Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution

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Abstract

Estrogen is an essential hormone for many reproductive and non-reproductive functions. The function of estrogen in the reproductive cycle of seabream (*Sparus aurata*), a protandrous hermaphrodite teleost fish, is complex but it is understood to be involved in sex inversion, a process that occurs in some individuals during the second reproductive season. Estrogen action is mediated by two estrogen receptor (ER) subtypes designated alpha and beta. As a step to understanding the mechanisms of estrogen action during natural and induced sex reversal in seabream, we have isolated two cDNAs encoding distinct forms of ER homologous to mammalian ERα and ERβ. The seabream ERα clone (sbERα1), which was truncated in the A/B domain, corresponded to a variant differing in five amino acids from another recently cloned sbERα. The ERβ clone (sbERβ) encoded a protein 559 amino acids long and showed only 40% identity to sbERα. Northern blot analysis of liver and ovary mRNA indicated the presence of several transcripts of the two receptor subtypes. PCR analysis showed that the two receptors differed in their expression pattern. sbERα had a more restricted distribution, occurring mainly in testis, liver and heart, and sbERβ was present in most tissues, being more abundant in ovary, testis, liver, intestine and kidney. The presence in seabream of two ERs with several ER transcripts and their pattern of distribution are consistent with the widespread effects of estrogen in different tissues.

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Introduction

Estrogen is a steroid hormone essential in several aspects of reproduction throughout the vertebrates, and also has many non-reproductive roles better known in mammals. Estrogen action is mediated by nuclear receptors, the first estrogen receptor (ER) being cloned from human more than 10 years ago (Walter *et al.* 1985, Green *et al.* 1986*b*) and was followed by the cloning of similar receptors from rat (Koike *et al.* 1987), chicken (Krust *et al.* 1986) and *Xenopus* (Weiler *et al.* 1987). More recently, a novel cDNA encoding a different estrogen receptor, ERβ, has been cloned in rat (Kuiper *et al.* 1996), mouse (Tremblay *et al.* 1997) and human (Mosselman *et al.* 1996, Enmark *et al.* 1997, Ogawa *et al.* 1998*b*), and has raised new questions regarding the mechanism of action and physiology of the ERs.

The ER belongs to the nuclear receptor superfamily and is included in the steroid receptor subfamily (Laudet 1997). Steroid receptor proteins are divided into six functionally independent domains, termed A to F from the amino to carboxyl terminus (Krust *et al.* 1986). The N-terminal region (domain A/B) has been demonstrated to have a cell-type and promoter specific transactivation function (AF-1) (Tora *et al.* 1989, Tzukerman *et al.* 1994). The central region (domain C or DNA-binding domain, DBD) is highly conserved among species and is responsible for DNA binding (Kumar *et al.* 1987). A nuclear localization signal, homologous to that of SV40 large antigen T, was identified in domain D (Picard *et al.* 1990). In the C-terminal region, the E domain (or ligand-binding domain, LBD) is required for ligand binding (Kumar *et al.* 1987) and includes a ligand-dependent transactivation function (AF-2) (Danielian *et al.* 1992). The function of the F domain is not completely clear, but it is proposed to have a modulatory role that affects the agonist/antagonist effectiveness of antiestrogens and the transcriptional activity of the ligand–receptor complex in cells (Montano *et al.* 1995).

ERβ cDNAs encode a protein with high amino acid identity with the ERα protein, particularly in the DBD (96–97%) and LBD (53–60%) (Tremblay *et al.* 1997, Ogawa *et al.* 1998*b*). Ligand binding studies using proteins synthesized *in vitro* have indicated that most estrogenic and anti-estrogenic compounds bind both forms of ER with a similar affinity (Kuiper *et al.* 1996), but may have different mechanisms regulating transcriptional activity (Tremblay *et al.* 1997); the two different forms of ER can also

dimerize and generate a functional unit (Pace *et al.* 1997). Important differences have been found in the tissue distribution and/or the relative levels of expression of ERβ and ERα mRNA. RT-PCR analysis of various rat tissues showed moderate to high expression of ERα in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal gland, while ERβ was more abundantly expressed in prostate, ovary, lung, bladder, brain, bone, uterus and testis (Kuiper *et al.* 1997). Besides the different pattern of tissue expression within the same organ, differential expression of both forms of ER has been noted in different cell types (Byers *et al.* 1997, Osterlund *et al.* 1998, Shughrue *et al.* 1998). This differential expression suggests tissue-specific roles for each ER subtype and that different effects may be mediated by homodimers or heterodimers of the two receptors. Studies with the ER-knockout mice αERKO, βERKO and αβERKO indicate that some biological functions of estrogen require the presence of both receptors (Krege *et al.* 1998, Couse & Korach 1999, Couse *et al.* 1999).

The ER has been cloned in several teleost fish, including rainbow trout, *Oncorhynchus mykiss* (Pakdel *et al.* 1990), killifish, *Oryzias* spp. (accession number D28954), tilapia, *Oreochromis aureus* (Tan *et al.* 1995), channel catfish, *Ictalurus punctatus* (Xia *et al.* 1999), Japanese eel, *Anguilla japonica* (Todo *et al.* 1996), red seabream, *Chrysophrys major* (Touhata *et al.* 1998), gilthead seabream, *Sparus aurata* (Munoz-Cueto *et al.* 1999) and goldfish, *Carassius auratus* (Tchoudakova *et al.* 1999). All fish ERs, excluding the Japanese eel and goldfish, are more related to ERα.

The function of estrogen in the reproductive cycle of seabream, a protandrous hermaphrodite teleost fish, is complex. During the first reproductive cycle this fish develops functional testis although administration of estrogen causes testicular regression (Condeça & Canario 1999) and eventually the development of functional ovaries (Happe & Zohar 1988). As a step to understanding the mechanisms of estrogen action during natural and induced sex reversal in seabream, we have isolated two cDNAs encoding distinct forms of ER homologous to mammalian ERα and ERβ respectively and studied their tissue expression.

Materials and Methods

Production of an ER cDNA probe

Total RNA was extracted from estradiol (E_2)-stimulated liver by an adaptation of the acid guanidinum thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987). Liver total RNA (5 μg) was reverse-transcribed using Moloney murine leukaemia virus (MMLV)-RT (Gibco BRL, Barcelona, Spain) and oligo$(dT)_{12–18}$ primer (Pharmacia Biotech, Lisbon, Portugal) in a final volume of 30 μl. Two degenerate PCR primers were designed to amplify a fragment of seabream ER cDNA which spanned conserved regions in the DNA and hormone-binding domains: forward primer, 5′-TAYGGNKTKTGGTCNTGYGA-3′ (YGVWSCE) and reverse primer 5′-TGYTCCATKCCKTTRTT RCT-3′ (SNKGMEH). PCR amplification was carried out with 5 μl of synthesized cDNA using 2·5 U of Taq polymerase (Gibco, BRL) and 50 pmol of each degenerate primer. PCR cycling 94 °C, 1 min 15 s; 50 °C, 2 min; 72 °C, 50 s was repeated 35 times, followed by a final 10 min extension at 72 °C. A fragment of the predicted size (1000 bp) was purified directly from the PCR reaction using Wizard PCR Preps DNA Purification System (Promega, Biocontec, Lisbon, Portugal), cloned into pGEM-T Easy Vector (Promega) and sequenced. This product (GenBank accession number AF 013104) was highly homologous to ER and was used as a probe to screen cDNA libraries of liver, pituitary and ovary of seabream.

Construction and screening of cDNA libraries

Three cDNA libraries were constructed in UNI-ZAP XR vector (Stratagene, Biocontec, Lisbon, Portugal) with reverse-transcribed cDNA of seabream E_2-stimulated liver, pituitary and ovary obtained from 5 μg of poly$(A)^+$ RNA and using the UNI-ZAP XR cDNA synthesis kit (Stratagene) according to supplier's instructions. Screening was carried out under high stringency conditions. Duplicate membranes (Hybond-C, Amersham, Lisbon, Portugal) were hybridized with the [^{32}P]-α-dCTP-labeled PCR product overnight at 65 °C in a solution containing 6 × SSC, 5 × Denhart's, 0·1% SDS and 0·1 mg/ml transfer RNA. Stringency washes were carried out at 65 °C with 0·1 × SSC containing 0·1% SDS. Several positive clones were obtained after first round screening of 4×10^5 liver or pituitary phages. Positive clones isolated from each of these libraries were sequenced (Licor DNA4200 sequencer, MWG Biotech-UK, UK) and shown to have identical sequence where they overlapped. The largest clone (Z22) of 3·4 kb, isolated from the liver library, was used for further analysis. Screening 2×10^5 phages of the ovary library with the same probe, yielded only one positive clone (Q45) of 2·2 kb which was isolated and characterized.

