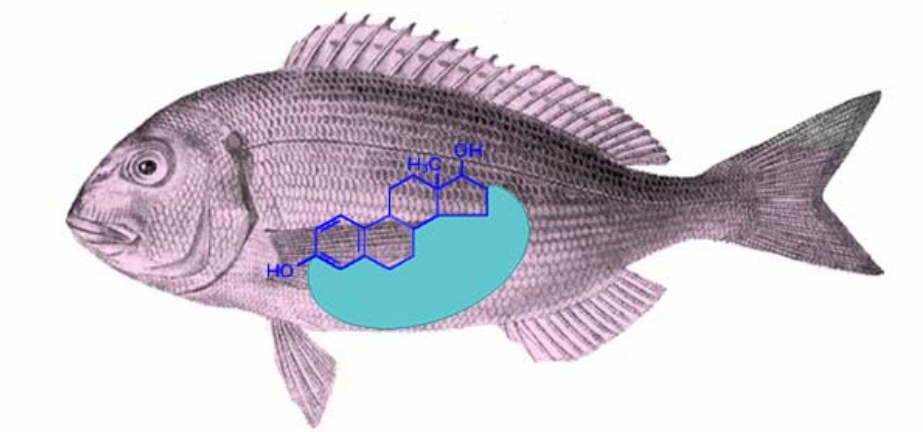
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências do Mar e do Ambiente

Diversity, expression and mechanism of action of estrogen receptors in sea bream, *Sparus auratus*

(Tese para a obtenção do grau de doutor no ramo de Biologia, especialidade de Biologia Molecular)



Patrícia Isabel Silvestre Pinto

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DATA: Janeiro 2006

TÍTULO DA TESE: Diversidade, expressão e mecanismo de acção dos receptores de estrogénio na dourada, *Sparus auratus*

RESUMO

A maioria das funções estrogénicas em vertebrados é mediada por dois receptores de estrogénio (ERα e ERβ), mas em peixes teleósteos existem dois genes ERβ.

Nesta tese, foi clonado um ERβ (sbERβb) na dourada (*Sparus auratus*), um peixe teleósteo hermafrodita, para além dos previamente clonados sbERα e sbERβa. O sbERβb liga com elevada especificidade e afinidade a agonistas e antagonistas estrogénicos. Os três genes (sbERα, βa, βb) têm uma distribuição parcialmente diferente em tecidos de dourada macho e fêmea e produzem múltiplos transcritos. Foram identificadas duas variantes de sbERα sem exão2, que têm diferente distribuição tecidular e regulação hormonal.

O estradiol-17β aumentou a expressão do sbERα e diminuiu a dos sbERβ no fígado, sugerindo um papel importante para o ERα na vitelogénese. Identificaram-se efeitos agonistas para o "antiestrogénio puro" ICI182,780 em várias respostas estrogénicas, provavelmente mediados por um aumento da expressão do sbERα no fígado. A imunolocalização das proteínas sbER em escamas de dourada sugeriu que os efeitos do estradiol na mobilização do cálcio ocorrem por acção directa nos osteoclastos. Usando hibridação subtractiva seguida de RT-PCR, demonstrou-se pela primeira vez no testículo de um peixe o aumento da expressão com estradiol de vários genes tipicamente responsivos em fígado (ex. vitelogeninas e coriogeninas).

PALAVRAS CHAVE:
Estradiol, receptor de estrogénios, peixe teleósteo, transcritos múltiplos, genes responsivos a estrogénios, antiestrogénio, disrupção endócrina.

THESIS TITLE: Diversity, expression and mechanism of action of estrogen receptors in sea bream, *Sparus auratus*

ABSTRACT

Two estrogen receptor subtypes (ERα and ERβ) mediate most estrogen actions in vertebrates, including fishes. Furthermore in teleost fishes two ERβ genes are differentially expressed. In this thesis, an ERβ (sbERβb) was cloned from the hermaphrodite teleost fish sea bream (*Sparus auratus*) which added to the previously cloned sbERα and sbERβa. sbERβb specifically binds estrogen agonists and antagonists with high affinity. The three sbER genes (α, βa, βb) have a partially overlapping but differential distribution in male and female sea bream tissues and produce multiple transcripts. Two exon2 deleted sbERα variants were also identified with different tissue distribution and hormonal regulation.

Estradiol-17β up-regulated the expression of sbERα and down-regulated both sbERβs in liver, suggesting a major role for ERα in vitellogenesis. Agonistic effects were identified for the "pure antiestrogen" ICI 182,780 in several estrogenic responses, probably mediated by sbERα up regulation in liver. The immunolocalization of sbER proteins in sea bream scales suggested that the calcium mobilising actions of E_2 in scales are via a direct action on osteoclasts. Subtractive hybridization followed by RT-PCR demonstrated for the first time in fish testis the E_2 up regulation of some typical liver E_2-induced genes (e.g. vitellogenins and choriogenins).

KEY-WORDS:

Estradiol, estrogen receptor, teleost fish, multiple transcripts, estrogen-responsive gene, antiestrogen, endocrine-disruption

LIST OF PUBLICATIONS, COMMUNICATIONS AND SEQUENCE SUBMISSIONS

Communications in meetings

Pinto, P.I.S., Passos, A.L., Power, D.M., Canário, A.V.M. "Cloning, expression and functional characterization of a second estrogen receptor beta in sea bream (*Sparus auratus*)"; Oral communication, 22nd Conference of European Comparative Endocrinologists (CECE), August 2004, Uppsala, Sweden.

Pinto, P.I.S., Sweeney, G.E., Canário, A.V.M. "Estrogen-responsive genes from sea bream (*Sparus auratus*) testis"; Oral communication, 5th International Symposium on Fish Endocrinology, September 2004, Castellon, Spain.

Pinto, P.I.S., Estêvão, M.D., Redruello, B., Power, D.M., Canário, A.V.M. "Distribution and cellular localization of sea bream estrogen receptors in calcified tissues"; poster communication, V Congresso da Associação Ibérica de Endocrinologia Comparativa, September 2005, Faro, Portugal.

Articles in international refereed journals

Pinto, P., A. L. Passos, D. M. Power and A. V. Canario (2005). Sea bream (*Sparus auratus*) estrogen receptors: phylogeny and tissue distribution. *Annals of the New York Academy of Sciences* **1040**: 436-438.

Pinto, P. I., A. L. Passos, R. S. Martins, D. M. Power and A. V. Canario (2006a). Characterization of estrogen receptor βb in sea bream (*Sparus auratus*): phylogeny, ligand-binding, and comparative analysis of expression. *General and Comparative Endocrinology* **145**(2): 197-207.

Pinto, P. I., H. R. Teodosio, M. Galay-Burgos, D. M. Power, G. E. Sweeney and A. V. Canario (2006b). Identification of estrogen-responsive genes in the testis of sea bream (*Sparus auratus*) using suppression subtractive hybridization. *Molecular Reproduction and Development* **73**(3): 318-329.

Conferences papers

Pinto, P. Passos, A.L., Power, D.M. and Canario, A.V.M. Cloning, expression and functional characterisation of a second estrogen receptor beta in sea bream (*Sparus auratus*). *Uppsala Journal of Medical Sciences* suppl 56, p79, 2004.

Submissions to nucleotide sequence databases

The sea bream ERβb cDNAs isolated in this thesis were submitted to the EMBL database with the following accession numbers: AJ580048 (clone 21), AJ580049 (clone 32) and AJ580050 (clone 54).
All expressed sequence tags isolated from the E_2-treated sea bream testis Subtractive Library were submitted to the EST database (dbEST) at NCBI with consecutive accession numbers from CX734847 to CX735033. The vitellogenin receptor EST cloned by PCR was submitted to the Genbank database with accession no. AY970973.

ACKNOWLEDGEMENTS

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Dedico esta dissertação ao Sr. Penisga, meu pai no céu.
Gostaria de lha ter podido entregar em mão.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

11KT	11-ketotestosterone
17,20βP	17,20β-dihydroxy-4-pregnen-3-one
18S	18S ribosomal RNA
Abs_x	Absorbance at x nanometres
AF-1, AF-2	(Trans) activation function 1 or 2
AI	Aromatase inhibitor
ANOVA	Analysis of variance
B_{max}	Maximum number of binding sites
bp	Base pairs
BPG axis	Brain-pituitary-gonads axis
bw	Body weight
cAMP	cyclic AMP
cDNA	Complementary DNA
Chg	Choriogenin
cm, mm, µm, nm	Centimetre, millimetre, micrometer, nanometre
CNS	Central nervous system
DAB	3,3-diaminobenzidine hydrochloride
DBD	DNA-binding domain
DEPC	Diethylpyrocarbonate
DES	Diethylstilbestrol
dH_2O, ddH_2O	Distilled water, double-distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E_1	Estrone
E_2	Estradiol-17β
EDC	Endocrine-disrupting compound
EDTA	Ethylenediaminetetraacetic Acid
EE_2	Ethynylestradiol
EF-1α	Elongation factor-1 α
ER	Estrogen receptor
ERE	Estrogen-response element
ERG	Estrogen-responsive gene
ERK	Extracellular-regulated kinase (MAP kinase)
EST	Expressed-sequence tag
h, min, sec	Hour, minute, second
hER	Human estrogen receptor
HRT	Hormone-replacement therapy
i.p.	Intra-peritoneal
ICI	ICI 182,780
IGF / EFF	Insulin-like growth factor / Epidermal growth factor
IHC	Immunohistochemistry
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ISH	*In situ* hybridization
KAc	Potassium acetate
Kb	Kilo bases
***K*d**	Dissociation constant

kDa	Kilo Dalton
kg, g, mg, µg, ng, pg	Kilogram, gram, milligram, microgram, nanogram, picogram
KO	Knock-out
L, ml, µl	Litre, millilitre, microlitre
LBD	Ligand-binding domain
LH	Luteinizing hormone
M, µM, pM	Molar, micromolar, picomolar
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCE	Molecular and comparative endocrinology group
MCS	Multiple cloning site
$MgCl_2$	Magnesium chloride
MMLV-RT	Mouse Moloney murine leukemia virus reverse transcriptase
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
MW	Molecular weight
N, n	Number of cycles, number of individuals
NaAc	Sodium acetate
NLS	Nuclear localization signal
NO	Nitric oxide
NR	Nuclear receptor
ON	Overnight
ORF	Open reading frame
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pfu	Plaque-forming units
PMSF	Phenylmethylsulphonyl fluoride
Poly(A)$^+$ RNA	Polyadenylated RNA (mRNA)
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
rtRT	Rainbow trout estrogen receptor
sb	Sea bream
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulators
stH_2O	Sterile water
Ta	Annealing temperature
TF	Transcription factor
TRACP	Tartrate-resistant acid phosphatase
U	Units
UTR	Untranslated region
UV	Ultraviolet
Vg	Vitellogenin
w/v, v/v	Weight/volume, volume/volume
wt	Wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER 1

General Introduction

1.1. General overview

Estrogen hormones are best known as key regulators of the reproductive system in female vertebrates, but their role in male physiology and in numerous non-reproductive processes started to be unravelled in the last decade, especially in mammals.

Most estrogen actions are mediated by interaction of the hormone with specific nuclear receptors, the estrogen receptors (ERs), which regulate the transcription of many physiological important target genes. Two different ER genes (ERα and ERβ) have been identified in tetrapods, three in some fish species (ERα and two ERβ genes), as well as many transcript and protein variants that in some cases modulate the wild-type ER functions. The mechanisms of estrogen action revealed to be highly complex, resulting from the interplay between different ER forms (including membrane ERs) and variants with growth factors, intracellular signalling cascade components and other transcription factors. With the development of high-throughput expression analysis techniques, many target genes involved in estrogen-regulated processes have been identified, but many remain to be identified. Moreover, the integration of this information to elucidate the mechanisms of estrogen action is still far from finished.

In fish, estrogens are best known to regulate the reproductive cycle, sex differentiation and yolk accumulation during oogenesis, but there is also evidence for estrogen actions in other processes such as calcium metabolism. Moreover, many disruptive effects on fish physiology have been attributed to estrogenic compounds found in the environment. However, the information about the tissues of action of estrogens and endocrine-disrupting compounds and the mechanisms or genes involved is still scarce and is essential for a better understanding of estrogen functions in fish physiology.

The present introduction will aim to describe the current knowledge about estrogen and ER functions and mechanisms of action in vertebrates. It will start by a brief overview of

the endocrine system and the array of different types of hormones and receptors that arose from evolution. After, the estrogens structure, biosynthesis and main functions in mammals and fish will be described, followed by an overview of the estrogen mechanisms of action, from classical nuclear ER-mediated direct transcriptional effects to an array of recently characterized alternative pathways. Then, it will focus on the ER phylogeny, structure, diversity and tissue distribution, as well as an overview of their target genes. Finally, the fish model used in the present work, the sea bream, will be presented, followed by the aim and an outline of the thesis.

1.2. The endocrine system

1.2.1. Definitions and historical background

The term "hormone" (from the greek verb "hormoa", to excite) was first used by Ernest Starling in 1905 as "chemical messengers which are carried from the organ where they are produced to the organ which they affect by means of the blood stream, to coordinate the activities and growth of different parts of the body" (Henderson 2005). In the last 100 years, our knowledge about endocrinology, the science that studies hormones, evolved into the concept of a complex array of hundreds of hormones, interacting between them and with the nervous system components to control physiological processes.

Some corrections have now to be made to the classical "gland"-based definition, since (1) hormones may also be produced in other tissues, not specialized for hormone production; (2) may move by circulation in blood, other body fluids or by diffusion between cells and (3) may act in a distant organ in the body (endocrine action), locally in the same cell type which produced it (autocrine) or in neighbour cells of a different type (paracrine) (Bentley 1998). A broader definition that can be used is "A hormone is a chemical, non-nutrient, intercellular messenger carrying information between two or more cells" (Bolander 1989).

In parallel with fundamental research, advances in Clinical Endocrinology identified several endocrine factors as responsible or predisposing for some important diseases such as some types of cancers, diabetes or osteoporosis, while the manipulation of hormone systems has lead to highly effective therapies (e.g. breast cancer treatments).

However, current knowledge of the endocrine system has largely been made possible by experiments on different animal species. Comparative Endocrinology, the discipline in which this work is integrated, concerns the study and comparison of the endocrine system in different vertebrate and invertebrate animal species, allowing the establishment of phylogenetic relationships between them and the evolution of the endocrine mechanisms themselves, as well as more applied applications such as exploring the diversity of molecule structure-biological activity relationships to find medically advantageous structural modifications. Moreover, the study of the endocrine system in a given species is, of course, primarily important to understand its physiology, and may be used in applied fields such as the control of reproduction (e.g. improvement of fish reproduction conditions in aquaculture) (Bentley 1998).

While in most part of the 20th century investigations concentrated on the identification of hormones and their physiological effects, the mechanisms of action have only started to be revealed in the last two decades. We are now at the stage of the "pathway biology", which includes mapping the cellular receptor(s), downstream signalling pathways and changes in gene expression or protein activities through which hormones act on cellular targets (Hillier 2005). This is the realm of this work.

1.2.2. General mechanisms of hormone action

The general mechanisms of hormone synthesis, release, transport and action are apparently similar between all vertebrates (Bentley 1998). Endocrine cells synthesize and release the hormone at appropriate occasions, upon the recognition of a stimulus. This may be an electrical signal or a chemical substance, such as a neurotransmitter, nutrient or other hormone, which signal for alterations in the external environment (e.g. temperature, social alterations) or inside the body (e.g. changes in nutrient or ion levels in body fluids). The released hormone travels in the circulation (or in the extracellular space) to their various effectors sites or target cells, where it is recognized by specific receptors located in the plasma membrane, cytoplasm or nucleus. Hormone binding causes a perturbation in the receptor (conformational change, phosphorylation or release of associated proteins), which is usually followed by a cascade of intracellular reactions or events that culminate in the hormone's physiological effect. This effect may be the result of changes in the activity of enzymes, alterations in ion levels within the cell or in particular intracellular compartments or changes in the transcription of responsive-genes, among others.

1.2.3. Types of hormones and receptors

Vertebrate hormones generally fall into two principal classes of chemical compound (Bentley 1998). Lipid hormones include fatty acid derivatives (e.g. prostaglandins) and the large group of steroid hormones, which include the estrogens. The other major group includes hormones made up by amino acids, ranging in complexity from single modified amino acids (e.g. thyroid hormones) to proteins composed of hundreds of amino acids (e.g. prolactin).

The structural and chemical properties of each hormone, especially its size and solubility, greatly determine which of the two major types of hormone receptor it binds and which intracellular signalling pathways are activated (Bentley 1998; Lodish et al. 2000). In

general, small lipophilic molecules (e.g. steroid or thyroid hormones) diffuse across the plasma membrane and bind to intracellular receptors in the nucleus or cytoplasm, which directly bind to DNA and affect transcription of their target genes (nuclear receptors, section 1.2.4). Water-soluble hormones (e.g. protein hormones and hydrophilic amino acid derivatives) cannot cross the plasma membrane and act through binding at the cell-surface to transmembrane receptors, generating a signal within the cell that is mediated by second messenger molecules, the most important being cyclic AMP (cAMP), cyclic GMP (cGMP), diacylglycerol (DAG), inositol triphosphate (IP_3) and Ca^{2+} (Bentley 1998; Lodish et al. 2000). Changes in concentration in one or more second messenger trigger a cascade of intracellular signalling responses, generally involving the activation of multiple effector enzymes such as protein kinases, which lead to the hormone's physiological effect. According to their mechanism of action, cell-surface receptors are divided in four major groups (Lodish et al. 2000): ion channels (e.g. acetylcholine receptor), receptors with intrinsic enzymatic activity (e.g. the receptors for many growth factors, with kinase activity), receptors directly associated with cytosolic protein tyrosine kinases (e.g. interferon receptors) and the G protein-coupled receptors (GPCRs), which act by activating a range of accessory proteins (G proteins) that route the hormonal signal to distinct (but interacting) intracellular signalling pathways.

