Cloning and expression of a functional estrogen receptor-α from African catfish (Clarias gariepinus) pituitary

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Abstract

An African catfish (Clarias gariepinus) estrogen receptor-α (cfERα) cDNA fragment was amplified by RT-PCR, in combination with a modified 3′-RACE procedure, on total RNA extracted from pituitary. This cDNA fragment was used to screen an African catfish pituitary cDNA library. A clone was obtained that contained an open-reading frame coding for a 620 amino acid cfERα protein with a deduced molecular mass of 68.1 kDa. In addition, a partial African catfish estrogen receptor-β (cfERβ) cDNA fragment was amplified by RT-PCR on total RNA extracted from testis.

Neighbor-joining analysis was used to infer a phylogenetic classification for cfERα and cfERβ. The tree obtained indicated that there are two major clusters of vertebrate ERs: ERα and ERβ. Within each cluster, teleost and tetrapod ER sister clades could be distinguished. The cfERα clustered with other teleost ERαs, whereas cfERβ clustered with other teleost ERβs.

The ligand-induced transcriptional activity of cfERα was demonstrated in a transient gene expression assay using cells in which an acute estrogenic response was created by co-transfecting cultures with recombinant cfERα cDNA expression vector constructs in the presence of an estrogen-dependent reporter plasmid.

Real-time, quantitative PCR revealed that cfERα transcripts were most abundantly expressed in pituitary, while in all other tissues tested the relative cfERα mRNA levels were less than ~5% of the level obtained in pituitary. Moreover, we found that, during pubertal development, the relative cfERα mRNA levels gradually increased in African catfish pituitary.


Introduction

Estrogens play a crucial role in the control of female and male reproductive physiology as well as other non-reproductive processes such as bone calcification and memory. Despite the diversity of estrogen target tissues and effects, and although there is increasing evidence that estrogens may also have rapid, non-genomic effects by interacting with membrane receptors or recognition sites (Loomis & Thomas 2000, Benten et al. 2001), the main actions of estrogens are mediated by an intracellular nuclear receptor, called the estrogen receptor (ER), on the transcriptional regulation of target genes. Ligands for the ER include estradiol (E2) as well as other steroidal and non-steroidal estrogens and antiestrogens (Murphy et al. 1997). The mechanisms of estrogen and estrogenic ligand action in the control of transcription have been recently reviewed (McDonnell 1999, Dutertre & Smith 2000, Kushner et al. 2000, Muramatsu & Inoue 2000, Pettersson & Gustafsson 2001). Ligand-activated ERs regulate gene expression directly by binding to a specific cis-element, called the estrogen-response element (ERE), in the promoters of target genes or indirectly via the interaction with other transcription factors.

Based on sequence similarity, all ERs display a structural organization similar to other nuclear receptors, and are divided into six domains termed...
A–F (Krust et al. 1986): a poorly conserved N-terminal A/B domain, including activation function 1 (AF1), a highly conserved central C domain (DNA-binding domain; DBD), flanked at its C-terminus by the D domain (hinge domain), the variably conserved E domain (ligand-binding domain; LBD), including the activation function 2 (AF2), and the F domain. ERs, together with other intracellular steroid hormone receptors, are grouped into subfamily III of the nuclear receptor superfamily, based on the construction of phylogenetic trees by two different methods (namely, distance matrix and parsimony analysis), tested by the bootstrap procedure (Laudet 1997).

In mammals, two ER subtypes, ERα and ERβ, have been characterized (Kuiper et al. 1996, Mosselman et al. 1996, Tremblay et al. 2000). The cloning of ER subtypes from several teleost fish species supported the notion that also in non-mammalian species ERα and ERβ subtypes exist (Chang et al. 1999, Tchoudakona et al. 1999, Hawkins et al. 2000, Socorro et al. 2000, Xia et al. 2000). Detailed phylogenetic analyses of ERs revealed that each receptor subtype contains two major sister clades, piscine (teleost) and tetrapod ERs (Xia et al. 2000).