In vitro *transcription and translation*

The complete Q45 cDNA inserted in the phagemid Bluescript SK(+/–) was translated *in vitro* in a rabbit reticulocyte lysate assay with 20 μCi of L-[^{35}S]methionine (Amersham). Reactions were performed using the 'TNT T3 Quick coupled Transcription/Translation System' following suppliers instructions (Promega). Translation products (5 μl) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions.

Sequence analysis

DNA sequences were analyzed using BLASTN and BLASTX (version 2·0, National Center for Biotechnology Information, Altschul *et al.* 1997) for database search, DNASIS version 5·0 for deduction of amino acid sequence of cDNA, ClustalX for multiple sequence alignment (version 1. 64b, Thompson *et al.* 1997), GeneDoc for sequence editing (Nicholas *et al.* 1997) and Phyllip (version 3.5c, Felsenstein 1989) for phylogenetic analysis. The following ER sequences were used for multiple sequence alignment and phylogenetic tree analysis: seabream clones isolated in the present study, accession numbers AF136979 (clone Z22) and AF136980 (clone Q45), seabream clone isolated by Munoz-Cueto *et al.* (1999, sbERα2), red seabream (rsER, Touhata *et al.* 1998), tilapia (tER, Tan *et al.* 1995), Nile tilapia (ntERα, accession number U75604), killifish (kER, accession number D28954), short and long forms of rainbow trout ERα (rtERαs and rtERαl, accession numbers AJ242741 and AJ242740 respectively), Japanese eel (eER, Todo *et al.* 1996), channel catfish (ccER, Xia *et al.* 1999), zebra finch (zfER, Jacobs *et al.* 1996), chicken (cER, Krust *et al.* 1986), *Xenopus* (xER, Weiler *et al.* 1987), rat ERα (rERα, Koike *et al.* 1987), sheep (sER, Madigou *et al.* 1996), human ERα (hERα, Green *et al.* 1986*b*), Nile tilapia ERβ (ntERβ, accession number U75605), goldfish ERβ (gfERβ, Tchoudakova *et al.* 1999), rat ERβ (rERβ, accession number AJ002602), Japanese quail ERβ (jqERβ, Lakaye *et al.* 1998), mouse ERβ (mERβ, Tremblay *et al.* 1997 and accession number AF067422) and human ERβ (hERβ, Ogawa *et al.* 1998*b*).

Northern blot analysis

Poly(A)$^+$ mRNA (5 μg) of adult seabream liver and ovary were separated on a 1% formaldehyde–agarose gel and transferred to Hybond-N (Amersham). The entire Q45 cDNA and a 341 bp fragment of clone Z22 obtained by PCR (see details below) were radiolabeled with [^{32}P]-α-dCTP (NEN, Zaventem, Belgium) using random priming (Redi-Prime, Amersham). Pre-hybridization was conducted for 3 h at 42 °C in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate, 0·1% SDS and 50 μg/ml calf thymus DNA. Hybridization was carried out overnight at the same temperature in an identical solution to which 2×10^6 c.p.m. per ml of denatured probe had been added. Stringency washes were carried out at 60 °C with 1 × SSC, 0·1% SDS five times during 10 min, and membranes were exposed to Biomax-MS film (Kodak, NY, USA) for an appropriate time.

RT-PCR analysis

Total RNA was extracted from ovary, testis, liver, brain, heart, bone, kidney, intestine, gills, muscle and skin of adult seabream using the Tri reagent protocol (Sigma-Aldrich Co., Madrid, Spain) and was reverse-transcribed as described above. PCR reactions were carried out using 5 μl of the synthesized cDNA. A 341 bp of clone Z22 and a 413 bp fragment of clone Q45 were amplified (35 cycles 94 °C, 1 min; 65 °C, 1 min 30 s; 72 °C, 1 min) using primers specific for each clone. Seabream homologous primers were designed to the highly variable N-terminal region of each receptor using Primer Premier software (version 4·1, Premier Biosoft International, Palo Alto, CA, USA) (Figs 1 and 2) to ensure receptor-specific PCR products. A fragment of 220 bp of the seabream β-actin was also amplified from the same volume of synthesized cDNA (35 cycles 94 °C, 1 min; 50 ° C, 1 min 30 s; 72 °C, 50 s) using the oligonucleotides 5′TTCCTCGGTATG GAGTCC3′ and 5′GGACAGGGAGGCCAGGA3′ (Santos *et al.* 1997).

Results

Cloning of two estrogen receptors

RT-PCR of sea bream liver using the degenerate primers to the DBD and LBD, amplified a 1000 bp fragment of cDNA which was isolated, cloned and sequenced. A search in the GenBank database indicated highest sequence identity to the majority of identified fish ERs and to other isolated vertebrate ERs (data not shown). This fragment was used to screen seabream cDNA libraries. Liver and pituitary cDNA library screening yielded several clones identical with the probe in the corresponding region. The complete sequence of the largest clone (Z22-isolated from the liver library), 3461 bp in length, was determined. Clone Z22 contained one ATG codon at nucleotide 92, but since it lacked an upstream in-frame stop codon and had a shorter A/B domain it was presumed truncated at the 5′ end. The 3′-untranslated region (UTR), including a poly(A)$^+$ tail, was 1826 bp long. The nucleotide sequence and deduced amino acid sequence of clone Z22 is shown in Fig. 1.

Ovary library screening yielded one positive clone (Q45), which was 2183 bp in length and had a different sequence from the probe (data not shown). Its nucleotide sequence is given in Fig. 2. Clone Q45 contained an in-frame ATG codon at nucleotide 286, preceded by an in-frame stop codon at nucleotide 232, suggesting it to be the likely start codon. A second downstream potential ATG start codon was located at nucleotide 384. Q45 contained a 285 bp 5′-UTR, followed by an open reading frame (ORF) with 1679 bp and a 216 bp 3′-UTR including a poly(A)$^+$ tail. The encoded protein was deduced to be 559 amino acids long and this was confirmed by *in vitro* translation of clone Q45 using the rabbit reticulocyte lysate assay. Analysis of the translation products on SDS-PAGE, 12% acrylamide gel revealed a protein doublet migrating

