 1). In addition to the encoding region, the cloned cDNA contained 667 nucleotides at the 5'-untranslated region and 107 nucleotides at the 3'-untranslated region (Fig. 1).

The predicted amino acid sequence of rtGR2 exhibited a high similarity to other GRs (Fig. 2), with the CI and CII domains of the rtGR2 being the most conserved (between 83 and 97%). This region in teleosts has unique characteristics, possessing additional amino acids between the two zinc fingers. However, the number of additional amino acids differs within and between teleost species, rtGR2 possessing four extra amino acids (TGAR, Fig. 2) while rtGR1 and the GR cDNA sequence of the Japanese flounder, *Paralichthys olivaceus* (pGR, GenBank, AB013444) possess nine extra amino acids (WRARQNTD, Fig. 2). High similarity was also found between the E domains of rtGR2 and the other fish GRs, rtGR1 and pGR (89 and 88% respectively), and this region had 73% similarity to the other tetrapod GRs (human GR (hGR), rat GR (rGR), *Xenopus* GR (xGR)) analysed. The A/B domain was the least well conserved region, showing 18–26% similarity between each rtGR, followed by decreasing similarity to other receptors: rtGR1 > pGR > rGR = hGR > xGR. The A/B domain is much shorter in rtGR2 (by 80 amino acids when compared with rtGR1) than the other GR and this accounts for the surprisingly small size of this novel GR (Fig. 2). In order to further characterize the protein encoded by this

Figure 2 (Upper) Amino acid sequence comparison between the steroid-binding region (E-domain) of the two rtGRs (rtGR1 and rtGR2) and other GRs: Japanese flounder, *Paralichthys olivaceus* (GenBank, AB013444; pGR), human GR (hGR), *Xenopus* GR (xGR) and rat GR (rGR). A line represents identical amino acids. (Lower) Percentage similarities between the different regions (A/B – transactivation domain, C – DNA-binding domain, D – hinge region, E – ligand-binding domain) of the rtGR2 and other GR.

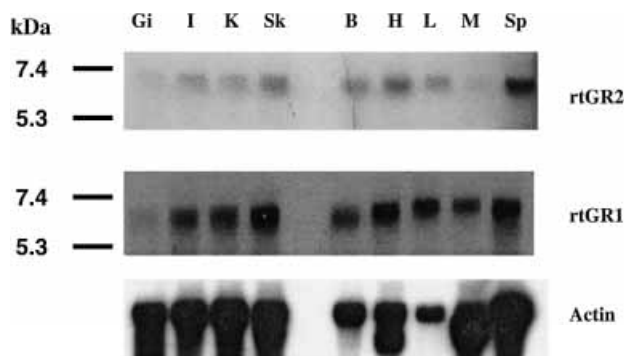


Figure 3 Northern blot analysis of 15 µg Poly A⁺ extracted from the gills (Gi), intestine (I), kidney (K), skin (Sk), brain (B), heart (H), liver (L), muscle (M) and spleen (Sp) of freshwater-adapted rainbow trout. The rtGR2 and rtGR1 probes were generated by PCR from the 5'-untranslated region of each receptor. The individual probes did not cross-react with the plasmids containing the full-length cDNA of the other receptor (assessed by Southern blot, data not shown). An actin probe was used to assess loading of Poly A⁺ into each lane.

gene, an *in vitro* translation assay with [³⁵S]methionine was carried out using rtGR1 and rtGR2 as template. Both expressed a number of products (see Fig. 5); for the rtGR2 template the largest was a 80 kDa protein, which was shorter than the 88 kDa protein expressed in the same conditions by rtGR1 template (see Fig. 5). This result indicates that the rtGR2 reading frame encodes for a shorter protein. Northern blot analysis of various tissues from freshwater-acclimated rainbow trout using probes specific for rtGR2 or rtGR1 revealed each receptor is encoded by a transcript of approximately 7.3 kb in all tissues assessed (Fig. 3).

