Molecular Cloning of Estrogen Receptors α and β in the Ovary of A Teleost Fish, the Tilapia (Oreochromis niloticus)

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Molecular Cloning of Estrogen Receptors α and β in the Ovary of A Teleost Fish, the Tilapia (*Oreochromis niloticus*)

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ABSTRACT—Estrogen receptors (ER) in mammals have recently been shown to be encoded by two distinct genes, ERα and ERβ. In this study, cDNAs encoding two tilapia ER subtypes, tER5.1 and tER4.3, were cloned from an ovarian cDNA library of a teleost fish, the tilapia *Oreochromis niloticus*. The tER5.1 and tER4.3 contain complete open reading frames encoding 585 and 557 amino acid residues, respectively. The two receptors share about 12% homology in the A/B domain, 96% in the DNA binding domain (C domain), 12% in the D domain, 57% in the ligand binding domain (E damain), and 20% in the F domain. Phylogenetic analysis of ER proteins from various vertebrate species indicated that vertebrate ERs consist of two major groups (ERα and ERβ); tER5.1 and tER4.3 belong to ERα and ERβ subtypes, respectively. Thus, we consider tER5.1 and tER4.3 to be the tilapia homologs of ERα (tERα) and ERβ (tERβ), respectively. In transient transfection assays using mammalian COS-7 cells, both tERα and tERβ showed estradiol-17β dependent activation of transcription from the estrogen-responsive ERE-Luc promoter. This is the first report of the presence of ERα and ERβ within a single non-mammalian vertebrate species.

INTRODUCTION

Estrogens play important roles in various aspects of reproduction in vertebrates. The actions of estrogens are mediated by their specific receptors which are localized in either the cytoplasm or nucleus of their target cells. The estrogen receptor α (ERα) is a member of a superfamily of transcription factors that induce transcription of target genes by binding to *cis*-acting enhancer elements in promoters of responsive genes. cDNAs encoding ERα have been cloned from several vertebrate species including mammals (Green *et al*., 1986; Greene *et al*., 1986; Koike *et al*., 1987; White *et al*., 1987), birds (Krust *et al*., 1986), amphibians (Weiler *et al*., 1987) and teleost fish (Pakdel *et al*., 1990; Tan *et al*., 1996; Touhata *et al*., 1998). More recently, a second type of ER (ERβ) has been reported in rat (Kuiper *et al*., 1996), human (Mosselman *et al*., 1996) and mouse (Tremblay *et al*., 1997). However, to date there have been no published works depicting the recently discovered ERβ in non-mammalian vertebrates.

Estradiol-17β is essential for normal ovarian development in vertebrates (Wallase, 1985). In fishes, as in other oviparous vertebrates, estradiol-17β is thought to be the main vitellogenic estrogen which stimulates hepatic production of vitellogenin, the precursor of egg proteins (Mommsen and Walsh, 1988, Specker and Sullivan, 1994). It has also been reported that inhibitors of aromatase, the enzyme responsible for the conversion of testosterone to estradiol-17β, cause genetic females to become phenotypic males (chicken, Elbrecht and Smith, 1992; reptiles, Dorizzi *et al*., 1994). Piferrer *et al*. (1994) found that treatment of chinook salmon, *Oncorhynchus tshawytscha*, with an aromatase inhibitor induced the development of functional, phenotypic males from genetic females. These findings strongly suggest that endogenous estradiol-17β act as natural inducers of ovarian differentiation in nonmammalian verterates. However, the mechanisms of estrogen action on ovarian differentiation in these animals remain unknown.

Tilapia, *Oreochromis niloticus*, is a gonochoristic teleost fish. Like many other teleost fishes, gonadal sex in tilapia can be manipulated by treatment of young fry with exogenous steroids (Nakamura and Takahashi, 1973). As an initial step

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toward understanding the mechanism of estradiol-17β action on ovarian differentiation in tilapia, we previously isolated cDNAs encoding aromatase (Chang *et al.*, 1997). More recently, we showed immunocytochemically that the four steroidogenic enzymes necessary for estradiol-17β biosynthesis, including aromatase, are present in the undifferentiated gonads of genetic females, but not males (Nakamura *et al.*, 1998). In this study, we isolated cDNA clones encoding tilapia homologs of ERα (tERα) and ERβ (tERβ) from an ovarian cDNA library. The transactivation function of the tilapia ERs was determined by expressing the cDNAs in transiently transfected COS-7 cells.