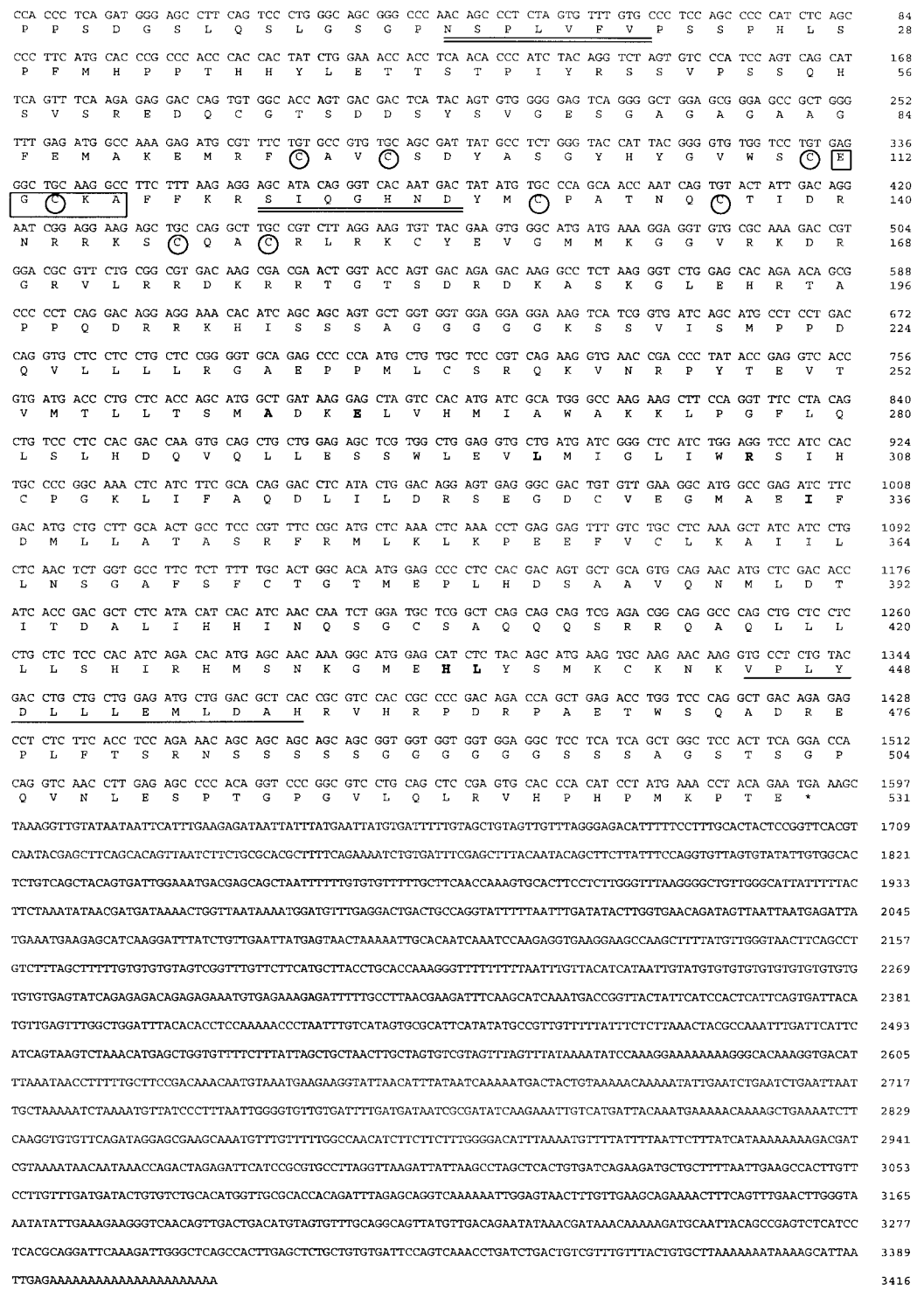


Figure 1 Nucleotide and deduced amino acid sequence of clone Z22 isolated from a seabream liver cDNA library (GenBank accession number AF136979). The eight cysteines of the DBD are circled and the residues corresponding to the D- and P-box are inside a rectangle. In the the LBD, the region corresponding to AF2 is underlined and the amino acids recognized to be involved in E_2 binding are in bold. Sequences of specific primers used for RT-PCR are double-underlined.

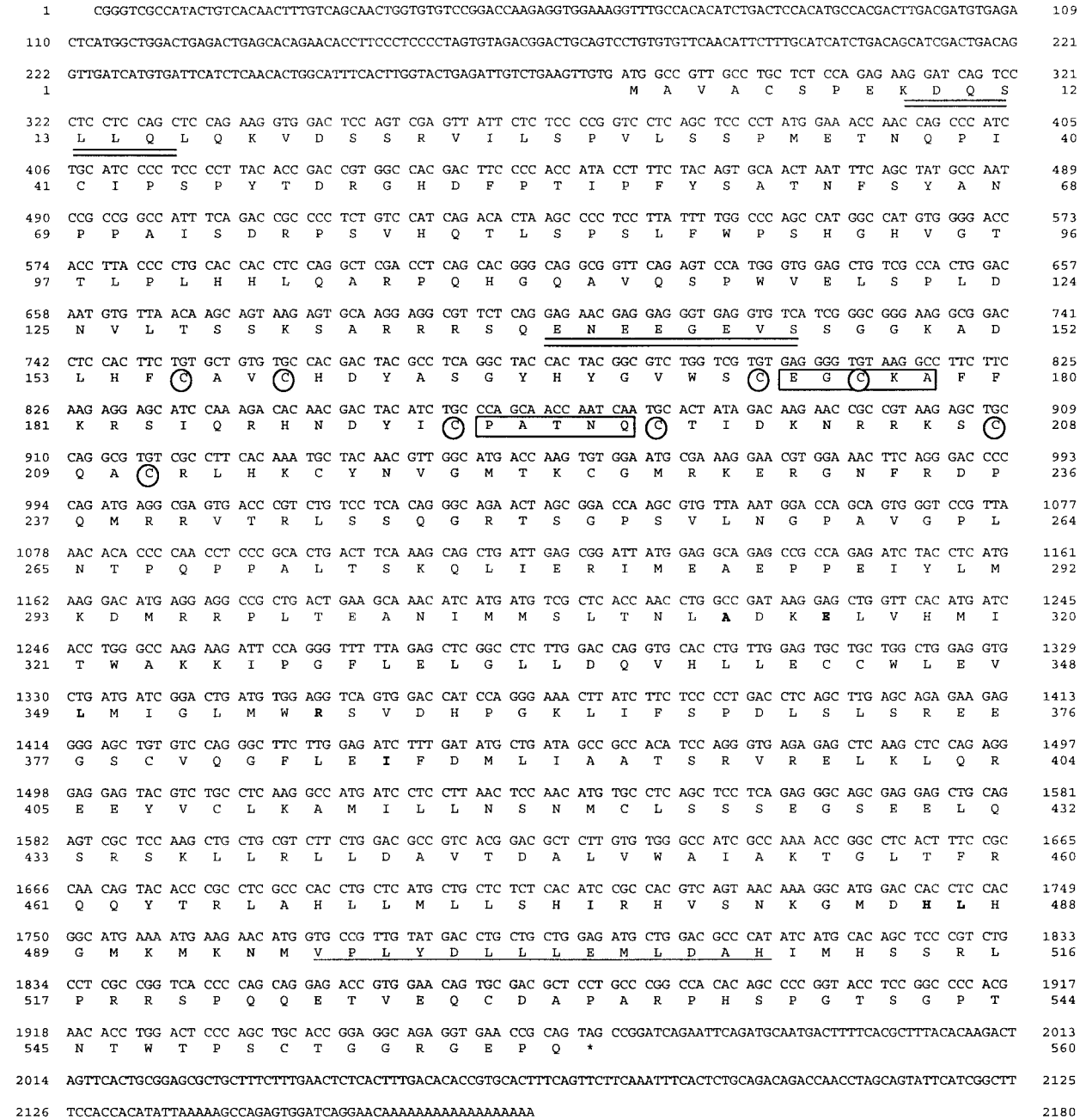


Figure 2 Nucleotide and deduced amino acid sequence of clone Q45 isolated from seabream ovary cDNA library (GenBank accession number AF136980). The eight cysteines of the DBD are circled and the residues corresponding to the D- and P-box are inside a rectangle. In the LBD, the region corresponding to AF2 is underlined and the amino acids recognized to be involved in E_2 binding are in bold. Sequence of specific primers used for RT-PCR are double-underlined.

close to the 61 kDa band of the luciferase positive control (not shown), thus confirming the predicted ORF. The existence of two translation products suggests that the two ATG start codons at nucleotides 286 and 384 of clone Q45 were being used.

Sequence analysis

Multisequence analysis of clones Q45 and Z22 with those of other fish and tetrapod ERs allowed identification of conserved features: the eight cysteine residues in the two zinc finger motifs common to all nuclear receptors

Table 1 Comparison of clone Z22 and clone Q45 proteins with other species' ERs (see Materials and Methods section for sequence references and abbreviations). Overall and domain percentages of amino acid identities are indicated but, since clone Z22 was truncated in the A/B domain, amino acids corresponding to the truncated region were excluded from the analysis. The total number of amino acids or the number of residues per domain are indicated in brackets