In mammals, there is considerable tissue specificity in the expression of ERα and ERβ. ERα is the dominant species expressed in pituitary, hypothalamus, uterus, liver, adipose tissue and skeletal muscle, whereas ERβ is the major form in ovary and prostate, as well as other regions in the brain including the limbic system, cerebellum and cerebral cortex (Couse et al. 1997, Shughrue & Merchenthaler 2000). In the pituitary, ERα is present primarily in lactotrophs and gonadotrophs (Friend et al. 1994, Scully et al. 1997, Mitchner et al. 1998). Both types of ER are differentially expressed in pituitary cell types, with overlapping expression in gonadotrophs where expression levels reflect sex differences and developmental changes (Friend et al. 1994, Wilson et al. 1998, Nishihara et al. 2000).

Information on the tissue distribution of ERs in teleosts is scarce and mainly described for liver, gonads and the central nervous system. In female rainbow trout (Oncorhynchus mykiss), ERα-immunoreactive cells were essentially located in brain regions involved in the neuroendocrine control of pituitary functions and having direct connections with the hypophysis (Anglade et al. 1994, Linard et al. 1996). In teleosts, ERβ has been detected mostly in liver and gonads, with the exception of the goldfish (Carassius auratus) ERβ2, which is highly expressed in pituitary, telencephalon and hypothalamus (Ma et al. 2000).

Recent data on ERαβ knock-out mice (i.e. mice with targeted disruption of both ER genes) clearly showed that both types of ER are required for E₂-mediated regulation of luteinizing hormone (LH) secretion in the hypothalamic–hypophyseal axis (Couse et al. 1999). Important functions regulated by E₂ via ERs in teleost fish are the positive and negative feedback on the brain–pituitary complex (Saligaut et al. 1998). Moreover, estrogens (in particular E₂) are known to play a direct role in the regulation of fish LHβ gene expression, and EREs have been identified in the promoter/enhancer region of all known fish LHβ promoters (Liu et al. 1995, Le Drean et al. 1996, 1997). In addition, the combinatorial action of ER with other transcription factors on LHβ gene expression has been established (Le Drean et al. 1996, 1997).

To better understand the molecular mechanisms mediating the regulatory role of E₂ on the expression of the African catfish (Clarias gariepinus) LHβ (cfLHβ) gene in the pituitary, and as a first step toward achieving the research tools necessary for such a study, we initiated the cloning of an African catfish ER cDNA. Here we describe the cloning of an African catfish ERα (cfERα) cDNA. In addition, the ligand-induced transcriptional activity of cfERα was shown on a synthetic promoter containing three EREs. Moreover, we determined the tissue distribution of the cfERα mRNA, and the relative cfERα mRNA expression levels in the pituitary during pubertal development, using real-time, quantitative PCR. Moreover, we also describe the cloning of a partial African catfish ERβ (cfERβ) cDNA, in order to rule out that an additional ER mRNA form of the African catfish was detected, in the real-time, quantitative PCR using the primers and probe designed on the cfERα cDNA sequence.

Materials and methods

Animals

African catfish were bred and raised in the laboratory by induced ovulation and artificial fertilization as described previously (de Leeuw et al. 1985), except that African catfish pituitary extract,
instead of human chorionic gonadotropin was used to induce ovulation. The fish were kept in a copper-free circulation system at 25 ± 2 °C, exposed to 14 h light:10 h darkness, and fed with Trouvit pellets (Trouw, Putten, The Netherlands). Animal culture and experimentation was consistent with the Dutch national regulations; experimental protocols were submitted to and approved by the appropriate University committee. In our hands, sexual maturation of male African catfish started at ~3 months of age when the first males were found in which spermatogonial proliferation had been initiated. At ~6 months of age spermatozoa were found in the testes of most fish. Fully mature males can be used for breeding purposes at 10–12 months of age.

**Primers**

The following primers were purchased from Life Technologies (Breda, The Netherlands): DBD66/100, 5'-GGAGTCGGTACCTGYGARGGCTGC AAGGGYTCTT-3'; DBD210/238, 5'-TCCTTGN GCATGCCACTTGAGCADCATT-3'; cfERα-1(S), 5'-GGTTGACATCTACGTTGCTCG GCC-3'; cfERα-2(S), 5'-CCGCGGATTTCGGCTGT CAAGCGCTGTCG-3'; 682, 5'-TTGGAGCTGGA TGCTCGGGGAC-3'; 690, 5'-ACGGCCGGA GCACCTCCCG-3'; adaptor-1, 5'-CGCTCTAG AGACTCGATCGACATCGA-3'; adaptor-2, 5'-CCGAGCTCGAATCGACATCGATTT-3'; GATC-N8-adaptor, 5'-TCCTTGTCCG-3'; 100, 5'-TTCAGGTACGGTACCGGCACCTTCGADGCACTTT-3'; 1160, 5'-ATGGGCTTGGGCTT-3'; 1163, 5'-CTGGTACCAT-3'; 1164, 5'-ARGCAYERTRABC CYTCRCA-3'; 1169, 5'-AGGCCAAAACAGAC ATGCACTT-3'; 1170, 5'-ATCTTCTTSGCCA GCTGATACT-3'; 1163, 5'-CCCTGCTGATYTC CNTCHCCBTA-3'; 1164, 5'-ARGCyyyyTRABC CYTCRCA-3'; 879 contained a Kozak consensus translation initiation sequence (Kozak 1987) (italics).

