

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

S Socorro, D M Power, P-E Olsson<sup>1</sup> and A V M Canario

Centro de Ciências do Mar, Universidade do Algarve, Campus de Gambelas, 8000 Faro, Portugal

<sup>1</sup>Department of Cell and Molecular Biology, Division of Physiology, Umea University, 901 87 Umea, Sweden

(Requests for offprints should be addressed to A V M Canario; Email: [acanario@ualg.pt](mailto:acanario@ualg.pt))

## 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 ER $\alpha$  and ER $\beta$ . The seabream ER $\alpha$  clone (sbER $\alpha$ 1), which was truncated in the A/B domain, corresponded to a variant differing in five amino

acids from another recently cloned sbER $\alpha$ . The ER $\beta$  clone (sbER $\beta$ ) encoded a protein 559 amino acids long and showed only 40% identity to sbER $\alpha$ . 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. sbER $\alpha$  had a more restricted distribution, occurring mainly in testis, liver and heart, and sbER $\beta$  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.

*Journal of Endocrinology* (2000) **166**, 293–306

## 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.* 1986b) 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, ER $\beta$ , 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.* 1998b), 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).

ER $\beta$  cDNAs encode a protein with high amino acid identity with the ER $\alpha$  protein, particularly in the DBD (96–97%) and LBD (53–60%) (Tremblay *et al.* 1997, Ogawa *et al.* 1998b). 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 ER $\beta$  and ER $\alpha$  mRNA. RT-PCR analysis of various rat tissues showed moderate to high expression of ER $\alpha$  in uterus, testis, pituitary, ovary, kidney, epididymis and adrenal gland, while ER $\beta$  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  $\alpha$ ERKO,  $\beta$ ERKO and  $\alpha\beta$ ERKO 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 ER $\alpha$ .

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 ER $\alpha$  and ER $\beta$  respectively and studied their tissue expression.

## Materials and Methods

### Production of an ER cDNA probe

Total RNA was extracted from estradiol (E<sub>2</sub>)-stimulated liver by an adaptation of the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987). Liver total RNA (5  $\mu$ g) was reverse-transcribed using Moloney murine leukaemia virus (MMLV)-RT (Gibco BRL, Barcelona, Spain) and oligo(dT)<sub>12-18</sub> primer (Pharmacia Biotech, Lisbon, Portugal) in a final volume of 30  $\mu$ l. 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'-TGYTCCATKCKKTRTT RCT-3' (SNKGMEH). PCR amplification was carried out with 5  $\mu$ l 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<sub>2</sub>-stimulated liver, pituitary and ovary obtained from 5  $\mu$ g of poly(A)<sup>+</sup> 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 [<sup>32</sup>P]- $\alpha$ -dCTP-labeled PCR product overnight at 65 °C in a solution containing 6  $\times$  SSC, 5  $\times$  Denhart's, 0.1% SDS and 0.1 mg/ml transfer RNA. Stringency washes were carried out at 65 °C with 0.1  $\times$  SSC containing 0.1% SDS. Several positive clones were obtained after first round screening of 4  $\times$  10<sup>5</sup> 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  $\times$  10<sup>5</sup> 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  $\mu$ Ci of L-[<sup>35</sup>S]methionine (Amersham). Reactions were performed using the 'TNT T3 Quick coupled Transcription/Translation System' following suppliers instructions (Promega). Translation products (5  $\mu$ l) 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 Phylip (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, sbER $\alpha$ 2), red seabream (rsER, Touhata *et al.* 1998), tilapia (tER, Tan *et al.* 1995), Nile tilapia (ntER $\alpha$ , accession number U75604), killifish (kER, accession number D28954), short and long forms of rainbow trout ER $\alpha$  (rtER $\alpha$ s and rtER $\alpha$ l, 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 ER $\alpha$  (rER $\alpha$ , Koike *et al.* 1987), sheep (sER, Madigou *et al.* 1996), human ER $\alpha$  (hER $\alpha$ , Green *et al.* 1986b), Nile tilapia ER $\beta$  (ntER $\beta$ , accession number U75605), goldfish ER $\beta$  (gfER $\beta$ , Tchoudakova *et al.* 1999), rat ER $\beta$  (rER $\beta$ , accession number AJ002602), Japanese quail ER $\beta$  (jqER $\beta$ , Lakaye *et al.* 1998), mouse ER $\beta$  (mER $\beta$ , Tremblay *et al.* 1997 and accession number AF067422) and human ER $\beta$  (hER $\beta$ , Ogawa *et al.* 1998b).

### Northern blot analysis

Poly(A)<sup>+</sup> 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 [<sup>32</sup>P]- $\alpha$ -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<sup>6</sup> 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  $\beta$ -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)<sup>+</sup> 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)<sup>+</sup> 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