In most cases, membrane receptor-mediated effects result in the activation of ion channels or existing enzymes, constituting the so-called rapid, non-genomic actions when compared with the long-term genomic actions of nuclear receptors in the control of transcription. However, it is now known that membrane receptors also control transcription of responsive-genes, through transcription factor activation (Lodish et al. 2000; Neves et al. 2002), and nuclear receptors also mediate non-genomic actions by interacting with components of the intracellular signalling cascades (Aranda and Pascual 2001).

1.2.4. Nuclear receptors

The nuclear receptor (NR) super-family constitutes a large group of phylogenetic and structurally related transcription factors that regulate diverse physiological functions in animals, from development to reproduction, homeostasis and metabolism (Gronemeyer and Laudet 1995). It includes receptors for hydrophobic molecules such as thyroid hormones, steroid hormones (glucocorticoids, mineralocorticoids, androgens, estrogens, progesterone, vitamin D, ecdysone, oxysterols, bile acids), retinoic acids, fatty acids, leukotrienes and prostaglandins, as well as different "orphan" receptors for which no natural ligand has yet been identified (Aranda and Pascual 2001; Escriva et al. 2004).

NRs typically act as ligand-inducible transcription factors by directly interacting as dimers with specific hormone-response elements in the promoter of target genes. A typical NR consists of a variable N-terminal region, a highly conserved DNA-binding domain, a hinge region and a moderately conserved C-terminal ligand-binding domain (Aranda and Pascual 2001). The mechanisms of action and domain structure are further detailed for the ERs in sections 1.4 and 1.5.

NRs have been found in diverse invertebrate and vertebrate clades, ranging from sponges to humans (Escriva et al. 2004). This diversity has recently been organized in the NUREBASE database and classified using a phylogeny-based nomenclature, which placed the ERs (official nomenclature NR3A) and other steroid hormone receptors in subfamily 3, together with the (up to now) orphan receptors estrogen-related receptors, ERRs (Nuclear Receptors Committee 1999; Duarte et al. 2002; Ruau et al. 2004).

The evolution of the NR superfamily apparently occurred from a common ancestral and followed two waves of gene duplication: the first, very early during metazoan evolution, resulted in the diversification into the present six subfamilies while the second, specifically in vertebrates, produced the different paralogues within each subfamily (e.g.

ERα and ERβ, section 1.5) (Escriva et al. 2004). While DNA-binding and dimerization functions probably co-evolved with NR diversification, the ligand-binding abilities appear to have been independently acquired within different NR groups during metazoan evolution (Escriva et al. 2004).

1.3. Estrogens

1.3.1. Estrogens structure and biosynthetic pathway

17β-estradiol (E_2) is the most abundant and potent among the natural estrogens, which also include estrone and estriol (Bentley 1998). E_2 biosynthesis has been found throughout the vertebrates and recently also in some invertebrate species (e.g. Di Cosmo et al. 2001; e.g. Pernet and Anctil 2002; Osada et al. 2004).

Like other steroid hormones, estrogens are derived from cholesterol and its biosynthetic pathway involves a complex series of enzymatic reactions, mainly catalyzed by cytochrome P450 oxidases (CYPs) and hydroxysteroid dehydrogenases (Payne and Hales 2004). This pathway is common to both estrogens and the "male hormones" androgens, since estrone and E_2 are synthesized by aromatization of androstenedione and testosterone, respectively, catalyzed by the P450 aromatase enzyme (CYP19) (Figure 1.1). Thus, aromatase may affect some estrogen-regulated processes (e.g. sex differentiation, see section 1.3.2.2) through the control of the androgen: estrogen ratio.

In common with mammals, the major sources of estrogen in female teleost fish are the somatic cells (granulosa layer) surrounding the developing oocytes, under the control of the brain-pituitary-gonads axis (BPG). According to the "two-cell" model, the theca cell layer synthesize androgens, in response to pituitary gonadotrophins, and these are then aromatized to estrogens in the granulosa cells (Baird 1984; Nagahama et al. 1995). The synthesized E_2 may then act locally in the ovary (Rosenfeld et al. 2001), travel in the circulation (mainly bound to plasma steroid-binding proteins) to act as endocrine factor on

its diverse target tissues, or exert positive/negative feedback effects on the BPG axis (Bentley 1998).

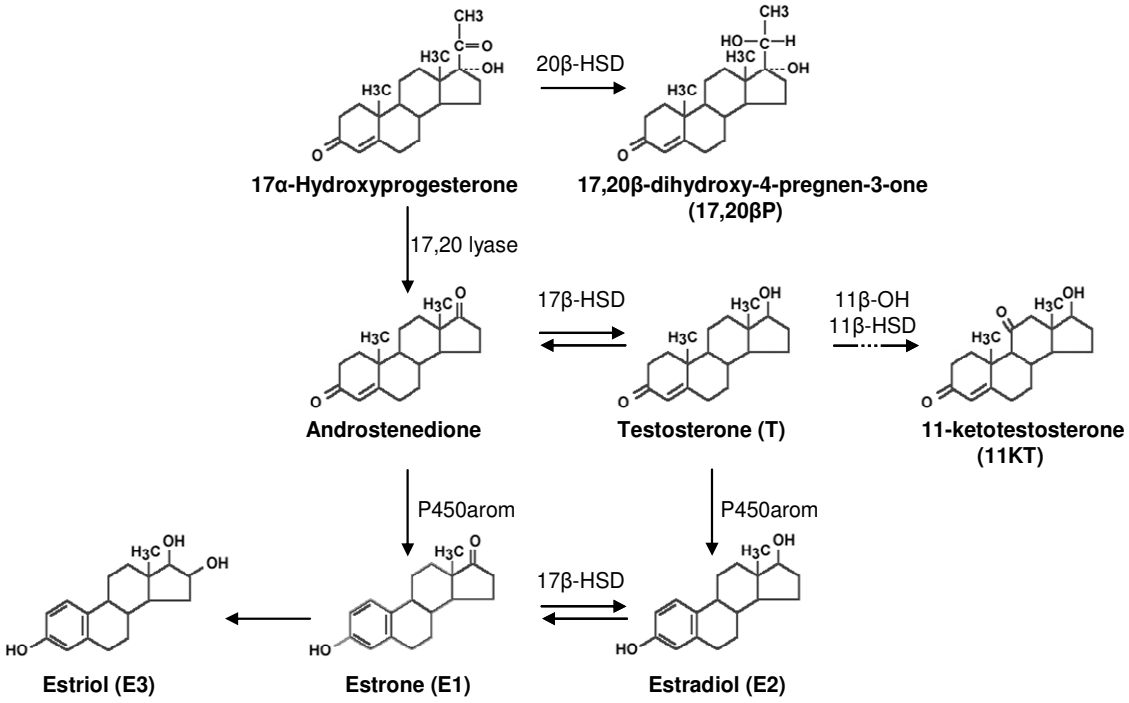

Figure 1.1. Final steps of androgen and estrogen biosynthesis in teleost fish, adapted from Borg (1994) and Bentley (1998). Enzyme abbreviations: 20β-HSD, 20β- hydroxysteroid dehydrogenase; 17,20lyase, 17,20 lyase (CYP17); 17β-HSD, 17β- hydroxysteroid dehydrogenase; P450arom, P450 aromatase (CYP19); 11β-OH, P450 11β-hydroxylase (CYP11B1); 11β-HSD, 11β- hydroxysteroid dehydrogenase.

In humans, aromatase is also expressed in a number of extra-gonadal sites, which become the major E_2 sources in postmenopausal women, including the mesenchymal cells of adipose tissue (including the breast), osteoblasts and chondrocytes of bone, the vascular endothelium, aortic smooth muscle cells and numerous sites in the brain, and in testis in men. The recognized E_2 actions in these tissues (section 1.3.2.1) appear to be mainly regulated by locally produced E_2 acting as paracrine or autocrine factor, rather than by circulating E_2 (Simpson 2003).

While tissue-specific expression of the aromatase gene (*cyp19*) in mammals is achieved through the use of three distinct promoters (Simpson 2003), teleost fish possess two

distinct aromatase genes, *cyp19a* and *cyp19b,* which are differentially regulated during development, in adult tissues and in response to E_2 (Callard et al. 2001). While aromatase (Aro) A is mainly expressed in the ovary, AroB is highly expressed and up regulated by E_2 in the teleost fish brain, which is characterized by exceptionally high levels of estrogen biosynthesis when comparing to the brain of other vertebrates. Since the high brain aromatase expression and activity is detected in particular regions of the forebrain where ERs are also expressed (telencephalon, preoptic area and hypothalamus), locally produced E_2 may directly act in the teleost fish brain, probably to mediate neurogenesis and to regulate reproductive functions (Callard et al. 2001; Pellegrini et al. 2005). Testicular synthesis of E_2 has been reported in several vertebrate species including mammals, amphibian and fish (Varriale et al. 1986; Fostier et al. 1987; Loomis and Thomas 1999; Carreau et al. 2003) and is probably responsible for its local actions in testicular physiology (see next section).

1.3.2. Estrogen biological functions

1.3.2.1. Mammals

Estrogens are best known for their essential role in the female reproductive system. In mammals, in particular, estrogens affect growth, differentiation and function of reproductive tissues such as the mammary gland, uterus, vagina and ovary, being required for female pubertal development, regulation of oestrous cycles, establishment and maintenance of pregnancy and lactation (Hewitt and Korach 2003; Barkhem et al. 2004). Although their proliferative effects in the uterine endometrium and mammary gland are required for embryo implantation or lactation, respectively, estrogens and hormones used in estrogen-replacement therapy may contribute to the etiology and progression of breast and endometrial cancer, in part by facilitating the proliferation of malignant cells (Henderson and Feigelson 1997; Colditz 1998; Archer 2004).

It is now evident that low levels of estrogens are also essential for normal male reproductive physiology, largely due to the demonstration that male fertility is impaired in mice lacking the ERα or the aromatase genes and in male patients with congenital aromatase deficiency (Couse and Korach 1999; Carreau et al. 2003). Estrogens primary function in the male reproductive tract appear to be the regulation of fluid resorption in the efferent ductules (Hess 2003), while a role in the control of testis development, spermatogenesis and steroidogenesis is also supported (O'Donnell et al. 2001; Carreau et al. 2003; Saunders 2005). In the prostate, estrogen appears to suppress proliferation and promote differentiation of the prostatic epithelium, and to have a modulatory effect on prostate cancer development and growth (Risbridger et al. 2003; Bradlow and Sepkovic 2004; Imamov et al. 2005).

In addition to their roles in reproduction, estrogens also exert a number of non-reproductive roles. Their role in the development and maintenance of the skeleton in both sexes is well recognized and estrogen-replacement therapy was conclusively shown to reduce osteoporosis in postmenopausal women (Rossouw et al. 2002; Liu and Muse 2005). Osteoporosis and bone growth abnormalities were also reduced by estrogen administration to both male and female patients with aromatase deficiency (Riggs et al. 2002; Barkhem et al. 2004; Syed and Khosla 2005). The molecular mechanisms by which estrogen exert its protective effects in bone include both genomic and non-genomic actions, which collectively result in the inhibition of bone remodelling, the inhibition of bone resorption and positive effects on bone formation (Riggs 2000; Syed and Khosla 2005). During puberty, estrogen, together with growth hormone and insulin-like growth factor-I (IGF-I) initiates the pubertal growth spurt in both sexes (Riggs et al. 2002).

In the cardiovascular system, estrogens exert both rapid and long-term effects that result on protection of the blood vessels against injury and atherosclerosis (Brown et al. 2000;

Barkhem et al. 2004; Edwards 2005; Hisamoto and Bender 2005). These effects include the increased production of the potent vasodilator molecule nitric oxide (NO) in cardiac miocytes and endothelial cells, decreased production of endogenous vasoconstrictors, inhibition of vascular smooth muscle cell proliferation in response to arterial injury, among others (Edwards 2005; Hisamoto and Bender 2005). Estrogens also exert indirect protective effects on the vasculature through the regulation of liver-specific gene expression, resulting in decreased total serum cholesterol and improved HDL:LDL cholesterol ratio (Brown et al. 2000; Barkhem et al. 2004). Although molecular and animal studies support estrogen protective roles against atherosclerosis and cardiovascular disease (CVD), in agreement with epidemiological studies reporting higher incidence of CVD in men and postmenopausal women when compared to premenopausal women, recent large randomized clinical trials failed to demonstrate a benefit of hormone (estrogen plus progestin)-replacement therapy (HRT) on CVD prevention in postmenopausal women. Additional clinical trials are required and are being performed to determine the optimal timing, dose and hormone composition for optimal therapeutic interventions (reviewed in Barkhem et al. 2004; Dubey et al. 2005; Hisamoto and Bender 2005).

In the central nervous system (CNS), estrogen controls reproductive functions through the brain-pituitary-gonads axis and affects libido and sex behaviour in both sexes (Shupnik 1996; Shupnik and Schreihofer 1997; O'Donnell et al. 2001; Lange et al. 2002). In the past decade, results from experimental and clinical studies have indicated that estrogens may also influence memory mechanisms, cognition, thermoregulation, postural stability, fine motor skills, mood, and affectivity (Maggi et al. 2004). In addition, a beneficial role of estrogens in selected brain pathologies such as multiple sclerosis, Alzheimer's disease and Parkinson's tremors have been supported by clinical and epidemiological studies, particularly in women, although this was not consistently confirmed by controlled

randomized clinical trials of HRT (reviewed by Barkhem et al. 2004; Maggi et al. 2004). Nevertheless, studies on molecular and animal models strongly support the estrogens neurotropic and neuroprotective roles against neurodegeneration and brain injury, and their ability to modulate the activity of all types of neural cells trough a multiplicity of mechanisms, leading to multiple reproductive and non-reproductive functions in the CNS (reviewed by Maggi et al. 2004).

The generation of knockout mice (KO) lacking one or both estrogen receptor genes (ERαKO, ERβKO or ERαβKO) or the aromatase gene (ArKO), together with natural models of ER/aromatase insufficiency, allowed to confirm the estrogen functions in certain tissues and to identify new functions. Moreover, these models lead to tremendous progress in elucidating the mechanisms involved in each action, the relative contribution of each ER subtype and in identifying differential responses between sexes (Barkhem et al. 2004; Hewitt et al. 2005; Simpson et al. 2005).

Some of the interesting phenotypes of ERKO mice include: incompletely differentiated epithelium in reproductive tissues (prostate, ovary, mammary gland); infertility of both male and female ERαKO and subfertility of female ERβKO; alterations in gonadotrophin levels in both sexes; decreased bone formation and bone volume in female ERαβKO; abolishment of NO production by endothelial vascular cells; disturbances in sexual behavior in male and female ERαKO; altered learning ability and social activities in male and female ERβKO; obesity and insulin resistance, among others (for reviews, consult Couse et al. 2001; O'Donnell et al. 2001; Rissman et al. 2002; Hewitt and Korach 2003; Barkhem et al. 2004; Maggi et al. 2004; Hewitt et al. 2005; Imamov et al. 2005; Simpson et al. 2005; and Syed and Khosla 2005). Many of these effects are also present in the ArKO mice (e.g. infertility, lack of sexual behavior and loss of bone mass in both sexes)

that also develops a metabolic syndrome with insulin resistance and obesity, supporting an important role for estrogens also in energy homeostasis (Simpson et al. 2005).

1.3.2.2. Fish

In teleost fish, like in mammals, E_2 plays an important role in regulating the female reproductive system, development of secondary sex characteristics and sex behaviour. In addition, in most fish and other oviparous animals E_2 has an essential role in regulating the synthesis of proteins required for oocyte growth (vitellogenesis and zonagenesis), as well as on prenatal development and sexual differentiation (Fairbrother 2000; Lange et al. 2002; Jalabert 2005).

Vitellogenesis is the period of large ovarian growth, during which the production of egg yolk precursors, vitellogenins (Vgs), is induced in the liver by direct action of E_2 synthesised in the ovary in response to gonadotrophins (Figure 1.2) (Nagahama et al. 1995; Arukwe and Goksøyr 2003). Vgs are transported in the blood, sequestered by Vg receptor-mediated endocytosis into growing ovarian follicles (Davail et al. 1998; Li et al. 2003a; Hiramatsu et al. 2004) and cleaved by cysteine proteinases into the egg yolk proteins lipovitellin and phosvitin (Carnevali et al. 1999; Fabra and Cerda 2004), providing the main source of nutrients for the developing embryo.

During the same period, liver-specific egg shell proteins (designated choriogenins, Chgs, to distinguish them from the oocyte-specific forms, zona pellucida proteins, ZP) are also produced in response of E_2, transported in the blood and deposited around the developing oocyte, forming the major part of the teleost egg shell that is responsible for egg protection and proper fertilization. This E_2-induced production of additional egg shell proteins in liver (zonagenesis) is specific to teleost fish and birds, which have thicker egg shells than mammals (Arukwe and Goksøyr 2003; Kanamori et al. 2003).