MATERIALS AND METHODS

Experimental animals and RNA extraction

Sexually mature tilapia were reared under natural light in laboratory glass aquaria with aerated water at 24°C. Total RNA from ovary was extracted with ISOGEN solution (Nippon Gene, Japan). Messenger RNAs were seperated from total RNA with oligo texTM-dT30 (Super) (JSR, Japan).

Isolation and sequence analysis of tilapia ERs

Two conserved amino acid regions in the DNA domain (DYMCPATNQ) and the hormone binding domain (LEMLDG(A)H) of steroid hormone receptors were selected according to Todo *et al.* (1996) and their degenerate oligonucleotides were used as primers for polymerase chain reaction (PCR): primer 1, 5' GACTA(CT)ATG-TG(CT)CC(CT)GCGAC(CT)AACCTG3' and primer 2, 5' GTGA-(CG)CGTCCAGCATCTCCAG 3'. As a template for PCR, first-strand cDNA was synthesized from 1 μg of mRNA isolated from ovary using Superscript II RnaseH (–) reverse transcriptase. The predicted PCR products were subcloned and sequenced using an ABI PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystem) with the ABI PRISMTM 377 DNA Sequencer (Perkin Elmer, USA).

PCR using primers 1 and 2 yielded a DNA fragment of 993 bp. The deduced amino acid sequence of this fragment had high homology to the C and E domains of other known ERs. Another primer (primer 3) 5' GC(AC)GA(TC)AAGGAGCTGGTTCACATGAT 3'corresponding to ADKELVHMI in the hormone-binding domain of the 993 bp fragment was synthesized. The PCR product was purified and labeled with [^{32}P] α-dCTP using a Random Primer Plus Extension Kit (NEN, USA). A tilapia ovarian cDNA library was constructed with a Uni-ZAP-cDNA synthesis Kit according to the method of Chang *et al.* (1997). A total of 3×10^5 phages were screened using the PCR product as a probe. After two rounds of screening, fourteen positive clones were subcloned into Bluescript SK(–) plasmids by *in vivo* excision.

Luciferase assay of ERs

The tER expression vectors were constructed by ligating the ORF sequences into the *Eco*RI site of pSG5 (Stratagene, La Jolla, Ca). For transfection, COS-7 cells were seeded in 24-well plates (Corning, NY) in phenol-red free Dulbecco's Modified Eagle's medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% charcoal-stripped fetal calf serum (FCS), and 50 μg/ml kanamycin. After 24 hr, cells were transfected with 5 ng of pcDNA tERs, 500 ng of reporter plasmid (ΔM-ERE-Luc, a generous gift of Dr. K. Umesono, Institute for Virus Research, Kyoto University) and 250 ng pRL-TK (to normalize for variation in transfection efficiency; contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter, Tokyo Ink), using Tfx-20 reagent (Promega) according to the manufacturer's instruction. After 1 hr of incubation, 1 ml of fresh medium containing 10% FCS in the presence or absence of various concentrations of estradiol-17β was applied to the cells. After 48 hr, luciferase activities of the cells were measured by a chemiluminescence assay using the PicaGene Dual TM SeaPansy Assay System Kit (Promega) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Isolation and characterization of tilapia ERs

Using a tER cDNA probe amplified by RT-PCR, fourteen positive clones were isolated from the tilapia ovarian cDNA library. Among these clones, only three clones (2.9, 4.3 and 5.1 kb) were amplified by primers 1 and 2. Two longer clones, named tER5.1 and tER 4.3, were selected to be sequenced (Fig. 1A). The tER5.1 insert is 5103 bp in size, and has a 1755 bp open reading frame with 5' and 3' untranslated regions of 115 bp and 3233 bp, respectively. The deduced protein is 585 amino acids (aa) with a calculated molecular weight of 64,365.8 Da. The tER4.3 insert is 4317 bp in size, and has a 1671 bp open reading frame with 5' and 3' untranslated regions of 295 bp and 2349 bp, respectively. The deduced protein is 557 aa with a calculated molecular weight of 61,758 Da.