Clone	Z22 (sbERα1)						Q45 (sbERβ)					
Species/Domain	Overall	A/B	C	D	E	F	Overall	A/B	C	D	E	F
sbERα2	99 (579)	94 (137)	100 (81)	100 (43)	100 (251)	100 (67)	36	10	75	9	54	10
rsER	93 (581)	94 (139)	98 (81)	97 (43)	97 (251)	70 (67)	36	10	76	11	54	8
ntERα	79 (585)	77 (133)	95 (81)	53 (43)	91 (251)	44 (77)	37	8	78	6	56	6
tER	77 (583)	76 (133)	95 (81)	40 (42)	89 (250)	41 (77)	36	8	77	9	55	6
KER	77 (620)	76 (180)	96 (81)	50 (44)	90 (251)	31 (64)	36	7	75	11	54	11
rtERαl	70 (622)	55 (187)	90 (82)	57 (45)	85 (251)	22 (57)	36	8	71	6	54	4
rtERαs	70 (577)	55 (142)	90 (82)	57 (45)	85 (251)	22 (67)	36	7	71	6	54	4
ccER	61 (581)	37 (145)	91 (82)	17 (43)	79 (251)	10 (60)	36	8	74	4	52	7
hERα	48 (595)	20 (179)	90 (83)	13 (39)	62 (251)	7 (43)	37	9	74	10	56	5
sER	48 (596)	23 (180)	90 (83)	11 (39)	62 (251)	7 (43)	38	9	74	10	56	0
rERα	48 (600)	25 (184)	90 (83)	8 (39)	61 (251)	4 (43)	37	9	74	7	56	5
cER	48 (589)	22 (173)	90 (83)	8 (39)	63 (251)	10 (43)	38	12	74	5	56	7
zfER	48 (587)	22 (171)	90 (83)	13 (39)	63 (251)	11 (43)	39	12	74	10	56	5
xER	48 (586)	23 (174)	91 (83)	8 (36)	61 (251)	8 (42)	38	14	74	5	55	2
mERβ	42 (549)	16 (162)	83 (83)	8 (29)	57 (247)	5 (28)	50	13	79	8	63	6
rERβ	42 (549)	16 (162)	81 (83)	8 (29)	57 (247)	2 (28)	50	12	78	8	64	4
hERβ	42 (530)	16 (143)	83 (83)	9 (29)	57 (247)	7 (28)	47	19	79	8	63	6
jqERβ	42 (472)	15 (99)	84 (83)	11 (26)	57 (246)	2 (18)	46	16	79	7	63	8
ntERβ	42 (557)	16 (149)	77 (89)	9 (25)	56 (249)	13 (45)	77	62	89	60	89	47
gfERβ	41 (568)	15 (164)	78 (91)	8 (28)	55 (249)	8 (36)	64	41	76	17	83	19
eER	41 (573)	20 (165)	78 (89)	11 (28)	55 (249)	8 (42)	58	38	82	10	77	10
Clone Q45 (sbERβ)	41 (559)	14 (50)	75 (89)	9 (25)	54 (249)	10 (46)						

(Schwabe *et al*. 1990); the D-box (EGCKA) and P-box (PATNQ), which have been recognized to be involved in binding to estrogen response elements (ERE) sequences (Koike *et al*. 1987); the ligand-dependent transactivation function (AF2) localized in the LBD (Danielian *et al*. 1992) is completely conserved in both clones (Figs 1 and 2); in addition, amino acids in the LBD shown in hERα to be involved in E_2 binding (Brzozowski *et al*. 1997) are also conserved in sea bream ERs (Figs 1 and 2). All receptor sequences (alpha and beta) shared 60 identical amino acids in the DBD and 86 in the LBD, in no other domain did this occur.

Comparison of amino acid sequence identities between the various ERs (Table 1) showed that clone Z22 was most similar to a recently cloned seabream ER (99%, Munoz-Cueto *et al*. 1999) and to most fish ERs (61–93%), and less to eERα, gfERβ, ntERβ and clone Q45 (41–42%). Identity to tetrapod ER was 48% and to tetrapod ERβ 42%. In contrast, clone Q45 showed 58–77% amino acid sequence identity to eER, gfERβ and ntERβ, and only 36–37% to other fish ERs. Identity to tetrapod ERβ was 47–50% and to tetrapod ERα 37–39%. In both sbERs domain C, followed by domain E, shared the highest amino acid sequence identity with other ERs (see Table 1), and sequence conservation was much lower and diminished sequentially from domains A/B, D and F.

Phylogeny analysis to determine the relationship between the various estrogen receptors was carried out using either the deduced whole receptor protein sequences or the various domains separately. A consensus tree with corresponding bootstrap values (from sampling 1000 trees) obtained by parsimony analysis (PAR) for the whole receptor sequence is shown in Fig. 3. This analysis produced four major groups consisting of fish and tetrapod receptor subtypes. With Neighbor Joining analysis (NJ) similar groupings were produced. A clear separation into four clades with maximum bootstrap percentages was obtained for the E domain with both PAR and NJ analysis. Analysis of the C domain originated three clades with PAR (placing tetrapod ERβ and clone Q45, eER and ntERβ in the same group) and two clades with NJ (placing clone Q45, eER and ntERβ with tetrapod ERα and tetrapod ERβ with the remaining fish ERs). Analysis of D domain yielded inconsistent results: PAR yielded similar clustering to that of the C domain but NJ yielded no clear separations. No significant clustering was obtained for the A/B and F domains with PAR or NJ.

Sequence identities and phylogeny analysis indicate that the two clones are closely related to identified fish ERs, and that clone Z22 is related to tetrapod ERα and clone Q45 is related to tetrapod ERβ. However, considering

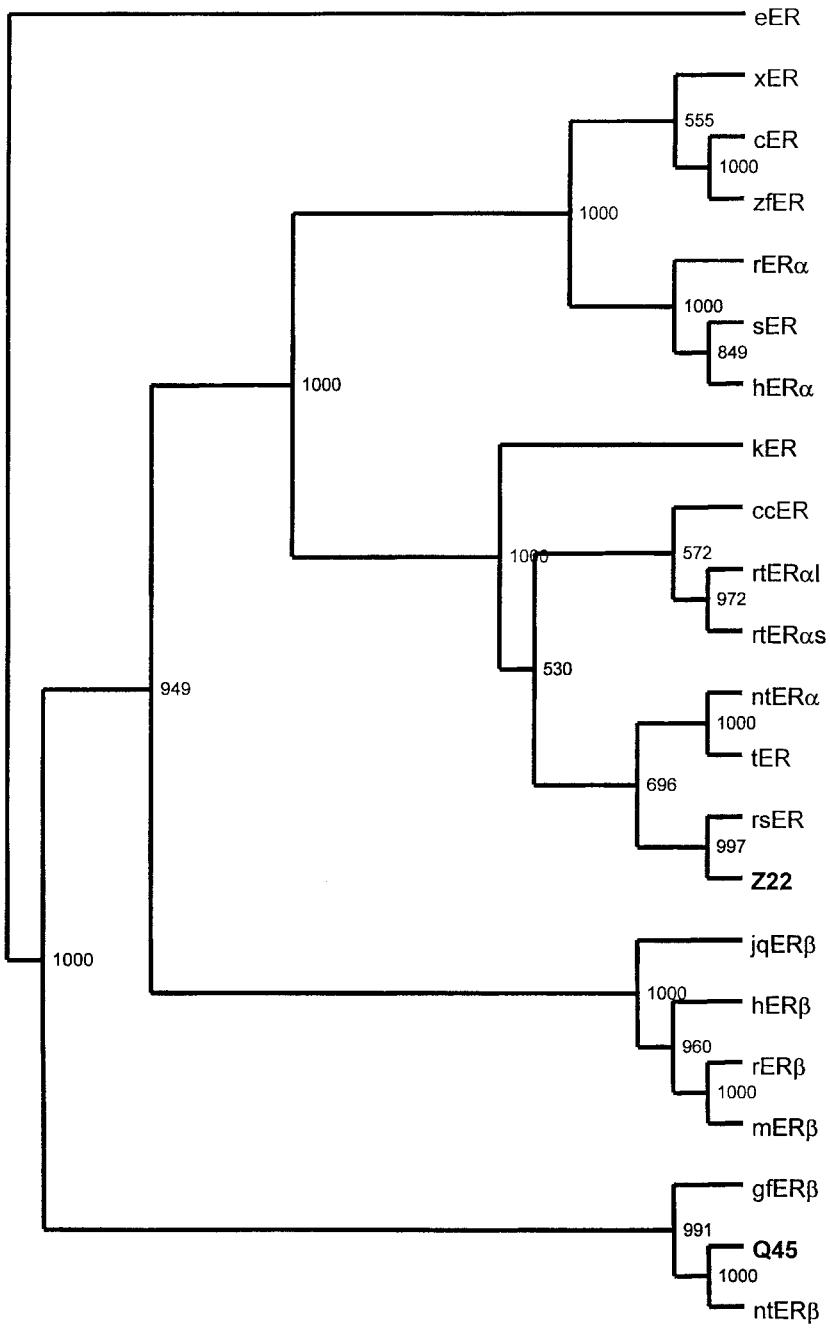


Figure 3 Phylogenetic unrooted tree – the most parsimonious consensus tree of estrogen receptors. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 1000 trees.

the generally low sequence identities between the fish and tetrapod estrogen receptors and the wide branching pattern of the phylogenetic trees, a more detailed analysis of amino acid conservation between the various receptor sequences was carried out. On the basis of the results of this analysis and those of the phylogenetic study clone Z22 and the related fish ERs have been assigned to a group denominated fish ERα and clone Q45 and related fish ERs to a group designated fish ERβ and will be referred to as sbERα and sbERβ respectively.

