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Fish glucocorticoid receptor with splicing variants in the DNA binding domain

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Abstract Here we describe the isolation of a rainbow trout cDNA containing an entire GR coding region. Although the encoded protein is highly homologous to other GRs, especially in its DNA binding domain, it contains a nine amino acid insertion between the two zinc fingers. This novel form is found in all rainbow trout tissues examined; however, the testis also contains a splice variant lacking this insert, making it completely continuous to other GRs. In transient transfection assays of cultured cells, the two rainbow trout GR variants activated transcription from the glucocorticoid-responsive mouse mammary tumor virus promoter to comparable levels.

Key words: Glucocorticoid receptor; MMTV promoter; Alternative splicing; DNA-binding domain; Rainbow trout; Steroid hormone

1. Introduction

Glucocorticoids are essential for normal development and maintenance of basal and stress-related homeostasis. They are also potent immunosuppressants and regulate a broad range of metabolic processes [1,2]. All of their effects are mediated by the glucocorticoid receptor (GR), which belongs to a large family of nuclear receptors having a related structure [3,4]. Sequence comparisons among multiple receptors and mutational analyses of the GR have identified functional domains responsible for transcriptional activation, DNA binding, nuclear localization, and hormone binding [5–8].

In salmonids, cortisol has been demonstrated to play an important role in stimulating an increase in sea water tolerance. Several laboratories reported that cortisol treatment of fish increases the number of chloride cells, Na^+/K^+-ATPase activity and the number of GR sites in the gill [9–13]. Thus, it is of great interest to analyze the molecular mechanism governing the osmoregulatory function of GR to efficiently promote fresh water to sea water adaptation.

We have undertaken the isolation of the rainbow trout homologue of the glucocorticoid receptor in order to study its potential role in the adaptation of salmonid fish from fresh water to sea water, and to further explore structure-function relationships through sequence comparisons. We found two forms of GR mRNA in rainbow trout. The major form, present in all tissues examined, encodes an 83.4-kDa protein that is highly homologous to mammalian GR, but contains an additional nine amino acids between the two zinc fingers of the DNA binding domain. The second form, found only in the testes, does not contain this insert. To investigate the significance of this difference between the two isoforms, we have examined their biological activities in a fish cell line using a transient expression assay.

2. Materials and methods

2.1. Isolation and characterization of rainbow trout GR cDNA

In order to isolate the rainbow trout GR cDNA, a probe was first generated by PCR. Oligonucleotide primers were designed based on the amino acid sequence of a highly conserved region of mammalian [14,15] and *Xenopus* [16] GRs, and then used to amplify rainbow trout pituitary cDNA as described previously [17]. Products of the expected size were subcloned into the pSK vector (Stratagene) and sequenced. The primers which yielded a 569 bp fragment highly homologous to the human sequence (63%) at the nucleotide level were as follows: primer 1: TGT/CAAA/GGTG/A/T/CTTT/CTTT/C; primer 2: TCG/A/T/CGGG/A/T/CGCA/GAAA/GTAG/A/T/CAGCAT.

A rainbow trout pituitary cDNA library in λZapII (Stratagene) was screened by hybridization with a probe made from the cloned PCR fragment as described previously [18].

2.2. Reverse transcriptase PCR

Reverse transcriptase PCR was conducted as described previously [19] with some modifications. Briefly, poly(A)-selected RNA was prepared from various rainbow trout tissues. The cDNAs were synthesized using a first strand synthesis kit (Stratagene). An aliquot of the first strand reaction was then subjected to PCR analysis using rainbow trout GR specific primers as indicated in Fig. 1.