**RNA isolation**

Pituitaries of male African catfish between 10 and 24 weeks of age were collected, and stored separately at −80 °C. The developmental stage of each male was assessed by testicular histology as described previously (Cavaco et al. 1997). Next, pituitaries from the same stage of development were pooled for RNA isolation with RNazol (Campro Scientific, Veenendaal, The Netherlands), according to the manufacturer’s instructions. To this end, pools of ten pituitaries (stage I; n = 8), five pituitaries (stage II; n = 5), or four pituitaries (stage III; n = 3) were used; from stage IV (n = 7) or adult (n = 6) males, single pituitaries were used. In addition, total RNA was isolated from several tissues (for each tissue, n = 3) of adult male African catfish, from pre-ovulatory, post-vitellogenic ovaries (n = 3) and post-ovulatory ovaries (n = 3) of adult female African catfish, and from ovaries (n = 3) of 1-week-old female African catfish, using the method described by Chirgwin et al. (1979).

**Isolation of cfERα cDNA clones**

Random-primed as well as GATC-N8-adaptor-primed pituitary cDNA was synthesized with Superscript II RNase H− reverse transcriptase (Life Technologies) using 2 µg pituitary total RNA, according to the manufacturer’s instructions. A cfERα cDNA was first identified as a 166 bp PCR product, using primers DBD66/100 and DBD210/283 (Lopes da Silva et al. 1995), based on homologies in the DBD of AGGTCA-binding factors, and random-primed pituitary cDNA as template. Next, two cfERα-specific primers (cfERα-1(S) and cfERα-2(S)) were designed and used in a modified 3′-RACE. To this end, PCR was performed between primers cfERα-1(S) and adaptor-1 on GATC-N8-adaptor-primed pituitary cDNA as template. Next, a nested PCR was performed between primers cfERα-2(S) and adaptor-2 on 1000-fold diluted PCR material between primers cfERα-1(S) and adaptor-1. PCR products of ~1·2, ~1·0, ~0·85 and ~0·7 kb were obtained, cloned and sequenced. Based on the DNA sequence of the longest PCR product cloned, two new cfERα-specific primers (682 and 690) were designed, and used in a PCR-based screening
of unidirectional, random-primed African catfish pituitary ZAP-Express cDNA sublibraries, and in order to generate a ~900 bp probe. Next, this PCR product was labeled by random-priming using a random-primed DNA labeling kit (Version 2.0; ICN, Costa Mesa, CA, USA) and $[^{32}P]$dATP (ICN), and used to screen the PCR-positive cDNA sublibraries. After rescreening at lower plaque density, six clones were isolated and excised in vivo as pBK-CMV phagemids.

**Isolation of a partial cfERα cDNA**

Random-primed brain, ovary and testis cDNAs were synthesized with Superscript II RNase H− reverse transcriptase (Life Technologies) using 2 µg of brain, ovary or testis total RNA, according to the manufacturer’s instructions. A cfERα cDNA was first identified as a ~420 bp PCR product, using primers 1163 and 1164 in all three types of cDNA. Next, the specific forward primer 1169 was designed and used in combination with primer 1160 in order to specifically PCR amplify additional cfERα cDNA sequences. The latter reaction yielded a PCR product of ~550 bp that was cloned and sequenced.

**DNA sequence analysis and phylogenetic analysis**

DNA sequence analysis was performed on an ABI 310 automated DNA sequencer using dye terminator chemistry (Applied Biosystems, Forster City, CA, USA). The DNA sequences were processed using Lasergene software (DNASTAR Inc., Madison, WI, USA). Multiple sequence alignment and phylogenetic analyses were performed with the Megalign program of the Lasergene software package, using the Clustal method (PAM250).