1	CCA	CCC	TCA	GAT	GGG	AGC	CTT	CAG	TCC	CTG	GGC	AGC	GGG	CCC	<u>AAC</u>	<u>AGC</u>	<u>CCT</u>	<u>CTA</u>	<u>GTG</u>	<u>TTT</u>	<u>GTG</u>	CCC	TCC	AGC	CCC	CAT	CTC	AGC	84		
1	P	P	S	D	G	S	L	Q	S	L	G	S	G	P	<u>N</u>	<u>S</u>	<u>P</u>	<u>L</u>	<u>V</u>	<u>F</u>	<u>V</u>	P	S	S	F	H	L	S	28		
85	CCC	TTT	ATG	CAC	CCG	CCC	ACC	CAC	CAC	TAT	CTG	GAA	ACC	ACC	TCA	ACA	CCC	ATC	TAC	AGG	TCT	AGT	GTC	CCA	TCC	AGT	CAG	CAT	168		
29	P	F	L	E	T	H	H	H	H	T	T	S	T	T	T	A	C	C	C	C	C	C	C	C	C	C	C	C	H	56	
169	TCA	GTT	TCA	AGA	GAG	GAC	CAG	TGT	GGC	ACC	AGT	GAC	GAC	TCA	TAC	AGT	GTG	GGG	GAG	TCA	GGG	GCT	GGA	GCG	GGA	GCC	GCT	GGG	252		
57	S	V	S	R	E	D	Q	C	G	T	S	D	D	S	Y	S	V	G	E	S	G	A	G	A	G	A	G	A	84		
253	TTT	GAG	ATG	GCC	AAA	GAG	ATG	CGT	TTC	<u>TGT</u>	GCC	GTG	<u>TGC</u>	AGC	GAT	TAT	GCC	TCT	GGG	TAC	CAT	TAC	GGG	GTG	TGG	TCC	<u>TGT</u>	<u>GAG</u>	336		
85	F	E	M	A	K	E	M	R	F	<u>C</u>	A	V	<u>C</u>	S	A	S	D	Y	A	S	G	Y	H	Y	I	V	W	S	<u>C</u>	<u>E</u>	112
337	GGC	<u>TGC</u>	<u>AAG</u>	GCC	TTC	TTT	AAG	AGG	AGC	ATA	CAG	GGT	CAC	AAT	GAC	TAT	ATG	TGC	CCA	GCA	ACC	AAT	CAG	TGT	ACT	ATT	GAC	AGG	420		
113	<u>G</u>	<u>C</u>	<u>K</u>	<u>A</u>	F	F	K	R	<u>S</u>	<u>I</u>	<u>Q</u>	<u>G</u>	<u>H</u>	<u>N</u>	<u>D</u>	Y	M	<u>C</u>	P	A	T	N	Q	<u>C</u>	T	I	D	R	140		
421	AAT	CGG	AGG	AAG	AGC	<u>TGC</u>	CAG	GCT	<u>TGC</u>	CGT	CIT	AGG	AAG	TGT	TAC	GAA	GTG	GGC	ATG	ATG	AAA	GGA	GGT	GTG	CGC	AAA	GAC	CGT	504		
141	N	R	R	K	L	<u>C</u>	Q	A	<u>C</u>	D	R	K	R	K	C	Y	E	V	G	M	M	K	G	L	P	G	R	K	D	R	168
505	GGA	CGC	GTT	CTG	CGG	<u>CGT</u>	GAC	AAG	CGA	CGA	ACT	GGT	ACC	AGT	GAC	AGA	GAC	AAG	GCC	TCT	AAG	GGT	CTG	GAG	CAC	AGA	ACA	CGC	588		
169	G	R	V	L	R	R	D	K	R	R	T	G	T	S	D	R	D	K	A	S	K	G	L	B	H	R	T	A	196		
589	CCC	CCT	CAG	GAC	AGG	AGG	AAA	CAC	ATC	AGC	AGC	AGT	GCT	GGT	GGT	GGA	GGA	GGA	AAG	TCA	TCG	GTG	ATC	AGC	ATG	CCT	CCT	GAC	672		
197	P	P	Q	T	S	R	S	A	I	S	S	P	A	G	G	G	G	G	A	A	A	S	V	W	S	P	D		224		
673	CAG	GTG	CTC	CTC	CTG	CTC	CGG	GGT	GCA	GAG	CCC	CCA	ATG	CTG	TGC	TCC	CGT	CAG	AAG	GTG	AAC	CGA	CCC	TAT	ACC	GAG	GTC	ACC	756		
225	Q	V	L	L	L	L	R	G	A	B	P	P	M	L	C	S	R	Q	K	V	N	R	P	Y	T	E	V	T	252		
757	GTG	ATG	ACC	CTG	CTC	ACC	AGC	ATG	GCT	GAT	AAG	GAG	CTA	GTC	CAC	ATG	ATC	GCA	TGG	GCC	AAG	AAG	CTT	CCA	GGT	TTC	CTA	CAG	840		
253	V	M	T	L	L	T	Q	A	<u>C</u>	D	R	K	R	K	C	Y	E	V	I	A	W	A	K	G	L	P	G	R	K	D	280
841	CTG	TCC	CTC	CAC	GAC	CAA	GTG	CAG	CTG	CTG	GAG	AGC	TCG	TGG	CTG	GAG	GTG	CTG	ATG	ATC	GGG	CTC	ATC	TGG	AGG	TCC	ATC	CAC	924		
281	L	S	L	H	D	Q	V	Q	L	L	B	S	S	W	L	E	V	L	M	I	G	L	I	W	R	S	I	H	308		
925	TGC	CCG	GGC	AAA	CTC	ATC	TTC	GCA	CAG	GAC	CTC	ATA	CTG	GAC	AGG	AGT	GAG	GGC	GAC	TGT	GIT	GAA	GGC	ATG	GCC	GAG	ATC	TTC	1008		
309	C	P	G	K	L	F	A	Q	D	L	I	L	D	R	S	E	G	D	C	V	E	G	M	A	E	I	F		336		
1009	GAC	ATG	CTG	CTT	GCA	ACT	GCC	TCC	CGT	TTC	CGC	ATG	CTC	AAA	CTC	AAA	CCT	GAG	GAG	TTT	GTC	TGC	CTC	AAA	GCT	ATC	ATC	CTG	1092		
337	D	M	L	L	A	T	A	S	R	F	R	M	L	K	L	K	P	B	E	F	V	C	L	K	A	I	I	L	364		
1093	CTC	AAC	TCT	GGT	GCC	TTC	TCT	TTT	TGC	ACT	GGC	ACA	ATG	GAG	CCC	CTC	CAC	GAC	AGT	GCT	GCA	GTG	CAG	AAC	ATG	CTC	GAC	ACC	1176		
365	L	N	S	Q	G	T	S	F	A	C	T	M	E	P	L	H	D	S	A	A	V	A	V	A	Q	M	L	D	T	392	
1177	ATC	ACC	GAC	GCT	CTC	ATA	CAT	CAC	ATC	AAC	CAA	TCT	GGA	TGC	TCG	GCT	CAG	CAG	CAG	TCG	AGA	CGG	CAG	GCC	CAG	CTG	CTC	CTC	1260		
393	I	T	D	A	L	I	H	H	I	N	Q	S	G	C	S	A	Q	Q	Q	Q	S	R	Q	A	Q	L	L	L	420		
1261	CTG	CTC	TCC	CAC	ATC	AGA	CAC	ATG	AGC	AAC	AAA	GGC	ATG	GAG	CAT	<u>CTC</u>	TAC	AGC	ATG	AAG	TGC	AAG	AAC	AAG	GTG	CCT	CTG	TAC	1344		
421	L	L	S	H	I	R	H	M	S	N	K	G	M	E	<u>H</u>	<u>L</u>	<u>Y</u>	<u>S</u>	<u>M</u>	<u>K</u>	<u>C</u>	<u>A</u>	<u>Q</u>	<u>M</u>	<u>T</u>	<u>S</u>	<u>L</u>	<u>Y</u>	448		
1345	GAC	CTG	CTG	CTG	GAG	ATG	CTG	GAC	GCT	CAC	CGC	GTC	CAC	CGC	CCC	GAC	AGA	CCA	GCT	GAG	ACC	TGG	TCC	CAG	GCT	ACA	AGA	GAG	1428		
449	<u>D</u>	<u>L</u>	<u>L</u>	<u>L</u>	<u>E</u>	<u>M</u>	<u>L</u>	<u>D</u>	<u>A</u>	<u>H</u>	<u>R</u>	<u>V</u>	<u>H</u>	<u>R</u>	<u>P</u>	<u>D</u>	<u>R</u>	<u>P</u>	<u>A</u>	<u>E</u>	<u>T</u>	<u>W</u>	<u>S</u>	<u>Q</u>	<u>A</u>	<u>D</u>	<u>R</u>	<u>E</u>	476		
1429	CCT	CTC	TTC	ACC	TCC	AGA	AAC	ASC	AGC	AGC	AGC	AGC	GGT	GGT	GGT	GGT	GGA	GGC	TCC	TCA	TCA	GCT	GGC	TCC	ACT	TCA	GGA	CCA	1512		
477	P	L	F	T	S	R	S	S	S	S	S	G	G	G	G	G	G	G	C	S	S	A	G	T	C	A	G	P	504		
1513	CAG	GTC	AAC	CTT	GAG	AGC	CCC	ACA	GGT	CCC	GGC	GTC	CTG	CAG	CTC	CGA	GTG	CAC	CCA	CAT	CCT	ATG	AAA	CCT	ACA	GAA	TGA	AAGC	1597		
505	Q	V	N	L	E	S	P	T	G	P	G	V	L	Q	L	R	V	H	P	H	P	M	K	P	T	E	*		531		
1598	TAAAGGTTGTATAATAATTCATTTGAAGAGATAATATTATGAATATATGATTTTGTAGCTGTAGTTGTTTGGGAGACATTTTCCCTTGGCACTACTCCGGTTCACGT	1709																													
1710	CAATACGAGCTTCAGCAGAGTTAATCTTCGCGCACCGCTTTTCAGAAAACTGTGATTTTCGAGCCTTACAATACAGCTCTTATTCCAGGTTAGTGTATATTGGGCAC	1821																													
1822	TCTGTCAGCTACAGTGTATGGAAATGACGAGCAGCTAATTTTGTGTGTTTTGTCTCAACCAAAAGTGCACTTCCTCTGGGTTTAAAGGGCGCTGTGGGCATTAATTTTAC	1933																													
1934	TTCTAAATATAACGATGATAAAAACCTGGTTAATAAAAATGGATGTTGAGGACTGACGTCAGGATTTTTTAATTTGATATACTTGGTGAACAGATAGTTAATTAATGAGATTA	2045																													
2046	TGAAATGAAGAGCATCAAGGATTTATCTGTGTAATTATGAGTAACTAAAATGCACAATCAAATCCAAGAGGTGAAGGAAGCCAAAGCTTTTATGTGGGTAACTTCAGCCCT	2157																													
2158	GTCTTTAGCTTTTTTGTGTGTGTAGTGGTTTGTCTTCACTGCTTACTGTCACCAAAAGGGTTTTTTTAAATTTGTTACATCATAAATGTATGTGTGTGTGTGTGTGTGTG	2269																													
2270	TGTGTAGTATCAGAGAGACAGAGAGAAATGTGAGAAAGAGATTTTGCCTTAAAGAAATTTCAAGCATCAAATGACCGGTTACTATTCACTCACTCACTCAGTGATTACA	2381																													
2382	TGTTGAGTTTGGCTGGATTTACACACTCCAAAAACCTAATTTTGCATAGTGGCGCATATATATGCGCTTGTTTTTTATTTCTCTFAAACTACGCCAAAATTTGATTCATTC	2493																													
2494	ATCAGTAACTTAAACATGAGCTGGTGTTCCTTTATTAGCTGCTAACTTGCTAGTGTGCTAGTTTGTATTATAAAAAATCCAAGGAAAAAAGGGCACAAAGGTGACAT	2605																													
2606	TTAAATAACCTTTTGCCTCCGACAAAACAAATGTAATGAAGAAGGTATTAACATTTATAATCAAATAAATGACTACTGTAAAAACAAAATATTGAATCTGAATCTGAATTAAT	2717																													
2718	TGCTAAAAATCTAAAAATTTATCCCTTTAATTTGGGGTGTGTGTATTTTATGATAATCGCGATAATCAAGAAATGTGTCATGATTACAAATGAAAAACAAAAGCTGAAAAATCTT	2829																													
2830	CAAGGTGTGTTTCAGATAGGAGCAAGCAAAATGTTGTTTTTGGCCAACTTCCTCTTTTGGGGACATTTAAAATGTTTTATTTTAATCTTTATCATAAAAAAGACGAT	2941																													
2942	CGTAAATAAACAATAAACAGACTAGAGATTCATCCGCTGCCCTTAGGTTAAGATTTAAGCCTAGCTCACTGTGATCAGAAGATGCTGCTTTTAAATGGAAGCCACTTGT	3053																													
3054	CCTTGTGTGATGATACTGTGTCTGCACATGTTGCGCACCAAGATTTAGAGCAGGTCAAAAAATTTGGAGTAACTTTGTTGAAGCAGAAAACTTTCAGTTTGAACCTGGGTA	3165																													
3166	AATATATTGAAAAGGGGTCAACAGTTGACTGACATGTAGTGTGTTGCAGGCAGTTATGTGTCAGAGAATATAAACGATAAAACAAAAGATGCAATTACAGCCGAGTCTCATCC	3277																													
3278	TCAGCAGGATTCAAAGATTGGGCTCAGCCACTTGAGCTGTGCTGTGTGATCCAGTCAAACCTGATCTGACTGTCTGTTTGTGTTTACCTGTGCTTAAAAAATAAAAGCATTA	3389																													
3390	TTGAGAAAAAATAAAAAAAAAAAAAA	3416																													