Phylogenetic analyses consistently group both trout GRs with other teleost fish GRs, with high bootstrap support (Fig. 4), strongly supporting a fish-specific duplication which led to the two trout GRs. Due to incomplete sequences, the phylogenetic relationship of these with other fish GRs cannot be resolved in a single analysis, but when more than two fish species are analysed the two rtGRs do not group. With variable bootstrap scores, depending on species and sites sampling, rtGR1 groups with *Paralichthys* GR and with *Takifugu* GSS1 (Fig. 4A), while rtGR2 groups with *Tilapia* GR, *Takifugu* GSS2 (Fig. 4A and B), and with *Tetraodon* GSS (bootstrap > 90%; not shown). When a maximum number of sites is used (Fig. 4A), the separation of rtGR1 and rtGR2 is highly

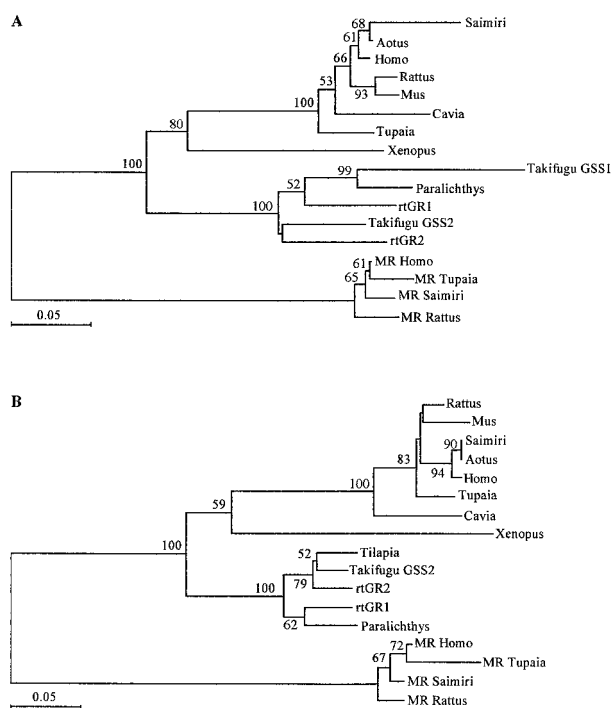


Figure 4 Neighbour-joining (Poisson model) phylogenetic trees of vertebrate GRs and MRs. Figures at nodes are bootstrap proportions in percent of 2000 replicates; scores under 50% are not reported; the scale bars represent 0.05 substitutions per site. (A) Including both *Takifugu* genome survey sequences (250 sites analysed). (B) Including the partial *Tilapia* GR sequence (151 sites analysed).

significant by a likelihood test ($P < 0.005$). Although the exact relationships of fish GRs cannot be determined with great confidence from the partial sequences available, there is good support for the duplication of GR being shared by several fish orders, and not specific to salmonids.

The functional characteristics of this new GR were compared with those observed with rtGR1. The steroid-binding characteristics of the two receptors were first tested in a rabbit reticulocyte lysate expression system and the calculated dexamethasone dissociation constants K_d for rtGR1 and rtGR2 are similar, being 5.5 ± 0.41 and 3.2 ± 0.79 nM respectively (Fig. 5).

The receptors transactivation activity in response to corticosteroids was measured using co-transfection assays. Initial analysis using various steroids indicated that cortisol and dexamethasone at concentrations of 10^{-6} M and above significantly stimulated luciferase activity of COS-7 cells