**Constructs, cell culture, transient transfections and luciferase assays**

The entire coding region of cfERα was PCR amplified using primers 879 and 283, cloned into the pcDNA3.1/V5-His TOPO mammalian expression vector (Invitrogen), and sequenced. This new cDNA construct was designated cfERα-pcDNA3. The human ERα (hERα) expression plasmid pSG5-HEGO (kindly provided by Dr P Chambon, IGBMC, Strasbourg, France) was used as a positive control. The estrogen-responsive reporter gene construct (3 × ERE-TATA-LUC), which contains three copies of a consensus ERE containing oligonucleotide and a TATA box in front of the luciferase cDNA (Kuiper et al. 1998), was kindly provided by Dr B van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands).

Human embryonic kidney (HEK) 293T cells (DuBridge et al. 1987) were routinely maintained in sodium pyruvate-free Dulbecco’s modified Eagle’s medium (DMEM), containing 2 mM glutamine, 4.5 g/l glucose, 4 mg/l pyridoxine HCl and

<table>
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<th>Target</th>
<th>GenBank</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>nM used in PCR</th>
<th>bp</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
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<td>cfGAPDH-Fw</td>
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<td>133</td>
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<td></td>
<td></td>
<td>cfGAPDH-Pr</td>
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<td>300</td>
<td>100</td>
<td>−3.32</td>
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**Table 1 Primers and TaqMan fluorogenic probes. Sequences are shown for the sense (-Fw) and antisense (-Rv) primers and the TaqMan probe (-Pr) as well as the size of the amplicon (bp), and the slope of the standard curve. Fluorogenic probes were synthesized with the fluorescent reporter dye FAM (cfERα) or VIC (cf28S rRNA and cfGAPDH) attached to the 5′-end and a quencher dye TAMRA to the 3′-end.**
Figure 1 Nucleotide sequence and deduced amino acid sequence of the cfERα cDNA. Numbers on the right of the nucleotide sequence indicate the nucleotide positions, starting with the first nucleotide of the cfERα cDNA. Numbers on the left of the deduced amino acid sequence indicate the amino acid positions in the cfERα protein, starting with the proposed initial methionine. The asterisk (*) indicates the stop codon. The A/B domain consists of amino acid residues 1–191, and contains the transcriptional AF-1 (amino acids 110–114; indicated by white letters in black boxes). The C domain (DBD; amino acid residues 192–257) is boxed, and the cysteine residues of the two zinc-finger motifs are indicated by white letters in black boxes. The D domain consists of amino acid residues 258–321. The E domain (LBD; amino acid residues 322–563) is also boxed, and contains the transcriptional AF-2 (amino acid residues 548–555; indicated by white letters in black boxes). The F domain consists of amino acid residues 564–620.
antibiotic/antimycotic solution (100 IU/ml–100 µg/ml), supplemented with 7.5% fetal calf serum (FCS) (all from Life Technologies) under a humidified 5% CO2 atmosphere at 37 °C. For transfection experiments the cells were plated 1 day before transfection in 24-well tissue culture plates at a density of 10^5 cells/well and cultured in phenol red-free transfection/stimulation medium (DMEM containing 110 mg/l sodium pyruvate, 1 g/l glucose, 4 mg/l pyridoxine HCl and antibiotic/antimycotic solution (100 IU/ml–100 µg/ml), supplemented with 5% charcoal-stripped FCS). Cells were transiently co-transfected by the calcium phosphate precipitation method (Chu & Sharp 1981) with 0.2 µg cfERβ9825-pcDNA3 or pSG5-HEGO, 0.4 µg pSV2-lacZ internal control plasmid (Hall et al. 1983), 1 µg 3'-ERE-TATA-LUC and 0.4 µg empty pcDNA3 vector. After 24 h, the transfection/stimulation medium was changed to transfection/stimulation medium without or with E2 (Sigma) at a concentration of 1–100 nM. The next day, cells were harvested and lysed in 150 µl ice-cold lysis buffer (250 mM Tris–HCl (pH 7.8), 0.5% Nonidet P-40). Fifty microliters of the cell lysates were used to measure the β-galactosidase activity in order to correct for variations in transfection efficiencies, whereas 50 µl of the cell lysates were used for a highly sensitive luciferase assay, as previously described (Kuiper et al. 1998).