**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 LBD, the region corresponding to AF2 is underlined and the amino acids recognized to be involved in E<sub>2</sub> binding are in bold. Sequences of specific primers used for RT-PCR are double-underlined.



1	CGGGTCGCCATACTGTCAACAACCTTTGTGTCAGCAACTGGTGTGTCCGGACCAAGAGGTGGAAAGGTTTGGCCACACATCTGACTCCACATGCCAGCACTGACGATGTGAGA	109
110	CTCATGGCTGGACTGAGACTGAGCACAGAACCCTTCCCTCCCCCTAGTGTAGACGGACTGCAGTCCCTGTGTGTCAACATTTCTTTCATCATCTGACAGCATCGACTGACAG	221
222	GTGTGATCATGTGATTCATCTCAACACTGGCAITTTTCACTTGGTACTGAGATTTGTCTGAAGTTGTG	321
1	ATG GCC GTT GCC TGC TCT CCA GAG AAG GAT CAG TCC	12
	M A V A C S P E K D Q S	
322	CTC CTC CAG CTC CAG AAG GTG GAC TCC AGT CGA GTT ATT CTC TCC CCG GTC CTC AGC TCC CCT ATG GAA ACC AAC CAG CCC ATC	405
13	L L Q L Q K V D S S R V I L S P V L S S P M E T N Q P I	40
406	TGC ATC CCC TCC CCT TAC ACC GAC CGT GGC CAC GAC TTC CCC ACC ATA CCT TTC TAC AGT GCA ACT AAT TTC AGC TAT GCC AAT	489
41	C I P S P Y T D R G H D F P T I P F Y S A T N F S Y A N	68
490	CCG CCG GCC ATT TCA GAC CGC CCC TCT GTC CAT CAG ACA CTA AGC CCC TCC TTA TTT TGG CCC AGC CAT GGC CAT GTG GGG ACC	573
69	P P A I S D R P S V H Q T L S P S L F W P S H G H V G T	96
574	ACC TTA CCC CTG CAC CAC CTC CAG GCT CGA CCT CAG CAC GGG CAG GCG GTT CAG AGT CCA TGG GTG GAG CTG TCG CCA CTG GAC	657
97	T L G P L H H A R R G C H G A V Q S P W V E L S P L D	124
658	AAT GTG TTA ACA AGC AGT AAG AGT GCA AGG AGG CGT TCT CAG GAG AAC GAG GAG GGT GAG GTG TCA TCG GGC GGG AAG GCG GAC	741
125	N V L T S S K S A R R R C R S Q E N E E G E V S S G G K A D	152
742	CTC CAC TTC TGT GCT GTG TGC CAC GAC TAC GCC TCA GGC TAC CAC TAC GGC GTC TGG TCG TGT GAG GGG TGT AAG GCC TTC TTC	825
153	L H F C A V C H D Y A S G Y H Y G V W S C E G C K A F F	180
826	AAG AGG AGC ATC CAA AGA CAC AAC GAC TAC ATC TGC CCA GCA ACC AAT CAA TGC ACT ATA GAC AAG AAC CGC CGT AAG AGC TGC	909
181	K R S I Q R H N D Y I C P A T N Q C T I D K N R R K S C	208
910	CAG GCG TGT CGC CTT CAC AAA TGC TAC AAC GTT GGC ATG ACC AAG TGT GGA ATG CGA AAG GAA CGT GGA AAC TTC AGG GAC CCC	993
209	Q A C R L C Y N V C G M T K C G M R K E R G N F R D P	236
994	CAG ATG AGG CGA GTG ACC CGT CTG TCC TCA CAG GGC AGA ACT AGC GGA CCA AGC GTG TTA AAT GGA CCA GCA GTG GGT CCG TTA	1077
237	Q M R R V T R L S S Q G R T S G P S V L N G P A V G P L	264
1078	AAC ACA CCC CAA CCT CCC GCA CTG ACT TCA AAG CAG CTG ATT GAG CGG ATT ATG GAG GCA GAG CCG CCA GAG ATC TAC CTC ATG	1161
265	N T P Q P P A L T S K Q L I E R I M E A E P P E I Y L M	292
1162	AAG GAC ATG AGG AGG CCG CTG ACT GAA GCA AAC ATC ATG ATG TCG CTC ACC AAC CTG GCC GAT AAG GAG CTG GTT CAC ATG ATC	1245
293	K D M R R P L T E A N I M M S L T N L A D K E L V H M I	320
1246	ACC TGG GCC AAG AAG ATT CCA GGG TTT TTA GAG CTC GGC CTC TTG GAC CAG GTG CAC CTG TTG GAG TGC TGC TGG CTG GAG GTG	1329
321	T W A K K I P G F L G C V H L L E C V H L E C T G W L E V	348
1330	CTG ATG ATC GGA CTG ATG TGG AGG TCA GTG GAC CAT CCA GGG AAA CTT ATC TTC TCC CCT GAC CTC AGC TTG AGC AGA GAA GAG	1413
349	L M I G L M W R S V D H P G K L I F S P D L S L S R E E	376
1414	GGG AGC TGT GTC CAG GGC TTC TTG GAG ATC TTT GAT ATG CTG ATA GCC GCC ACA TCC AGG GTG AGA GAG CTC AAG CTC CAG AGG	1497
377	G S C V Q G F L E I F D M L I A A T S R V R E L K L Q R	404
1498	GAG GAG TAC GTC TGC CTC AAG GCC ATG ATC CTC CTT AAC TCC AAC ATG TGC CTC AGC TCC TCA GAG GGC AGC GAG GAG CTG CAG	1581
405	E E Y V C L K A M I L L N S N M C L S S E G S E E L Q	432
1582	AGT CGC TCC AAG CTG CTG CGT CTT CTG GAC GCC GTC ACG GAC GCT CTT GTG TGG GCC ATC GCC AAA ACC GGC CTC ACT TTC CGC	1665
433	S R S K L L R L L D A V T D A L V W A I A K T G L T F R	460
1666	CAA CAG TAC ACC CGC CTC GCC CAC CTG CTC ATG CTG CTC TCT CAC ATC CGC CAC GTC AGT AAC AAA GGC ATG GAC CAC CTC CAC	1749
461	Q Q Y T R L A H L L M L L S H I R H V S N K G M D H L H	488
1750	GGC ATG AAA ATG AAG AAC ATG GTG CCG TTG TAT GAC CTG CTG CTG GAG ATG CTG GAC GCC CAT ATC ATG CAC AGC TCC CGT CTG	1833
489	G M K M K N M V P L Y D L L L E M L D A H I M H S S R L	516
1834	CCT CGC CGG TCA CCC CAG CAG GAG ACC GTG GAA CAG TGC GAC GCT CCT GCC CGG CCA CAC AGC CCC GGT ACC TCC GGC CCC ACT	1917
517	P R R R S P Q G E T V E Q C D A P A R H S P G T S P G C P T	544
1918	AAC ACC TGG ACT CCC AGC TGC ACC GGA GGC AGA GGT GAA CCG CAG TAG CCGGATCAGAATTCAGATGCAATGACTTTTCACGCTTTACACAAGACT	2013
545	N T W T P S C T G G R G E P Q *	560
2014	AGTTCACTGCGGAGCGTCTTTCTTTGAACCTCTCACTTTTGACACACCGTGCACTTTTCAGTCTCTTCAAATTTCACTCTGCAGACAGACCAACCTAGCAGTATTCATCGGCTT	2125
2126	TCCACCACATATTA AAAAGCCAGAGTGGATCAGGAA CAAAAAAAAAAAAAAAAAAAA	2180