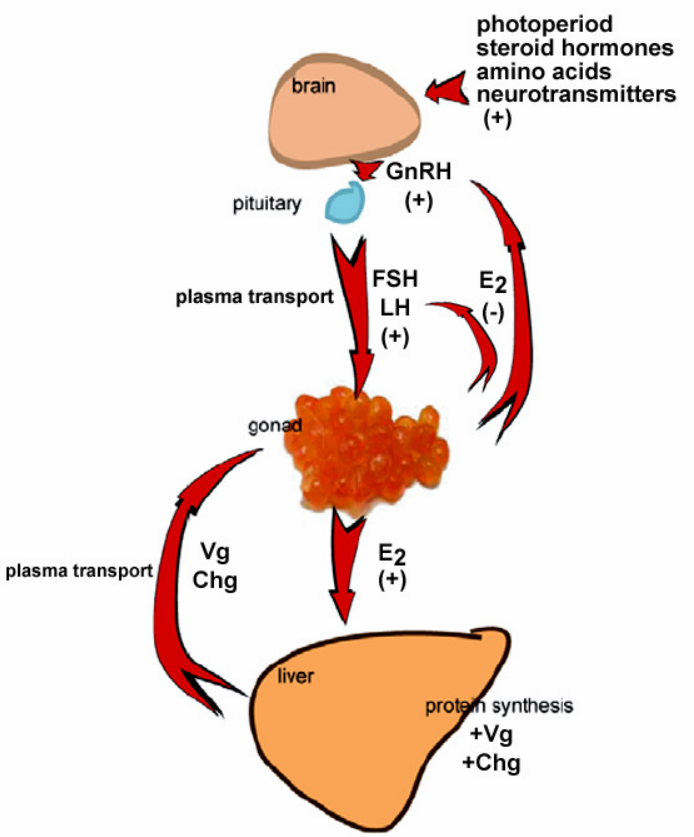

Figure 1.2. Schematic representation of the brain-pituitary-gonads (BPG) axis during the oocyte growth phase in female teleosts. (+/-) indicate stimulatory/inhibitory effects. E_2 is synthesized in the follicular cells of the ovary in response to gonadotrophin stimulation (follicle stimulating hormone, FSH and luteinizing hormone, LH), enters the blood circulation and acts on the liver, inducing the synthesis and secretion of egg yolk precursors, vitellogenins (Vg) and egg shell proteins, choriogenins (Chg). These proteins are transported in the plasma and incorporated in the growing oocyte follicles in the ovary. The production of gonadotrophins in the pituitary and their releasing factors (GnRH) in the brain are regulated through the positive/negative feedback mechanisms of E_2 (negative at this phase). Adapted from Arukwe and Goksøyr (2003).

E_2 controls vitellogenesis and zonagenesis at the transcriptional level, demonstrated *in vivo* and *in vitro* for numerous species (e.g. Celius et al. 2000; Takemura and Kim 2001; Lee et al. 2002), extended to the post-transcriptional level in the case of the Vg mRNA that is stabilized by E_2 (Flouriot et al. 1996). This response is accompanied by the self-regulation of ER mRNAs by E_2, increasing cell sensitivity to E_2 and boosting Vg/Chg expression to much higher levels in a second wave of transcription (Pakdel et al. 1991; Bowman et al. 2002; Mosconi et al. 2002; Sabo-Attwood et al. 2004).

In general, E_2 plasma levels in fish are low in the previtellogenic phase, increase continuously during the vitellogenic phase, accompanied by Vg protein plasma levels, and drop simultaneously with the rise of the maturation-inducing steroid 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), remaining low through spawning (reviewed in Fostier et al. 1983; Nagahama 1994; Jobling 1995; Nagahama et al. 1995). The same profile was observed for the liver levels of vitellogenin and different ER isoform mRNAs, further pointing for their involvement in the control of vitellogenesis (Sabo-Attwood et al. 2004).

As in mammals, in fish E_2 indirectly affects many reproductive functions though negative and positive feedback mechanisms on the brain-pituitary-gonads (BPG) axis. E_2 feedback in fish appears to modulate the release of both gonadotrophin releasing hormones (GnRHs) and gonadotrophins (GTHs) (Figure 1.2), as well as the pituitary GnRH responsiveness (Redding and Patiño 1993; Trudeau 1997; Melamed et al. 1998). However, feedback regulations in fish vary with the reproductive stage and with the species (Schmitz et al. 2005). While in immature or sexually regressed fish the feedback is normally positive, leading to GTH release and onset of gonadal maturation or recrudescence, during late stages of gonadal recrudescence E_2 switches to a negative feedback (Figure 1.2) as shown by gonadectomy and estrogen replacement in several fish species (Weil and Marcuzzi 1990; Redding and Patiño 1993; Khan et al. 1999).

Compared to mammals, the mechanisms of sex differentiation in teleost fish are complex, diverse, and may be influenced in most species by environmental factors or exogenous steroid exposure, and this plasticity has been explored to manipulate fish sex in aquaculture (Baroiller and D'Cotta 2001; Piferrer 2001; Nagahama et al. 2004). Moreover, fish reproductive strategies are diverse and reflect numerous adaptations to a large variety of aquatic environments (Jalabert 2005). E_2 is now known to play a critical role in ovarian

differentiation and in sex change of adult fish from different sexuality types (see below) (Devlin and Nagahama 2002; Nagahama et al. 2004).

In a wide range of gonochoristic species, exogenous E_2 administration during the sensitive period (just prior or during the gonadal sex differentiation) induced feminization of the population (Baroiller and D'Cotta 2001; Piferrer 2001). This was further confirmed in genetically manipulated all-male (XY) populations, in several fish species possessing a XX-XY heterogametic sex determination, whereas the inhibition of E_2 synthesis at the labile period, using aromatase inhibitors (AI), resulted in complete sex reversal of all-female (XX) populations to functional males. This masculinizing effect was counteracted by co-administration of E_2, supporting the importance of aromatase and E_2 in ovarian differentiation but also the importance of their absence for testicular development (Piferrer et al. 1994; Guiguen et al. 1999; Kitano et al. 2000; Kobayashi et al. 2003; Nakamura et al. 2003).

Moreover, E_2 presence or absence, respectively, appears to be important for the natural process of sex change in adult protandrous (which mature first as males and then sex change to female) and protogynous hermaphrodite species (female-to-male sex change). In several protandrous species, including the sea bream, E_2 administration caused sex reversal of differentiated male fish to functional females (Happe and Zohar 1988; Chang et al. 1995a; Ruan et al. 1996; Condeca and Canario 1999; Lee et al. 2001; Wu et al. 2005). In the black porgy, *Acanthopagrus schlegeli*, the E_2-induced sex change was accompanied by high gonadal and brain aromatase activity and high plasma LH levels, while the administration of AI to 2-year-old male black porgy during one year suppressed brain aromatase activity and inhibited the natural sex change (Lee et al. 2001; Lee et al. 2004; Wu et al. 2005).

In some protogynous species, AI treatment induces a complete female-to-male sex change, blocked by co-treatment with E_2, which is consistent with the sudden drop in gonadal aromatase expression and E_2 plasma levels observed at the onset of the natural sex change (Bhandari et al. 2003; Nakamura et al. 2003; Bhandari et al. 2004; Bhandari et al. 2005). Moreover, some fish species from the Gobiidae family are able to change sex repeatedly in both directions, and manipulation of E_2 levels via the aromatase pathway was able to induce adult sex change in each direction, further suggesting that aromatase may be the key enzyme regulating sex differentiation (Kroon et al. 2005).

Although sex manipulation in gonochoristic species is usually only successful at the sensitive period of sex differentiation, there are some reports of E_2-induced feminization in differentiated males (Mori et al. 1995; Andersen et al. 1996) and of AI-induced sex change in differentiated females (Nakamura et al. 2003), showing that the differentiated gonads of gonochoristic species still have some sexual bipotency. However, in most cases E_2 administration after sex differentiation causes deleterious effects in testicular structure and functions, similar to the feminizing effects observed when fish are exposed to environmental estrogenic contaminants (xenoestrogens) (Piferrer 2001; Devlin and Nagahama 2002; Nagahama et al. 2004). The lability of the sex differentiation systems in fish make them very sensitive to exposure to these endocrine disrupting compounds (EDCs), which are able to mimic or inhibit estrogen actions (Nagahama et al. 2004). Xenoestrogen exposure in fish was shown to cause the permanent disruption of male fish gonad ducts, incomplete sex change (ovotestis), inhibition of testicular growth and Vg or Chg hepatic synthesis, which have been used as endocrine disruption biomarkers (Sumpter and Jobling 1995; Jobling et al. 1996; Jobling 1998; Celius et al. 2000).

Positive effects of E_2 in testis have also been observed, especially when used at lower doses, suggesting a role for E_2 in normal fish testis physiology. In black porgy, low doses of E_2 stimulated spermatogenesis in male fish, while a higher dose caused sex change (Chang et al. 1995b). E_2 appear to regulate mainly the early mitotic spermatogenic stage, promoting *in vivo* and *in vitro* spermatogonial stem cell renewal in the testis of Japanese huchen (*Hucho perryi*) and Japanese eel (*Anguilla japonica*) but with only slight effects on spermatogonial proliferation, which was mainly promoted by the androgen 11-ketotestosterone (11KT) (Miura et al. 1999; Amer et al. 2001; reviewed in Miura and Miura 2003). In agreement, natural E_2 plasma levels in male rainbow trout, *Oncorhynchus mykiss*, showed highest levels at the initiation of gonadal recrudescence, and *in vivo* AI treatment at this stage dramatically inhibited spermatogenesis (Le Gac et al. 2004).

A role in regulating the secretion of 17,20βP (responsible for sperm maturation) during spermiation has also been proposed, based on the decrease in E_2 plasma levels at this part of the reproductive cycle in trout and huchen, concomitant with the 17,20βP rise (Vizziano et al. 1996; Amer et al. 2001; Le Gac et al. 2004). Using *in vitro* treatment of trout testicular fragments, E_2 was able to significantly reduce 17,20βP secretion, especially in fragments at the spermiation stage (Vizziano et al. 1996). Moreover, E_2 appears to decrease (while AI increases) 11KT and testosterone plasma levels, *in vivo* or *in vitro*, in several fish species, while decreasing the expression of steroidogenic enzymes involved in androgen synthesis in the rainbow trout testis (Chang et al. 1995a; Loomis and Thomas 2000; Govoroun et al. 2001; Bhandari et al. 2004; Bhandari et al. 2005).

A role in testicular development is also supported since E_2 was shown to enhance interstitial cell proliferation in the immature rainbow trout testis (blocked by the estrogen antagonist ICI 182,780), before the beginning of spermatogenesis (Bouma et al. 2003).

Apart from the direct roles in reproductive functions and sex differentiation, E_2 is also involved in calcium metabolism in fish, although it appears to have a resorptive role, rather than the antiresorptive role it has in mammals. In fish, the elevated Vg levels at the vitellogenic stage of the reproductive season or in response to E_2 treatment are paralleled by increased plasma calcium and phosphate levels (Bromage et al. 1982; Norberg et al. 1989; Li et al. 1993; Guerreiro et al. 2002). This may be justified by an increased calcium demand, since Vgs are calcium binding-proteins (providing the main source of mineral for the embryo) and its synthesis does not occur in the absence of calcium (Yeo and Mugiya 1997). E_2 appears to regulate the increase of plasma calcium levels by different mechanisms, including the increase of whole body calcium uptake (Guerreiro et al. 2002); the calcium mobilisation from internal stores, especially the scales, where it appears to increase osteoclastic activity (Mugiya and Watabe 1977; Persson et al. 1995; Armour et al. 1997); and probably a decrease in mineralization, since E_2 was shown to down regulate osteonectin, a marker for mineralization, in scales (Lehane et al. 1999).

However, a recent paper also reports E_2-stimulated osteoblastic activity in goldfish regenerating scales, especially in the early reproductive stages, suggesting that at least in this species E_2 could affect both osteoblastic (mineralization) and osteoclastic (calcium mobilization) activities, depending on the reproductive stage (Yoshikubo et al. 2005).

Estrogens also appear to affect the cardiovascular system in fish, although its role is not consensual. Some studies report a relationship between E_2 levels and incidence and size of coronary lesions in fish (House et al. 1979; Christiansen et al. 1998), while in rainbow trout E_2 caused a rapid vasodilative response and reduced the vasoconstriction induced by acetylcholine, thus supporting a protective role like in mammals (Agnisola et al. 2004). Isolated reports also showed E_2 effects on several other processes including the metabolism of several substances (Wiegand and Peter 1980; Ng et al. 1984; Woo et al.

1993; Palace et al. 2001), thyroid activity (Leatherland 1985), smoltification (Ikuta et al. 1987), hearing sensitivity to vocal reproductive behaviours (Sisneros et al. 2004), olfactive response (Stacey and Sorensen 2002), among others.

1.4. Mechanisms of Estrogen actions

The classical model of estrogen action (Figure 1.3-1), which explains their long-term direct genomic effects, involves estrogen binding to ERs located in the nucleus (Shank and Paschal 2005), after which the ER become "activated" through a process involving its dissociation from protein chaperones (e.g. heat shock protein 90), conformational change(s), receptor dimerization and binding to specific estrogen-responsive elements (EREs) in the promoter of target genes (Nilsson et al. 2001; Bjornstrom and Sjoberg 2005; Edwards 2005). The DNA-bound receptors then alter the rate of transcription of target genes through the selective recruitment of a range of coregulator proteins (coactivator or corepressor) that, among other functions, interact with the basal transcription machinery (Kumar et al. 2004). A more detailed description about ligand or DNA binding and ER-mediated transactivation can be found in section 1.5.3.

However, it is now accepted that estrogens and/or ERs can regulate gene expression by a number of distinct mechanisms besides the classical pathway, best studied for E_2 in mammals, including indirect genomic actions, ligand-independent ER activation and non-genomic actions (Figure 1.3) (good reviews by Driggers and Segars 2002; Segars and Driggers 2002; Bjornstrom and Sjoberg 2005; Edwards 2005; Levin 2005).

In indirect genomic actions (or ERE-independent, see Figure 1.3-2), nuclear E_2/ER complexes associate with target promoters through protein-protein interactions with other transcription factors (TF), which then modulate gene transcription by binding their

cognate DNA-elements (Gottlicher et al. 1998; Kushner et al. 2000; Bjornstrom and Sjoberg 2005).

For instance, many genes are activated by E_2 through ER interaction with Jun/Fos TFs at AP-1 sites (e.g. the IGF-I gene; Bjornstrom and Sjoberg 2005), while other TFs activated by E_2/ER include Sp1, NF-κB, C/EBPβ and STAT (Gottlicher et al. 1998; Bjornstrom and Sjoberg 2005). The trans-repression (down regulation) of several genes also occur through interaction of ERs with other TFs at alternative response elements (e.g. Galien and Garcia 1997; Chu et al. 2004; e.g. Schmitz et al. 2005).

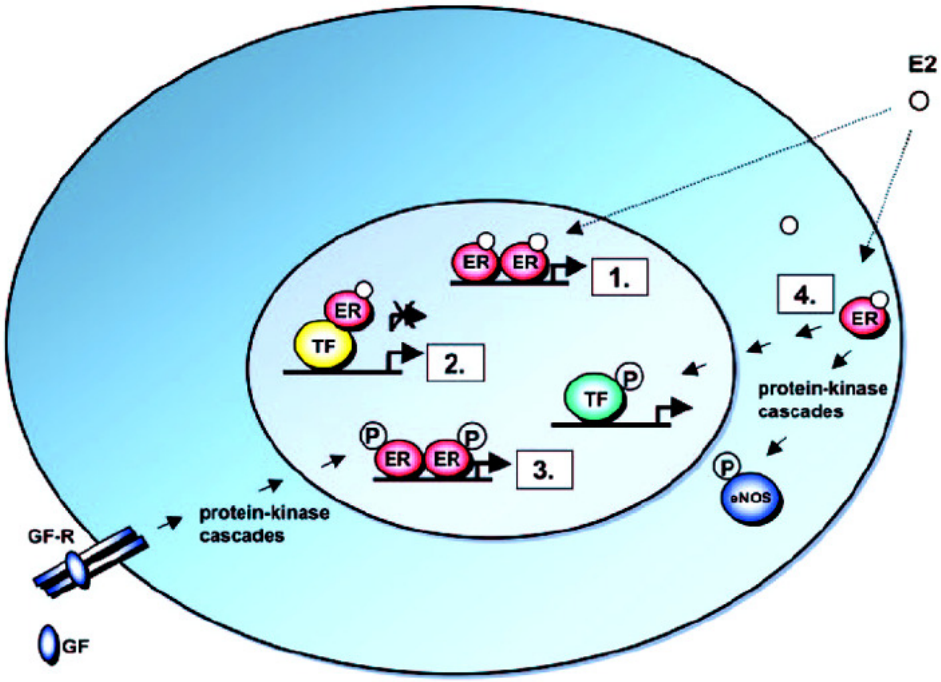

Figure 1.3. Representation of estrogen/ER signalling mechanisms. 1. Classical genomic mechanism, in which nuclear E_2-ER complexes bind directly to EREs in target gene promoters. 2. Indirect (ERE-independent) genomic actions, in which nuclear E_2-ER complexes indirectly influence transcription through protein-protein interactions to a transcription factor complex (TF) that contacts the target gene promoter. 3. Ligand-independent genomic actions, in which protein-kinase cascades are activated following binding of, for instance, a growth factor (GF) to its membrane receptor (GF-R), leading to phosphorylation (P) and activation of nuclear ERs at EREs. 4. Nongenomic actions, in which membrane-localized E_2-ER complexes activate protein-kinase cascades, leading to altered functions of proteins in the cytoplasm (e.g. activation of eNOS) or to regulation of gene expression through phosphorylation (P) and activation of a TF. From Bjornstrom and Sjoberg (2005).

Ligand-independent ER activation has been shown to occur in several cell types, *in vivo* and *in vitro*, in response to dopamine, growth factors (e.g. epidermal growth factor, EGF, and IGF-I), cytokines, protein kinase A (PKA) or C (PKC) activators and cell-cycle regulators (Smith 1998; Nilsson et al. 2001; Shupnik 2004; Olesen et al. 2005). These actions are mediated by cognate membrane receptor binding and activation of different cytoplasmatic protein-kinase signalling cascades, which culminate in the phosphorylation (and activation) of ERs and/or ER coactivators and resultant transcriptional activation in the nucleus (Figure 1.3-3) (reviewed in Weigel and Zhang 1998; Segars and Driggers 2002; Shupnik 2004) One of the main signalling pathways used in growth factor-induced ER activation is the ras-raf-Mitogen-activated protein kinase (MAPK) pathway, contributing to the bidirectional cross-talk occurring between growth factor-receptor tyrosine kinases and ERs in many target cells, with important consequences in cancer cell growth and neuroprotection (Kato et al. 2000; Driggers and Segars 2002; Klotz et al. 2002; Levin 2003; Mendez et al. 2005).