Phylogenetic analysis (Neighbor-joining method) of ERs from various vertebrate species is shown in Fig. 2. The rainbow trout glucocorticoid receptor sequence was used as the outgroup. According to this analysis, ERs can be classified into two major groups: 1) mammalian ERαs and previously identified nonmammalian ERs (except for Japanese eel ER and tERβ), and 2) mammalian ERβs and Japanese eel ER. tER5.1 and tER4.3 belong to the ERα and ERβ groups, respectively. Thus, we consider tER5.1 and tER4.3 to be the respective tilapia homologs of ERα and ERβ. Data on sequence comparison among various vertebrate ERs support this classification (see below).

Transactivation function of tERα and tERβ

A transactivation assay was used to determine whether tERα and tERβ show an estrogen-dependent transcriptional activity. The tERα and tERβ expression vector was co-transfected with an estrogen-regulated reporter vector containing the luciferase gene with the promoter into COS-7 cells. Estrogen-dependent induction of luciferase activity was examined by incubation with various concentrations of estradiol-17β. Estradiol-17β at concentrations of 0.1–1000 mM effectively stimulated luciferase activity ($p<0.01$) with no marked differences between tERα and tERβ, although a slightly higher luciferase activity was found in COS-7 cells transfected with tERβ (Fig. 3). These results indicate that the cloned tERα and tERβ cDNAs encode functional ERs.

Sequence comparison among vertebrate ERs

Amino acid sequences of tERα and tERβ show an overall homology of 43%. Using the nomenclature of Krust *et al.* (1986), the tER sequence can be divided into five domains based on its sequence homology to other steroid hormone receptors. The two tERs share 12% homology in the A/B

A

```
tER 5.1 ( α)      1 MYPEESRGSGGVATVDFLEGTYDYAAPTPAPTPLYSHSTTGCYSAPLDAHGPLSDGSLQS  60
tER 4.3 ( β)      1 MMAAAS-SPEKLLQLQEVDSSRAGSRILSPILGSSSPGLSHETSQPICIRSPYTDLGHDF  59
                    *    *                                *       * *     *  *

tER 5.1 ( α)     61 LGSGPTSPLVFVPSSP----------RLSPFMHPPSHHYLETTSTPVYRSSHQPVPREDQ 110
tER 4.3 ( β)     60 TTIPFYSPTIFSYGGPSISECSSVHQSLSASLFWPSHGRVGTPITLHCPQGRSQQGQSAQ 119
                          **  *    *           **     ***    *  *              *

tER 5.1 ( α)    111 -------CGTRDEAYSVGELGAGAGGFEMTKDTRFCAVCSDYASGYHYGVWSCEGCKAFF 163
tER 4.3 ( β)    120 TPWDSVITTSKSVRRRSQESEESMVSSGGKADLHYCAVCHDYASGYHYGVWSCEGCKAFF 179
                              *            *   **** ********************

tER 5.1 ( α)    164 KRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGMRKDRGRVLRREKRR 223
tER 4.3 ( β)    180 KRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCYEVGMTKCGIRKERGNYRNSQARR 239
                    ********** ****************************** * * ** **       **

tER 5.1 ( α)    224 ACDRDKPAKDLPHTRASPQDGRKRAMSSSSTSGGGGRSSLNNMPPDQVLLLLQGAEPPIL 283
tER 4.3 ( β)    240 --------LTRLSSQGKTAEPKGITGPAEGSLNKPEK---PALTPEQLIERILEAEPPEI 288
                                                          * *       ****

tER 5.1 ( α)    284 SSRQKMSRPYTEVTIMTLLTSMADKELVHMITWAKKLPGFLQLSLHDQVLLLESSWLEVL 343
tER 4.3 ( β)    289 YLVKDAKRPLTEASVMMLLTNLADKELVHMISWAKKIPGFVELSLVDQVHLLECCWLEVL 348
                        ** **   * ***  ********* **** ***  *** *** ***  *****

tER 5.1 ( α)    344 MIGLIWRSIQCPGKLIFAQDLILDRNEGTCVEGMAEIFDMLLATASRFRVLKLKPEEFVC 403
tER 4.3 ( β)    349 MIGLMWRSVDHPGKLIFCPDLSLSREEGSCVQGFVEIFDMLIAATTRVRELKLQREEYVC 408
                    **** ***   ******  ** * * ** ** *  ****** *   * * ***  ** **

tER 5.1 ( α)    404 LKAIILLNSGAFSFCTGTMEPLHDSAAVQHMLDTITDALIFHISHLGCSAQQQSRRQAQL 463
tER 4.3 ( β)    409 LKAMILLNSNMCLSSSDCSEDLQSRSKLLRLLDAMTDALVLAIGKTGLTFRQQYTRLAHL 468
                    *** *****          * *         **  ****   *   *    **  * * *

tER 5.1 ( α)    464 LLLLSHIRHMSNKGMEHLYSMKCKNKVPLYDLLLEMLDAHRIHRPVKPFQSWSQGDRDSP 523
tER 4.3 ( β)    469 LMLLSHIRHVSNKGMDHLHCMKMKNIVPLYDLLLEMLDAHIMHSSCLPHQPPQQDSKDQS 528
                    * ******* ***** **  ** ** **************  *    * *   *   *

tER 5.1 ( α)    524 TASSTSSSGGGGGDDEGASSAGSSSGPQGSHESPRRENLSRAPTGPGVLQYRGSHSDCTR 583
tER 4.3 ( β)    529 EVPAPLHSSAGG-----PSNTWTPSSARAGGESQ------------------------- 557
                          *  **      *     *      **

tER 5.1 ( α)    584 IP 585
tER 4.3 ( β)    558 -- 557
```