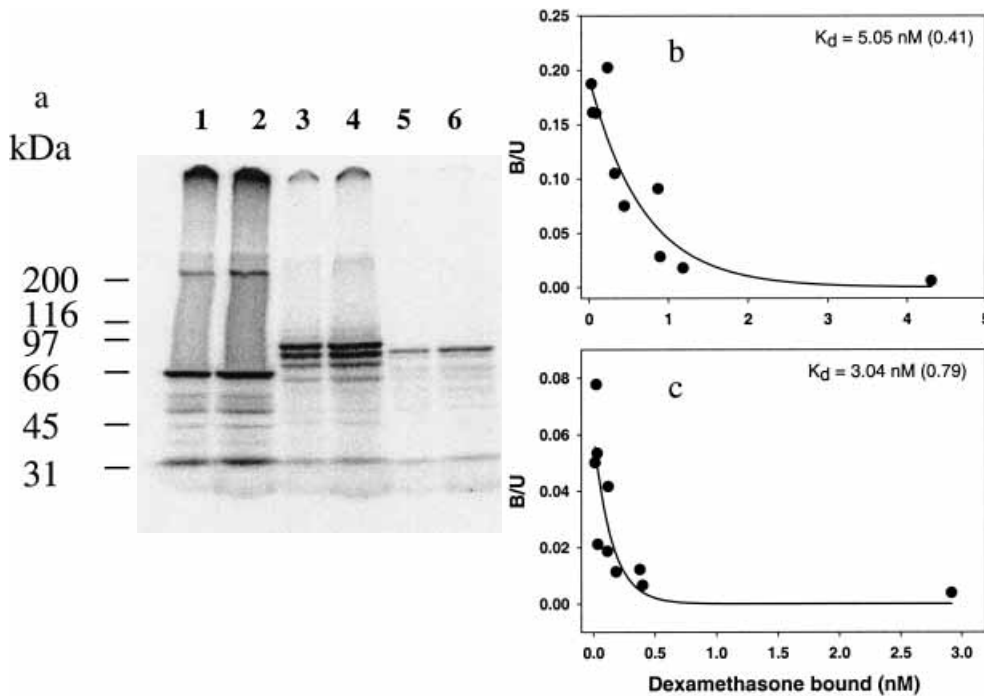


Figure 5 (a) For *in vitro* translation, the TNT-coupled reticulocyte system was used with rtGR1 template (lanes 3, 4), or rtGR2 template (lanes 5, 6), or control luciferase template (lanes 1, 2). SDS-PAGE electrophoresis was performed on 5 μ l (lanes 1, 3 and 5) or 10 μ l (lanes 2, 4 and 6) of lysate incubation medium. The dried gel was autoradiographed for 8 h. The control luciferase template expressed the 66 kDa expected protein. [3 H]dexamethasone binding to rtGR1 (b) or rtGR2 (c). Bound (B) and unbound (U) steroids were separated via the dextran–charcoal method, and bound steroid was measured by counting the radioactivity of the supernatant. B as a function of U was analysed using the methods of Claire *et al.* (1978) and the dissociation constant at equilibrium, K_d , calculated.

transfected with either rtGR1 or rtGR2 (Fig. 6). 11-Deoxycortisol and corticosterone only partially stimulated rtGR2 transactivational activity: 18.8 and 10.1% of transactivational activity at 10^{-6} M cortisol respectively. However, these steroids, at this concentration, were without effect on rtGR1 transactivational activity. The GR antagonist RU486 blocked both rtGR1 and rtGR2 transactivational activity in the presence of 10^{-7} M dexamethasone (Fig. 7). However, rtGR1 was more sensitive to RU486, transactivational activity being reduced by 86.4% at 10^{-7} M RU486, whereas 10^{-6} M RU486 is required to achieve a similar inhibition (80.5%) with rtGR2 (Fig. 7). Preliminary transactivation studies with CHO cells showed that none of the other steroids tested (progesterone, testosterone, 11-ketotestosterone, oestradiol, 17-dihydroprogesterone, oestrone, 20 β -dihydroxycortisone) induced transactivational activity (data not shown). This cell line was also used to

investigate the ligand sensitivities of the two receptors in more detail. rtGR2 was found to be more sensitive than rtGR1, the EC_{50} being 0.72 ± 0.87 and 46 ± 12 nM for cortisol respectively, and 0.35 ± 1.1 and 4.3 ± 1.4 nM for dexamethasone respectively (Fig. 8).

Discussion

In the present study, we isolated a novel corticosteroid receptor in rainbow trout, whose DNA-binding domain and ligand-binding domain show high homologies with other GRs, while being different from the GR (rtGR1) isolated by Ducouret *et al.* (1995). Northern blot analysis shows that the new receptor (rtGR2) gene is expressed in the same tissues as rtGR1 (Fig. 3). These observations were complemented by functional analysis of the two trout receptors,