A third mechanism involves non-genomic actions (Figure 1.3-4) initiated at the cell surface through estrogen binding to membrane receptors (recent reviews Bjornstrom and Sjoberg 2005; Edwards 2005; Levin 2005). Non-genomic actions have been identified in several species from fish to human and in several cell types and tissues (Revelli et al. 1998; Loomis and Thomas 2000; Beck and Hansen 2004; Remage-Healey and Bass 2004; Edwards 2005). For instance, E_2 was shown to decrease the *in vitro* 11KT production in Atlantic croaker (*Micropogonias undulatus*) testicular fragments, through a rapid (<5min) action that was not blocked by transcription or translation inhibitors and occurred even in response to the membrane-impermeant BSA-conjugated E_2. The identification of a saturable, high affinity, rapid association estrogen-binding moiety in testicular membranes preparations further supported a membrane-initiated action (Loomis and Thomas 2000).

The identified non-genomic actions of estrogens involve the activation of different cytoplasmatic signalling cascades (many through G protein activation), such as MAPK, PI3Kinase/Akt, PKC or cAMP/PKA, and result in the generation of calcium currents, NO release, protein phosphorylation and eventual transcriptional activation through TF phosphorylation (reviewed in Segars and Driggers 2002; Bjornstrom and Sjoberg 2005; Levin 2005; Song et al. 2005).

However, the identity of the membrane receptors mediating these effects is still unclear. Substantial evidence support that they are mediated by a subpopulation of the classic, nuclear ERs (reviewed by Levin 2005), that localize to the inner face of the plasma membrane through interactions with other proteins. These appear to include the scaffold protein caveolin, G proteins, Shc, Src kinase, ras, PI3Kinase, and MNAR (modulator of non-genomic activity of ER), forming a multiprotein complex that facilitate ER membrane localization and signalling (Bjornstrom and Sjoberg 2005; Levin 2005; Song et al. 2005). Alternatively, in several cell types ERs have also been localized within isolated caveolae, plasma membrane invaginations which facilitate signal transduction, in association with several signalling molecules. One well-studied example is the rapid activation of nitric oxide synthase (eNOS) in both isolated plasma membranes and caveolae fractions of endothelial cells, involving ER association with eNOS, caveolin, Src, PI3K and other signalling molecules that lead to eNOS phosphorylation by the PI3-kinase/Akt pathway and consequent NO release (Chambliss and Shaul 2002; Edwards 2005). In fish, other mechanisms are probably involved in the reported E_2 rapid vasodilator effects, which were not affected by a NOS inhibitor (Agnisola et al. 2004).

Moreover, an N-terminal variant of the nuclear ERα (ER46kDa) was also shown to localize to the plasma membrane and caveolae of human endothelial cells, which appears to modulate eNOS activation more efficiently than the full-length ERα (Li et al. 2003b).

However, the observation of rapid estrogen actions in nuclear ER-negative cells and effects that are not blocked by ER antagonists suggest the involvement of novel membrane receptors unrelated to the classical ERs (Segars and Driggers 2002; Thomas et al. 2005). Recently, the orphan G protein-coupled receptor GPR30 was identified as a high affinity, low capacity receptor for E_2, expressed in plasma membranes of breast cancer cells that lack classical ERs but bind E_2, and capable of G protein activation and cAMP generation in response to E_2 (Thomas et al. 2005). However, GPR30 was not expressed in known cellular models of E_2 non-genomic actions, thus suggesting that both nuclear ER (or ER variants) and membrane-based estrogen-responsive proteins may mediate rapid estrogen actions, whose significance could depend on the cellular context (Segars and Driggers 2002; Thomas et al. 2005).

Many estrogen membrane-initiated actions culminate in transcriptional activation through the activation of diverse TFs by protein-kinase cascades (including the nuclear ER itself), providing a mechanism through which membrane ERs may modulate or amplify nuclear ER actions on the same promoter (Segars and Driggers 2002; Bjornstrom and Sjoberg 2005; Levin 2005). Moreover, in some target genes both type of actions may converge on multiple response elements for different TFs, whose relative contributions *in vivo* may depend on the cell type (one good example is the estrogen regulation of the cyclin D1 gene, reviewed by Bjornstrom and Sjoberg 2005).

For more information about the mechanisms involved in the estrogen actions in their main target systems there are several valuable recent reviews (CNS, Maggi et al. 2004; testis, Akingbemi 2005; female reproductive system, Hewitt et al. 2005; cardiovascular system, Hisamoto and Bender 2005; breast cancer, Song et al. 2005; bone, Syed and Khosla 2005).

1.5. Estrogen Receptors (ERs)

1.5.1. Evolution and ER forms

Since the cloning of the first ER cDNA in the 1980's (Green et al. 1986), two forms of ER (ERα and ERβ) encoded by different genes have been found in many vertebrate species, which share the same structure (section 1.5.3) but encode proteins that are complementary but functionally distinct (Matthews and Gustafsson 2003).

Until recently, no orthologues for ERs or other steroid receptors (SR) had been found in invertebrates either by PCR screening or in available complete genomes, suggesting they were specific to vertebrates (Escriva et al. 1997; Escriva et al. 2004). However, the recent isolation of an ER orthologue in the marine gastropod *Aplysia californica* (although unable to bind estrogen) indicate that SRs are more ancient and widespread than initially thought (Thornton et al. 2003a). It was proposed that all SRs diversified from a primordial gene with estrogen receptor-like functionality, before the origin of bilaterian animals, which was lost in the lineage leading to arthropods and nematodes and became independent of estrogen regulation in the mollusc *Aplysia* lineage (Thornton et al. 2003a). The two ERα and β paralogues were probably originated by duplication of the ancestral ER gene during the second wave of NR diversification (section 1.2.4, Escriva et al. 2004), after the arthropod/vertebrate split.

In several teleost fish, two ERβ forms have recently been isolated (Tchoudakova et al. 1999; Hawkins et al. 2000; Ma et al. 2000; Bardet et al. 2002; Menuet et al. 2002; Halm et al. 2004; Sabo-Attwood et al. 2004), which appear to be encoded by distinct genes arisen by duplication of an ancestral ERβ gene before the divergence of teleosts (Robinson-Rechavi et al. 2001), but their significance in fish physiology is still unclear.

1.5.2. Genomic organization of ER genes and generation of ER variant transcripts

The exon-intron organization of ERα and ERβ genes has been described for several mammalian and fish species (Table 1.1). All genes are composed by eight coding exons encoding proteins with 485-622 amino acids, except for fish ERα which have an additional intron dividing exon 4, and share well conserved intron-exon boundaries (Ponglikitmongkol et al. 1988; Le Roux et al. 1993; Tan et al. 1996; Enmark et al. 1997; Lassiter et al. 2002). Despite the high conservation in the coding exon organization, it is now known that ER genes contain multiple regulatory features (Table 1.1), which allow their transcription into a wide range of variant transcripts in a cell-context specific manner (Tan et al. 1996; Pakdel et al. 2000; Reid et al. 2002; Hirata et al. 2003). These features are most well characterized in human ER genes, represented in Figure 1.4.

Table 1.1. List of characterized ER genes, with comparison of the gene size spanned by and the number of coding exons (kb). Indicated for each gene are the size of the full-length protein, in amino acid number and kDa, and the presence of additional gene features that allow the generation of variant transcripts. * An extra intron divides exon 4 in fish ERα genes.

Gene	Reference	Spans (kb)	Coding exons	Protein (size, MW)	Alternative 5'-UTR exons	Others
Human ERα	(Ponglikitmongkol et al. 1988)	>140kb	8	595, 66kDa	≥ 8 (Kos et al. 2001; Okuda et al. 2003)	"Intronic" leader exon S (Hirata et al. 2002)
Human ERβ	(Enmark et al. 1997)	40 kb	8	485, 54kDa 530, 59kDa (Ogawa et al. 1998a)	≥ 7 (Hirata et al. 2001; Lewandowski et al. 2002)	"Intronic" leader exon M (Shoda et al. 2002); ≥ 5 alternative exon 8's (Hirata et al. 2003)
Mouse ERβ	(Enmark et al. 1997)	40 kb	8	530, 59kDa		
Mouse ERα	(Swope et al. 2002)	>220 kb	8	599, 66kDa	≥ 6 (Kos et al. 2000)	
Rainbow trout ERα	(Le Roux et al. 1993)	> 30 kb	9 *	577, 65kDa 622,71kDa (Pakdel et al. 2000)	≥ 2 (Pakdel et al. 2000)	
Tilapia ERα	(Tan et al. 1996)	40.4 kb	9 *	583, 65kDa		Alternative transcription start sites and polyadenylation signals (Tan et al. 1996)
Zebrafish ERβa	(Lassiter et al. 2002)	> 20 kb	8	553, 62kDa		

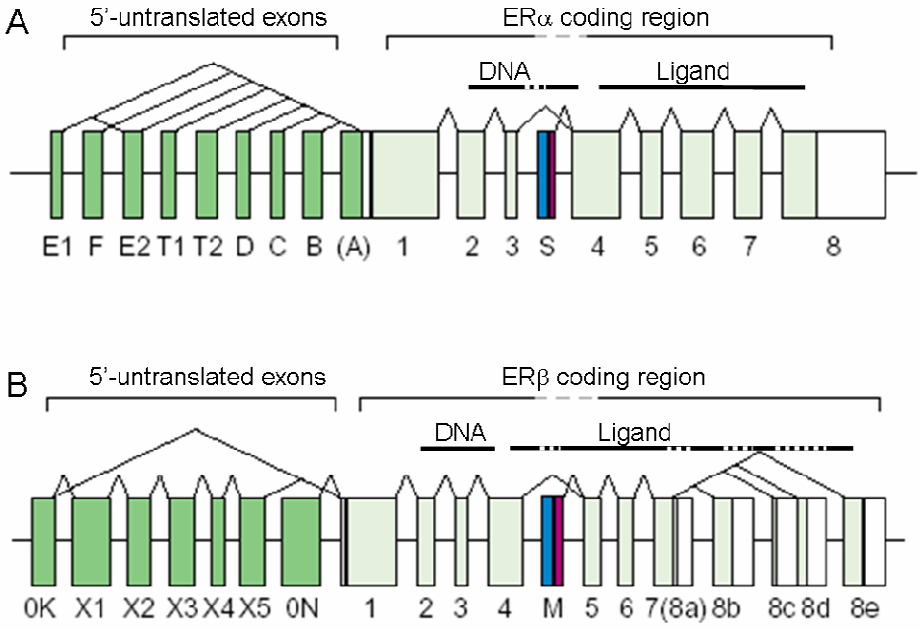

Figure 1.4. Structure of human ERα (A) and ERβ (B) genes. Exons are represented by boxed structure and introns by solid lines. Coding and untranslated regions are indicated, including the regions encoding the ER protein domains responsible for DNA or ligand binding (section 1.5.3). In addition to coding exons 1-8, at least eight alternative untranslated 5'-exons have been identified in the upstream region of exon 1 of the ERα gene, seven in the ERβ gene. In ERα, transcription may also be initiated from a region upstream of the splicing acceptor site of exon 1, termed exon (A), or from an "intronic" exon S located between exons 3 and 4. In ERβ it can also be initiated from the "intronic" exon M between exons 4 and 5. The ERβ coding region comprises exons 1-7 and five alternative exon 8s. Moreover, the translation at exon 1 may be initiated at alternative in-frame ATG start codons, producing two proteins with different N-terminal sizes *in vivo*, as indicated on Table 1.1. From Hirata et al. (2003).

For instance, the transcription of the human ERα gene is regulated in a tissue-specific manner by alternative promoter and 5'-untranslated exon usage and splicing (Figure 1.4), resulting in the expression of different levels of multiple ER transcripts with different 5'-untranslated regions (UTR) but encoding the same protein (Kos et al. 2001; Okuda et al. 2003). This regulation is also used by the human ERβ and mouse ERα genes (Table 1.1) and appear to be involved in the differential regulation of ER protein translation. In mouse ERα, the different 5'-UTRs were shown to contain different numbers of short upstream open reading frames (suORFs), which have an inhibitory effect on the translation of the downstream ER ORF (Kos et al. 2002). suORFs appear to be a common feature in the

5'UTR of ERα and β mRNAs and have been identified in several mammalian and non-mammalian species (e.g. Green et al. 1986; Krust et al. 1986; Koike et al. 1987; Weiler et al. 1987; Pakdel et al. 1990; Madigou et al. 1996; Tan et al. 1996; Todo et al. 1996; Muñoz-Cueto et al. 1999; Tchoudakova et al. 1999; Ma et al. 2000; Xia et al. 2000).

In addition, a large number of alternative ERα or β transcripts encoding protein variants different from the full-length ER have been described in different human tissues, particularly in cancer cell lines and tumours (e.g. Poola et al. 2000; Ye et al. 2000; Poola et al. 2002; Aschim et al. 2004; Perlman et al. 2005). These transcripts are originated by multiple (or combination of) mechanisms including the deletion of one or more exons, exon duplication, transcription initiation or termination at "intronic" exons or the use of alternative coding exons, as is the case of the multiple exon 8 system of the human ERβ gene producing proteins with different C-terminal (Figure 1.4) (Hirata et al. 2003; Wang et al. 2005).

Some of the corresponding variant proteins have been detected *in vivo* (Ogawa et al. 1998b; Fuqua et al. 1999; Flouriot et al. 2000; Denger et al. 2001b; Tiffoche et al. 2001; Critchley et al. 2002; Saunders et al. 2002; Li et al. 2003b; Wang et al. 2005) and synthesized *in vitro* (e.g. Bollig and Miksicek 2000; Hirata et al. 2002; e.g. Shoda et al. 2002), but in general their *in vivo* functional significance is still unclear.

Nevertheless, significant progress into the functional characterization of some of these variant proteins has been made in the last years using *in vitro* reporter gene transactivation assays and co-expression with wild-type ERs. While most of them are unable to activate transcription of ERE-driven reporter genes, several variants are able to inhibit the transcriptional activity of the wild-type (wt) ERα and/or ERβ proteins (dominant negative effect), which in some cases is achieved through the formation of wt ER - variant heterodimers with reduced DNA binding ability (Inoue et al. 1996; Ogawa et al. 1998b;

Bollig and Miksicek 2000; Flouriot et al. 2000; Inoue et al. 2000; Peng et al. 2003; Poola et al. 2005). Other functional characteristics include stimulation of wt ER transcriptional activities at ERE promoters (dominant positive) (Lin et al. 2003); an increased ligand-independent transcriptional activity when compared with the wt ER (Poola et al. 2005), transcriptional activity at non-consensus response element like AP-1 (Bollig and Miksicek 2000) or localization and rapid signalling at the plasma membrane (Li et al. 2003b). These functions appear to be dependent on the promoter, the cell context, the wt ER subtype involved and the relative wt / variant protein levels (Ogawa et al. 1998b; Bollig and Miksicek 2000; Flouriot et al. 2000; Lin et al. 2003).

In fish, the expression of multiple ERα or ERβ transcripts of different length has been detected by Northern blot in several species (Socorro et al. 2000 and references therein), but their identity and functional significance remain mostly unknown. A number of ERα mRNA variants have been identified in channel catfish, *Ictalurus punctatus*, including transcripts encoding ERs with N-terminal truncations, internal insertions or deletions, and an antisense ERα mRNA of unknown function (Patiño et al. 2000). In rainbow trout, two ERα transcripts with different 5'-UTR are expressed in a tissue-specific manner, generated by alternative splicing and promoter usage (Table 1.1) and encode variant proteins with different N-terminal length (translated from alternative ATG start codons) which differ in their transactivation properties (section 1.5.3.2) (Metivier et al. 2000; Pakdel et al. 2000; Menuet et al. 2001). In tilapia, *Oreochromis aureus*, transcripts with different 5' or 3'UTRs may be generated from the ERα gene through the use of alternative transcription start sites or polyadenylation signals (Tan et al. 1995). One of the transcripts has an extremely long 3'UTR (>1/2 of the transcript length), a characteristic of many ER mRNAs (Tan et al. 1995) that has been demonstrated to exert a destabilizing effect in the human ERα mRNA, contributing to its short half-life (Keaveney et al. 1993; Kenealy et al. 2000).

1.5.3. ER Protein

1.5.3.1. Domain structure

Like other NRs, all ER proteins share a common modular structure organized into six independent but interacting functional domains termed A to F (Figure 1.5) (Krust et al. 1986). These consist of the variable A/B domain in the N-terminal region, followed by a central conserved DNA-binding domain (DBD or C-domain) through which ERs dimerize and contact with specific estrogen-response elements (ERE) in target promoters. The D (hinge) domain is important for nuclear localization and links the DBD and the relatively well-conserved ligand-binding domain (LBD or E-domain). The LBD contains the binding site for estrogens and other ligands as well as the main dimerization surface, and is followed by an F domain with unclear functions (reviewed by Aranda and Pascual 2001; Nilsson et al. 2001).

Two distinct regions within the ER molecule, apart from the DBD, specifically contribute to transcriptional activity by serving as docking sites to numerous coregulator proteins, the ligand-independent transactivation function 1 (AF-1) located in the N-terminal A/B domain, and the strictly ligand-dependent AF-2 function in the LBD (Tora et al. 1989). The contributions of AF-1 and AF-2 functions are dependent on the promoter context and cell-type, and in most cell contexts and promoters both AF-1 and AF-2 function in a synergistic manner and are required for full receptor activity (Tora et al. 1989; Berry et al. 1990; Tzukerman et al. 1994; Tremblay and Giguere 2001).

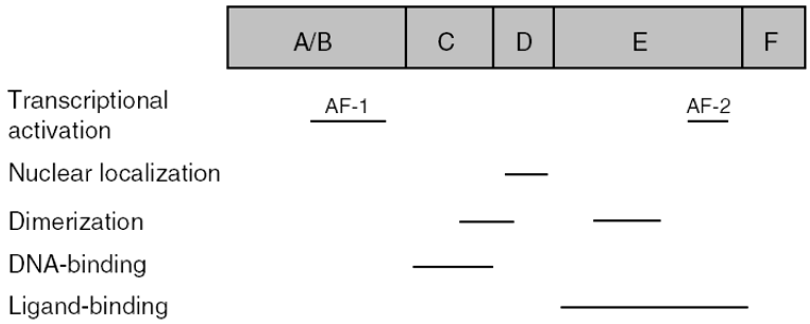

Figure 1.5. Schematic view of the typical ER protein structure. The functions assigned for each region are represented by solid lines. AF= activation function. From Barkhem et al. (2004).