B

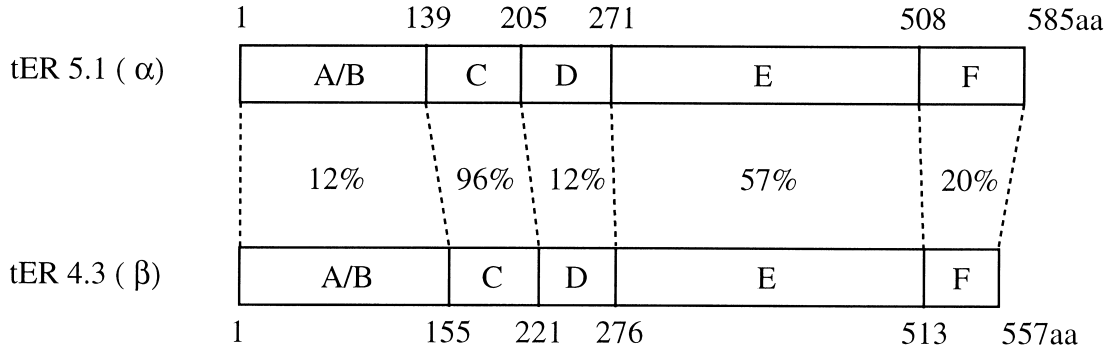

Fig. 1. Structures of tERs. (**A**) Comparison of deduced amino acid sequences of full-length cDNAs of tERα and ERβ. Identical amino acids are marked by asterisks under the sequence. Gaps are introduced into the sequence (–) to obtain maximal alignment of identical amino acids. Numbers on the side represent the position of amino acid residues in each sequence. (**B**) Comparison of the structures between tERα and tERβ. The functional A to F domains are presented schematically with the numbers of amino acid residues indicated. The percentage of amino acid identity is depicted.