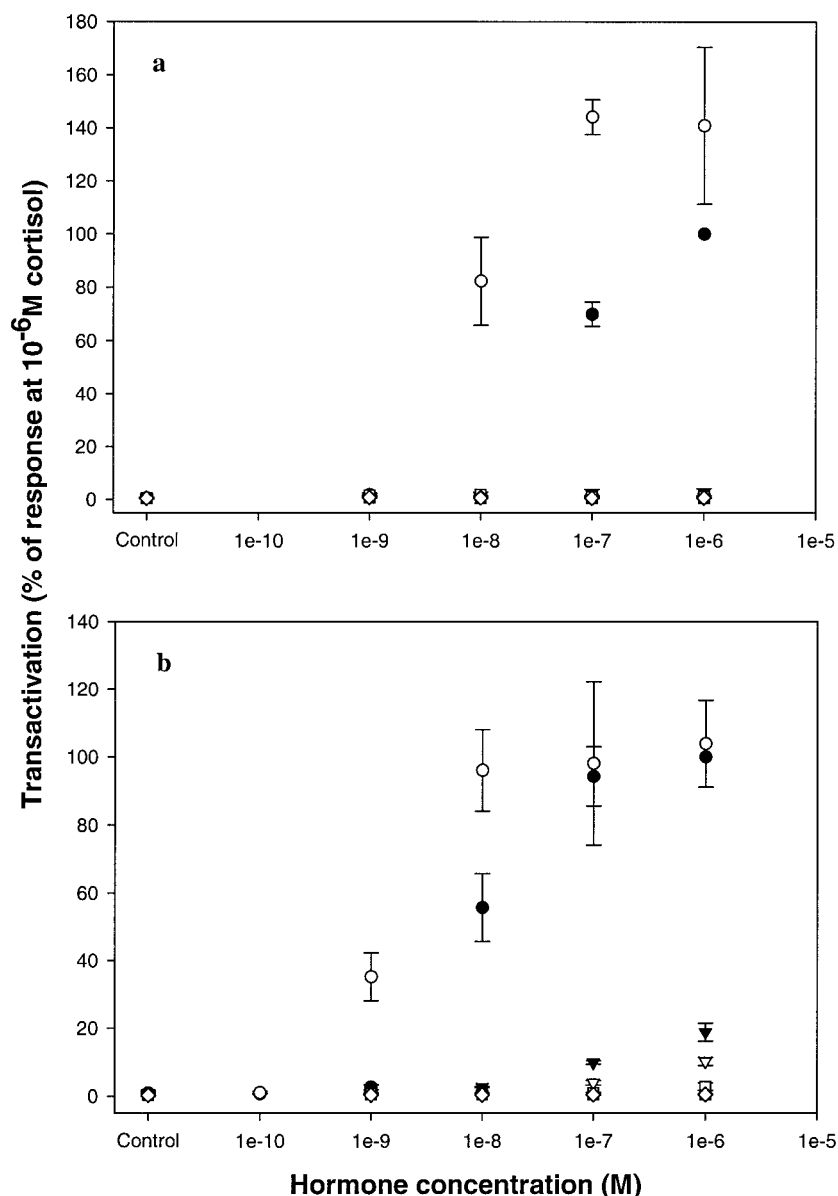


Figure 6 Transactivation activity of COS-7 cells co-transfected with either rtGR1 (a) or rtGR2 (b) expression vectors, as well as the reporter plasmid pFC31 Luc, and the pSV β plasmid which expresses β -galactosidase. After transfection, cells were treated with various concentrations of steroids (\bullet cortisol; \circ dexamethasone; \blacktriangledown 11-deoxycortisol; \blacktriangledown corticosterone; \blacksquare cortisone; \square aldosterone; \blacklozenge 11-deoxycorticosterone; \blacklozenge $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one). Transactivation was determined by luciferase activity, normalized to the internal β -galactosidase control, and expressed as a percent of the GR1 or GR2 construct activities measured in the presence of 10^{-6} M cortisol. Values represent means \pm S.E.M. of between three and seven separate experiments, with each experiment performed in triplicate.