Table 2 Number of strictly conserved amino acids in ER within and between groups created on the basis of phylogenetic analysis. The average number of amino acids within each group are given in parentheses

	ERα Tetrapod	ERβ Tetrapod	ERα Fish	ERβ Fish
ERα Tetrapod (595)	66			
ERβ Tetrapod (549)	0	55		
ERα Fish (580)	18	2	28	
ERβ Fish (560)	6	17	0	29

The amino acid conservation contrasts (i.e. amino acids that are uniquely conserved within a group and do not appear at that position in any sequence outside that group (Nicholas *et al.* 1997)) for the two types of fish receptors and the tetrapod ERα and ERβ are shown in Table 2. The number of amino acids of ERα and ERβ that are exclusively conserved in the tetrapods is proportional to the size of the ERs, suggesting similar evolutionary rates for the two receptor types. The levels of conservation contrasts found for the two types of receptors in teleosts are half those of the tetrapods and may reflect faster evolutionary rates. Conservation contrast between α and β types of ER in fish and tetrapods is very low or absent (0–6 amino acids), but is high between tetrapod ERα and fish ERα (18 amino acids) and between tetrapod ERβ and fish ERβ (17 amino acids). The analysis of conservation contrasts unequivocally showed that fish ERs are related to tetrapod α and β and proved to be much more sensitive than a simple comparison of sequence identities to relate fish ERs to existing types in tetrapods. Uniquely conserved amino acids for each of the tetrapod and fish ER types are found mainly in the A/B (32–48%) and E (36–67%) domains. Uniquely conserved amino acids within the ERα and in ERβ groups are found mainly in the E domain (61 and 65% respectively).

Motif analysis of all the tetrapod and fish sequences using the Prosite database did not show a specific pattern for any of the fish receptor types. In addition to the general ER features described above, a highly conserved amino acid sequence RRKS, corresponding to a potential cAMP- and cGMP-dependent protein kinase phosphorylation site, is found in the C domain. Two highly conserved N-myristoylation sites are also present in the C domain and have amino acid sequences GVWSCE and GM(M,V,T)K(C,G)G. In the E domain, a totally conserved amino acid sequence SNK, potential protein kinase C (PKC) phosphorylation site is present.

There were also some apparent ER type specific motifs. In the A/B domain of ERα a mitogen-activated protein kinase phosphorylation site with the consensus motif P-$X_{(1,2)}$-SP is found which is not apparent in ERβ (Fig. 4). However, in tetrapod ERβ potential mitogen-activated protein kinase (MAPK) phosphorylation sites are located downstream of the corresponding region in ERα, while in fish ERβ potential MAPK sites are located upstream, except for eERβ which has two sites and sbERβ which apparently lacks a MAPK phosphorylation site. Finally, in both fish and tetrapod ERα LBD, a completely conserved tyrosine kinase phosphorylation site (KGMEHLY) is present.

Transcripts size of sbERα and sbERβ

Northern blot analysis was performed to characterize the sbER mRNAs. After hybridization with a 314 bp PCR fragment encompassing the major part of the N-terminal region of sbERα two mRNA transcripts of approximately 6 and 4·5 kb were identified in liver and a single transcript of 4·5 kb was detected in ovary. Four prominent ovary mRNA transcripts of approximately 6, 2·6, 0·5 and 0·3 kb hybridized with the full-length sbERβ cDNA, while in liver only the 0·3 kb transcript was detected (Fig. 5).

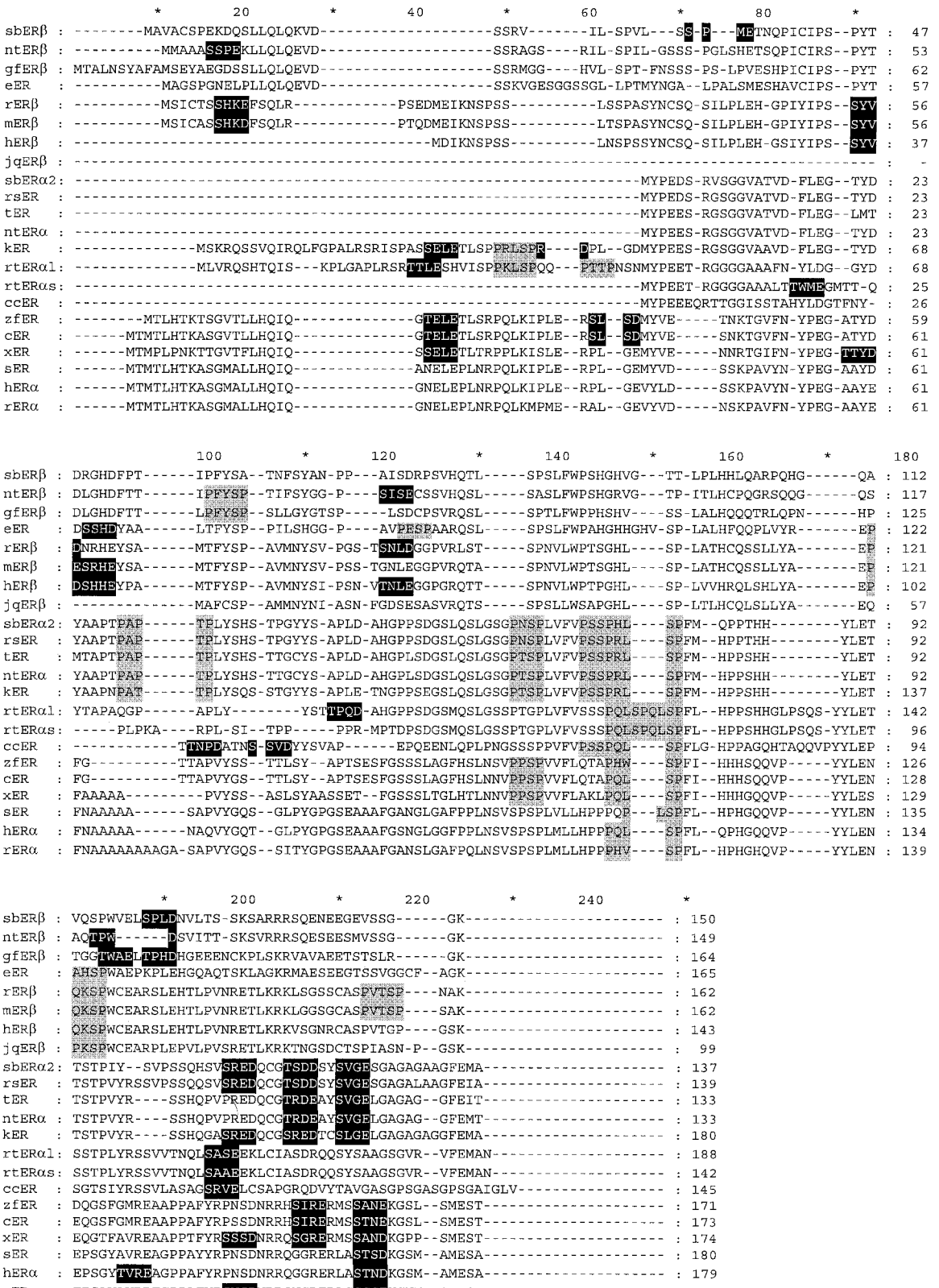
Tissue distribution of sbERα and sbERβ

In order to examine the distribution of sbERα and sbERβ mRNA, the sensitive method of RT-PCR analysis was performed with ERα- and β-specific primers. The identity of the amplified PCR products was confirmed by cloning and subsequent sequencing. By performing RT-PCR on the same samples with β-actin primers and using this to normalize the results with primers for sbERα and β it was possible to obtain semi-quantitative results which demonstrated important differences in the level of expression and tissue distribution of both receptors (Fig. 6). sbERβ was expressed in all tissues analyzed, except gills; high levels of expression were detected in ovary and testis and also in kidney, intestine and liver. In other tissue samples expression was much lower, although heart had a slightly stronger signal. SbERα was only detected in testis, liver and heart with similar levels of expression.

Discussion

Two clones were isolated from seabream cDNA libraries and both showed high homology to known estrogen receptor sequences. Clone Z22, despite being the largest of several clones obtained from the pituitary and liver cDNA libraries, was assumed truncated so that the 5′UTR and part of the A/B domain was missing. The deduced

Figure 4 Multiple alignment of domain A/B of estrogen receptor. Potential MAPK phosphorylation sites are shaded in light gray and casein kinase II phosphorylation sites in black (see Materials and Methods for sequence references and abbreviations).