1.5.3.2. A/B domain

This modulatory region is the most variable when comparing ER proteins from different subtypes and species, both in sequence and in length (132-186 amino acids) and greatly contributes for the partial agonistic transcriptional activity observed for some antiestrogens (e.g. tamoxifen) on certain promoter- or cell-contexts (Tzukerman et al. 1994; McInerney and Katzenellenbogen 1996).

The AF-1 activity can be enhanced through phosphorylation of specific residues within the A/B domain, in response to different signal transduction pathways (section 1.4), or in response to ligand-binding (reviewed by Driggers and Segars 2002; Lannigan 2003). In response to EGF activation, the MAP kinase ERK (extracellular-regulated kinase, or MAPK) phosphorylates the human ERα (hERα) Ser^{118} residue, increasing AF-1-mediated transcriptional activity in a ligand-independent manner, through increased recruitment of coactivators (e.g.p72/p68) and dissociation from corepressors (e.g. SRMT) (Kato et al. 1995; Bunone et al. 1996; Deblois and Giguere 2003). Other residues identified in hERα may be phosphorylated by other signalling pathways (e.g. PKA), while E_2 binding further enhances hERα phosphorylation at residues Ser^{104}, Ser^{106} and Ser^{118} and is required for full AF-1 activation (Metivier et al. 2001; Driggers and Segars 2002; Lannigan 2003).

The ERβ subtype was considered to contain a weaker AF-1 function when compared to ERα on a variety of promoters and cell types (Cowley and Parker 1999; Delaunay et al. 2000). However, it was demonstrated to contain AF-1 ligand-independent activity potentiated by growth factor-activated MAPK phosphorylation at Ser^{106} (analogous to ERα Ser^{118}) and Ser^{124}, *in vivo* and *in vitro*, with increased association to the SRC-1 coactivator (Tremblay et al. 1999; Kato et al. 2000; Tremblay and Giguere 2001).

Most teleost fish ERα contain shorter A/B domains (132-146 amino acids) relative to tetrapod ERα (173-183 amino acids), suggesting that they could lack a defined A domain. However, longer form ERα (ERαL) with 179-186 amino acids A/B domains have now been reported in some species (e.g. Patiño et al. 2000). Rainbow trout (rt) ERαL differs functionally from the short ERα (ERαS) form by the presence of a strictly E_2-dependent transcriptional activity, whereas rtERαS exhibits a basal level of estrogen-independent (AF-1) activity (Pakdel et al. 2000). Using rtERαL, rtERαS, an A domain-truncated hERα and chimeric trout/human ERα receptors, it was further demonstrated that the A domain of ERα proteins plays an inhibitory role in the ligand-independent activity of the receptor, apparently mediated by a direct interaction with the C-terminal region of the receptor, which is reduced upon ligand binding (Metivier et al. 2000). Furthermore, the authors found a minimal region of 11 amino acids containing a potential α helix as necessary and sufficient for autonomous transactivation in both hERα and rtERα, which was conserved and interchangeable between the two distant species, supporting a conserved role in AF-1 activity (Metivier et al. 2000).

1.5.3.3. C-domain

The DBD is the most conserved region between ERs from different subtypes or species, and among NRs (Aranda and Pascual 2001). It contains two zinc fingers, each formed by four conserved cysteines that coordinate tetrahedrically one zinc ion, folded to form a

compact, interdependent structure. Located at the base of the first zing finger, the P-box (sequence CEGCKA) contains the amino acids responsible for the specific recognition and contact with the DNA estrogen response elements (ERE) (Schwabe et al. 1990; Schwabe et al. 1993). The consensus ERE is composed of two half-sites of the core sequence AGGTCA oriented in a palindromic order and separated by three non-specific nucleotides, i.e. AGGTCAnnnTGACCT (Gruber et al. 2004). The D-box in the second zinc finger is involved in ERE half-site spacing recognition and harbours a dimerization interface that, together with sequences in the LBD, allows the interaction between two ER molecules. The ligand-bound ER dimer cooperatively binds DNA on a pair of ERE half-sites, with the P-box of each monomer contacting one half-site, to allow the transcriptional modulation of the target gene (Ruff et al. 2000).

However, many natural ER-regulated promoters/enhancers in target genes contain regulatory elements that deviate from the consensus perfect palindromic ERE. These include: a) imperfect palindromic EREs in which one or more nucleotides of the recognition sequence are changed, resulting in decreased ER affinity and transcriptional activation, b) direct repeats of multiple ERE half-sites that cooperatively bind ER, and c) ERE half-sites that act in synergism or overlap with other regulatory elements within the promoter region of the gene (e.g. AP-1 or Sp1) (Gruber et al. 2004; O'Lone et al. 2004). Moreover, different EREs may alter not only the ER binding affinity but also its conformation, selective coregulator recruitment and both ligand-independent transcriptional activity and agonist or antagonist-induced transcriptional activity. Thus the response element itself is an allosteric effector of ER action, and may contribute to differential hormone responses verified for different promoters (reviewed by Klinge et al. 2004; O'Lone et al. 2004; Geserick et al. 2005).

Despite high conservation in the DBD sequence and identical P- and D-boxes, differential subtype affinities and response to a subset of natural EREs have also been reported between the two mammalian ER subtypes α and β (Klinge 2001; Kulakosky et al. 2002; Yi et al. 2002a; Yi et al. 2002b). Moreover, substitution of the rtERα DBD with those of the highly homologous hERα enhanced the transactivation ability of rtERα (Petit et al. 2000). These results highlight the importance of other residues from the DBD, outside the P- and D-boxes, and of ERE modulation in the differential ER-DNA interaction and transcriptional activation between ER subtypes and species.

The DBD was also shown to influence ligand activation profiles at AP-1 response-elements, although intact DNA binding activity was not required. A conserved lysine in the DBD was required for antiestrogen-ERβ activation at AP-1 and prevents estrogen-ERα from becoming superactive at AP-1 sites (Webb et al. 1995; Uht et al. 2004).

1.5.3.4. D-domain

This short and low conserved domain contains the nuclear localization signals (NLS) that are responsible for the ER nuclear translocation, which appears to occur after ER synthesis and independently of ligand binding (Picard et al. 1990; Ylikomi et al. 1992; Shank and Paschal 2005). The importance of these signals are evidenced by the cytoplasmatic localization of ERα protein variants lacking the NLSs, in contrast with the nuclear localization of wt ERα and variant ERα that retain the NLSs (e.g. Bollig and Miksicek 2000). This region was also shown to interact with corepressors in response to an ER antagonist (Oesterreich et al. 2000), and to be involved in direct protein-protein interactions with c-Jun and JunB transcription factors, affecting the transcriptional regulation at an AP-1 promoter (Teyssier et al. 2001). Moreover, specific residues at the D domain are targets for several post-translational modifications such as phosphorylation by

PKA, acetylation by p300 coactivators or sumoylation, which influence transcriptional activation, hormone sensitivity or ligand antagonist/agonist properties (Wang et al. 2001; Michalides et al. 2004; Sentis et al. 2005).

1.5.3.5. E-domain

The moderately conserved E-domain or LBD contains several functions, mostly ligand induced, which include ligand binding, receptor dimerization and the ligand-dependent transactivation function AF-2, and is the largest domain among ERs.

A wide repertoire of structurally distinct compounds binds ERs. These include natural estrogens (E_2, estrone, estriol); synthetic estrogens (ethynylestradiol, diethylstilbestrol, etc.) and antiestrogens (e.g. tamoxifen); phytoestrogens, which are estrogenic compounds produced in plants that display estrogenic activity in animals (e.g. genistein) and a wide variety of xenoestrogens (e.g. PCBs) (Kuiper et al. 1998; Pike et al. 1999; Matthews and Zacharewski 2000). While many of these compounds act solely as ER agonists, displaying estrogen-like actions, others appear to act as antagonists, mainly through competition with agonists for ER binding and inducing an ER inactive conformation (e.g. ICI 182,780 or ICI 164,384, considered pure antiestrogens). Other compounds, the selective ER modulators (SERMs, see section 1.7), may act as agonists, antagonists or as partial agonists/antagonists with mixed activity, depending on the cell, the promoter context and/or the ER subtype (e.g. tamoxifen, raloxifen)(Frasor et al. 2004).

The three-dimensional structures of the LBDs of mammalian ERα and ERβ have been determined in the presence of agonists or antagonists (Brzozowski et al. 1997; Shiau et al. 1998; Pike et al. 1999; Pike et al. 2001; Shiau et al. 2002). Both LBDs have a similar globular structure, formed by 12 α-helices (H1-H12) folded as a three antiparallel-layered helical sandwich packed around a central hydrophobic pocket, which contains the ligand-

binding site. The size of the binding cavity (nearly twice the molecular volume of E_2), its shape and plasticity may explain the promiscuity of ER, binding to diverse ligands in contrast to other steroid receptors (Brzozowski et al. 1997; Pike et al. 1999). H12 contains several conserved residues directly involved in ligand-dependent transactivation (AF-2 function), as shown by mutagenesis, and the position it adopts in response to different ligands determines whether the ER is activated or not, i.e., the ligand agonist/antagonist behaviour (see below) (Danielian et al. 1992; Ruff et al. 2000).

The amino acids lining the ligand-binding pocket (Brzozowski et al. 1997; Pike et al. 1999) have been mostly conserved between species and ER-subtypes, including both fish ERβs, which may explain similar affinities for E_2 (Kuiper et al. 1997; Matthews et al. 2000; Hawkins and Thomas 2004). However, different binding affinities or transactivation potencies for several estrogenic compounds have also been described, which have been attributed to species-specific or subtype-specific amino acid substitutions within the LBD, particularly in the positions identified to be involved in ligand-binding (Kuiper et al. 1997; Barkhem et al. 1998; Matthews et al. 2000; Hawkins and Thomas 2004). For instance, human ERα and ERβ ligand-binding pockets differ in two positions, which have been hypothesized to contribute for the higher affinity of genistein to hERβ (Pike et al. 1999). Rainbow trout ERα, in contrast to hERα, exhibited decreased E_2 binding affinities and transactivation abilities with increasing temperatures (4-37ºC) and reciprocal mutagenesis in the two conservative amino acid substitutions that differ between their ligand-binding pockets resulted in an effective, although not complete, exchange of phenotypes (Matthews et al. 2001). Differential binding characteristics were also identified between the two fish ERβ forms in Atlantic croaker, with ERβa showing lower relative binding affinity for some synthetic estrogens and antiestrogens than ERβb, ERα and ERs from other species. Mutation in one amino acid position conserved in fish ERβas (identified as

important for ligand binding in hERs) to the ERβb/ERα residues shifted the ERβa binding properties towards those of ERβb/ERα forms (Hawkins and Thomas 2004).

The resolved structures of ER LBDs revealed that although agonists and antagonists bind to the same binding site, each type of ligand induces different conformational changes. When the LBD is complexed with full agonists like E_2, DES or THC (an ERα-subtype specific agonist), the transactivation helix H12 packs tightly over the ligand-binding site and forms part of the coactivator-binding groove together with residues from helices 3, 4, 5 and 12. This "agonist-bound, active conformation" allows the docking of coactivators on the exposed AF-2 region, leading to transcriptional activation (Brzozowski et al. 1997; Shiau et al. 1998; Mak et al. 1999; Shiau et al. 2002). In contrast, when ERα or ERβ LBDs are complexed with antagonists (e.g. raloxifen), H12 is displaced from its agonist position and partially occludes the coactivator-binding groove, thus preventing coactivator recruitment and blocking AF-2 transcriptional activation ("antagonist-bound, inactive conformation") (Brzozowski et al. 1997; Pike et al. 1999). Alternative conformations include the completely disordered H12 conformation in the ERβ-ICI 164,384 (pure antagonist) complex, or the suboptimal H12 position in the ERβ-genistein (partial agonist) complex (Pike et al. 1999; Pike et al. 2001). In all complexes, H12 acts as a molecular switch, mediating the allosteric control of AF-2 mediated transcription by the ligand nature (Nettles and Greene 2005). Although no crystal structure was yet resolved for any fish ER, homology-modelling of the LBDs of the three zebrafish (*Danio rerio*) ERs predicted their folding in a very similar way to their human ER homologues in the presence of agonists and antagonists (Costache et al. 2005).

A large number of coactivators have been identified through their ligand-dependent interaction with NRs. These include, among many others, members of the p160 family of coactivators (e.g. SRC 1-3, TIF2, pCIP, etc.), most of them interacting with the AF-2

surface via their conserved amino acid LXXLL motifs (NR box) (good recent reviews by Kumar et al. 2004; Smith and O'Malley 2004). Different coactivator complexes appear to interact sequentially with activated NRs to exert a series of functions needed to activate transcription, including histone acetylation, DNA unwinding, chromatin remodeling and recruitment of the basal transcription machinery, (Smith and O'Malley 2004). Many of the coactivators identified as AF-2 also activate the AF-1 function in a ligand-independent manner (e.g. SRCs), but there are also AF-1 specific coactivators (e.g. p68/p72), coactivators with subtype-selective affinity and coactivators whose subtype preference depend on the ligand (Matthews and Gustafsson 2003; Kumar et al. 2004). Both AF-1 and AF-2 transactivation is dependent on the balance between the coactivator and corepressor interaction, although corepressor recruitment and functions in ER-mediated transcription are less well studied.

While other NRs are constitutively bound to DNA and actively repress transcription via corepressor binding, ERs were long considered to be maintained inactive (and unbound to DNA) in the absence of ligand, through binding of chaperone proteins. A more recent model proposed that unliganded ER may bind DNA and both coactivator and corepressor complexes, through AF-1 and AF-2, but the balance between these complexes may be influenced by intracellular signalling thus affecting the basal receptor activity (especially via AF-1) (Smith and O'Malley 2004). Upon agonist binding, corepressors appear to be released concomitant with coactivator recruitment, while antagonists promote the recruitment of corepressors (e.g. NCoR, SMRT or REA), thereby additionally antagonizing the ER-mediated action (Smith and O'Malley 2004). Although the interaction between ERs and corepressors is not yet clear, the corepressor-binding site may partially overlap with the coactivator-binding site but it is also likely that different ER regions may interact with different corepressors (Dobrzycka et al. 2003; Nettles and Greene 2005).

Recent evidences propose that corepressors not only mediate ER inactivation by antiestrogens, but probably play important biological roles by controlling the magnitude of the estrogenic response, repressing DNA-bound ER in the absence of ligand, and conferring active repression of agonist-ER down regulated genes, which until now is not well understood (Dobrzycka et al. 2003). Corepressors appear to exert these actions through different mechanisms, mostly studied for ERα, including the formation of multiprotein complexes affecting chromatin remodeling, histone deacetylation and basal transcription, competition with coactivators, interference with DNA binding or ER dimerization, alteration of ER stability, sequestration of ER in the cytoplasm and effects on RNA processing (Dobrzycka et al. 2003).

The LBD also contains the main ligand-induced dimerization function, which together with the weaker and constitutive dimerization function contained in the DBD allow the formation of ligand-stabilized ER dimers on DNA (Kumar and Chambon 1988). Resolved LBD structures revealed that ligand-bound homodimers have the same arrangement whatever the ligand or the ER subtype, and similar to that of other NRs, which is a "head-to-head" arrangement with each protomer assembled along the twofold dimer axis, but slightly tilted from it. The large dimerization interface involves residues from helix H8 up to H11, with the most important dimer contacts formed by H10 and H11, also involved in ligand-binding (Pike et al. 1999). Besides forming homodimers, mammalian ERα and β have also been shown to form α/β heterodimers *in vivo* and *in vitro*, which appear to use the same dimerization interface as in homodimers (Cowley et al. 1997; Pace et al. 1997; Pettersson et al. 1997; Ogawa et al. 1998a). The possibility of interplay between the two ER subtypes *in vivo* is one more variant to ER-mediated actions, although the role of the ERαβ heterodimer remains mostly unknown. Some authors reported that ERβ reduces ERα-mediated transcription activation, *in vivo* and *in vitro*, suggesting that one of the roles

of ERβ is to function as a dominant inhibitor of ERα in tissues where both are expressed (Hall and McDonnell 1999; Pettersson et al. 2000; Weihua et al. 2000; Lindberg et al. 2003). However, recent *in vitro* studies using single chain homogenous heterodimer populations demonstrated that this repression occurs mainly through competition of β/β or α/α homodimers for ERE binding rather than through the formation of α/β heterodimers, and the ERα subtype properties appear to be dominant in the αβ heterodimer-mediated genomic actions (Li et al. 2004).

Several ER variant proteins have been shown to form heterodimers with ERα or ERβ and to repress their transcriptional activities (section 1.5.2). For instance, the ERβx isoform (or ERβ2) possessing an alternative C-terminal end, cannot bind E_2 nor activate gene transcription but heterodimerizes preferentially with ERα, rather than ERβ, inhibiting ERα DNA binding and exerting a dominant negative effect on ligand-dependent ERα reporter gene activity (Ogawa et al. 1998b). Moreover, it was recently described that the wt ERα was also able to inhibit the transcriptional activity of one ERβ variant (ERβ5) (Poola et al. 2005).

Although the molecular requirements involved in estrogen non-classical actions are still poorly understood, the LBD appears to be sufficient and to contain the critical elements for both ERα membrane translocation and rapid signaling to ERK, PI3K, G protein and EGF-receptor transactivation, including the Ser^{522} residue and the dimerization function (Evinger and Levin 2005). However, it is likely that different ER motifs are required to activate different signalling molecules, since it was shown that dimerization is not required for eNOS activation, which appears to rather depend on the NLSs but, in another way, the NLSs appear to prevent MAPK activation at the membrane (Chambliss et al. 2005).