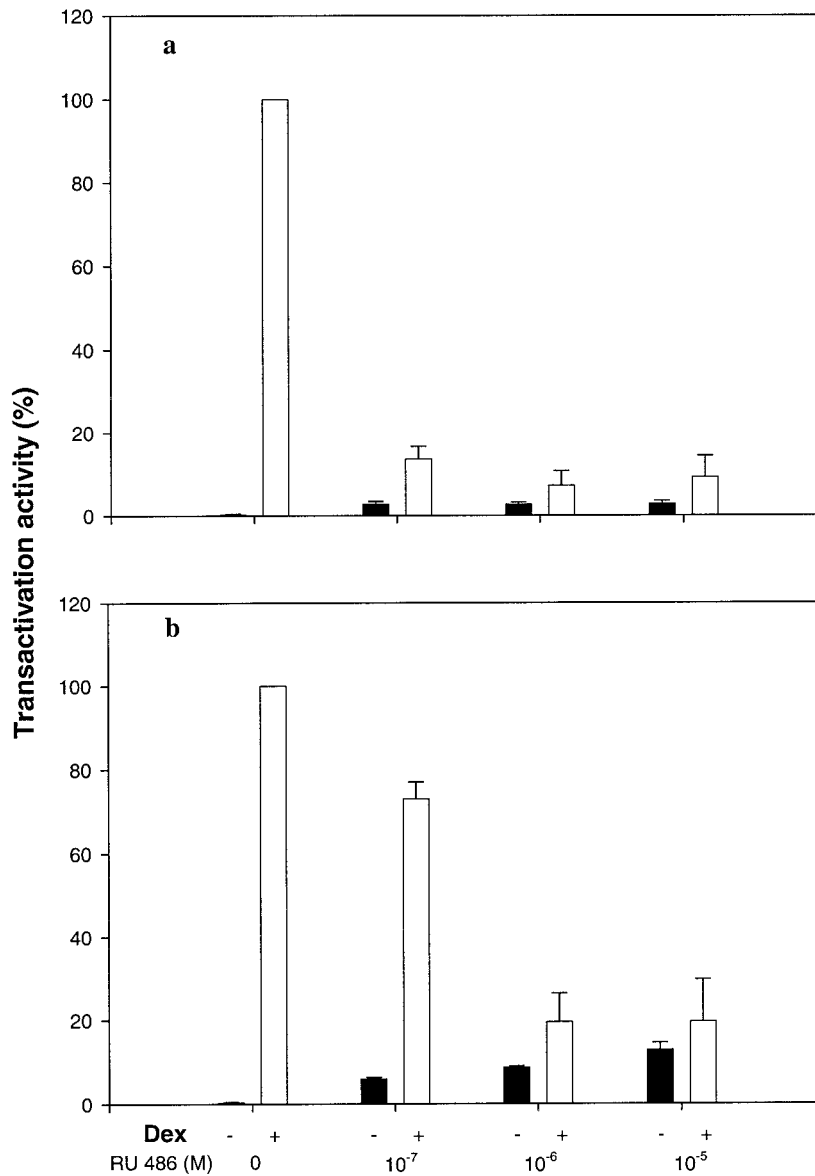


Figure 7 Transactivation activity of COS-7 cells co-transfected with either rtGR1 (a) or rtGR2 (b) expression vectors, as well as the reporter plasmid pFC31 Luc, and the pSV β plasmid which expresses β -galactosidase. After transfection, cells were treated with varying concentrations of RU486 in the presence or absence of 10^{-7} M dexamethasone (Dex). Transactivation was determined by luciferase activity, normalized to the internal β -galactosidase control, and is expressed as a percent of the GR1 or GR2 construct activities measured in the presence of 10^{-7} M dexamethasone. Values represent means \pm S.E.M. of an experiment performed in triplicate.

rtGR1 and rtGR2. Both receptors show a similar binding affinity for the synthetic corticosteroid dexamethasone (Fig. 5). However, analysis of the transactivation properties of the two rtGR forms

revealed that, although both exhibited a cortisol-dependent transactivation activity, rtGR2 is characterized by a higher sensitivity to cortisol ($EC_{50} = 0.72 \pm 0.87$ nM) compared with rtGR1