**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<sub>2</sub> 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

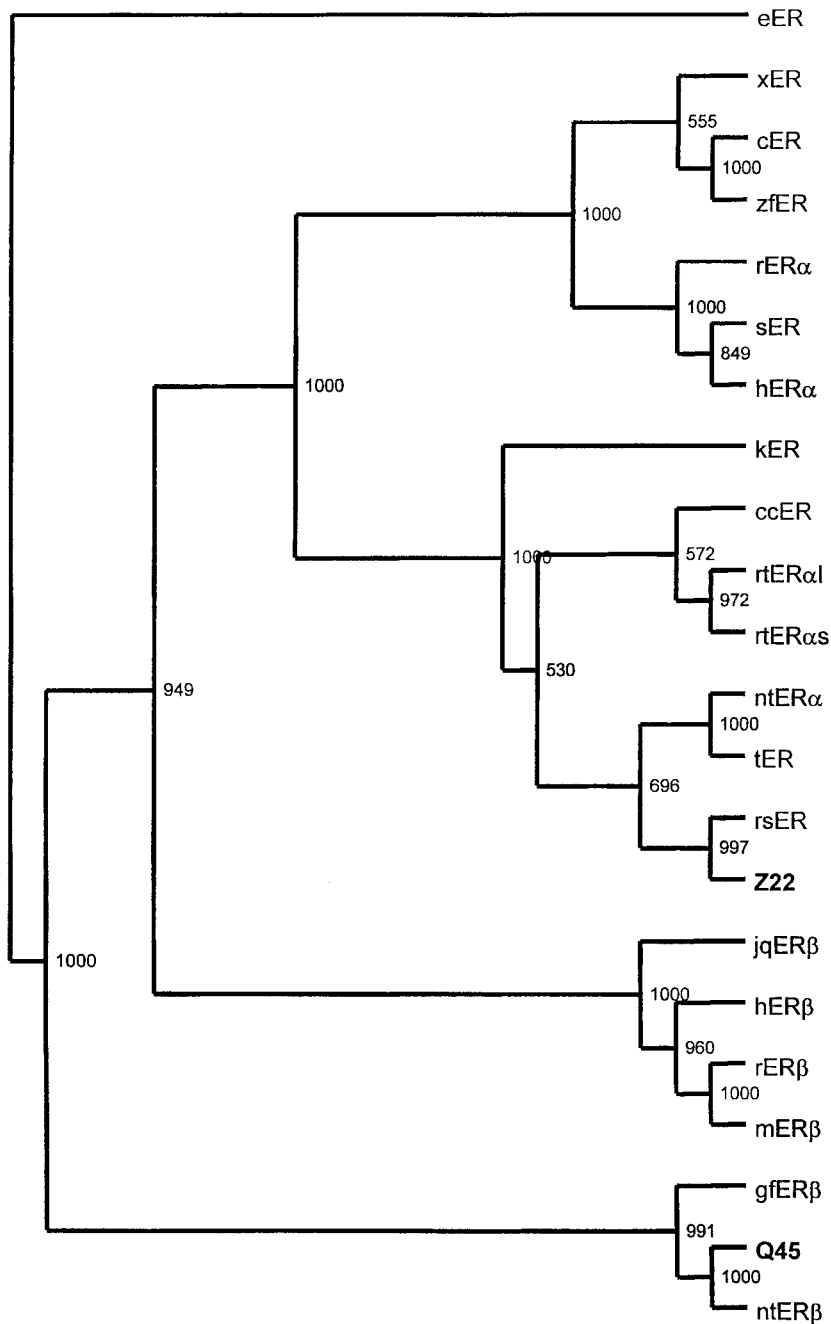
Clone Species/Domain	Z22 (sbER $\alpha$ 1)						Q45 (sbER $\beta$ )					
	Overall	A/B	C	D	E	F	Overall	A/B	C	D	E	F
sbER $\alpha$ 2	99 (579)	94 (137)	100 (81)	100 (43)	100 (251)	100 (67)	36	10	75	9	54	10
rsER	93 (581)	94 (139)	98 (81)	97 (43)	97 (251)	70 (67)	36	10	76	11	54	8
ntER $\alpha$	79 (585)	77 (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
rtER $\alpha$ l	70 (622)	55 (187)	90 (82)	57 (45)	85 (251)	22 (57)	36	8	71	6	54	4
rtER $\alpha$ s	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
hER $\alpha$	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
rER $\alpha$	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
mER $\beta$	42 (549)	16 (162)	83 (83)	8 (29)	57 (247)	5 (28)	50	13	79	8	63	6
rER $\beta$	42 (549)	16 (162)	81 (83)	8 (29)	57 (247)	2 (28)	50	12	78	8	64	4
hER $\beta$	42 (530)	16 (143)	83 (83)	9 (29)	57 (247)	7 (28)	47	19	79	8	63	6
jqER $\beta$	42 (472)	15 (99)	84 (83)	11 (26)	57 (246)	2 (18)	46	16	79	7	63	8
ntER $\beta$	42 (557)	16 (149)	77 (89)	9 (25)	56 (249)	13 (45)	77	62	89	60	89	47
gfER $\beta$	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 (sbER $\beta$ )	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 hER $\alpha$  to be involved in E<sub>2</sub> 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 eER $\alpha$ , gfER $\beta$ , ntER $\beta$  and clone Q45 (41–42%). Identity to tetrapod ER was 48% and to tetrapod ER $\beta$  42%. In contrast, clone Q45 showed 58–77% amino acid sequence identity to eER, gfER $\beta$  and ntER $\beta$ , and only 36–37% to other fish ERs. Identity to tetrapod ER $\beta$  was 47–50% and to tetrapod ER $\alpha$  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 ER $\beta$  and clone Q45, eER and ntER $\beta$  in the same group) and two clades with NJ (placing clone Q45, eER and ntER $\beta$  with tetrapod ER $\alpha$  and tetrapod ER $\beta$  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 ER $\alpha$  and clone Q45 is related to tetrapod ER $\beta$ . 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 designated fish ER $\alpha$  and clone Q45 and related fish ERs to a group designated fish ER $\beta$  and will be referred to as sbER $\alpha$  and sbER $\beta$  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

	ER $\alpha$ Tetrapod	ER $\beta$ Tetrapod	ER $\alpha$ Fish	ER $\beta$ Fish
ER $\alpha$ Tetrapod (595)	66			
ER $\beta$ Tetrapod (549)	0	55		
ER $\alpha$ Fish (580)	18	2	28	
ER $\beta$ 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 ER $\alpha$  and ER $\beta$  are shown in Table 2. The number of amino acids of ER $\alpha$  and ER $\beta$  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  $\alpha$  and  $\beta$  types of ER in fish and tetrapods is very low or absent (0–6 amino acids), but is high between tetrapod ER $\alpha$  and fish ER $\alpha$  (18 amino acids) and between tetrapod ER $\beta$  and fish ER $\beta$  (17 amino acids). The analysis of conservation contrasts unequivocally showed that fish ERs are related to tetrapod  $\alpha$  and  $\beta$  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 ER $\alpha$  and in ER $\beta$  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 ER $\alpha$  a mitogen-activated

protein kinase phosphorylation site with the consensus motif P-X<sub>(1,2)</sub>-SP is found which is not apparent in ER $\beta$  (Fig. 4). However, in tetrapod ER $\beta$  potential mitogen-activated protein kinase (MAPK) phosphorylation sites are located downstream of the corresponding region in ER $\alpha$ , while in fish ER $\beta$  potential MAPK sites are located upstream, except for eER $\beta$  which has two sites and sbER $\beta$  which apparently lacks a MAPK phosphorylation site. Finally, in both fish and tetrapod ER $\alpha$  LBD, a completely conserved tyrosine kinase phosphorylation site (KGMEHLY) is present.