1.5.3.6. F-domain

This C-terminus domain is extremely variable in sequence and is only present in some NRs, although its size appears to be conserved between steroid receptor subtypes (Nichols et al. 1998). It appears that one of its roles in ER could be to modulate the functions of other domains, in particularly the adjacent LBD. It may contribute for the discrimination between agonists and antagonists, which appears to depend not only on H12 positioning but also in the position of the F domain, and to modulate the magnitude of the transcriptional activity or repression of each type of compound in a cell-context dependent manner (Montano et al. 1995; Nichols et al. 1998; Schwartz et al. 2002). Moreover, it may inhibit the dimerization function through interaction with the LBD, thus suggesting that the full-length ER contains dimerization restraints that were not detected in the available crystallographic studies of the LBD, which did not include the F domain (Peters and Khan 1999). It is also involved in the interaction with coregulators required for the E_2-dependent activation of ERα at Sp1 sites (Kim et al. 2003).

1.5.4. ER tissue distribution

In mammalian species, ERα and ERβ display overlapping but distinct patterns of mRNA and protein expression throughout a broad range of tissues and cell-types, including the ovary, uterus, vagina, mammary gland, testis, epididymis, prostate, bladder, pituitary, several regions of the brain, liver, thyroid, adrenal, gastrointestinal tract, kidney, lung, heart, blood vessels, bone and immune system. Some tissues appear to express predominantly ERα (e.g. the uterus and pituitary) while others express predominantly ERβ (e.g. the prostate, ovary, lung, bladder) (Couse et al. 1997; Kuiper et al. 1997; Saunders et al. 1997; Nilsson et al. 2001; Nie et al. 2002; Thornton et al. 2003b; Koehler et al. 2005). However, within a particular tissue, the expression of each receptor subtype may localize to different cell types. For instance, localization studies in the ovary of several mammalian species supported that the ERβ protein is the main form expressed in granulosa cells at all stages, with low expression in theca cells and corporea lutea, whereas ERα appears to be expressed in thecal and interstitial cells and at low levels in the granulosa cells of antral follicles only, which suggests that each subtype may play distinct functions within a specific tissue (Fitzpatrick et al. 1999; Rosenfeld et al. 2001; Saunders 2005).

Nonetheless, ERα and ERβ proteins have been co-localized in many cell-types including specific types of neurons, the stroma and glandular layers of the endometrium or Leydig cells in the testis, among others, which are potential targets for heterodimerization and interplay between the two receptors (section 1.5.3.5.) (Shughrue et al. 1998; O'Donnell et al. 2001; Saunders 2005). However, several discrepancies in the ERα or β patterns between species have been reported; for instance, while several studies have detected ERα in mouse and rat Leydig cells, the ERα localization in Leydig cells of humans and primates has not been consistently detected (O'Donnell et al. 2001). Moreover, several ER variant proteins have also been co-detected with the wt ERs in several tissues and cell-

types, opening the possibility of *in vivo* interference with wt ER-mediated signalling (Friend et al. 1997; Fuqua et al. 1999; Critchley et al. 2002; Saunders et al. 2002; Scobie et al. 2002; Li et al. 2003b).

In teleost fish, tissue distribution studies have been mainly restricted to the liver, central nervous system and gonads and often to only one or two ER subtypes. In general, teleost ERα showed a more restricted distribution, mainly expressed in pituitary and liver of both sexes, while ERβa and ERβb (using the nomenclature adopted in this study, see discussion of chapter 3) were more widespread, with highest levels found in pituitary and gonads of both sexes (ERβa) or pituitary, liver and intestine (ERβb) (Halm et al. 2002; Menuet et al. 2002; Choi and Habibi 2003; Hawkins and Thomas 2004). Nevertheless, the distribution of the three forms largely overlaps, with co-expression in brain, pituitary, liver and gonads but different patterns of relative expression between tissues, and species-specific differences were also found between their patterns (Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004; Hawkins and Thomas 2004).

In situ hybridization (ISH) has been mainly used to map the mRNA distribution of one or more fish ER subtypes in the brain of several fish species, which localized the three forms in neuroendocrine regions such as the preoptic area, the mediobasal hypothalamus and also in the ventral telencephalon, in agreement with the localization of rtERα protein in the same areas by immunohistochemistry (IHC) (Anglade et al. 1994; Hawkins et al. 2000; Menuet et al. 2002; Andreassen et al. 2003; Menuet et al. 2003; Forlano et al. 2005; Hawkins et al. 2005). Although the expression of each subtype partially overlapped in all species analyzed, with some cells of the preoptic area and hypothalamus of zebrafish showing co-expression of the three receptors (unpublished results by Adrio at al., cited in Pellegrini et al. 2005), differential patterns of localization within these areas of the brain were also found in zebrafish and Atlantic croaker (Hawkins et al. 2000; Menuet et al.

2002; Hawkins et al. 2005). ISH experiments have also been reported for ERs in fish testis, although with discrepant results that may reflect species-specific or seasonal differences in expression. In channel catfish, ERα and ERβb were co-localized in mature sperm and in the germinal (secondary spermatocytes and spermatids) and non-germinal epithelia of the mature testis (Wu et al. 2001), while in other species ERα mRNA localized only to putative Sertoli cells and the ERα protein was localized to Leydig cells and primary spermatogonia in rainbow trout testis by IHC (Miura et al. 1999; Legler et al. 2000; Bouma and Nagler 2001; Andreassen et al. 2003).

Fragmentary reports demonstrated the localization of ERα mRNA in distinct regions of the pituitary, in the liver and in theca, granulosa and interstitial cells of the ovary (Kah et al. 1997; Menuet et al. 2001; Andreassen et al. 2003), but additional RNA and protein localization studies for the three ER subtypes are needed to clarify their functions in the different estrogen target tissues in fish. The available studies suggest that, like in mammals, the three fish ER subtypes may be expressed in different cell types of the same tissue, suggesting differential functions, or co-expressed in some cell-types.

1.6. Estrogen responsive genes (ERGs)

With the advances in high-throughput techniques for global analysis of gene expression, an increasing number of genes whose expression is up or down regulated by estrogens (estrogen-responsive genes, ERGs) have been accumulated in the last years, although the induction mechanisms are in most cases unknown, and it is predicted that many remain to be identified. Many of these genes have been recently grouped in the ERG database (http://defiant.i2r.a-star.edu.sg/projects/Ergdb-v2/index.htm, v.2 updated in October 2005) (Tang et al. 2004). These have been identified in diverse mammalian tissues and cell lines, mainly cancer cell lines (Watanabe et al. 1998; Charpentier et al. 2000; Seth et al. 2002a;

Seth et al. 2002b; Frasor et al. 2003; Hewitt et al. 2003; Nagai et al. 2003; Ikeda and Inoue 2004; Abba et al. 2005; Selvaraj et al. 2005), and their expression profiles have been analyzed in various developmental stages or in response to different estrogenic compounds (e.g. Reese et al. 2001; Inoue et al. 2002; Frasor et al. 2004; Terasaka et al. 2004; Ise et al. 2005). Recent studies also demonstrated different ERG subsets regulated through either ER subtype α or β, or trough α/β ER heterodimers (Matthews and Gustafsson 2003; Monroe et al. 2003; Monroe et al. 2005).

Moreover, with the availability of complete genomes, an increasing number of promoters/enhancers containing ERE or ERE-like elements have been identified. A recent paper reviewed the known mammalian primary ER-regulated genes (genes that are up regulated by estrogens due to indirect actions, e.g. expression of other TFs, were not included), containing promoters which have been demonstrated to respond to estrogens and to associate with ERs *in vivo* or *in vitro* (O'Lone et al. 2004), and classified them according to their response elements. Although palindromic EREs were the most represented regulatory sequences, many genes contain ERE variants (section 1.5.3.3) and about one third of the known, primary human estrogen target genes lack any ERE-like elements and associate only indirectly with ER through interaction with intermediary TFs (section 1.4).

The set of primary ERGs listed included genes identified in several tissues and cell lines, encoding diverse types of protein products such as receptors (e.g. the ERα itself, EGF-receptor or the progesterone receptor); ligands (e.g. complement C3); hormones (e.g. angiotensin, oxytocin, IGF-I); transcription factors (e.g. c-Fos); enzymes (e.g. cathepsin D); chaperones (e.g. hsp27) and structural molecules (e.g. keratin 19). These products function in a multitude of cellular pathways, serving a variety of cellular roles: cell cycle progression (e.g. Ebag9 and cyclin D1), cellular maintenance (e.g. telomerase reverse

transcriptase, TERT), cell growth and survival (e.g. TGFα, BCL2 antiapoptotic factor) or signal transduction (e.g. the estrogen, progesterone, retinoic acid, EGF or IGF-I receptors) (reviewed by O'Lone et al. 2004).

In fish, the most well known ERGs are the genes encoding ERα (see below) and several vitellogenin and choriogenin forms. These genes are expressed in the liver of mature females in response to E_2 or in male liver exposed to xenoestrogens (section 1.3.2.2), and their temporal and dose-response expression in response to E_2 or other estrogenic compounds have been characterized in several fish species (e.g. Celius et al. 2000; Takemura and Kim 2001; Bowman et al. 2002; Lee et al. 2002; Yamaguchi et al. 2005).

A few other genes have also been shown to be up regulated by E_2 in fish, the LH-β subunit and the glycoprotein hormone α subunit in pituitary (Xiong et al. 1994; Yaron et al. 2001), aromatase B in the brain (Pellegrini et al. 2005) and a spermatogonial stem-cell renewal factor in testis (Miura et al. 2003). Identified down regulated genes include osteonectin in scales (Lehane et al. 1999) and in the liver the serum proteins albumin (Flouriot et al. 1998), retinol-binding protein (RBP, Funkenstein 2001) and transthyretin (TTR, Funkenstein et al. 2000), several cytochrome P450 enzymes (CYP2 M1, CYP2K1 and CYP3A27, Buhler et al. 2000) and the growth factors IGF-I and IGF-II (Carnevali et al. 2005). Analysis of these genes identified different types of response elements, including palindromic EREs, ERE half-sites and response elements for other TFs (e.g.Melamed et al. 1998; Callard et al. 2001; Ueno et al. 2004).

Only recently have some systematic studies been used to identify ERGs in the liver of several fish species, by differential display, suppressive subtractive hybridization or macroarrays, and the patterns of expression of identified genes analyzed in response to xenoestrogens (Denslow et al. 2001; Bowman et al. 2002; Larkin et al. 2002; Larkin et al. 2003; Brown et al. 2004). Up regulated genes identified included Vgs, Chgs, ERα, liver

aspartic proteinase, CRIM1, ubiquitin 3, one UDP-glucuronosyltransferase, aldose reductase, coagulation factor XI and protein disulfite isomerase, while down regulated genes included transferrin, β-actin, AMBP and fibrinogen β. However, ERGs in other fish tissues apart from liver remain mostly unknown.

The ER genes themselves are well-recognized ERGs, since exposure to estrogens or estrogen agonists/antagonists has been shown to alter the steady-state levels of the mRNAs coding for several ER subtypes in many tissues and cell lines, in mammalian (e.g. Shyamala et al. 1992; Zou and Ing 1998; Zhou et al. 2002; Matthews and Gustafsson 2003; Pinzone et al. 2004) and non-mammalian species (e.g. Varriale and Tata 1990; Ninomiya et al. 1992; Menuet et al. 2004; Sabo-Attwood et al. 2004).

Although in most cases the ER mRNAs (especially ERα) are up regulated by estrogens, which has been considered as a mechanism to enhance estrogen responsiveness in target tissues, down regulation may also occur and differential regulation has been found between different tissues, cell-types, ER subtypes and among different species (e.g. Zou and Ing 1998; Nephew et al. 2000; Ihionkhan et al. 2002; Sabo-Attwood et al. 2004). However, in most cases the mechanisms involved in the ER regulation by estrogens are not known. Many of the multiple promoters of the human ERα and β genes (section 1.5.2) contain EREs and some were shown to be estrogen-responsive (Matthews and Gustafsson 2003; Pinzone et al. 2004); however, it is not know if the remaining promoters are unresponsive, which could be a key factor in the differential regulation of ER mRNAs among tissues by tissue-specific promoter usage. Moreover, differential regulation was found between the wt ER and the mRNAs coding for several ER variant proteins that have distinct functional properties than that of the wt ERs, and this may contribute to the final estrogenic response (Tena-Sempere et al. 2004; Bryant et al. 2005; Varayoud et al. 2005).

While, in most cases, the ERα protein appears to be the most important form mediating the estrogen up regulation of wt ER α or β and variant ER mRNAs, one variant ER protein was recently shown to auto-regulate its promoter (Denger et al. 2001a; Matthews and Gustafsson 2003; Bryant et al. 2005).

Estrogens also affect ER protein levels by post-transcriptional or post-translational mechanisms. In some mammalian systems, short-term exposure to estradiol induces proteasome-dependent degradation of ERα and ERβ proteins, down regulating their levels, which for ERα was shown to be followed by mRNA up regulation and repletion of ER protein levels (Ihionkhan et al. 2002; Tschugguel et al. 2003; Pinzone et al. 2004). Moreover, estrogens autoregulate the stability of ERα mRNAs in fish liver and in the mammalian endometrium, through unknown mechanisms, which may greatly account for the increase in ER mRNA levels (reviewed by Ing 2005).

In fish, the up regulation of ERα mRNA in liver, characteristic of oviparous species, has been demonstrated for several fish species (MacKay et al. 1996) and shown in rainbow trout to require, for full transcriptional activation, the synergism between an ERE half-site and an imperfect ERE and the cooperation between ERα and the COUP transcription factor (Petit et al. 1999). Only recently has the estrogen regulation been compared between the three ER genes in the liver of two fish species, showing strong ERα up regulation and slight up or down regulation of the ERβ genes, depending on the subtype and species (Menuet et al. 2004; Sabo-Attwood et al. 2004). Moreover, ERα up regulation required not only the ERα but also one of the ERβ proteins, while the other was not involved, suggesting that fish ERβs play different roles in the transcriptional activation of the ERα gene (Menuet et al. 2004).

1.7. Molecular determinants of the tissue-specificity of estrogenic responses

During the above literature review, it became evident that estrogenic actions are complex and multifactorial processes, involving both nongenomic and genomic effects that may be directly or indirectly mediated by different receptor types, subtypes and variants, localized at several sub-cellular pools.

The different receptors may have different affinities and transcriptional responses to different ligands, cofactors and promoters (better studied for the two mammalian ER subtypes, reviewed by Matthews and Gustafsson 2003), and ultimately different *in vivo* functions, as indicated by the phenotypes of gene-null animals for each subtype (main references in section 1.3.2.1), while their differential regulation by estrogens and other compounds results in different patterns of expression among target tissues (sections 1.5.4 and 1.6). Moreover, ER subtypes and variants may differentially interact with and modulate the actions of intracellular signalling cascades and other transcription factors (section 1.4), as well as with other ER subtypes/variants in the cell-types where they are co-expressed (section 1.5.3.5), and several of these components may contribute to finely regulate the estrogenic action at certain target genes.

It also became evident that the nature of the ligand differentially affects the conformation of the receptor and subsequent recruitment of coactivators and corepressor proteins to both AF-1 and AF-2 functions, which together with the cell-type-specific array of coactivators and corepressors appears to be the main determinant for the final effect on transcription of a given ligand, i.e., stimulating or repressive (section 1.5.3.5, recent review by Nettles and Greene 2005). Furthermore, the DNA response elements themselves were shown to allosterically modulate the ER conformation and determine the differential transcriptional responses of certain ligands at different promoters (see section 1.5.3.3 and Geserick et al. 2005), as well as determining whether the ER or other transcription factors will mediate

the estrogenic action. In addition, ERs may also be activated in a ligand-independent manner in response to other signalling pathways.

Thus, the ER-mediated modulation of the transcriptional activity of ERGs is tightly regulated *in vivo* and may be influenced by a variety of factors, including the nature of the extracellular stimuli (e.g. the structure of the ligand), the characteristics of the promoter, the balance between the expression levels of ER subtypes and variants, other transcription factors, intracellular signalling molecules and coactivator or corepressor proteins, or the cellular localization of ERs, and many of these factors are dependent on the cell-context. This is the basis for the tissue- and promoter-specific differential effects on transcription (agonistic or antagonistic) of selective ER modulators, SERMs. The tissue-specificity of SERMs has been extensively studied in mammals and exploited to develop new drugs for the treatment of estrogen-related diseases in some tissues (e.g. breast cancer and osteoporosis) without deleterious side-effects in other tissues (Diel 2002; McDonnell 2004; Henke and Heyer 2005). However, in fish the information on the mechanisms of estrogen action and genes involved is still scarce, and the presence of a third ER subtype is an additional variant that must be investigated.

1.8. Aim and outline of this thesis

The aim of this thesis is to investigate the diversity, expression and mechanisms of action of estrogen receptors in sea bream, in order to contribute to the elucidation of estrogen and estrogen receptor functions in the physiology of fish.

The gilthead sea bream (*Sparus auratus*), a marine teleost fish from the Sparidae family, was chosen as experimental model to investigate the above referred aspects. The sea bream is commonly found throughout the Mediterranean Sea and along the Eastern Atlantic coast, from Great Britain to Cape Verde and around the Canary Islands. It is one of the most important commercial species for Eastern Atlantic and Mediterranean fisheries and aquaculture (www.fishbase.org) and due to its high economical value, it has been extensively used as a experimental model to study its growth, reproduction, physiology and endocrine regulation. Furthermore, a range of molecular tools for this species is available in several European marine research centers, such as tissue-specific cDNA libraries and a high number of expressed sequence (ESTs) tags from a sea bream genome-mapping project currently undergoing (http://www.bridgemap.tuc.gr/index.htm).

Sea bream belongs to the Perciforme order, one of the most successful groups of fish, which is characterized by multiple alternative reproductive strategies, including different types of hermaphroditism (Devlin and Nagahama 2002), making them good models to study gonadal development and differentiation.