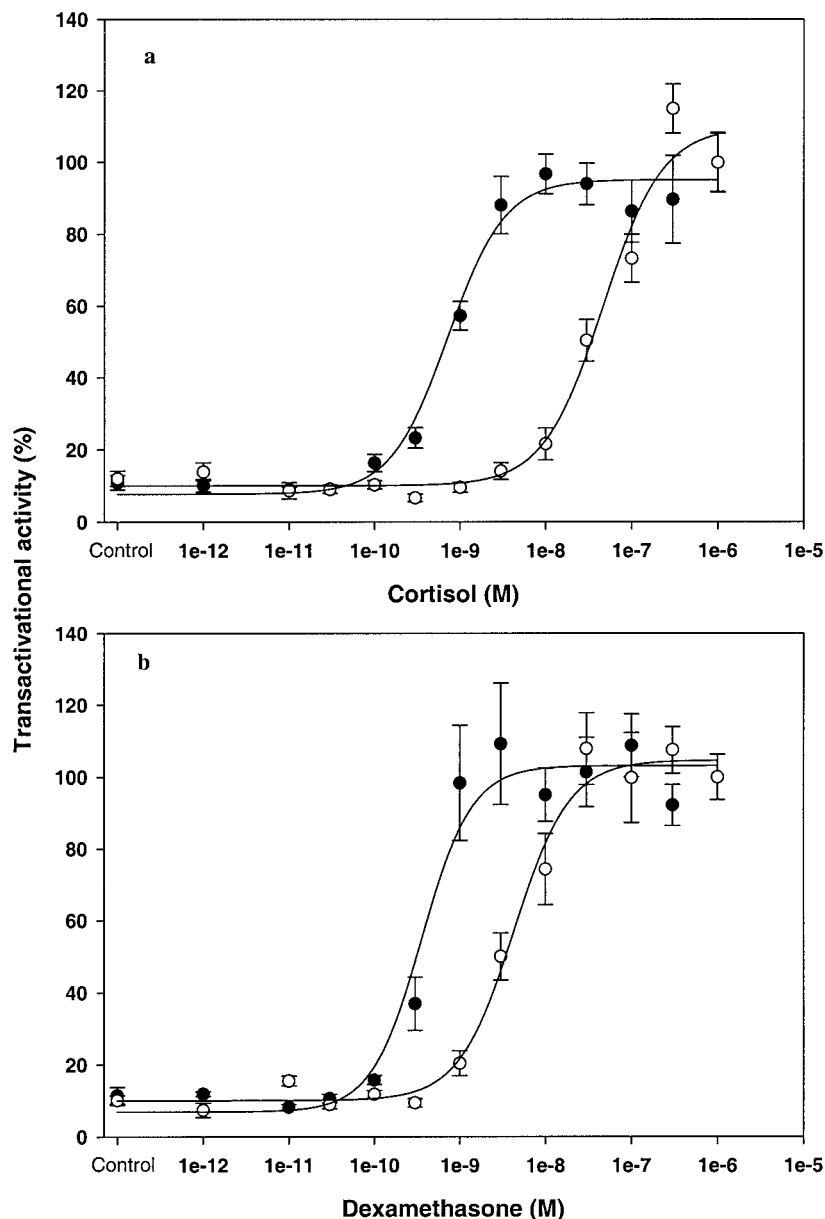


Figure 8 Transactivation activity of CHO cells co-transfected with either rtGR1 (○) or rtGR2 (●) expression vectors, as well as the reporter plasmid pFC31 Luc, and the pSVβ plasmid which expresses β-galactosidase. After transfection cells were treated with varying concentrations of cortisol (a) or dexamethasone (b). Transactivation was determined by luciferase activity, normalized to the internal β-galactosidase control, and expressed as a percent of the GR1 or GR2 construct activities measured in the presence of 10^{-6} M of the appropriate steroid. For cortisol, rtGR1 $EC_{50}=46\pm 12$ nM and rtGR2 $EC_{50}=0.72\pm 0.87$ nM; and for dexamethasone, rtGR1 $EC_{50}=4.3\pm 1.4$ nM and rtGR2 $EC_{50}=0.35\pm 1.1$ nM. Values represent means \pm S.E.M. of three separate experiments, with each experiment performed in triplicate.

($EC_{50}=46 \pm 12$ nM). This difference in sensitivity was not cell line-specific, being observed in transfected CHO-K1 and COS-7 cells (Figs 6 and 8). The sensitivity of rtGR2 is also reflected in the fact that it is only this receptor that shows partial transactivational activity in the presence of 10^{-6} M 11-deoxycortisol and corticosterone (Fig. 6), and that the corticosteroid antagonist RU486 is a more potent inhibitor of rtGR1 transactivational activity (Fig. 7). The presence of two GRs that act with different potency as ligand-inducible transcription factors provides a new perspective on whether these functional differences support different physiological regulations.