#### Transcripts size of sbER $\alpha$ and sbER $\beta$

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 sbER $\alpha$  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 sbER $\beta$  cDNA, while in liver only the 0.3 kb transcript was detected (Fig. 5).

#### Tissue distribution of sbER $\alpha$ and sbER $\beta$

In order to examine the distribution of sbER $\alpha$  and sbER $\beta$  mRNA, the sensitive method of RT-PCR analysis was performed with ER $\alpha$ - and  $\beta$ -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  $\beta$ -actin primers and using this to normalize the results with primers for sbER $\alpha$  and  $\beta$  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). sbER $\beta$  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. SbER $\alpha$  was only detected in testis, liver and heart with similar levels of expression.

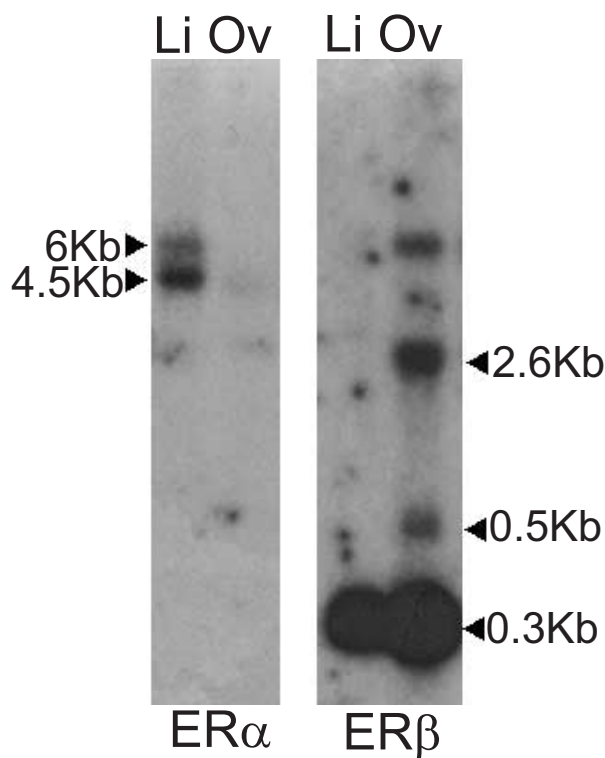
## Discussion

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).