2.3. Plasmid construction

A set of primers was generated bearing both the N- and C-terminal ends of the rtGR coding sequence flanked by *Eco*RI sites. These primers were used to generate a 2350 bp *Eco*RI PCR fragment containing the entire open reading frame, which was then subcloned into *Eco*RI digested pcDNA3 (Stratagene). The resulting plasmid carrying the insert in the sense orientation was designated pCMV-rtGR-I. A mutant version of the plasmid lacking nine codons in the DNA binding domain was generated by deoxyoligonucleotide-directed mutagenesis, as described previously [20,21]. The template DNA for mutagenesis was prepared from M13 mpl9 containing the rtGR *Eco*RI cDNA fragment described above. Mutations were confirmed by DNA sequencing. The insert was then excised from the RF DNA by digestion with *Eco*RI and subcloned into pcDNA3, yielding plasmid pCMV-rtGR-II. The pMSG-CAT reporter plasmid was purchased from Pharmacia Co.

2.4. Transient GR expression in cultured cells

RBCF-1 cells, derived from goldfish fin [22], were propagated as a monolayer culture at 37°C in Dulbecco's modified Eagle's (Gibco):-Ham F12 (1:1) medium supplemented with 10% FCS [23]. A mixture of 0.01 μg of receptor plasmid (pCMV-rtGR-I or II) and 0.2 μg of reporter plasmid (pMSG-CAT) were cotransfected into subconfluent cultures of RBCF-1 cells in each well of 6-well dishes (Falcon) using calcium phosphate coprecipitation [24,25]. After 4 h, the precipitate was removed, and the cultures incubated for 48 h in fresh medium containing various concentrations of steroids supplemented with 1% FCS. The cells were then harvested and CAT activities measured using a CAT-ELISA assay system (Boeringer) according to the manufacturer's instructions.

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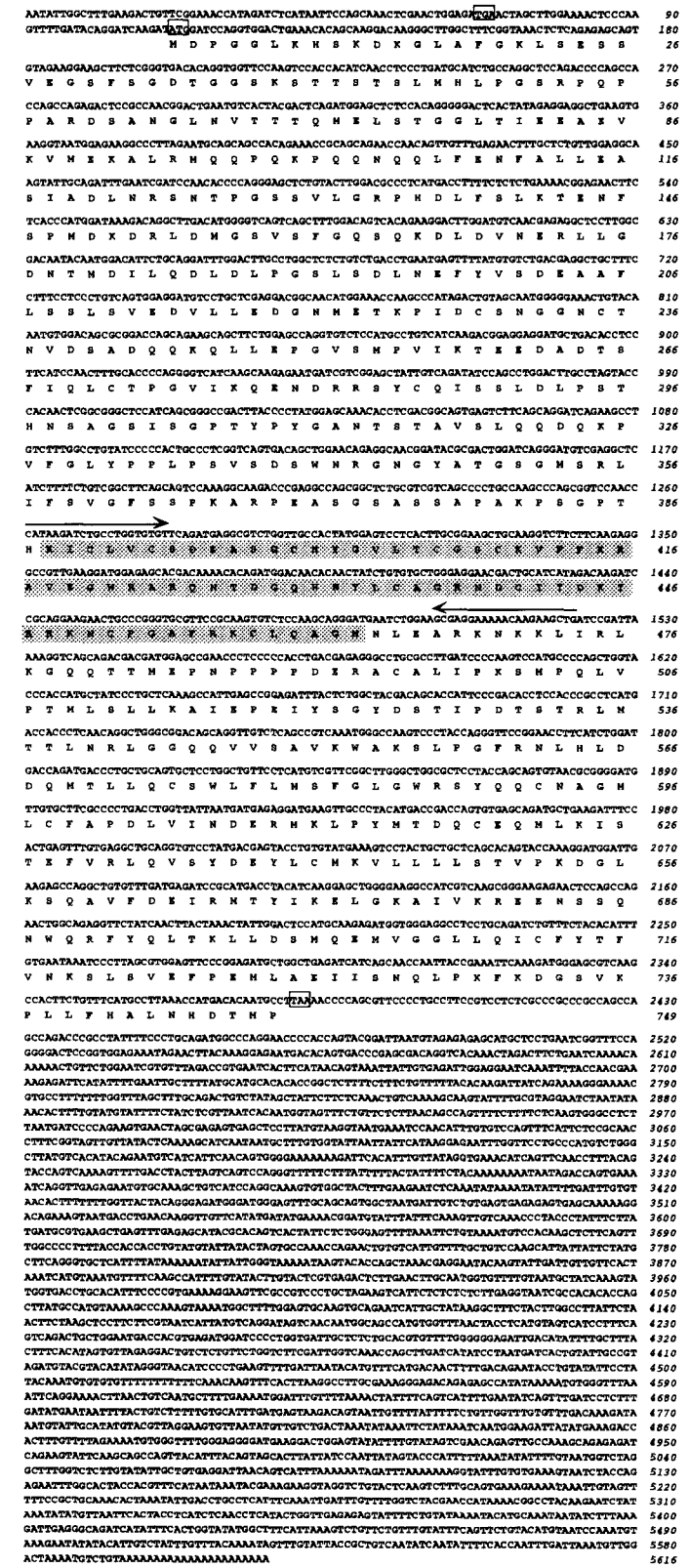