The rationale behind claiming this novel steroid receptor is a new form of GR in trout comes from analysis of the sequence, which displays high similarity to other GRs at nucleotide and amino acid levels (Fig. 2). However, the disparity in sequence conservation between rtGR1 and rtGR2 in the A/B domain (Fig. 2) indicates that they are not products of alternate splicing, but are products of separate genes. Alternate splicing accounts for the two human GR isoforms, α and β (Oakley *et al.* 1996), both of which are widely expressed (Oakley *et al.* 1997), but hGR β is transcriptionally inactive and acts as a negative inhibitor of hGR α and hMR activity (Bamberger *et al.* 1995, 1997, Oakley *et al.* 1996). In rainbow trout, Takeo *et al.* (1996) described two GR cDNA isoforms, one being identical to that previously reported (Ducouret *et al.* 1995), while the other partial cDNA sequence lacked the additional nine amino acids present in rtGR1 between the two zinc fingers of the C domain (Ducouret *et al.* 1995, Takeo *et al.* 1996). RT-PCR revealed the second isoform was only present in the testis, while rtGR1 was found in all tissues. PCR using genomic DNA confirmed that the second isoform was a product of alternate splicing, but its function is unclear (Takeo *et al.* 1996).

The new form, rtGR2, appears to have originated through gene duplication, consistent with the known abundance of duplicated genes in teleost fishes (Robinson-Rechavi *et al.* 2001a). Our phylogenetic analysis shows that GRs probably duplicated before the divergence of salmonids (trout) and percomorphs (*Tilapia*, *Tetraodon*, *Takifugu*, *Paralichthys*), so it does not appear to be due to salmonid tetraploidy (Allendorf & Thorgaard 1984). Although longer sequences are

needed to resolve the phylogeny of fish GRs, our results imply two GR genes in all salmoniforms and percomorphs. The disputed hypothesis of a fish-specific genome duplication (Robinson-Rechavi *et al.* 2001b, Taylor *et al.* 2001) predicts that the two GRs should in fact be found in all teleost fishes, but we cannot categorically conclude this given the available data.

The cellular or molecular mechanisms by which each rtGR can act independently are probably complex, but are not thought to be due to differences in ligand-binding sensitivities. Of the 23 amino acids identified as playing a key role in the GR/dexamethasone interactions within the ligand-binding pocket (Bledsoe *et al.* 2002), all of those that form hydrogen bonds with the hydrophilic residues of dexamethasone are conserved between the trout and other GRs. Of the remaining 18 amino acids that form hydrophobic bonds with the ligand only four differ between the trout GRs and the other GRs, Leu for a Met at rtGR2 position 493 (Leu493 Met, Fig. 2), Ser497 Ala, Phe627 Tyr and Val639 Ile, and of these only one position differs between the two trout GRs, an Ile in rtGR1 replaces Phe at 627 of rtGR2 (Ray *et al.* 1999, Bledsoe *et al.* 2002). Substituting the Tyr with Phe (equivalent to the Phe627 Tyr of rtGR2) or Val in the rat GR has no effect on the affinity of dexamethasone binding (Ray *et al.* 1999). This highly conserved region probably accounts for the similar high-affinity binding of the trout GRs for dexamethasone, rtGR2 $K_d=3.5 \pm 0.79$ and rtGR1 $K_d=5.5 \pm 0.41$ nM; K_d values that are similar to mammalian GRs (e.g. hGR 3.7 nM, Hellal-Levy *et al.* (1999), or 4.6 nM (Ray *et al.* 1999)).

The difference in transactivational sensitivities is predicted to be a consequence of alterations in the amino acid sequences in key regions of rtGR1 and rtGR2 involved in DNA recognition and transactivation. The startling difference between the rtGRs and tetrapod GRs is the additional amino acids between the zinc fingers of the DNA-binding domain (Fig. 2). The amino acid sequence that makes up the zinc fingers is highly conserved, and the N-terminal zinc finger determines target gene specificity (Green *et al.* 1988, Umesono & Evans 1989), while the other zinc finger is involved in dimerization (Dahlem-Wright *et al.* 1991). Amino acid insertion or substitution in the N-terminal zinc finger can either stimulate or perturb receptor activity (Green *et al.* 1988), and this region differs

between rtGR1 and rtGR2, with rtGR1 possessing nine (Ducouret *et al.* 1995, Takeo *et al.* 1996) and rtGR2 possessing four extra amino acids (Fig. 2).