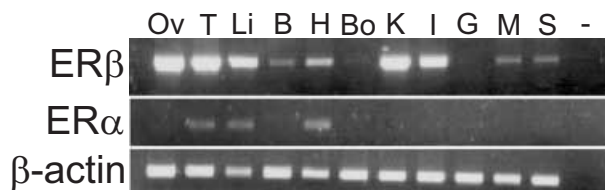
sbERβ	-----MAVACSPKQDSSLQLQKVD-----	20	-----SSRV-----	40	-----IL-SPVL-----	60	-----SSP-----	80	-----METNQPICIPS--PYT	47
ntERβ	-----MMAAA <b>SSPE</b> KLLQLQEVN-----		-----SSRAGS-----		-----RIL-SPIL-GSSS-PGLSHETSQPICIRS--PYT					53
gfERβ	MTALNSYAFAMSEYABGSSDSSLQLQEVN-----		-----SSRMGG-----		-----HVL-SPT-FNSSS-PS-LPVESHPIICIPS--PYT					62
eER	-----MAGSPGNELPLLQLQEVN-----		-----SSKVGESGGSSGL-LPTMYNGA--LPALSMESHAVCCIPS--PYT							57
rERβ	-----MSICTS <b>SHKE</b> FSQLR-----		-----PSEDMEIKNSPSS-----		-----LSSPASYNCSQ-SILPLEH-GPIYIPS-- <b>SYV</b>					56
mERβ	-----MSICASS <b>SHKD</b> FSQLR-----		-----PTQDMEIKNSPSS-----		-----LTSFASYNCSQ-SILPLEH-GPIYIPS-- <b>SYV</b>					56
hERβ	-----MSICASS <b>SHKE</b> FSQLR-----		-----MDIKNSPSS-----		-----LNSFSSYNCSQ-SILPLEH-GSIYIPS-- <b>SYV</b>					37
jqERβ	-----		-----		-----					-
sbERα2	-----		-----		-----MYPEDS-RVSGGVATVD-FLEG--TYD					23
rsER	-----		-----		-----MYPEDS-RGSGGVATVD-FLEG--TYD					23
tER	-----		-----		-----MYPEES-RGSGGVATVD-FLEG--LMT					23
ntERα	-----		-----		-----MYPEES-RGSGGVATVD-FLEG--TYD					23
ker	-----MSKRQSSVQIRQLFGPALRSRISPASS <b>SELE</b> TLSP <b>PLSPR</b> -----		-----DPL--GDMYPEES-RGSGGVAADV-FLEG--TYD							68
rterα1	-----MLVRQSHQIS--KPLGAPLRSR <b>TL</b> LS <b>SHV</b> IS <b>PKLS</b> PQ-----		-----PTTPNSNMYPEET-RGGGGAFAFN-YLDG--GYD							68
rterαs	-----		-----		-----MYPEET-RGGGGAAL <b>TWME</b> GMTT-Q					25
ccER	-----		-----		-----MYPEEQRTGGISSTAHYLDGTFNY--					26
zfER	-----MTLTKTSGVTL <b>LHQIQ</b> -----		-----GTELETLSP <b>RQ</b> LKIP <b>LE</b> -- <b>RSL</b> -- <b>SDMYVE</b> --TNKTKGVFN-YPEG-ATYD							59
cER	-----MTMTLHTKASGVTL <b>LHQIQ</b> -----		-----GTELETLSP <b>RQ</b> LKIP <b>LE</b> -- <b>RSL</b> -- <b>SDMYVE</b> --SNKTKGVFN-YPEG-ATYD							61
xER	-----MTMPLPNKTTGVTL <b>LHQIQ</b> -----		-----SSELETLSP <b>RQ</b> LKIP <b>LE</b> -- <b>RPL</b> --GEMVVE--NMRTGIFN-YPEG- <b>ITYD</b>							61
sER	-----MTMTLHTKASGMALL <b>LHQIQ</b> -----		-----ANELEPLNR <b>PQ</b> LKIP <b>LE</b> -- <b>RPL</b> --GEMVVD--SSKPAVYN-YPEG- <b>AAVD</b>							61
hERα	-----MTMTLHTKASGMALL <b>LHQIQ</b> -----		-----GNELEPLNR <b>PQ</b> LKIP <b>LE</b> -- <b>RPL</b> --GEVYLD--SSKPAVYN-YPEG- <b>AAVE</b>							61
rERα	-----MTMTLHTKASGMALL <b>LHQIQ</b> -----		-----GNELEPLNR <b>PQ</b> LKIP <b>LE</b> -- <b>RAL</b> --GEVYVD--NSKPAVFN-YPEG- <b>AAVE</b>							61
sbERβ	DRGHDFPT-----IPFYSA--TNFSYAN-PP--AISDRPSVHQT-----	100	-----SPSLFWP <b>SHGHV</b> G--TT-LPLHHLQAR <b>PQH</b> G-----	120	-----TT-LPLHHLQAR <b>PQH</b> G-----	140	-----	160	-----	180
ntERβ	DLGHDFTT-----IPFY <b>SP</b> --TIPSYGG-P-- <b>STSE</b> CSSVH <b>QSL</b> -----		-----SASLFWP <b>SHGRV</b> G--TP-ITLHC <b>PQGR</b> SQ <b>Q</b> G-----		-----TP-ITLHC <b>PQGR</b> SQ <b>Q</b> G-----					112
gfERβ	DLGHDFTT-----L <b>PFYSP</b> --SLLGYGTSP--LSDCP <b>SVRQSL</b> -----		-----SPTLFWP <b>PHSHV</b> --SS-LAL <b>HQQ</b> TRL <b>QPN</b> -----		-----SS-LAL <b>HQQ</b> TRL <b>QPN</b> -----					117
eER	<b>DSH</b> H <b>YAA</b> -----LTFYSP--PILSHGG-P-- <b>AV</b> ES <b>EAARQSL</b> -----		-----SPSLFWP <b>AGHHGHV</b> -SP-LAL <b>HQQ</b> PL <b>VYR</b> -----		-----SP-LAL <b>HQQ</b> PL <b>VYR</b> -----					122
rERβ	<b>DN</b> RHE <b>YSA</b> -----MTFYSP--AVMNYSV-PGS- <b>TSN</b> LD <b>GGPVR</b> LT-----		-----SPNVLWPT <b>SGHL</b> --SP-LATH <b>CS</b> SL <b>LYA</b> -----		-----SP-LATH <b>CS</b> SL <b>LYA</b> -----					121
mERβ	<b>BS</b> RHE <b>YSA</b> -----MTFYSP--AVMNYSV-PSS-TGN <b>LEGG</b> PVR <b>QTA</b> -----		-----SPNVLWPT <b>SGHL</b> --SP-LATH <b>CS</b> SL <b>LYA</b> -----		-----SP-LATH <b>CS</b> SL <b>LYA</b> -----					121
hERβ	<b>DS</b> H <b>H</b> Y <b>PA</b> -----MTFYSP--AVMNYSI-PSN- <b>V</b> T <b>N</b> LD <b>GGPGR</b> TT-----		-----SPNVLWPT <b>PGHL</b> --SP-LV <b>VHR</b> QL <b>SHLYA</b> -----		-----SP-LV <b>VHR</b> QL <b>SHLYA</b> -----					102
jqERβ	-----MAFCSP--AMMNYNI-ASN-FGD <b>SE</b> SA <b>SVRQTS</b> -----		-----SPSL <b>LWS</b> AP <b>AGHL</b> --SP-L <b>TL</b> H <b>QC</b> SL <b>L</b> LYA-----		-----SP-L <b>TL</b> H <b>QC</b> SL <b>L</b> LYA-----					57
sbERα2	YAAPT <b>AP</b> -----TPLYSHS-TPGYYS-APLD-AHG <b>PP</b> SD <b>GS</b> LQ <b>SLG</b> SG <b>NS</b> PL <b>V</b> FP <b>SS</b> PHL-----		-----SEFM--QP <b>PTH</b> -----		-----SEFM--QP <b>PTH</b> -----					92
rsER	YAAPT <b>AP</b> -----TPLYSHS-TPGYYS-APLD-AHG <b>PP</b> SD <b>GS</b> LQ <b>SLG</b> SG <b>NS</b> PL <b>V</b> FP <b>SS</b> PHL-----		-----SEFM--HP <b>PTH</b> -----		-----SEFM--HP <b>PTH</b> -----					92
tER	MTAPT <b>AP</b> -----TPLYSHS-TTG <b>CYS</b> -APLD-AHG <b>PL</b> SD <b>GS</b> LQ <b>SLG</b> SG <b>PT</b> SP <b>L</b> VFP <b>SS</b> PHL-----		-----SEFM--HP <b>SSH</b> -----		-----SEFM--HP <b>SSH</b> -----					92
ntERα	YAAPT <b>AP</b> -----TPLYSHS-TTG <b>CYS</b> -APLD-AHG <b>PL</b> SD <b>GS</b> LQ <b>SLG</b> SG <b>PT</b> SP <b>L</b> VFP <b>SS</b> PHL-----		-----SEFM--HP <b>SSH</b> -----		-----SEFM--HP <b>SSH</b> -----					92
ker	YAAPT <b>AP</b> -----TPLY <b>SQ</b> S-STG <b>YYS</b> -APLE-TNG <b>PP</b> SE <b>GS</b> LQ <b>SLG</b> SG <b>PT</b> SP <b>L</b> VFP <b>SS</b> PHL-----		-----SEFM--HP <b>SSH</b> -----		-----SEFM--HP <b>SSH</b> -----					137
rterα1	YTAP <b>QGP</b> -----APLY-----YST <b>TPQD</b> -AHG <b>PP</b> SD <b>GS</b> MQ <b>SLG</b> SS <b>PT</b> GP <b>L</b> VFP <b>V</b> SS <b>PQ</b> SP <b>Q</b> SP <b>FL</b> -----		-----HPPSH <b>HGL</b> PS <b>Q</b> S--YYLET		-----HPPSH <b>HGL</b> PS <b>Q</b> S--YYLET					142
rterαs	-----PLPKA-----RPL-SI--TPP-----PPR-MPT <b>DP</b> SD <b>GS</b> MQ <b>SLG</b> SS <b>PT</b> GP <b>L</b> VFP <b>V</b> SS <b>PQ</b> SP <b>Q</b> SP <b>FL</b> -----		-----HPPSH <b>HGL</b> PS <b>Q</b> S--YYLET		-----HPPSH <b>HGL</b> PS <b>Q</b> S--YYLET					96
ccER	-----T <b>NP</b> AT <b>N</b> -----S <b>V</b> DYYS <b>VAP</b> -----EPQ <b>EN</b> LQ <b>PL</b> NG <b>SS</b> PP <b>V</b> FP <b>SS</b> PHL-----		-----SE <b>FL</b> G-HP <b>PA</b> Q <b>HTA</b> Q <b>V</b> PPY <b>LEP</b> -----		-----SE <b>FL</b> G-HP <b>PA</b> Q <b>HTA</b> Q <b>V</b> PPY <b>LEP</b> -----					94
zfER	FG-----TTAP <b>VYSS</b> --TTLSY--APT <b>SE</b> FG <b>SS</b> L <b>AG</b> F <b>HS</b> L <b>NS</b> V <b>FP</b> SV <b>FL</b> Q <b>TAP</b> H-----		-----SPFI--HH <b>HS</b> Q <b>V</b> P-----		-----SPFI--HH <b>HS</b> Q <b>V</b> P-----					126
cER	FG-----TTAP <b>VYGS</b> --TTLSY--APT <b>SE</b> FG <b>SS</b> L <b>AG</b> F <b>HS</b> L <b>NN</b> V <b>FP</b> SV <b>FL</b> Q <b>TAP</b> H-----		-----SPFI--HH <b>HS</b> Q <b>V</b> P-----		-----SPFI--HH <b>HS</b> Q <b>V</b> P-----					128
xER	FAAAAA-----PV <b>YSS</b> --ASLS <b>YAA</b> SS <b>ET</b> --FG <b>SS</b> LT <b>GL</b> HT <b>LN</b> N <b>V</b> FP <b>SV</b> FL <b>AK</b> L <b>P</b> Q-----		-----SPFI--HH <b>H</b> Q <b>V</b> P-----		-----SPFI--HH <b>H</b> Q <b>V</b> P-----					129
sER	FNAAAAA-----SAP <b>VY</b> Q <b>S</b> --GL <b>PY</b> GP <b>GE</b> EA <b>AF</b> GAN <b>GL</b> F <b>AP</b> PL <b>NS</b> V <b>SP</b> PL <b>V</b> LL <b>HP</b> PP <b>Q</b> -----		-----SE <b>FL</b> G-HP <b>H</b> Q <b>V</b> P-----		-----SE <b>FL</b> G-HP <b>H</b> Q <b>V</b> P-----					134
hERα	FNAAAAA-----NAQ <b>VY</b> Q <b>T</b> --GL <b>PY</b> GP <b>GE</b> EA <b>AF</b> FG <b>NS</b> GL <b>GF</b> PP <b>LN</b> SV <b>SP</b> PL <b>ML</b> LL <b>HP</b> PP <b>Q</b> -----		-----SE <b>FL</b> G-HP <b>H</b> Q <b>V</b> P-----		-----SE <b>FL</b> G-HP <b>H</b> Q <b>V</b> P-----					135
rERα	FNAAAAA <b>AAGA</b> -SAP <b>VY</b> Q <b>S</b> --S <b>IT</b> YGP <b>GE</b> EA <b>AF</b> GAN <b>SL</b> GA <b>FP</b> QL <b>NS</b> V <b>SP</b> PL <b>ML</b> LL <b>HP</b> PP <b>H</b> -----		-----SE <b>FL</b> G-HP <b>H</b> Q <b>V</b> P-----		-----SE <b>FL</b> G-HP <b>H</b> Q <b>V</b> P-----					139
sbERβ	VQSPWV <b>EL</b> SP <b>LD</b> N <b>V</b> L <b>T</b> S--SKSARRRSQ <b>ENE</b> GEVSSG-----	200	-----GK-----	220	-----	240	-----			150
ntERβ	AQ <b>TPW</b> -----DSV <b>IT</b> T--SKSVRRRSQ <b>E</b> ESMVSSG-----		-----GK-----		-----					149
gfERβ	TGGT <b>WAE</b> L <b>TP</b> H <b>D</b> H <b>GE</b> EN <b>CK</b> PL <b>S</b> K <b>R</b> V <b>A</b> VA <b>ET</b> ST <b>SLR</b> -----		-----GK-----		-----					164
eER	A <b>HS</b> W <b>AE</b> PK <b>LE</b> H <b>G</b> QA <b>Q</b> TS <b>K</b> L <b>AG</b> K <b>MA</b> E <b>SE</b> EGT <b>SS</b> VGG <b>CF</b> --AGK-----		-----NAK-----		-----					165
rERβ	Q <b>K</b> SP <b>W</b> CEAR <b>S</b> LE <b>HT</b> LP <b>V</b> NR <b>ET</b> L <b>K</b> R <b>L</b> SG <b>SS</b> CA <b>SP</b> VT <b>SP</b> -----		-----NAK-----		-----					162
mERβ	Q <b>K</b> SP <b>W</b> CEAR <b>S</b> LE <b>HT</b> LP <b>V</b> NR <b>ET</b> L <b>K</b> R <b>L</b> SG <b>SS</b> CA <b>SP</b> VT <b>SP</b> -----		-----SAK-----		-----					162
hERβ	Q <b>K</b> SP <b>W</b> CEAR <b>S</b> LE <b>HT</b> LP <b>V</b> NR <b>ET</b> L <b>K</b> R <b>K</b> V <b>S</b> GN <b>R</b> CA <b>SP</b> VT <b>GP</b> -----		-----GSK-----		-----					143
jqERβ	E <b>K</b> SP <b>W</b> CEAR <b>P</b> LE <b>P</b> V <b>L</b> PS <b>R</b> ET <b>L</b> K <b>R</b> KT <b>NG</b> SD <b>CT</b> SP <b>IA</b> SN-P--GSK-----		-----		-----					99
sbERα2	TSTPIY--SVPSS <b>Q</b> HS <b>V</b> S <b>R</b> ED <b>CC</b> GT <b>SD</b> DS <b>Y</b> SV <b>G</b> ES <b>G</b> AG <b>A</b> AG <b>F</b> EMA-----		-----		-----					137
rsER	TSTPVYRSVSPSS <b>Q</b> Q <b>S</b> VS <b>R</b> ED <b>CC</b> GT <b>SD</b> DS <b>Y</b> SV <b>G</b> ES <b>G</b> AG <b>A</b> AG <b>F</b> EIA-----		-----		-----					139
tER	TSTPVYR--SSHQ <b>P</b> V <b>RE</b> D <b>CC</b> TR <b>DE</b> AV <b>S</b> V <b>G</b> EL <b>G</b> AG <b>A</b> G--GFEIT-----		-----		-----					133
ntERα	TSTPVYR--SSHQ <b>P</b> V <b>RE</b> D <b>CC</b> TR <b>DE</b> AV <b>S</b> V <b>G</b> EL <b>G</b> AG <b>A</b> G--GFEMT-----		-----		-----					133
ker	TSTPVYR--SSHQ <b>G</b> AS <b>R</b> ED <b>CC</b> SR <b>ED</b> CT <b>SL</b> G <b>EL</b> G <b>A</b> G <b>A</b> GG <b>F</b> EMA-----		-----		-----					180
rterα1	SSTPLYR <b>SS</b> V <b>T</b> N <b>Q</b> L <b>S</b> A <b>EB</b> E <b>K</b> L <b>C</b> I <b>A</b> S <b>D</b> R <b>Q</b> S <b>Y</b> S <b>A</b> AG <b>S</b> G <b>V</b> R--VFEMAN-----		-----		-----					188
rterαs	SSTPLYR <b>SS</b> V <b>T</b> N <b>Q</b> L <b>S</b> A <b>AE</b> E <b>K</b> L <b>C</b> I <b>A</b> S <b>D</b> R <b>Q</b> S <b>Y</b> S <b>A</b> AG <b>S</b> G <b>V</b> R--VFEMAN-----		-----		-----					142
ccER	SGT <b>S</b> Y <b>R</b> SS <b>V</b> L <b>S</b> AG <b>S</b> V <b>EL</b> CS <b>AP</b> GR <b>Q</b> D <b>V</b> Y <b>AV</b> G <b>S</b> GP <b>S</b> GA <b>IG</b> LV-----		-----		-----					145
zfER	DQGS <b>F</b> GM <b>R</b> E <b>A</b> AP <b>F</b> Y <b>R</b> PN <b>S</b> DN <b>RR</b> HS <b>I</b> R <b>ER</b> MS <b>S</b> AN <b>E</b> K <b>G</b> SL--SMEST-----		-----		-----					171
cER	EQGS <b>F</b> GM <b>R</b> E <b>A</b> AP <b>F</b> Y <b>R</b> PN <b>S</b> DN <b>RR</b> HS <b>I</b> R <b>ER</b> MS <b>S</b> T <b>N</b> E <b>K</b> G <b>SL</b> --SMEST-----		-----		-----					173
xER	EQGT <b>F</b> AV <b>R</b> E <b>A</b> AP <b>T</b> F <b>Y</b> R <b>S</b> SN <b>DR</b> Q <b>S</b> GR <b>ER</b> MS <b>S</b> AN <b>E</b> K <b>G</b> PP--SMEST-----		-----		-----					174
sER	EP <b>S</b> G <b>V</b> AV <b>R</b> E <b>A</b> GP <b>P</b> Y <b>R</b> PN <b>S</b> DN <b>RR</b> Q <b>G</b> GR <b>ER</b> LA <b>ST</b> SD <b>K</b> GM <b>S</b> --AMESA-----		-----		-----					180
hERα	EP <b>S</b> G <b>V</b> AV <b>R</b> E <b>A</b> GP <b>P</b> Y <b>R</b> PN <b>S</b> DN <b>RR</b> Q <b>G</b> GR <b>ER</b> LA <b>ST</b> ND <b>K</b> GM <b>S</b> --AMESA-----		-----		-----					179
rERα	EP <b>S</b> AV <b>AV</b> RD <b>T</b> GP <b>P</b> Y <b>R</b> PN <b>S</b> DN <b>RR</b> Q <b>G</b> GR <b>ER</b> LA <b>ST</b> SS <b>S</b> E <b>K</b> GM <b>S</b> --IMESA-----		-----		-----					184