Sea bream is a protandrous hermaphrodite, i.e., it first matures as functional male, but may undergo a sex reversal to female in one of the subsequent spawning seasons (Zohar et al. 1978). Spawning in southern Portugal occurs between October and February. The sequence of gonadal events, steroid plasma levels and steroidogenic changes that occur during sex reversal have been characterized (Zohar et al. 1978; Condeca 2001), and E_2 treatment has been shown to induce testicular regression and development of functional

ovaries (Happe and Zohar 1988; Condeca and Canario 1999). In both natural and E_2-induced sex reversal, the regression of the testicular (ventral) region appears to occur first, followed by an effective growth and development of the ovarian (dorsal) part of the bisexual gonad (Condeca 2001). An ERα and an ERβ cDNAs were isolated and important changes in their hepatic and gonadal expression detected in response to E_2 treatment, suggesting they have an important role in relation to reproduction and sex reversal (Socorro et al. 1999; Socorro 2001). E_2 actions in calcium uptake were reported in sea bream, but the role of ERs was not investigated (Guerreiro et al. 2002).

The thesis was organized as follows:

CHAPTER 2 describes the general materials and methods used throughout this thesis, while the results obtained are described and discussed in chapters 3, 4, 5, 6 and 7.

CHAPTER 3 describes the molecular cloning of a second highly polymorphic sea bream ER (sbERβb), the characterization of its ligand-binding properties and a comparative analysis of its structure and mRNA expression with the previously cloned sbERα and sbERβa. Phylogenetic and Southern blot analyses were used to demonstrate that the three forms are encoded by different genes. The *in vitro* phosphorylation of each recombinant sbER protein (chapter 5) by MAP kinase 2 was investigated to evaluate if they are responsive to this intracellular signalling pathway.

In **CHAPTER 4**, the existence of internal variant transcripts for sbERs was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers designed based on the intron/exon organization for each sbER gene, which was predicted by comparative analysis with the *in silico* identified *Fugu rubripes* ER genes. The mRNA tissue distribution and hepatical expression in response to estrogen or estrogen antagonist was

compared between the identified variants, all from the ERα subtype, and the wild-type sbERα.

CHAPTER 5 describes the generation and characterization of specific antibodies for each sbER subtype, as well as the production of each recombinant sbER protein, which provided the material for specificity tests of the anti-sbER antibodies and for ligand-binding and *in vitro* phosphorylation studies (chapter 3). The ER-subtype specific antisera were used to investigate the cellular localization of each ER protein in sea bream scales by immunohistochemistry, while the *in situ* hybridization of TRACP was used to identify the ER-immunoreactive cells as putative osteoclasts. To further understand the estrogen actions on sea bream calcium metabolism, the tissue distribution of sbER mRNAs was investigated by RT-PCR in several calcified tissues.

CHAPTER 6 describes the hepatic and testicular regulation of mRNA expression of sbERs in response to estradiol or the antiestrogen ICI 182,780. The expression of the ERGs vitellogenin 2 and choriogenin L were also analyzed, as well as the calcium levels as an indicator of estrogenic response, in order to give insight into the mechanisms of actions of estrogens and ICI 182,780 in these tissues.

In **CHAPTER 7** Suppressive Subtractive Hybridization was used to identify candidate estrogen-responsive genes in the testis of male E_2-treated sea bream, in order to give insight into the functions and mechanisms of E_2 actions in the testis. 152 different genes were identified by sequence analysis and grouped according to the putative biological function. The E_2 up regulation of some of the genes was confirmed by reverse transcriptase polymerase chain reaction in fish treated with different doses of E_2.

CHAPTER 2

General Methods

This chapter contains the general materials and methods used throughout the thesis. Common laboratory techniques and general procedures for reagent preparation are based upon protocols from *Molecular cloning: a laboratory manual* (Sambrook et al. 1989), unless otherwise mentioned. Additional methods that have been used for specific tasks are presented in the respective chapter (chapters 3-7). Solutions, media, vectors, strains and primers used are listed in Appendices I-V.

2.1. Safety considerations

Protective clothes and disposable latex gloves were used for most manipulations, especially those involving hazardous chemicals like phenol, acrylamide, formaldehyde, DEPC and ethidium bromide, which were handled in a fume hood when possible. Handling and disposal of radioactivity (^{32}P, ^{35}S and ^{3}H) was carried out in accordance with Portuguese and European Regulations (European Union directive no. 89/391/CEE from 12/06/89; Decreto-lei no.301/2000 from 18/11/00; Decreto-lei no.348/89 from 12/10/89 and Decreto-Regulamentar no.9/90 from 19/04/90).

2.2. Animal maintenance

All animal maintenance and handling procedures were carried out in compliance with the recommendations of the Association of Animal Behaviour (ASAB 2003).

Immature (approx. 50 g body weight) or mature sea bream (200g -3 kg) were obtained from TIMAR Cultura de Águas (Olhão, Portugal) and maintained in Ramalhete Marine Experimental Station (University of Algarve, Faro, Portugal) in through-flow seawater tanks under natural annual conditions of water temperature, photoperiod and salinity. Due to the high proportion of animals with both sexes that usually exist at the second reproductive cycle in sea bream, samples collected from 200-400 g male sea bream (first and second reproductive cycle, respectively) were only used in this study after visual

inspection of the gonads for the inexistence of dorsal ovary tissue. In some experiments, histology was used to confirm that the ovary was a low proportion of the gonad and the testis was dominant and active (chapter 7). Sea bream females (3-5 kg) were sampled to provide tissues when required.

2.3. Hormone treatments and sampling

All experiments were conducted in September-November (at the beginning of the reproductive season), and involved sampling of tissues from control fish or from fish treated with coconut oil implants containing different doses of E_2 and/or the estrogen antagonist ICI 182,780. The specific doses, period of treatment and sea bream used in each experiment are described in the methods section of chapter 6.

Animals were randomly distributed between different tanks (one tank per treatment) and left to acclimatize for at least seven days prior to the start of experiments. Treatment was administered in fish that had been anaesthetized with 2-phenoxyethanol (1:10,000 2-phenoxyethanol:seawater; Sigma-Aldrich, Madrid, Spain) as an intra-peritoneal (i.p.) implant of coconut oil (200 µl /100 g body weight, Sigma) containing different doses of hormone or coconut oil alone (control fish). Fish were returned to their tanks and left undisturbed during the experimental period (1-2 days).

On the day of sampling, fish were anaesthetized (1:5,000 2-phenoxyethanol:seawater), weighed, measured and blood samples collected from the caudal vein with heparinised (ammonium heparin, Sigma-Aldrich, 150 U/ml) 1 ml syringes fitted with 25G needles. Plasma was obtained by centrifugation of whole blood (10,000 rpm for 5 min) and stored at -20ºC. Fish were killed by decapitation and tissues were immediately sampled, snap frozen in liquid nitrogen and stored at –80 ºC for subsequent DNA or RNA extraction. Tissues for histology were immersed in Bouin-Holland fixative (Sigma-Aldrich) and stored at room temperature.

2.4. Total RNA extraction

All work involving RNA was performed using disposable latex gloves, autoclaved materials and DEPC-treated water (Appendix I) to avoid RNase contamination. Total RNA was extracted from 100-500 mg of frozen tissues (-80ºC) using TRI Reagent (Sigma-Aldrich, modified guanidine thiocyanate / phenol / chloroform method) and an Ultra Turrax mechanical homogenizer (IKA Labortechnik, Staufen, Germany). All additional steps recommended in the protocol to avoid DNA contamination were performed, such as the initial centrifugation before chloroform addition and precipitation of DNA with 0.1 volumes of isopropanol. The quality and quantity of the extracted RNA, resuspended in 20-500 µl of DEPC-treated water, was assessed by measuring its absorbance at 260 and 280 nm (1Abs_{260}=40µg/ml of RNA; ratio Abs_{260}/Abs_{280} estimates RNA purity) and analyzing 1 µg of RNA by agarose gel electrophoresis (Appendix II.2).

2.5. mRNA purification

Poly(A)$^+$ RNA was purified from 0.5-1.25 mg of total RNA extracts using the mRNA purification kit (Amersham Biosciences, Buckinghamshire, England, now part of GE Healthcare), following the manufacturer's instructions. This purification kit is based on the interaction of the polyadenylic acid tail of mRNA (polyA tail) to oligo(dT)-cellulose spun columns under high-salt conditions (0.5 M NaCl), washing of unbound RNA in 0.5-0.1M NaCl and elution of Poly(A)$^+$ RNA in TE pH 7.4 buffer, pre-warmed to 65ºC. Eluted Poly(A)$^+$ RNA was ethanol precipitated, stored at -80ºC, when needed pelleted by centrifugation (Appendix II.4) and resuspended in 30 µl of DEPC water. Quality and quantity of Poly(A)$^+$ RNA were assessed by measuring Abs_{260} and Abs_{280} with a capillary and analysing 1-5 µl on a denaturing agarose-formaldehyde gel (Appendix II.3).

2.6. Complementary DNA (cDNA) synthesis

cDNAs were synthesized from 4 μg of heat-denatured RNA (65ºC for 10 min) in a 30 μl reaction containing 10 mM DTT, 1 mM of dNTPs mixture, 1 μg of random hexamer primers ($pd(N)_6$, Amersham), 6 U of RNAguard RNase inhibitor (Amersham Biosciences) and 40 U of MMLV RT (reverse transcriptase) in 1x RT buffer (Invitrogen, Carlsbad, CA, USA). Reactions were incubated for 2 hours at 37ºC, heated at 65ºC for 10 minutes to inactivate the enzyme and stored at -20ºC.

2.7. Polymerase-chain reaction (PCR)

A typical PCR reaction consisted of 1 μl of cDNA or an appropriate dilution of plasmid DNA, 25 pmol of each primer, forward and reverse (complete list in Appendix V), 200 μM each dNTP, 1.5-3 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Promega, VWR, Portugal), 1x PCR buffer and molecular biology grade water (Sigma-Aldrich) to a final volume of 50 μl.

Thermocycling conditions consisted of an initial denaturing step of 94ºC for 3 min, followed by 15-40 cycles of 94ºC for 40 sec (denaturing), 42-61ºC for 30-60 sec (annealing) and 72ºC for 50-180 sec (usually 60 sec/Kb expected, extension). A final elongation step was performed at 72ºC for 5 to 10 min and PCR products were analysed by agarose gel electrophoresis (Appendix II.1).

2.8. Cloning of PCR products

PCR products were purified directly from the PCR reaction (if one single band was visible on gel) or from an excised agarose gel band using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). Purified DNA was eluted in 30-50 μl of stH2O and 1 μl analysed on an agarose gel before cloning or using as a probe.

The purified DNAs were cloned into pGEM-T Easy or pGEM-T vectors (Promega). These vectors are supplied linearized and possess a single 3' thymidine (T) residue at both ends (see Appendix III), which facilitates ligation to the 3'single deoxyadenosine (A) residues added by the 5´→3´ exonuclease activity of some DNA polymerases, including *Taq* polymerase, to PCR products (T/A cloning).

Ligation was performed overnight (ON) at 4ºC using 15ng of vector, 10-200 ng of purified PCR product, 5 µl of 2x Rapid ligation buffer, 1.5U of T4 DNA ligase (Promega, supplied with the vector) and stH_2O to a final volume of 10µl.

For transformation, 5 µl of ligation reaction was mixed with a 100µl aliquot of *E. coli* competent cells (Appendix II.5), incubated on ice for 30 min and heat-shocked for 2 min at 42ºC followed by 2 min on ice. Transformed cells were plated on LB agar plates with ampicillin / IPTG / X-Gal (Appendix I) and grown ON at 37ºC.

The next morning, transformed colonies were selected by "blue/white selection", available for both pGEM-T and pGEM-T Easy vectors. These vectors contain the IPTG-inducible *lac* operon promoter followed by the multiple cloning site (MCS) located inside the coding region for β-galactosidase. This enzyme is responsible for X-Gal degradation producing a blue colour. Thus, when grown on plates with IPTG/X-Gal, cells containing a plasmid without insert turn blue, while positive transformants with a DNA insert in the MCS will remain white, due to the interruption of the enzyme coding sequence which means that the active enzyme is not produced.

2.9. Small-scale plasmid purification (miniprep)

In order to characterize positive transformants, plasmid DNA was isolated by the alkaline-lysis method, as described in Sambrook et al. (1989). Single colonies were inoculated into 2 ml of LB broth with 80 µg/ml ampicillin and grown ON at 37ºC with vigorous shaking.

1.5 ml of culture was spun for 1 min in a microcentrifuge at maximum speed and the pellet resuspended in 100 µl of ice-cold GTE buffer (Appendix I). To induce cell lysis, 200 µl of a freshly prepared solution of 0.2N NaOH / 1% SDS were added, mixed by inversion and incubated 5 min on ice. 150 µl of ice-cold 3M KAc (Appendix I) were added to neutralize the lysate, mixed by inversion and incubated on ice for 5 min. Cellular debris were spun down for 10 min at maximum speed and the supernatant transferred to a fresh tube. RNase A was added to a final concentration of 20 µg/ml and incubated for 20 min at 37ºC. An equal volume of phenol pH 8.0: chloroform (mix 1:1) was added to the mixture, mixed by vortexing and centrifuged for 2 min. The supernatant was ethanol precipitated (Appendix II.4) and resuspended in 50 µl of stH_2O.

To confirm the presence of an insert, 1-2 µl of purified plasmids was digested (Appendix II.6) in a 10µl reaction with 6U of *Eco*RI (for pGEM-T Easy vector) or 5U each of *Nco*I and *Sal*I (pGEM-T) and analysed on 1% agarose gels (Appendix II.1). When a mini plasmid preparation of higher quality was required (e.g. for sequencing reactions) the Wizard Plus Miniprep DNA Purification System (Promega) was used according to the manufacturer's instructions.

2.10. Sequencing and sequence analysis

All sequencing reactions were made by the dideoxy chain termination method and analysed by automatic DNA sequencers - ABI Prism 377 (Sistemas Genomicos, Valencia, Spain) or ABI 3100 (Molecular Biology Unit, Biosciences Department, Cardiff University, UK).

Bioinformatic web resources and software routinely used for sequence analysis were:

- BlastN, BlastX and BlastP at http://www.ncbi.nlm.nih.gov/BLAST/ for sequence comparison against the non-redundant public databases (Altschul et al. 1997);

- Fugu BLAST Server at http://fugu.biology.qmul.ac.uk/blast/ for *in silico* identification of orthologous genes in the *Fugu rubripes* genome;

- Ensembl BLASTView at http://www.ensembl.org/Multi/blastview for *in silico* identification of orthologous genes in *Tetraodon nigroviridis* and *Danio rerio* genomes;

- BCM Search Launcher Sequence Utilities at http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html for basic DNA sequence manipulation (format conversion, sequence inversion-reversion, six-frame translation, restriction analysis);

- ClustalX (version 1.8; Thompson et al. 1994) for multi-sequence alignments;

- GeneDoc (version 2.6; Nicholas et al. 1997) for multi-sequence alignment analysis, shading and editing, sequence identities calculation, identification of functional motifs and analysis of group sequence contrasts.

The use of other programs or web resources is indicated in each chapter.

2.11. Labelling cDNA probes

The radioactive labelling of cDNA probes with ^{32}P was required for the screening of cDNA libraries, Northern blot and Southern blot. DNA fragments obtained by PCR or by plasmid digestion with adequate enzymes were labelled by the random-priming method using the Rediprime DNA labelling System (Amersham Biosciences). Briefly, 45 µl of DNA diluted in stH_2O was denatured for 5 min at 95ºC and then added to the labelling mix. 20-50 µCi of [α-^{32}P]-dCTP (specific activity 3,000 Ci/mmol, Amersham Biosciences) were added to the tube, mixed by pipetting and incubated for 10 min at 37ºC. The labelled probe was denatured at 95-100ºC for 5 min, snap cooled on ice and added to the hybridization solution, pre-warmed at hybridization temperature.

2.12. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to compare the expression of ERs and ERGs between different tissues or treatments (chapters 3-7). This method uses the amplification of an internal control gene (an endogenous mRNA expressed at a relatively high and constant level in all samples) to account for the amount of RNA and the efficiency of reverse transcription in each cDNA sample. The amplification signal of the target gene in each sample is normalized with the internal control, allowing the comparison of the relative expression levels between different samples (but not between genes quantified by different pairs of primers). The gene coding for the 18S ribosomal RNA was chosen as internal control, after preliminary tests in which 18S showed a more homogeneous expression between different tissues and samples treated with different E_2 doses than β-actin or elongation factor 1α (EF-1α).

All semi-quantitative RT-PCR experiments were conducted as follows:

(1) Optimization of annealing temperatures (Ta) and cycle number (N) for both target gene and 18S to ensure detection in the exponential phase of amplification (optimized Ta and N are indicated in Appendix V and in the methods of each chapter, respectively).

(2) Amplification of the 18S (495 bp fragment) in 25 µl reactions containing 1 µl of each cDNA, 10 pmol each primer (Appendix V), 60 µM each dNTP, 1 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase. Cycling conditions were 2 min at 94 ºC, 15-20 cycles of 45 sec at 94 ºC, 30 sec at 59 ºC and 45 sec at 72 ºC, and 5 min at 72 ºC.

(3) Amplification of the target gene in 25 µl reactions containing 1µl of each cDNA, 12.5 pmol each primer (Appendix V), 200 µM each dNTP, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase. Cycling conditions were the same for all genes: 3 min at 94ºC, N cycles of 40 sec at 94ºC, 30 sec at Ta and 50 sec at 72ºC, followed by 5 min at 72 ºC.

(4) Analysis of 5μl of each PCR reaction in ethidium bromide stained 1-1.5% agarose gels (Appendix II.1) and quantification of band intensities by densitometry using the ImageMaster 1D Prime v3.00 software (Amersham Biosciences).

(5) Calculation of the relative expression for each sample as log10 (target/18S).

(6) Statistical analysis of relative expression values using the general linear model (GLM) module of SYSTAT (v.10, SPSS Inc, Chicago, USA). Post-hoc differences between treatments were evaluated by pairwise comparisons using the Tukey test. Pearson correlations of expression levels among genes were calculated and probabilities determined with Bonferroni corrections. Statistical significance was established at $P<0.05$.