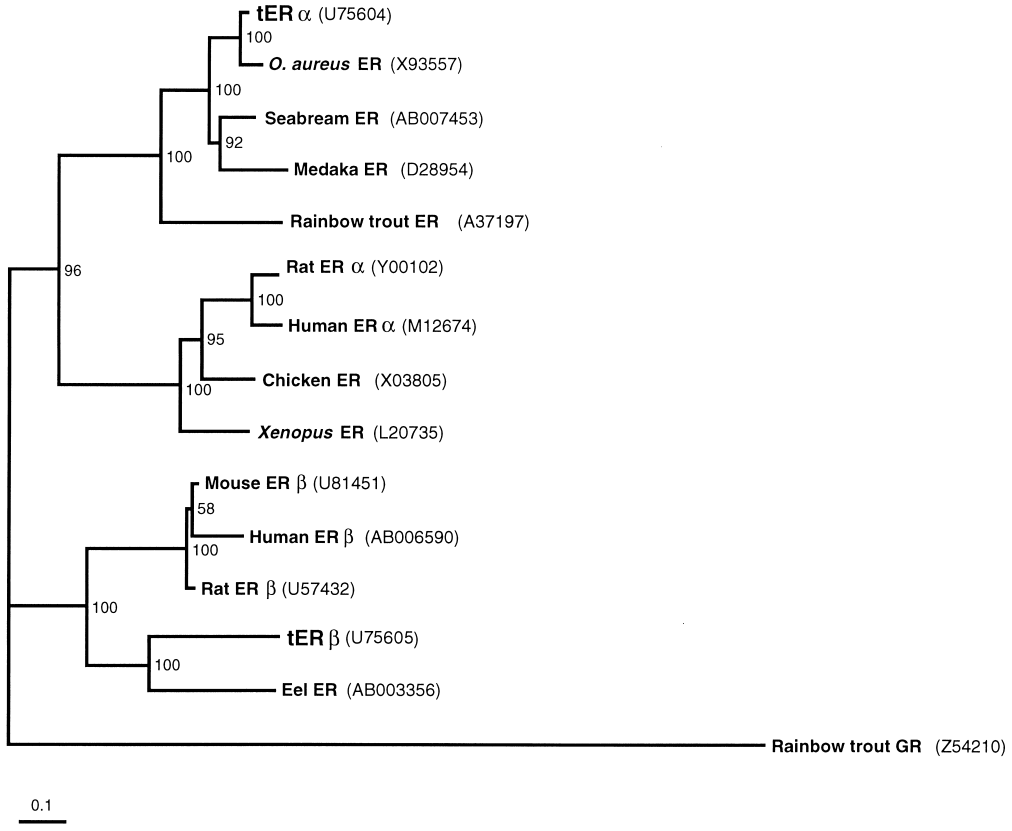

Fig. 2. A phylogenetic tree of ER proteins. The tree was constructed using the neighbor-joining (NJ) method and deduced amino acid sequences of ER from teleosts and tetrapods. The sequences were extracted from the EMBL/GenBank/DDBJ and PIR/Swiss-Prot databases. The accession number is shown with each sequence name. Lengths of horizontal lines indicate the genetic distance. One hundred bootstrap repetitions were performed and values are shown at the inner nodes. (GR, glucocorticoid receptor)

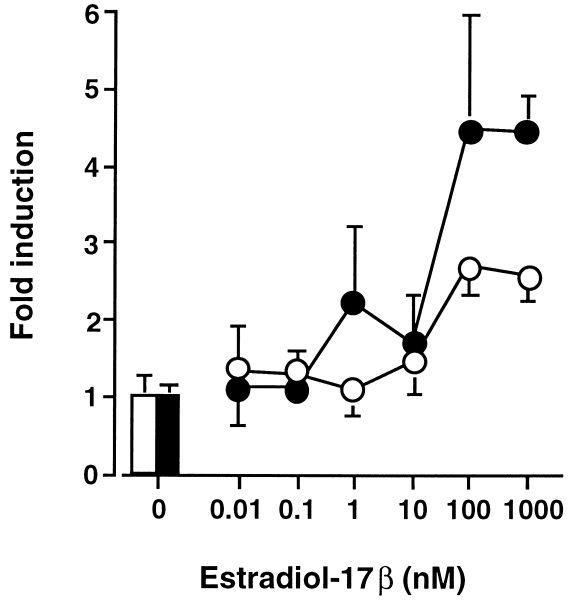

Fig. 3. Activation of tER5.1 (α) or tER4.3 (β) by estradiol-17β COS-7 cells were transiently transfected with the ERE-luciferase vector together with tER expression vectors. Cells were incubated with increasing concentations of estradiol-17β (0.01–1000 nM) or with no ligand for 48 hr. Each point represents the mean of triplicate determinations. Vertical bars represent the S.E.M.