### Real-time quantitative PCR

Primers and fluorogenic probes specific for cfERα mRNA, and specific for the endogenous controls (African catfish 28S rRNA (cf28S) or African catfish GAPDH mRNA (cfGAPDH)), were designed with Primer Express software (Applied Biosystems), according to the manufacturer’s guidelines as described previously (Livak 1999), and purchased from Applied Biosystems. Reverse transcription and TaqMan PCR assays were performed as described previously (Bogerd et al. 2001), using the concentrations of primers and probes listed in Table 1. The relative cfERα mRNA expression levels were calculated using the arithmetic comparative method (the ΔΔCt method), which involves normalization of the Ct values for the cfERα mRNA to the internal reference (i.e. cf28S RNA or cfGAPDH mRNA) Ct values. The relative amounts of cfERα mRNA in the cDNA samples from different tissues and

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**Figure 2** Nucleotide sequence and deduced amino acid sequence of the partial cfERβ cDNA. The C domain (DBD) is boxed, and the cysteine residues of the two zinc-finger motifs are indicated by white letters in black boxes. The first part of the E domain (LBD) is also boxed.
Figure 3 Phylogenetic tree of ERs. The Clustal method was used to perform multiple sequence alignment. The phylogenetic tree was constructed using the Megalign program of the Lasergene software package. The following (deduced) full-length amino acid sequences were used: Oncorhynchus mykiss (rainbow trout) ERβ (accession number P57782), Carassius auratus (goldfish) ERβ (Q9W669), Danio rerio (zebrafish) ERβ (AAK16742), Clarias gariepinus (African catfish) ERβ (cERβ; partial), Micropterus undulatus (Atlantic croaker) ERγ (P57783), Sparus aurata (gilthead seabream) ERβ (Q9W6M2), Oreochromis niloticus (Nile tilapia) ERβ (Q9YH32), Anguilla japonica (Japanese eel) ERβ (O13012), Micropogonias undulatus ERβ (P57781), Ictalurus punctatus (channel catfish) ERβ (Q9IAK1), Carassius auratus ERβ (Q9IAL9), rat ERβ (CAA05631), mouse ERβ (O08537), hERβ (Q92731), Callithrix jacchus (white-tufted-ear marmoset) ERβ (Q95171), sheep ERβ (Q9XSB5), pig ERβ (AAK15151), Japanese quail ERβ (O93511), chicken ERβ (Q9PTU5), common starling ERβ (Q9PVE2), Chrysocephalus major (red seabream) ERα (O42132), Sparus aurata ERα (Q9PVZ9), Oreochromis aureus (blue tilapia) ERα (P50240), Oreochromis niloticus ERα (Q9YH33), Oryzias latipes (medaka) ERα (P50241), Oncorhynchus mykiss ERα (P16058), Ictalurus punctatus ERα (Q9YH7), Clarias gariepinus ERα (cERα; X84743), Danio rerio ERα (AAK16740), rat ERα (CAA43411), mouse ERα (NP_031982), golden hamster ERα (Q9QZJ5), hERα (P03372), pig ERα (Q92940), horse ERα (Q9TV98), chicken ERα (P06212), Taeniopygia guttata (zebra finch) ERα (Q91250), and Xenopus laevis (African clawed frog) ERα (P81559), while human estrogen-related receptor 1 (ERR1) (P11474) was used as an outgroup. The scale beneath the phylogenetic tree indicates the number of substitution events, and is a measure of distance between sequences.
from the pituitary primary cell cultures were calculated using normalization to the cf28S internal reference values. However, normalization to the cfGAPDH internal reference values was used for calculation of the relative cfERα mRNA amounts in the pituitary cDNA samples during pubertal development. Optimization and validation were performed according to the manufacturer’s guidelines and as described previously (Bogerd et al. 2001). Briefly, the PCR efficiency and whether the relationship between $C_\text{t}$ and log starting copy number was linear for all primer/probe sets was tested using undiluted pituitary cDNA, and three serial 10-fold dilutions of pituitary cDNA. For all primer/probe sets used, the slopes of the standard curves were close to –3·32 (Table 1), indicating maximal PCR amplification. In addition, a validation experiment (in order to test if the amplification efficiency of the target and of the internal reference were approximately the same) has been performed, resulting in absolute values of the slopes <0·1 for the $\Delta C_\text{t}$ vs the log input pituitary cDNA for all primer/probe combinations, according to the manufacturer’s instructions (Applied Biosystems; data not shown). The relative expression levels of cfERα mRNA in unknown samples are expressed relative to the expression in the control group since the mean of the control group levels was used as calibrator for the calculations.