The regions of the GR essential for transactivation, the $\tau 1$ core region of the A/B domain (Almlöf *et al.* 1998) and the $\tau 2$ region of the N-terminal region of the highly conserved E domain (Milhon *et al.* 1997), differ between the two trout GRs. The $\tau 1$ domain is considered the main region responsible for transactivation forming interactions with various proteins of the transcriptional apparatus (Ford *et al.* 1997). The analogous region in the two trout GRs shows the major disparity in sequence homology. The putative $\tau 1$ core of rtGR2 shows only 40% similarity between the two receptors and within this region there are variations in the proportion and position of Glu and Asp and Ser residues essential for transactivation (Hollenberg & Evans 1988, Dahlem-Wright *et al.* 1994, 1995, Beato *et al.* 1995). The $\tau 2$ region is smaller and highly conserved, and has been shown to bind to HSP90 (Giannoukos *et al.* 1999) there are only two amino acid substitutions in this region at rtGR2 434 and 435 (an Asp for Glu and a Thr for Ala respectively). Part of the C-terminus, termed AF2, is also implicated in transactivation (Williams & Sigler 1998). Within this region a substitution of Tyr735, an amino acid that interacts with the D ring of dexamethasone, with Ser (Tyr735 Ser) greatly increased the EC₅₀ and decreased maximal transactivation activity of the MMTV reporter (Ray *et al.* 1999). It is precisely at this position where rtGR1 and rtGR2 differ: Iso and Phe respectively. In the rat the Tyr735 Phe substitution does not affect dexamethasone binding affinity or transactivation EC₅₀, but reduces maximal transactivational activity of an MMTV reporter (Ray *et al.* 1999). It is tempting to suggest that these differences in amino acid sequence may account for the lower sensitivity of rtGR1 compared with rtGR2. Concomitantly, given the large differences in sequence within the putative $\tau 1$ region it will be of interest to identify how these two receptors interact with the transcriptional machinery within a teleost fish model.

Circulating cortisol concentration in non-stressed fish are generally <10 ng/ml (27 nM), while this level rises to >100 ng/ml (270 nM) in stressful situations (Bern & Madsen 1992, Wendelaar Bonga 1997); this would indicate that cortisol preferentially binds to rtGR2 in low or mild stressful

conditions, and to both rtGR2 and rtGR1 at times of extreme stress. This would also suggest that rtGR2 is the main receptor regulating gene transcription. However, it is difficult to correlate differing *in vitro* GR ligand sensitivities with the situation *in vivo*. Empirical evidence for distinct physiological processes attributable to different cortisol concentrations are not immediately evident and most studies are equivocal, showing that the effect of cortisol usually follows a dose-dependent response (e.g. McCormick 1990, Bury *et al.* 1998, Takagi & Björnsson 1999). However, Marsigliante *et al.* (2000) have recently demonstrated by isoelectric focusing the presence of two putative CR receptor types, with pI values of 6.1 and 6.7, in the gills of the European yellow eel. The identification of a greater quantity of the pI 6.1 form in the gills of freshwater-acclimated eels compared with seawater-acclimated individuals suggests that there is a physiological role for this form in saltwater adaptation in fish. However, it is not possible to determine from this study whether the different pI values are due to binding to two distinct GR populations or a GR and MR. The rainbow trout MR has a similar affinity for cortisol as the GR (Colombe *et al.* 2000), and it may be that previous cortisol-binding studies in fish tissues refer to the binding of this hormone to one or more CRs. In-depth analysis of the involvement of rtGR1 and rtGR2 in varying physiological situations is required to determine the true biological roles of these receptors.

In conclusion, we have isolated a second rtGR that is more sensitive to cortisol, based on transactivation studies, than the first rtGR to be identified (Ducouret *et al.* 1995). This is the first time that two transcriptionally active GRs have been identified in the same species. The control of both teleost glucocorticoid and mineralocorticoid actions was initially perceived to be under the control of one hormone (cortisol)/one receptor (GR) system, but the identification of two GRs and a putative rtMR (Colombe *et al.* 2000) indicates that the control of energy metabolism and mineral balance is more complex in fish than originally thought.

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