domain, 96% in the C domain (the DNA binding domain), 12% in the D domain, 57% in the E domain (the ligand binding domain), and 20% in the F domain (Fig. 1B). Thus, domains C and E are highly conserved between tERα and tERβ, whereas A/B, D and F show greater variability.

The percent homologies of ERs from various vertebrate species relative to the tERs are as follows: tERα, tilapia, *O. aureus* (94%), rainbow trout (62%), medaka (78%), seabream (83%), Japanese eel (41%), *Xenopus* (44%), chicken (43%), mammalian ERαs (44–45%) and mammalian ERβs (45–47%); tERβ, tilapia, *O. aureus* (42%), rainbow trout (40%), medaka (43%), seabream (43%), Japanese eel (59%), *Xenopus* (43%), chicken (46%), mammalian ERαs (41–43%) and mammalian ERβs (49–52%). Most intriguing is that tER4.3 shows higher homology to mammalian ERβs than to mammalian ERαs. Furthermore, tER4.3 is most homologous to Japanese eel ER.

The DNA binding domains of tERα and tERβ are virtually identical, differing by only two amino acids. The extremely high degree of amino acid sequence identity between tERα and tERβ in the C domain suggests that tERα and tERβ bind to the same DNA elements. Furthermore, they may be capable of forming heterodimers. Our recent co-transfection experiments demonstrate the heterodimer complex formation of two

tilapia androgen receptors in the testis of the Japanese eel (Ikeuchi *et al.*, unpublished). It is possible that, *in vivo*, tERα and tERβ may cooperate in regulating estrogen-responsive gene expression in cell types where they are co-expressed.

The functional region necessary for hormone-binding, dimerization and transcription activation (E-domain) is also well conserved in the two tERs. Consistent with all of other members of the steroid nuclear receptor superfamily, a heptad repeat of hydrophobic residues is present in this region of the tERs. This region is thought to be critical for both receptor dimerization and hormone binding (Fawell *et al.*, 1990).

One structural difference between mammalian ERα and ERβ subtypes is the size of the A/B domain: the A/B domain of ERα subtypes is longer than ERβ subtypes. In contrast, the A/B domain of tERα (138 aa) is shorter than that of tERβ (154 aa). Interestingly, the Japanese eel ER (a probable β type of ER) was reported to have a relatively long A/B domain with 166 aa (Todo *et al.*, 1996). Amino acid sequences of the A/B domains of the various ERs appear to be poorly conserved. Likewise, only a 12% identity was observed in the A/B domain of tERα and tERβ. However, percent identities between tERα and other ERs (α subtypes) previously reported in teleost species were found to be high (94–50%). tERβ did not show significant homology to any ERs except for Japanese eel ER (45%). The low homology of the A/B domains between tERα and tERβ suggest that their transcriptional activation of different estrogen-responsive genes may show distinctly different patterns.

In conclusion, two functional ERs, tERα and tERβ, were identified in tilapia ovarian follicles. The identification of two distinct subtypes of ERs in tilapia ovary raises many questions, most notably relating to their respective functions. For example, one type may regulate constitutive expression of genes necessary for the female phenotype whereas the other may be responsible for differential expression during the reproductive cycle. Similarly, one may be more critical developmentally; therefore, it is important to examine the expression pattern of these two ERs in the ovarian follicles of tilapia during various stages of development and maturation. Consequently, the cloning of tERα and tERβ represents an important step forward in understanding the mechanism of estrogen-induced ovarian formation in tilapia.

While this work was under review two reports describing fish ERβ were published (catfish, Xia *et al.*, Gen. Comp. Endocrinol., 113, 360–368, 1999; goldfish, Tchoudakova *et al.*, 113, 388–400, 1999).

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