### Statistical analysis

Graphs show means ± s.e.m. with $n$ given in the respective figure legends. Before analysis, all data from the real-time, quantitative PCR were transformed to the natural logarithmic scale to obtain equal residual variation among the treatments. Statistical analyses were performed using two-way ANOVA, followed by Fisher’s protected least significant difference (PLSD) test (StatView 4·5 for Windows; Abacus Concepts, Berkeley, CA, USA). Differences were considered statistically significant when $P<0·05$. Data from the luciferase assay are presented as means ± s.e.m., after normalization to the β-galactosidase activity.

### Results

#### Cloning of a cfERα and cfERβ cDNAs

Screening of the African catfish pituitary cDNA library with a ~900 bp cfERα-specific probe,
followed by in vivo excision, resulted in the isolation of six cfERα cDNA clones. The longest cDNA consisted of 2102 bp (Fig. 1), of which the first 22 nucleotides make up the 5′-untranslated region, followed by 1863 nucleotides coding for the cfERα protein, and the 217 nucleotide 3′-untranslated region. The deduced cfERα amino acid sequence contains characteristic domains of other ERs (Fig. 1), including amino acids involved in receptor transactivation (Danielian et al. 1992), dimerization (Tanenbaum et al. 1998) and ligand binding (Ekena et al. 1996, Brzozowski et al. 1997, Hung et al. 1999).

The cloning of a partial cfERβ cDNA (Fig. 2) was initiated only very recently in order to evaluate the specificity of the primers and probe used for quantitative analysis of the cfERα mRNA levels (see below).

### Phylogenetic and structural analysis

The phylogenetic tree representing the multiple sequence alignment between the full-length (deduced) amino acid sequences of cfERα, (the partial) cfERβ and other ER members of the steroid hormone receptor subfamily is shown in Fig. 3. The tree clearly showed that cfERα clustered together with known teleost ERα proteins, while cfERβ clustered with known teleost ERβ proteins. Moreover, the tree revealed a clear division between teleost ER and tetrapod ER sister clades within each major ER subtype. Inside the teleost ERα clade, cfERα has the highest similarity to the channel catfish (Ictalurus punctatus) sequence (i.e. 85% similarity). In addition, the cfERα also shared a high overall similarity to other known teleost and tetrapod ERα sequences: zebrafish (Danio rerio; 63%), rainbow trout (Oncorhynchus mykiss; 54%), red seabream (Chrysophrys major; 56%), medaka (Oryzias latipes; 52%), Nile tilapia (Oreochromis niloticus; 50%), blue tilapia (Oreochromis aureus; 49%) and tetrapods (44–46%). Inside the teleost ERβ clade, cfERβ has the highest similarity to the rainbow trout (Oncorhynchus mykiss) and goldfish (Carassius auratus; ERβ1) sequences (i.e. 77% similarity). In addition, the cfERβ also shared a high overall similarity to other known teleost and tetrapod ERβ sequences: zebrafish (Danio rerio; 73%), Nile tilapia (Oreochromis niloticus; 65%), Atlantic croaker (Micropogonias undulatus; ERβ and ERγ; 64%) and gilthead seabream (Sparus aurata; 64%), Japanese eel (Anguilla japonica; 63%), channel catfish (Ictalurus punctatus; 57%), goldfish (Carassius auratus; ERβ2) (56%) and tetrapods (53–57%).

### Functional characterization of cfERα

The transcriptional activation properties of the cloned cfERα and the hERα proteins were compared in HEK-293T cells. To this end, the
cells transiently co-transfected with a luciferase enzyme reporter gene construct fused to three copies of a consensus ERE in front of a TATA box, together with either the cfERα-pcDNA3 or pSG5-HEGO expression vector constructs, were cultured in the presence and absence of two different concentrations (10^{-7} and 10^{-9} M) of E_2.

The transcriptional activity of the estrogen-responsive reporter gene in the presence of cfERα or hERα expression vectors was increased by E_2 (Fig. 4). Dose-dependent differences in the E_2-induced luciferase activity were only observed for hERα, while the two different E_2 concentrations resulted in similar folds of induction mediated by cfERα.