Fig. 1. Nucleotide and deduced amino acid sequence of a cDNA encoding rainbow trout glucocorticoid receptor. The open reading frame is defined by translation start and stop codons (boxed in the nucleotide sequence). The putative DNA binding domain is shown in shaded boxes. Arrows show positions and directions of synthetic oligonucleotide primers for RT–PCR analysis.

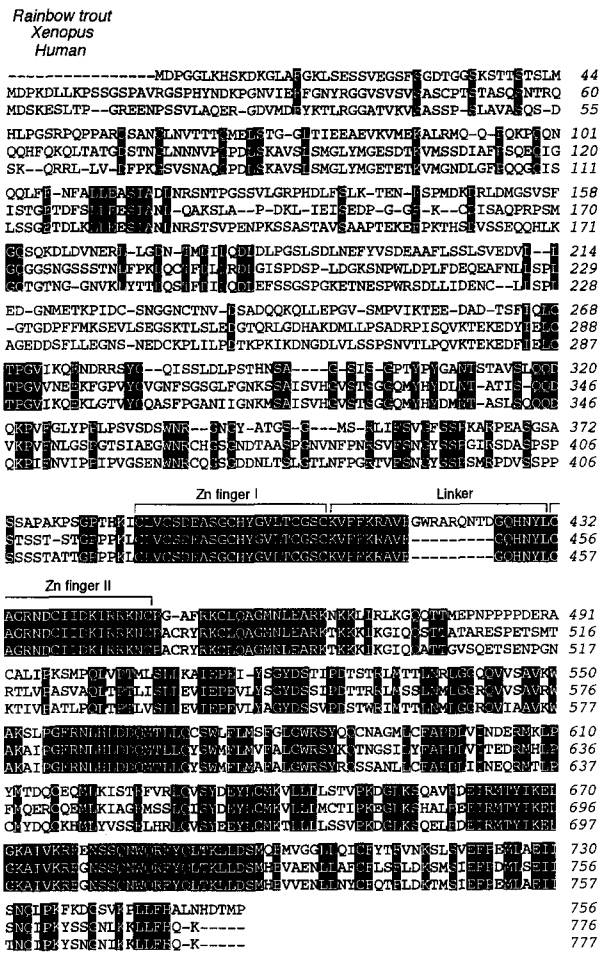

Fig. 2. Comparison of the deduced amino acid sequence of rainbow trout GR, human GR and *Xenopus* GR. Sequence identities are shown by black boxes. The two zinc fingers are bracketed [27]. Gaps are indicated by dashes.

3. Results and discussion

3.1. Isolation of a rainbow trout cDNA encoding the GR

As a first step in isolating the rainbow trout GR cDNA clone, we designed a set of degenerate oligonucleotide primers based on an amino acid sequence that is highly conserved among various species. These primers were then used to generate PCR fragments from rainbow trout pituitary cDNA to isolate a homologous fragment to serve as a screening probe. A PCR product of the length predicted from the human GR gene was isolated, cloned and sequenced. The sequence encoded 190 amino acids that had a 67% sequence identity with the corresponding region of the human GR.