**Figure 5** Northern blot analysis of seabream ER $\alpha$  and ER $\beta$ . Liver (Li) and ovary (Ov) poly(A)<sup>+</sup> mRNA (5  $\mu$ g) were probed with a 341 bp cDNA fragment encoding sbER $\alpha$  and full-length sbER $\beta$ .

amino acid sequence differed by only five amino acids from a recently published sbER sequence, below designated sbER $\alpha$ 2 (Munoz-Cueto *et al.* 1999). Sequence identities (see Table 1) were also highest with other teleost ERs and ER $\alpha$  from tetrapod species. Lowest identities were found with tetrapod ER $\beta$  and the teleost eER, ntER $\beta$ , gfER $\beta$  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 ER $\alpha$  and ER $\beta$  analyzed by RT-PCR.  $\beta$ -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, ntER $\beta$ , gfER $\beta$  and tetrapod ER $\beta$  and less with tetrapod ER $\alpha$  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, ntER $\beta$ , gfER $\beta$  and Q45 appear to be more related to tetrapod ER $\beta$ , while the other fish ERs, including Z22, appear to be more related to tetrapod ER $\alpha$ . 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, ntER $\beta$ , gfER $\beta$  were  $\beta$  subtype ERs (designated sbER $\beta$ ) and clone Z22 (designated sbER $\alpha$ 1) and the remaining fish ERs were of the  $\alpha$  subtype.