**Tissue distribution of cfERα mRNA**

Real-time, quantitative PCR was used to determine the relative cfERα mRNA expression levels in various tissues (Fig. 5). Since the primers and probe used for the quantitative detection of cfERα mRNA might potentially recognize a putative cfERβ mRNA, we cloned a putative, partial cfERβ cDNA (Fig. 2). Alignment of the cfERα cDNA sequence with the cfERβ cDNA sequence, in the region where the primers and probe for the cfERα mRNA detection were designed (Fig. 6), revealed that these primers and probe are not able to detect the cfERβ mRNA. Moreover, using the cfERβ cDNA in the real-time, quantitative PCR did yield a C_0 value of 40, providing evidence for no PCR amplification of the cfERβ mRNA with the cfERα primers/probe set (data not shown).

The highest cfERα mRNA level was found in pituitary (set at 100%; Fig. 5), while in all other tissues tested the relative cfERα mRNA expression levels were less than ~5% of the level obtained in pituitary (Fig. 5).

**Changes in cfERα mRNA levels during pubertal development**

The relative cfERα mRNA levels in the pituitary were measured during pubertal development (Fig. 7), and revealed that cfERα mRNA expression levels significantly increased during the transition from stage I to stage II as well as during the transition from stage IV to the adult stage. In stage III, the cfERα mRNA levels remained the same as detected in stage II, but dropped in stage IV.

**Discussion**

In our attempts to get more insight into the molecular components involved in modulating the transcriptional activity of the cfLHβ gene, we report here that at least one ER-like protein (cfERα) is produced by the pituitary of the African catfish. Phylogenetic analysis revealed that cfERα clustered with other known teleost ERα proteins, indicating that cfERα is a new member of the ERα protein family. Highest similarity of cfERα was found with the channel catfish ERα. Similar to other ERs, cfERα has a domain structure, consisting of six domains designated A–F (Weinberger et al. 1985, Krust et al. 1986, Kumar et al. 1987).

The ability of the cfERα cDNA to produce a functional protein was verified by transient transfection in HEK-293T cells, which lack endogenous ERs. Similar to hERα, cfERα was able to increase the activity of an estrogen-responsive reporter gene product (i.e. luciferase) in the presence of E_2, although the magnitude of E_2-induced luciferase activity was higher for hERα than for cfERα. Moreover, hERα but not cfERα showed a higher luciferase activity with 10^{-7} M (77-fold induction) than with 10^{-9} M (29-fold induction) E_2.
The tissue distribution of the cfERα transcript was essentially the same as found for the channel catfish ERα (Xia et al. 2000). ERα expression in the gilthead seabream was only detected in testis, liver and heart (Socorro et al. 2000). To our surprise we detected very low cfERα mRNA levels in the liver of a male African catfish, since most of the teleost ER were first cloned from this tissue. However, also in the liver of male channel catfish very low ERα mRNA levels were found, while clear ERα mRNA levels were detected in the liver of female channel catfish (Xia et al. 2000). Also, the presence of specific ERs in the cytosol of male rainbow trout liver has been reported and has been connected with the regulation of estrogen-dependent production of hepatic vitellogenin (Maitre et al. 1985). Moreover, regulation of the hepatic ERα mRNA by E2 has been reported for several teleost species (MacKay 1996). In African catfish, since most of the teleost species reported for goldfish (Ma et al. 2000) and for Atlantic croaker (Hawkins et al. 2000), and this needs further experimentation. Phylogenetic analysis of this partial cfERβ indicated that it clustered with other known teleost ERβ proteins. However, the cloning of the full-length cDNA as well as testing its functionality are needed.

The cfERα levels in pituitary increased during pubertal development in a stepwise mode; notable increases were seen between stage I and II and between stage IV and the adult stage. The highest increase in the cfERα mRNA levels, observed between stage I and stage II, coincides with the period in which strong activation of the gonadotrophs takes place, as described previously (Schulz et al. 1997). This period of pubertal development coincides and precedes the period (transition from stage I to II and II to III) in which the higher increases for the cfLHβ mRNA levels have been monitored.

In conclusion, the availability of a cfERα cDNA will allow us to study its functional relationship, if any, with cfLHβ gene expression in African catfish pituitary. Further studies are necessary to explain the biological and evolutionary significance of cfERβ (and maybe cfERγ, if it exists) in this process.

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