Using a probe generated from this fragment, approx. 5×10^5 plaques from a rainbow trout pituitary cDNA library were screened, and several strongly hybridizing clones were obtained. One clone was found to contain the complete coding sequence of the presumptive rainbow trout glucocorticoid receptor (rtGR) (Fig. 1).

3.2. Structure of rainbow trout GR

The rainbow trout cDNA contains an ATG initiation codon followed by an open reading frame that encodes a 749 amino acid protein of a molecular mass of 83.4 kDa. The overall amino acid sequence has 50 and 46% homology to human and *Xenopus* GR, respectively, (by comparison, human and *Xenopus* GR are 61% homologous) (Fig. 2). Comparison of the four different functional domains of the rtGR to those of human GR shows a homology of 28% for the transcriptional activation domain (aa 1–419), 92% for the DNA binding domain (aa 420–485), 18% for the nuclear localization domain (aa 497–524) and 72% for the hormone binding domain (aa 527–776) (Fig. 2). Two striking features are the low homology (18%) for the nuclear localization domain (by comparison, human and *Xenopus* are 50% homologous for this region), and the presence of nine additional amino acids between the two zinc fingers in the DNA binding domain, despite otherwise very high homology for this region (92%). No such insertion has been previously reported in this region for any of the steroid hormone receptors thus far analyzed. This same nine amino acid insertion was present in each of 3 other independent clones obtained from the pituitary library, ruling out the possibility of a cloning artifact.

3.3. Rainbow trout GR contains alternative splicing variants located in the DNA binding domain

We performed the highly sensitive RT-PCR technique to survey the tissue distribution of rtGR mRNA in rainbow trout, and also to determine if the nine amino acid insertion might be the result of tissue-specific alternative splicing. PCR fragments spanning the DNA binding domain were amplified from rainbow trout liver, heart, spleen, stomach, brain, pituitary, ovary, testis, skin and bladder cDNAs. In each case a major product was detected (Fig. 3), whereas no amplification was seen when the AMV reverse transcriptase was omitted from the cDNA synthesis reaction (data not shown). In the case of testis, a second, smaller, product was also produced. To define more precisely the nature of this variant, both bands derived from testis cDNA were cloned and sequenced. As expected, the longer product was identical to the rtGR sequence isolated from the pituitary library. The sequence of the smaller product was identical to it except for the lack of the 27 bp insert corresponding to the nine amino acids de-

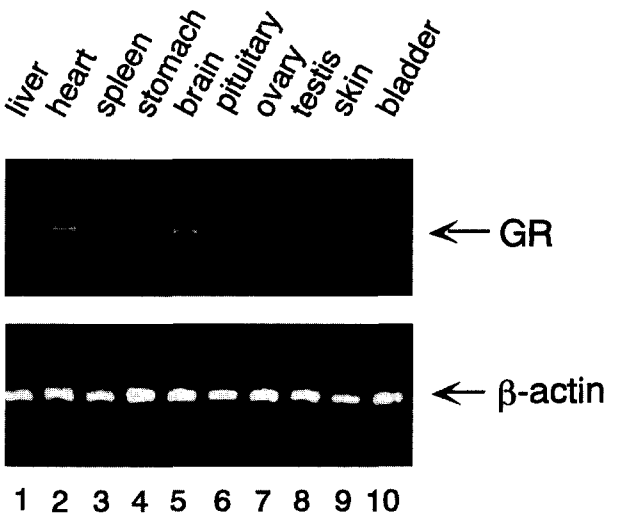

Fig. 3. Expression and alternative splicing of rainbow trout GR mRNA. Poly $(A)^+$ RNA from various tissues was used to generate cDNA for PCR amplification using rtGR specific primers as described in Section 2. The PCR products were resolved on an agarose gel and stained with ethidium bromide. Primers specific for the rainbow trout β-actin mRNA were used as a control.