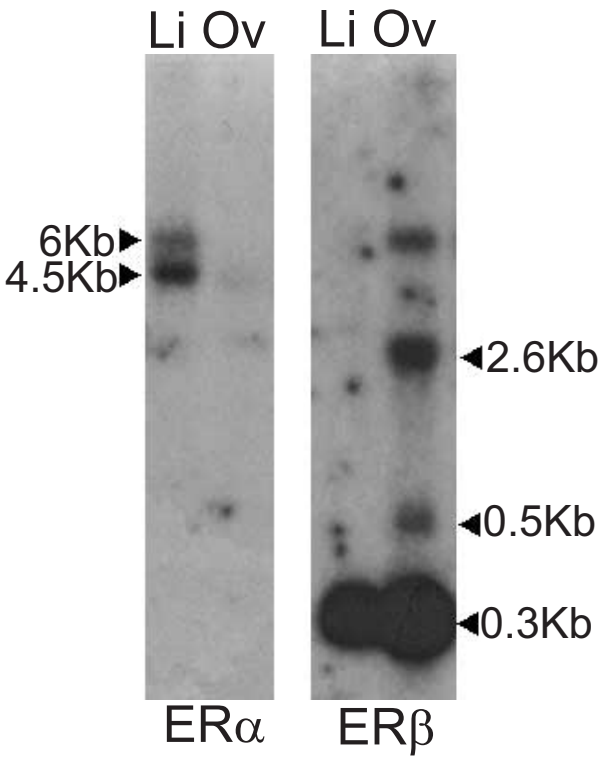


Figure 5 Northern blot analysis of seabream ERα and ERβ. Liver (Li) and ovary (Ov) poly(A)$^+$ mRNA (5 µg) were probed with a 341 bp cDNA fragment encoding sbERα and full-length sbERβ.

amino acid sequence differed by only five amino acids from a recently published sbER sequence, below designated sbERα2 (Munoz-Cueto *et al.* 1999). Sequence identities (see Table 1) were also highest with other teleost ERs and ERα from tetrapod species. Lowest identities were found with tetrapod ERβ and the teleost eER, ntERβ, gfERβ and clone Q45.

Clone Q45, obtained from the ovarian cDNA library, encodes a protein of 526 or 559 amino acids depending on which of two potential start codons are used. That either of the two start codons can be used was confirmed by the production of two proteins *in vitro* with rabbit

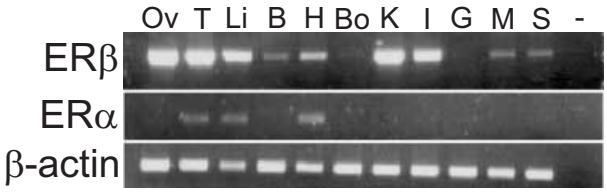


Figure 6 Tissue distribution of ERα and ERβ analyzed by RT-PCR. β-Actin was used as a control. Ov, ovary; T, testis; Li, liver; B, brain; H, heart; Bo, bone; K, kidney; I, intestine; G, gills; M, muscle; S, skin; -, mRNA not reversed transcribed.

reticulocytes. In contrast to clone Z22, Q45 shared more identical amino acids with eER, ntERβ, gfERβ and tetrapod ERβ and less with tetrapod ERα or with the group of teleost ERs most like Z22.

Phylogenetic analysis of the ER receptors groups them into four clusters each consisting of fish or tetrapod receptor subtypes (Fig. 3). The teleost clade consists of eER, ntERβ, gfERβ and Q45 appear to be more related to tetrapod ERβ, while the other fish ERs, including Z22, appear to be more related to tetrapod ERα. This pattern of relatedness was also obtained from the analysis of the more conserved C and E domains. Further confirmation of the degree of relatedness between fish and tetrapod ERs was obtained by analysis of amino acid conservation contrasts (Table 2) among the four major clades identified by phylogenetic analysis. On the basis of these results it was concluded that clone Q45 and eER, ntERβ, gfERβ were β subtype ERs (designated sbERβ) and clone Z22 (designated sbERα1) and the remaining fish ERs were of the α subtype.

The size of the deduced ER protein obtained from the various fish and tetrapod cDNA sequences is variable (Table 1). In tetrapod ERα it varies from 586 (*Xenopus*) to 600 (rat) and in fish ERα from 574 (rainbow trout short form) to 622 (rainbow trout long form). Tetrapod ERβ varies from 549 (mouse, rat) to 589 (zebra finch) and fish ERβ from 557 (Nile tilapia) to 573 (Japanese eel). ERβ is generally shorter than ERα (Table 1, see also Tchoudakova *et al.* 1999) although longer forms have recently been identified in mammals (Leygue *et al.* 1998). Some of the longer forms are derived from extra coding sequence at the 5′ region which is proposed to result from a single base change in transcripts upstream of the start codon causing a frame shift (Leygue *et al.* 1998). The length of the A/B domain is most variable (Table 1, Fig. 4), with 133–187 amino acids in fish ERα, 171–184 in tetrapod ERα, 149–165 in fish ERβ and 143–162 in tetrapod ERβ (excluding the partial clones of jqERβ and sbERα1). Clearly the largest differences are found in teleost ERα and this may be explained by the recent identification of short and long forms of ERα in the rainbow trout (F Pakdel, R Metivier, G Flouriot & Y Valotaire, unpublished observations) which differ by up to 53 amino acids in the A/B domain. The cDNA for sbERα1 differs from sbERα2 (Munoz-Cueto *et al.* 1999) by five amino acids in the A/B domain (Fig. 4). sbERα2 has Gln^{83} (equivalent to Gln^{122} in hERα) instead of His (present in all other ERs), $Ala\text{-}Asn^{85}$ instead of Pro-Thr and lacks Arg-Ser after Tyr^{98}, indicating that multiple variants, differing in the A/B domain, of ERα also occur in seabream. Two variants of ERα have also been identified in catfish (Xia *et al.* 1999). A number of ERα and β variants have also been identified in other species, and in fish (e.g. Chu & Fuller 1997, Murphy *et al.* 1997, Flouriot *et al.* 1998, Lu *et al.* 1998, Maruyama *et al.* 1998, Leygue *et al.* 1999), up to three variants of gfERβ may

exist (Tchoudakova *et al.* 1999) and four variants of tER (Tan *et al.* 1996).

The length of the C and E domains of all ERs has been highly conserved (ERα 81–83 and 250–251 amino acids; ERβ 83–91 and 246–249 amino acids). However, despite small variations, tetrapod ERβ has the shortest D domain, 25–29 amino acids compared with 31–33 for fish ERβ, 36–39 in tetrapod ERα and 42–45 amino acids in fish ERα. The largest F domains are found in the α receptor subtype (57–77 for fish ERα, 42–43 for tetrapod ERα, 36–45 for fish ERβ and 18–28 for tetrapod ERβ) and there appears to be a trend for larger F domains in teleosts, particularly in the more advanced teleosts (Table 1). The significance of these differences is not clear but it has been suggested that this domain may be important in determining the final conformation of the receptor–ligand complex, thus affecting the potential for interaction with cofactors or transcription factors in a particular cell context (Montano *et al.* 1995). It appears that evolutionary differences of receptor function are largely reflected in the F domain.

General ER features revealed by motif analysis included the nuclear receptor DNA-binding region signature with the eight cysteines constitutive of the zinc-finger motifs and the D- and P-box sequences which have been recognized to be necessary for DNA binding (Koike *et al.* 1987, Schwabe *et al.* 1990). Also completely conserved among all receptors are amino acids in domain E of the ligand-dependent transactivation function (Danielian *et al.* 1992), as well as amino acids known to be involved in E_2-binding (Brzozowski *et al.* 1997). In domain C the complete conservation of two N-myristoylation sites overlapping with the binding region signature potentially allows covalent addition of the C14-saturated fatty acid myristate to their N-terminal glycine residue, which must be an important feature in DNA binding. Although in the E domain of all ER isolated there is a conserved potential PKC phosphorylation site, available evidence suggests that only PKC δ isoform (not PKC α or ε) in the AF1 region participate in the signaling pathways that lead to estrogen receptor phosphorylation (Lahooti *et al.* 1998).