The size of the deduced ER protein obtained from the various fish and tetrapod cDNA sequences is variable (Table 1). In tetrapod ER $\alpha$  it varies from 586 (*Xenopus*) to 600 (rat) and in fish ER $\alpha$  from 574 (rainbow trout short form) to 622 (rainbow trout long form). Tetrapod ER $\beta$  varies from 549 (mouse, rat) to 589 (zebra finch) and fish ER $\beta$  from 557 (Nile tilapia) to 573 (Japanese eel). ER $\beta$  is generally shorter than ER $\alpha$  (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 ER $\alpha$ , 171–184 in tetrapod ER $\alpha$ , 149–165 in fish ER $\beta$  and 143–162 in tetrapod ER $\beta$  (excluding the partial clones of jqER $\beta$  and sbER $\alpha$ 1). Clearly the largest differences are found in teleost ER $\alpha$  and this may be explained by the recent identification of short and long forms of ER $\alpha$  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 sbER $\alpha$ 1 differs from sbER $\alpha$ 2 (Munoz-Cueto *et al.* 1999) by five amino acids in the A/B domain (Fig. 4). sbER $\alpha$ 2 has Gln<sup>83</sup> (equivalent to Gln<sup>122</sup> in hER $\alpha$ ) instead of His (present in all other ERs), Ala-Asn<sup>85</sup> instead of Pro-Thr and lacks Arg-Ser after Tyr<sup>98</sup>, indicating that multiple variants, differing in the A/B domain, of ER $\alpha$  also occur in seabream. Two variants of ER $\alpha$  have also been identified in catfish (Xia *et al.* 1999). A number of ER $\alpha$  and  $\beta$  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 gfER $\beta$  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 (ER $\alpha$  81–83 and 250–251 amino acids; ER $\beta$  83–91 and 246–249 amino acids). However, despite small variations, tetrapod ER $\beta$  has the shortest D domain, 25–29 amino acids compared with 31–33 for fish ER $\beta$ , 36–39 in tetrapod ER $\alpha$  and 42–45 amino acids in fish ER $\alpha$ . The largest F domains are found in the  $\alpha$  receptor subtype (57–77 for fish ER $\alpha$ , 42–43 for tetrapod ER $\alpha$ , 36–45 for fish ER $\beta$  and 18–28 for tetrapod ER $\beta$ ) 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<sub>2</sub>-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  $\delta$  isoform (not PKC  $\alpha$  or  $\epsilon$ ) in the AF1 region participate in the signaling pathways that lead to estrogen receptor phosphorylation (Lahooti *et al.* 1998).

In hER $\alpha$ , five phosphorylation sites have been mapped, four of which are in the A/B domain (Ser<sup>104</sup>, Ser<sup>106</sup>, Ser<sup>118</sup> and Ser<sup>167</sup>). Ser<sup>118</sup> and Ser<sup>167</sup> 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.* 1995b, Kato *et al.* 1995) and the second by casein kinase II (Arnold *et al.* 1995a). In mER $\alpha$  the corresponding Sers identified in hER $\alpha$  are phosphorylated and two additional sites, Ser<sup>156</sup> and Ser<sup>158</sup>, 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 ER $\alpha$ , but not in ER $\beta$  (Fig. 4). However, the serine residue in mouse ER $\beta$  located in the corresponding ER $\alpha$  consensus MAPK phosphorylation site can also be phosphorylated by MAPK (Tremblay *et al.*

1997). In sbER $\beta$ , ntER $\beta$  and gfER $\beta$  the sequences corresponding to hER $\alpha$  Ser<sup>118</sup>-Pro<sup>119</sup> are, respectively, Thr<sup>96</sup>-Thr<sup>97</sup>, Thr<sup>101</sup>-Pro<sup>102</sup> and Ser<sup>109</sup>-Ser<sup>110</sup> (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 ER $\beta$  and other tetrapod ER $\beta$ , or whether a ligand-independent transactivation function is absent or, if present, is activated by another mechanism.

The presence of several transcripts for both sbER $\alpha$  and sbER $\beta$  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 sbER $\alpha$ , 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 sbER $\alpha$ 1 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 ER $\alpha$  forms in the seabream is a consequence of alternative splicing. In the case of sbER $\beta$ , at least four mRNAs (6, 2.6, 0.5 and 0.3 kb) were detected in seabream ovary after hybridization with the full-length ER $\beta$  cDNA. In liver only the 0.3 kb transcript was detected. Only the two larger transcripts can potentially generate the entire coding sequence of sbER $\beta$ . 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 sbER $\alpha$  and sbER $\beta$  (Fig. 6) was different and sbER $\beta$  was widespread and had a generally higher level of expression than sbER $\alpha$ . The highest expression of sbER $\beta$  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 sbER $\beta$  could be detected. ER $\beta$  and ER $\alpha$  were co-expressed in testis, liver and heart. No signal of ER $\alpha$  was visible in any other tissues analyzed, but its presence cannot be excluded. In goldfish ER $\beta$  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.* 1998a,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 ER $\beta$  but further studies will be required to completely exclude the possibility that ER $\alpha$  is also present.

It is notable that in sea bream ER $\beta$  was clearly expressed in ovary and testis while ER $\alpha$  was most abundant in testis. This pattern of expression may indicate, in this species, a different function for each form of sER in male and female reproductive physiology. Recent data on  $\alpha\beta$ ERKO mice clearly show that only ER $\alpha$  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 ER $\beta$  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 ER $\alpha$  and ER $\beta$  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.

## Acknowledgements

This work was sponsored by NATO's Scientific Affairs Division in the framework of the Science for Stability Programme. Silvia Socorro was in receipt of a grant Praxis XXI BD/9241/96.

## 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, Jégo 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 1995a 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 1995b *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  $\beta$  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  $\beta$  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-17 $\beta$  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  $\alpha$  and  $\beta$ . *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, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M & Gustafsson JA 1997 Human estrogen receptor  $\beta$ -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- $\alpha$  gene 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 estrogen 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  $\beta$ . *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, Peltouhikko 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  $\alpha$  and  $\beta$ . *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  $\delta$ . *Journal of Molecular Endocrinology* **20** 245–259.
- Lakaye B, Foidart A, Grisar T & Balthazart J 1998 Partial cloning and distribution of estrogen receptor  $\beta$  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  $\beta 1$ ,  $\beta 2$ , and  $\beta 5$  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  $\beta$  mRNA variants in human and murine tissues. *Molecular and Cellular Endocrinology* **138** 199–203.
- Madigou T, Tifföche 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.
- Maryama K, Endoh H, Sasaki-Iwaoka H, Kanou H, Shimaya E, Hashimoto S, Kato S & Kawashima H 1998 A novel isoform of rat estrogen receptor  $\beta$  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 ER $\beta$ : 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 1998a Cross-inhibition of both estrogen receptor  $\alpha$  and  $\beta$  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 1998b The complete primary structure of human estrogen receptor  $\beta$  (hER $\beta$ ) and its heterodimerization with ER $\alpha$  *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  $\beta$  in rat bone. *Endocrinology* **138** 4509–4512.
- Osterlund M, Kuiper G, Gustafsson JA & Hurd YL 1998 Differential distribution and regulation of estrogen receptor- $\alpha$  and - $\beta$  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  $\beta$  binds DNA in a manner similar to and dimerizes with estrogen receptor  $\alpha$ . *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  $\beta$  forms estrogen response element-binding heterodimers with estrogen receptor  $\alpha$ . *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*)  $\beta$ -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  $\beta$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ) 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  $\beta$  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  $\beta$ . *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.

Received 20 January 2000

Accepted 29 March 2000