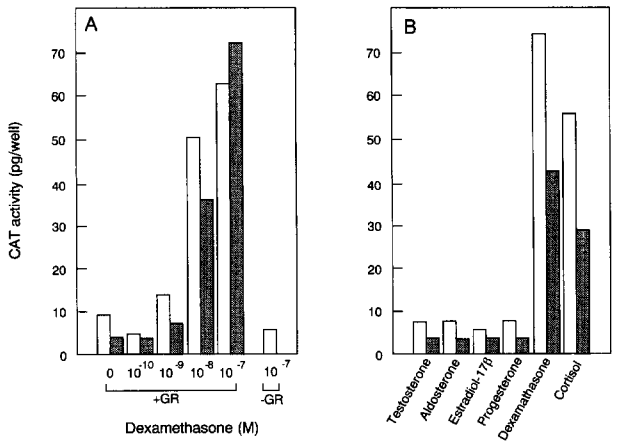

Fig. 4. Transcriptional activity of rainbow trout GR and its splicing variant in RBCF-1 cells. RBCF-1 cells were cotransfected with the pMSG-CAT reporter plasmid and either pCMV-rtGR-I (empty bars) or pCMV-rtGR-II (stippled bars) as described in Section 2. (A) Dexamethasone dependence of GR-mediated activation of CAT activity in the presence (+GR) or absence (−GR) of pCMV-GRI/II. Following transfection, cells were incubated with dexamethasone at the indicated levels prior to harvest. (B) Ability of various steroids to activate transcription via the rtGRs. Following transfection, cells were incubated with the indicated steroids at 10^{-7} M prior to harvest. For both (A) and (B), averaged values are presented from three independent transfections.

scribed above, making its encoded amino acid sequence completely continuous with other known GR sequences. The most likely explanation for this result is alternative splicing. To further check for this possibility, we performed PCR using the same primers on rainbow trout genomic DNA. Only the longer product was observed (data not shown). Thus, we conclude that whereas most rainbow trout tissues produce a GR carrying the additional nine amino acids in its DNA binding region, through alternative splicing the testis also makes an isoform more like other known steroid receptors. Thus, we conclude that whereas most rainbow trout tissues produce a GR carrying the additional nine amino acids in its DNA binding region, through alternative splicing the testis also makes an isoform more like other known steroid receptors. There is precedence for the presence of alternative spliced forms of GR from human studies [26]. In one case [14], the last 50 amino acids of the hGR are replaced with an alternative 15 amino acid sequence, producing an isoform unable to bind to glucocorticoid hormones and activate transcription. In two other cases [27], splicing variants deleted for different exons in the hormone binding domain were isolated from a glucocorticoid resistant myeloma cell line. In each of these cases the hGR is altered in its hormone binding domain; consequently, the rtGR is the first instance of a splice variant in the DNA binding domain.

3.4. Biological activities of rainbow trout GR and its splicing variant

To determine whether the nine amino acid insertion present in the rainbow trout GR affects its biological activity, two rtGR constructs, rtGR-I (major form, with insert) and rtGR-II (testis-specific alternative splicing product) were inserted into CMV expression vectors. The reporter construct pMSG-CAT, carrying the glucocorticoid-responsive MMTV promoter [28], was used to monitor GR activity. Transfection of the pMSG-CAT reporter into the RBCF1 fish cell line resulted in very low basal expression of CAT, which could not be induced by the dexamethasone (Fig. 4A). In contrast, dexamethasone stimulated CAT activity upon co-transfection of either the rtGR-I or rtGR-II expression plasmids (Fig. 4A). Induction was also observed with cortisol, but not with testosterone, aldosterone, estradiol-17β or progesterone (Fig. 4B). These results indicate that both rainbow trout GR-I and GR-II are capable of producing a fully functional receptor that mediates hormone dependent transcriptional activation. More extensive studies are now in progress to try to determine whether the alternatively spliced forms have different properties. For example, since the spacing between the DNA binding zinc fingers is altered, they may differentially respond to variations in the GRE. It is expected that further investigation of this novel GR will provide new insights into the functioning and specificity of this class of receptors, as well as enable studies of osmoregulation in fish.

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