In hERα, five phosphorylation sites have been mapped, four of which are in the A/B domain (Ser^{104}, Ser^{106}, Ser^{118} and Ser^{167}). Ser^{118} and Ser^{167} are the major estrogen-inducible phosphorylation sites (Ali *et al.* 1993, Arnold *et al.* 1994, Le Goff *et al.* 1994). The first can be phosphorylated *in vitro* by MAPK (Arnold *et al.* 1995*b*, Kato *et al.* 1995) and the second by casein kinase II (Arnold *et al.* 1995*a*). In mERα the corresponding Sers identified in hERα are phosphorylated and two additional sites, Ser^{156} and Ser^{158}, have been identified which are phosphorylated by casein kinase II (Lahooti *et al.* 1995). A conserved MAPK phosphorylation site consensus sequence is found in all ERα, but not in ERβ (Fig. 4). However, the serine residue in mouse ERβ located in the corresponding ERα consensus MAPK phosphorylation site can also be phosphorylated by MAPK (Tremblay *et al.* 1997). In sbERβ, ntERβ and gfERβ the sequences corresponding to hERα Ser^{118}-Pro^{119} are, respectively, Thr^{96}-Thr^{97}, Thr^{101}-Pro^{102} and Ser^{109}-Ser^{110} (Figure 4). Since the replacement of Ser by Thr potentially allows phosphorylation in this position it would be of interest to know whether the mitogen-activated phosphorylation pathway is used by fish ERβ and other tetrapod ERβ, or whether a ligand-independent transactivation function is absent or, if present, is activated by another mechanism.

The presence of several transcripts for both sbERα and sbERβ were demonstrated by Northern blot (Fig. 5), just as found in many other fish and mammalian species (Weiler *et al.* 1987, Lazennec *et al.* 1995, Mosselman *et al.* 1996, Todo *et al.* 1996, Tremblay *et al.* 1997, Tchoudakova *et al.* 1999). Two mRNA transcripts of sbERα, 6 and 4·5 kb in length, were detected in liver. In the ovary only the 4·5 kb transcript was detected. The 4·5 kb mRNA should correspond to the complete sequence of the sbERα1 clone isolated in the present study while the bigger transcript probably correspond to a mRNA with a longer 3′-UTR. The transcript isolated by Munoz-Cueto *et al.* (1999) also from liver was a smaller 3 kb transcript differing in the length of 3′-UTR and is shorter by two amino acids in the A/B domain. It will be of interest to determine if the difference in length of the 3′-UTR detected between the two ERα forms in the seabream is a consequence of alternative splicing. In the case of sbERβ, at least four mRNAs (6, 2·6, 0·5 and 0·3 kb) were detected in seabream ovary after hybridization with the full-length ERβ cDNA. In liver only the 0·3 kb transcript was detected. Only the two larger transcripts can potentially generate the entire coding sequence of sbERβ. The significance of the smaller transcripts in seabream is uncertain and small transcripts have also been reported in eel (Todo *et al.* 1996), mouse (Tremblay *et al.* 1997) and human (Mosselman *et al.* 1996). Additional hybridization studies using partial probes will be necessary to characterize the nature of each mRNA.

Tissue distribution of sbERα and sbERβ (Fig. 6) was different and sbERβ was widespread and had a generally higher level of expression than sbERα. The highest expression of sbERβ was detected in ovary and testis, moderate expression was observed in kidney, intestine and liver and lower expression in brain, heart, muscle and skin. Gill was the only tissue in which no signal for sbERβ could be detected. ERβ and ERα were co-expressed in testis, liver and heart. No signal of ERα was visible in any other tissues analyzed, but its presence cannot be excluded. In goldfish ERβ expression has been reported to be restricted largely to the liver, brain, ovary and testis (Tchoudakova *et al.* 1999).

Unfortunately, data on tissue distribution of fish ER are scarce and essentially restricted to liver and central nervous system of salmonids (Anglade *et al.* 1994). Both sbERs are expressed in seabream liver, although the clones isolated from liver cDNA library were of the alpha subtype.

Whether vitellogenesis in fish is mediated by heterodimerization of the two ER subtypes, as shown for human ERs (Pettersson *et al.* 1997, Ogawa *et al.* 1998*a,b*), requires investigation. Another action demonstrated for estrogens in teleost fish is the positive and negative feedback on the brain–pituitary complex (Saligaut *et al.* 1998) and in this context the distribution of ER has been characterized (Anglade *et al.* 1994, Linard *et al.* 1996). RT-PCR analysis of seabream brain only detected ERβ but further studies will be required to completely exclude the possibility that ERα is also present.

It is notable that in sea bream ERβ was clearly expressed in ovary and testis while ERα was most abundant in testis. This pattern of expression may indicate, in this species, a different function for each form of sbER in male and female reproductive physiology. Recent data on αβERKO mice clearly show that only ERα is required for normal testicular function. However, the presence of both ER subtypes is required for maintenance of germ and somatic cells in the postnatal ovary and their absence causes the appearance of sex reversal features including structures resembling seminiferous tubules, degeneration of granulosa cells and appearance of Sertoli-like cells (Couse *et al.* 1999). Whether this model can be applied to seabream reproductive physiology and natural sex reversal requires elucidation.

The significance in sea bream of the expression of ERβ in heart, bone, kidney and intestine, all known targets for estrogen action in mammals (Kuiper *et al.* 1997, Onoe *et al.* 1997), is unclear since little information exists about the effects of estrogen on these tissues in teleosts.

In conclusion, the pattern of distribution of ERα and ERβ in seabream parallels to a great extent what has been observed in mammals and, in common with mammals, seabream also produces a range of receptor transcripts in a tissue-specific manner, consistent with the reported widespread effects of estrogen in different tissues and developmental stages.

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References

Ali S, Metzger D, Bornert JM & Chambon P 1993 Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. *EMBO Journal* **12** 1153–1160.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25** 3389–3402.

Anglade I, Pakdel F, Bailhache T, Petit F, Salbert G, Jego P, Valotaire Y & Kah O 1994 Distribution of estrogen receptor-immunoreactive cells in the brain of the rainbow trout (*Oncorhynchus mykiss*). *Journal of Neuroendocrinology* **6** 573–583.

Arnold SF, Obourn JD, Jaffe H & Notides AC 1994 Serine 167 is the major estradiol-induced phosphorylation site on the human estrogen receptor. *Molecular Endocrinology* **8** 1208–1214.

Arnold SF, Obourn JD, Jaffe H & Notides AC 1995*a* Phosphorylation of the human estrogen receptor by mitogen-activated protein kinase and casein kinase II: consequence of DNA binding. *Journal of Steroid Biochemistry and Molecular Biology* **55** 163–172.

Arnold SF, Obourn JD, Yudt MR, Carter TH & Notides AC 1995*b* *in vivo* and *in vitro* phosphorylation of the human estrogen receptor. *Journal of Steroid Biochemistry and Molecular Biology* **52** 159–171.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA & Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389** 753–758.

Byers M, Kuiper GG, Gustafsson JA & Park-Sarge OK 1997 Estrogen receptor β mRNA expression in rat ovary: down-regulation by gonadotropins. *Molecular Endocrinology* **11** 172–182.

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.

Chu S & Fuller PJ 1997 Identification of a splice variant of the rat estrogen receptor β gene. *Molecular and Cellular Endocrinology* **132** 195–199.

Condeça JAB & Canario AVM 1999 The effect of estrogen on the gonads and on *in vitro* conversion of androstenedione to testosterone, 11-ketotestosterone and estradiol-17β in *Sparus aurata* (Teleostei, Sparidae). *General and Comparative Endocrinology* **116** 59–72.

Couse JF & Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews* **20** 358–417.

Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ & Korach KS 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. *Science* **286** 2328–2331.

Danielian PS, White R, Lees JA & Parker MG 1992 Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. (Published erratum appears in *EMBO Journal* 1992 **11** 2366.) *EMBO Journal* **11** 1025–1033.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M & Gustafsson JA 1997 Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **82** 4258–4265.

Felsenstein J 1989 PHYLIP – Phylogeny Inference Package (Version 3·2). *Cladistics* **5** 164–166.

Flouriot G, Griffin C, Kenealy M, Sonntag-Buck V & Gannon F 1998 Differentially expressed messenger RNA isoforms of the human estrogen receptor-αgene are generated by alternative splicing and promoter usage. *Molecular Endocrinology* **12** 1939–1954.

Green S, Kumar V, Krust A, Walter P & Chambon P 1986a Structural and functional domains of the estrogen receptor. *Cold Spring Harbor Symposia on Quantitative Biology* **51** 751–758.

Green S, Walter P, Greene G, Krust A, Goffin C, Jensen E, Scrace G, Waterfield M & Chambon P 1986b Cloning of the human oestrogen receptor cDNA. *Journal of Steroid Biochemistry* **24** 77–83.

Happe A & Zohar Y 1988 Self-fertilization in the protandrous hermaphrodite *Sparus aurata*: development of the technology. In *Reproduction in Fish – Basic and Applied Aspects in Endocrinology and Genetics*, pp 177–180. Eds Y Zohar & B Breton. Les colloques de l'INRA, no. 44. Tel-Aviv, Israel: INRA: Paris.

Jacobs EC, Arnold AP & Campagnoni AT 1996 Zebra finch estrogen receptor cDNA: cloning and mRNA expression. *Journal of Steroid Biochemistry and Molecular Biology* **59** 135–145.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D & Chambon P 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** 1491–1494.

Koike S, Sakai M & Muramatsu M 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research* **15** 2499–2513.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β. *PNAS* **95** 15677–15682.

Krust A, Green S, Argos P, Kumar V, Walter P, Bornert JM & Chambon P 1986 The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO Journal* **5** 891–897.

Kuiper G, Enmark E, Peltohuikko M, Nilsson S & Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *PNAS* **93** 5925–5930.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863–870.

Kumar V, Green S, Stack G, Berry M, Jin JR & Chambon P 1987 Functional domains of the human estrogen receptor. *Cell* **51** 941–951.

Lahooti H, White R, Hoare SA, Rahman D, Pappin DJC & Parker MG 1995 Identification of phosphorylation sites in the mouse oestrogen receptor. *Journal of Steroid Biochemistry and Molecular Biology* **55** 305–313.

Lahooti H, Thorsen T & Aakvaag A 1998 Modulation of mouse estrogen receptor transcription activity by protein kinase C δ. *Journal of Molecular Endocrinology* **20** 245–259.

Lakaye B, Foidart A, Grisar T & Balthazart J 1998 Partial cloning and distribution of estrogen receptor β in the avian brain. *Neuroreport* **9** 2743–2748.

Laudet V 1997 Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *Journal of Molecular Endocrinology* **19** 207–226.

Lazennec G, Huignard H, Valotaire Y & Kern L 1995 Characterization of the transcription start point of the trout estrogen receptor-encoding gene: evidence for alternative splicing in the 5′ untranslated region. *Gene* **166** 243–247.

Le Goff P, Montano MM, Schodin DJ & Katzenellenbogen BS 1994 Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *Journal of Biological Chemistry* **269** 4458–4466.

Leygue E, Dotzlaw H, Lu B, Glor C, Watson PH & Murphy C 1998 Estrogen receptor beta: mine is longer than yours? *Journal of Clinical Endocrinology and Metabolism* **83** 3754–3755.

Leygue E, Dotzlaw H, Watson PH & Murphy LC 1999 Expression of estrogen receptor β1, β2, and β5 messenger RNAs in human breast tissue. *Cancer Research* **59** 1175–1179.

Linard B, Anglade I, Corio M, Navas JM, Pakdel F, Saligaut C & Kah O 1996 Estrogen receptors are expressed in a subset of tyrosine hydroxylase-positive neurons of the anterior preoptic region in the rainbow trout. *Neuroendocrinology* **63** 156–165.

Lu B, Leygue E, Dotzlaw H, Murphy LJ, Murphy LC & Watson PH 1998 Estrogen receptor β mRNA variants in human and murine tissues. *Molecular and Cellular Endocrinology* **138** 199–203.

Madigou T, Tiffoche C, Lazennec G, Pelletier J & Thieulant ML 1996 The sheep estrogen receptor: cloning and regulation of expression in the hypothalamo-pituitary axis. *Molecular and Cellular Endocrinology* **121** 153–163.

Maruyama K, Endoh H, Sasaki-Iwaoka H, Kanou H, Shimaya E, Hashimoto S, Kato S & Kawashima H 1998 A novel isoform of rat estrogen receptor β with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regulator of estrogen action. *Biochemical and Biophysical Research Communications* **246** 142–147.

Montano MM, Müller V, Trobaugh A & Katzenellenbogen BS 1995 The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Molecular Endocrinology* **9** 814–825.

Mosselman S, Polman J & Dijkema R 1996 ERβ: identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392** 49–53.

Munoz-Cueto JA, Burzawa-Gerard E, Kah O, Valotaire Y & Pakdel F 1999 Cloning and sequencing of the gilthead sea bream estrogen receptor cDNA. *DNA Sequence* **10** 75–84.

Murphy LC, Dotzlaw H, Leygue E, Douglas D, Coutts A & Watson PH 1997 Estrogen receptor variants and mutations. *Journal of Steroid Biochemistry and Molecular Biology* **62** 363–372.

Nicholas KB, Nicholas HB Jr & Deerfield DW II 1997 GeneDoc: analysis and visualization of genetic variation. *EMBNEW. NEWS* **4** 14.

Ogawa S, Inoue S, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998*a* Cross-inhibition of both estrogen receptor α and β pathways by each dominant negative mutant. *FEBS Letters* **423** 129–132.

Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998*b* The complete primary structure of human estrogen receptor β (hERβ) and its heterodimerization with ERα *in vivo* and *in vitro*. *Biochemical and Biophysical Research Communications* **243** 122–126.

Onoe Y, Miyaura C, Ohta H, Nozawa S & Suda T 1997 Expression of estrogen receptor β in rat bone. *Endocrinology* **138** 4509–4512.

Osterlund M, Kuiper G, Gustafsson JA & Hurd YL 1998 Differential distribution and regulation of estrogen receptor-α and -β mRNA within the female rat brain. *Molecular Brain Research* **54** 175–180.

Pace P, Taylor J, Suntharalingam S, Coombes RC & Ali S 1997 Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. *Journal of Biological Chemistry* **272** 25832–25838.

Pakdel F, Le Gac F, Le Goff P & Valotaire Y 1990 Full-length sequence and *in vitro* expression of rainbow trout estrogen receptor cDNA. *Molecular and Cellular Endocrinology* **71** 195–204.

Pettersson K, Grandien K, Kuiper GG & Gustafsson JA 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Molecular Endocrinology* **11** 1486–1496.

Picard D, Kumar V, Chambon P & Yamamoto KR 1990 Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. *Cell Regulation* **1** 291–299.

Saligaut C, Linard B, Mananos EL, Kah O, Breton B & Govoroun M 1998 Release of pituitary gonadotrophins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*) –modulation by estradiol and catecholamines. *General and Comparative Endocrinology* **109** 302–309.

Santos CRA, Power DM, Kille P, Llewellyn L, Ramsurn V, Wigham T & Sweeney GE 1997 Cloning and sequencing of a full-length sea bream (*Sparus aurata*) β-actin cDNA. *Comparative Biochemistry and Physiology* **117B** 185–189.

Schwabe JW, Neuhaus D & Rhodes D 1990 Solution structure of the DNA-binding domain of the oestrogen receptor. *Nature* **348** 458–461.

Shughrue PJ, Lane MV, Scrimo PJ & Merchenthaler I 1998 Comparative distribution of estrogen receptor β (ERα) and β (ERβ) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63** 498–504.

Tan NS, Lam TJ & Ding JL 1995 Molecular cloning and sequencing of the hormone-binding domain of *Oreochromis aureus* estrogen receptor gene. *DNA Sequence* **5** 359–370.

Tan NS, Lam TJ & Ding JL 1996 The first contiguous estrogen receptor gene from a fish, *Oreochromis aureus*: evidence for multiple transcripts. *Molecular and Cellular Endocrinology* **120** 177–192.

Tchoudakova A, Pathak S & Callard GV 1999 Molecular cloning of an estrogen receptor β subtype from the goldfish, *Carassius auratus*. *General and Comparative Endocrinology* **113** 388–400.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG 1997 The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25** 4876–4882.

Todo T, Adachi S & Yamauchi K 1996 Molecular cloning and characterization of Japanese eel estrogen receptor cDNA. *Molecular and Cellular Endocrinology* **119** 37–45.

Tora L, White J, Brou C, Tasset D, Webster N, Scheer E & Chambon P 1989 The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59** 477–487.

Touhata K, Kinoshita M, Toyohara H & Sakaguchi M 1998 Sequence and expression of a cDNA encoding the red seabream estrogen receptor. *Fisheries Science* **64** 131–135.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F & Giguere V 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. *Molecular Endocrinology* **11** 353–365.

Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW & McDonnell DP 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Molecular Endocrinology* **8** 21–30.

Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, Staub A, Jensen E, Scrace G, Waterfield M & Chambon P 1985 Cloning of the human estrogen receptor cDNA. *PNAS* **82** 7889–7893.

Weiler IJ, Lew D & Shapiro DJ 1987 The *Xenopus laevis* estrogen receptor: sequence homology with human and avian receptors and identification of multiple estrogen receptor messenger ribonucleic acids. *Molecular Endocrinology* **1** 355–362.

Xia Z, Patiño R, Gale WL, Maule AG & Densmore LD 1999 Cloning, *in vitro* expression, and novel phylogenetic classification of a channel catfish estrogen receptor. *General and Comparative Endocrinology* **113** 360–368.

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