All primers were designed to span at least two exons to discriminate complementary from genomic DNA and reverse transcriptase-minus reactions were used to control for genomic DNA contamination in extracted RNA. The specificity of some pairs of primers was validated by cross-amplification tests between miniprep dilutions of clones of the same gene family (ERs, choriogenins, vitellogenins, fibrinogens) or between different transcript variants of the same gene (sbERα), where no products were in general detectable.

CHAPTER 3

Characterization of estrogen receptor βb in sea bream (*Sparus auratus*): Phylogeny, ligand-binding and comparative analysis of expression.

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3.1. Introduction

Estrogens actions are mediated by specific nuclear estrogen receptors (ERs) (Klinge 2001; Nilsson et al. 2001) or via membrane recognition sites or receptors, in the case of non-genomic rapid effects (Revelli et al. 1998; Watson and Gametchu 2001). ERs are expressed in target tissues and regulate the transcription of estrogen-responsive genes by binding as dimers to specific estrogen response elements (ERE) found in their regulatory regions, a mechanism involving recruitment and interaction of ligand-bound receptors with coactivators and/or corepressors (Nilsson et al. 2001). In addition, there is increasing evidence of ER indirect association with target gene promoters through interaction with proteins within transcription factor complexes (Kushner et al. 2000) and of ligand-independent transcriptional activation of ERs (Lannigan 2003). For example, in response to growth factor activation the mitogen-activated protein kinase (MAPK) ERK2 (extracellular-regulated kinase) was shown to phosphorylate specific serine residues in the A/B domain of mammalian ER proteins, resulting in increased coactivator recruitment and transcriptional activation (Kato et al. 2000; Tremblay and Giguere 2001; Lannigan 2003).

In tetrapods, two ERs have been identified which are encoded by different genes, ERα and ERβ. Despite their similar overall structure the two ER forms have overlapping but distinct ligand binding affinities, tissue distribution, transcriptional activities and knockout phenotypes (Matthews and Gustafsson 2003). Both are present in teleost fish, where two ERβ forms, encoded by distinct genes, have recently been isolated (Tchoudakova et al. 1999; Hawkins et al. 2000; Ma et al. 2000; Bardet et al. 2002; Menuet et al. 2002). Sequence analysis and phylogenetic studies of ERβ suggest the two forms arose by duplication of an ancestral gene (Robinson-Rechavi et al. 2001; Bardet et al. 2002). The existence of two ERβ subtypes raises questions about ERβ-mediated estrogen function in fish and it remains to be established if these receptors have distinct or redundant functions.

In the protandrous hermaphrodite teleost, sea bream (*Sparus auratus*), a ERα and a ERβ (sbERα and sbERβa, formerly sbERβ) have previously been isolated (Socorro et al. 2000). Analysis of mRNA expression revealed the presence of multiple transcripts of both receptors. The ERα form had a restricted distribution and was expressed mainly in testis, liver and heart, while the ERβ form was present in most tissues but was most abundant in ovary, testis, liver, intestine and kidney (Socorro et al. 2000). Important changes in hepatic and gonadal expression of these receptors occur in response to estradiol (E_2) treatment suggesting they have an important role in relation to reproduction and sex change (Socorro et al. 1999). The objectives of the present study were 1) to investigate the presence of a second ERβ gene in sea bream, 2) to characterize its ligand-binding properties, and 3) to compare the phosphorylation by MAP kinase 2, the number/size of transcripts and pattern of tissue distribution between sbER subtypes.

3.2. Specific methods

3.2.1. Cloning of a sbERβb cDNA probe

A sbERβb homologous probe was obtained by PCR (section 2.7) using two degenerate primers ERf1 and ERr1 (Appendix V) designed to conserved regions of an alignment of all available fish ERβ sequences. The 50 µl PCR reaction contained 2 µl of ovary cDNA from a female sea bream (3 kg), 20 pmol of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase. Cycling conditions were 3 min at 94 ºC, 30 cycles of 40 sec at 94 ºC, 1 min at 51 ºC and 1 min at 72 ºC, followed by 5 min at 72ºC. A fragment of the predicted size (320 bp) was amplified, gel purified, cloned in pGEM-T Easy vector and sequenced (sections 2.8-2.10). The fragment shared high homology to other fish ERβ and was used as a probe to screen a sea bream pituitary cDNA library.

3.2.2. Sea bream pituitary library

A sea bream pituitary cDNA library previously constructed in the MCE group (Santos et al. 1999) was used to isolate the full-length cDNAs for sbERβb. This library was constructed from 30 individual pituitaries of adult fish (approx. average size 400 g) using the UNI-ZAP XR cDNA cloning kit (Stratagene). This kit allows directional cloning of cDNA clones and, once a positive bacteriophage is identified, excision of the pBlueScript SK(+/-) phagemid (Appendix III), containing the cDNAs directionally inserted between the *Eco*RI and *XhoI* restriction sites of the MCS.

3.2.3. Library screening

Two independent screenings of the sea bream pituitary library were performed with: (i) the sbERβb homologous probe and (ii) a 1000 bp heterologous probe of European sea bass (*Dicentrachus labrax*) ERβb (Accession no. AJ489524). Briefly, recombinant phages were plated, transferred to duplicate nylon membranes, hybridized with ^{32}P-labelled probes and positive clones identified and isolated as described below. After a second round of screening, two positive clones were isolated in screening (i) and one positive in screening (2). All solutions and media are described in Appendix I, while host bacteria characteristics and preparation are in Appendix IV.

3.2.3.1. Plaque lifts

In each primary screening, 300,000 recombinant phages were plated in 150 mm square NZY agar plates at a density of 50,000 plaque-forming units (pfu) each. 10 µl aliquots of the sea bream pituitary library diluted 1:100 in SM buffer were mixed with 600 µl of prepared XL1-B host cells and incubated for 15 min at 37ºC. Each aliquot of infected bacteria was mixed with 10 ml of NZY Top agar (previously melted and cooled to 48ºC) and plated out on NZY agar plates. Plates were incubated for 5-9 h at 37ºC and chilled for

2h at 4ºC. Phage were transferred to duplicate nylon membranes (Hybond-NX, Amersham Biosciences) for 2 min (first filter) or 4 min (duplicate filter) and pricked with a needle at the same four asymmetric points for orientation. DNA on filters was denatured by incubating for 5 min in a denaturing solution, neutralized for 5 min in a neutralizing solution and equilibrated for 5 min in 6xSSC buffer. Filters were air dried and fixed to the membrane by baking for 2h at 80ºC.

3.2.3.2. Hybridization

Both probes were PCR-amplified from 1:50 plasmid dilutions with specific primers, purified using the GFX purification kit and labelled by random-priming with 50 µCi of [α-^{32}P]-dCTP (sections 2.7, 2.8 and 2.11).

Filters were pre-hybridized for at least 2h in hybridization bottles (6 filter/bottle) containing Church-Gilbert solution at 58ºC (sbERβb homologous probe) or at 52ºC (heterologous probe), and then hybridized ON after addition of each ^{32}P labelled probe. High stringency washes were performed in the homologous probe screening (15 min at 58 ºC with 1xSSC / 0.1% SDS followed by 4 times 15 min with 0.1xSSC / 0.1% SDS, 58ºC) while less stringent washes were applied for heterologous probe-hybridized filters (4x 15 min with 1xSSC / 0.1% SDS at 52ºC). After filter exposure for 1-5 days to Biomax MS film (Kodak, New York, USA), the position of the putative positives (duplicated signals) was identified in the plates. Agar cores of phage of interest were collected into 500 µl SM buffer with 20 µl chloroform and eluted ON at 4ºC.

3.2.3.3. Secondary screening

1-10 µl of a SM buffer dilution (1:1000) of each phage eluate were plated with 200 µl of host cells (500-1000 pfu/plate). After hybridization and exposure as indicated above, putative positives were recovered from SM buffer/chloroform. Since all putative positives

were isolated on the plate, collected cores containing unique pfu were excised with no need of tertiary screening.

3.2.3.4. *In vitro* excision of pBlueScript from Uni-ZAP XR

100 µl of each eluted phage stock were combined with 200 µl of XL1-B host cells and 1 µl of ExAssist helper phage (Invitrogen) and incubated at 37ºC for 15 min. 3 ml of LB broth were added and the mixture incubated for 3 h at 37ºC with shaking. Tubes were then heated for 20 min at 70ºC, centrifuged, 1000xg for 15 min and the supernatant decanted to a new tube and stored at 4ºC. 10 µl of excised pBlueScript phagemid were incubated for 15 min at 37ºC with 200 µl of freshly prepared SOLR host cells and 30 µl of this mix plated on LB agar/ampicillin plates. After incubation ON at 37ºC, one single colony per excision was transferred to 2 ml LB broth/ ampicillin, grown ON at 37ºC with shaking and plasmid DNA purified by alkaline-lysis (section 2.9). Plasmid DNA was analysed by digestion with 6U *Eco*RI and 5U *Xho*I and then run in a 1% agarose gel (Appendix II).

3.2.4. Sequence data and phylogenetic analysis

The clones isolated from the cDNA library were sequenced in both directions with T3 and T7 primers (flanking the MCS of pBlueScript SK (+/-), Appendix III) and identified as ERβ by BlastX and BlastN. A combination of sub-cloning and primer walking was used to obtain the full-length sequence of the three isolated sbERβb cDNA clones. In each sub-cloning step, cloned inserts were digested with one internal cutting enzyme and one in the MCS and then religated ON in pBlueScript II KS(+/-) vector (Appendix III) with compatible ends. Competent cells were transformed with each construct and plasmid DNA purified and sequenced with T3 and T7 (sections 2.8-2.10).

Each library isolated sbERβb clone was sequenced to give at least 3-fold coverage and sequence polymorphisms were carefully confirmed. Sequences were assembled with the

CAP contig assembling program (Huang 1992). *In silico* identification of putative ER genes in available fish genomes was performed using BlastN as indicated in section 2.10. A phylogenetic tree including the amino acid sequences for sbERβb clone 21, the three putative *Fugu* ERs (chapter 4) and ERs from several groups of vertebrates (Table 3.1) was constructed using the maximum parsimony method with 1000 bootstrap replicates (PAUP software, v.4, Swofford 2002). *Homo sapiens* ER related receptor 1 (hERR1, P11474) was used as outgroup. GeneDoc was used to calculate sequence identities and to identify functional motifs and amino acids uniquely conserved within each fish ERβ group.

Table 3.1. Species, abbreviations and Genbank accession numbers of ER amino acid sequences used in multi-sequence alignments and phylogenetic tree construction.

Common name	Scientific name	Abbreviation	Accession no.
Human	*Homo sapiens*	hERa, hERb	P03372, Q92731
Mouse	*Mus musculus*	mERa, mERb	P19785, O08537
Rat	*Rattus norvegicus*	rERa, rERb	P06211, Q62986
Chicken	*Gallus gallus*	cERa, cERb	P06212, Q9PTU5
Xenopus	*Xenopus laevis*	xER	P81559
Crocodile	*Caiman crocodilus*	crocER	BAB79436
Dogfish shark	*Squalus Acanthias*	sharkERb	AAK57823
African catfish	*Clarias gariepinus*	afcfER	CAC37560
Channel catfish	*Ictalurus punctatus*	cfERa, cfERb	AAG24543, Q9IAK1
Goldfish	*Carassius auratus*	gfERa, gfERb1, gfERb2	AAL12298, Q9W669, Q9IAL9
Zebrafish	*Danio rerio*	zfERa, zfERba and zfERbb	P57717, CAC93849, CAC93848
Gilthead sea bream	*Sparus auratus*	sbERa, sbERba, sbERbb	Q9PVZ9, Q9W6M2, CAE30469 (clone 21)
Atlantic croaker	*Micropogonias undulatus*	acERba, acERbb	P57783, P57781
Red sea bream	*Pagrus major*	rsER	BAA22517
Bastard halibut	*Paralichthys olivaceus*	hlERa, hlERb	BAB85622, BAB85623
Medaka	*Oryzias sp.*	mdERa, mdERb	BAA25900, BAB79705
Blue tilapia	*Oreochromis aureus*	tERa	P50240
Nile tilapia	*Oreochromis niloticus*	ntERa, ntERb	Q9YH33, Q9YH32
Eel	*Anguilla japonica*	eERb	O13012
Rainbow trout	*Oncorhynchus mykiss*	rtERa, rtERb	P16058, P57782
Common carp	*Cyprinus carpio*	carpERb	BAB91218
Taiwan Shoveljaw carp	*Varicorhinus barbatulus*	vbarbER	CAC85366
Lake Candidus dace	*Candidia barbatus*	cbarbER	CAC85356
Japanese puffer fish	*Fugu rubripes*	fgERa, fgERba, fgERbb	Predicted, see chapter 4

3.2.5. *In vitro* phosphorylation by MAP kinase

Putative MAP kinase 2 phosphorylation sites were identified in the sbER amino acid using GeneDoc to search the motif P-X(1,2)-[ST]-P, where P=proline, X(1,2)= one or two amino acids of any nature, and [ST]=serine or threonine. This motif was not found in the N-terminal-tag sequence that is an integral part of sbER recombinant fusion proteins, which were expressed in *E. coli* BL21 as described in chapter 5. The cell lysis pellets of the ON induced cultures, found to contain the bulk of the ER proteins, were resuspended and dialysed (ERα, ERβb) or concentrated by ultrafiltration (ERβa) as described in section 5.2.2.3. Approximately 1 μg (roughly estimated by its Abs_{280}) of pellet solution containing each ER recombinant protein were incubated for 30 min at 30ºC with 50 ng of MAP kinase 2/ERK2 enzyme (Upstate, New York, USA) and 5 μCi of [^{32}P]-γ-dATP, in ADBI buffer (Appendix I.10) containing 100 μM ATP and 15 mM $MgCl_2$. Control phosphorylation reactions contained 5 μg of myelin basic protein (MBP, positive control), 1 μg of pellet from the sbERα pre-induced control culture (to control for ERK2 substrates in the *E. coli* extract) or 1 μg of pellet solution from the sbERα ON induced culture in the absence of enzyme (to control for intrinsic kinase activity in the *E. coli* extract).

The reactions were stopped by the addition of 0.5 volumes of 2x SDS sample buffer and proteins were denatured for 5 min at 100ºC before separation by SDS-PAGE (section 5.2.4; 8% for the negative controls and recombinant sbERs, predicted molecular weight, MW, of 67-79 kDa or 12 % for MBP, MW 18-23 kDa). Phosphorylated bands were then detected by exposure of dried gels to film.

3.2.6. Ligand-binding characterization of sbERβb

To determinate the concentration of protein necessary for ligand binding studies (50% total binding), a preliminary binding assay was conducted with different concentrations (estimated by the Abs_{280}) of the dialysed pellet solution containing the recombinant

sbERβb protein (chapter 5, section 5.2.2). The protein dilutions were incubated with 250 pM of [^{3}H]-E_2 (specific activity 5698 Gbeq/mmol, Amersham) in 100 µl of 0.05 M phosphate buffer with 1g/l gelatine (Appendix I) and incubated ON at 4 ºC in duplicate tubes. Free steroids were removed with dextran-coated charcoal (Chard, 1990) and total binding was determined by counting the supernatant. For saturation E_2-binding assays, 0.04-8 nM of [^{3}H]-E_2 was used and specific binding was obtained by subtracting non-specific binding (determined in the presence of 100-fold unlabelled E_2 excess) from total binding for each [^{3}H]-E_2 concentration tested. The dissociation constant (*K*d) and maximum number of binding sites (B_{max}) was determined by non-linear regression (Sigma Plot version 8.0, SPSS, Chicago, IL, USA) from three independent assays performed in duplicate. The pre-induced control extract displayed no E_2 binding activity.

The ability of selected steroids to displace [^{3}H]-E_2 (1nM) from its binding site was determined by adding to the reaction mixture unlabeled competitors (ICI 182,780 from Tocris, Cookson Ltd., Bristol, UK, all others from Sigma-Aldrich) at 10-fold dilutions from 0.1 to 10^5 nM. The IC_{50} for each steroid (the concentration that inhibits 50% of the maximum [^{3}H]-E_2 binding) was determined using non-linear regression curves for single site competitive binding analysis (Sigma Plot) for each of four independent experiments, performed in duplicate. The IC_{50} values were used to calculate the relative binding affinities of each steroid in relation to E_2. Significance of differences between the $Log_{10}IC_{50}$ of different steroids ($P<0.05$) were evaluated by one-way analysis of variance (ANOVA) using SigmaStat (v.3.00, SPSS Inc, Chicago, USA), followed by the post-hoc Tukey test.

3.2.7. Southern blot

3.2.7.1. Genomic DNA extraction and digestion

Genomic DNA was extracted from 50 mg of sea bream liver using a standard proteinase K and phenol: chloroform extraction. Briefly, tissue was homogenised in liquid nitrogen with mortar and pestle in 1 ml of extraction buffer (Appendix I), and 1 mg/ml proteinase K were added, vortexed and incubated for 3-5h at 65ºC with frequent mixing by inversion. 100 µg/ml of RNase A were added and incubated for 1 h at 37ºC. The mixture was centrifuged for 3 min at 5,000xg and the supernatant extracted for 10 min with 1 volume of a phenol pH 8.0: chloroform: isoamylic alcohol mixture (25:24:1), followed by 10 min of centrifugation as above. The upper aqueous phase was then extracted for 5 min with 1 volume of chloroform: isoamylic alcohol (24:1) and centrifuged for 10 min. The DNA in the upper aqueous phase was precipitated for 30 min at room temperature with 2/3 volumes of isopropanol, centrifuged as above for 15 min, washed twice with 75% ethanol, air dried and resuspended in 250 µl TE buffer pH 8.0. DNA quality and quantity was verified by spectrophotometry at 260 and 280 nm ($1Abs_{260}$= 50µg/ml DNA) and 0.7% agarose gel electrophoresis (Appendix II).

For each sbER, 10 µg of genomic DNA were digested ON at 37 ºC with 45U of either *Kpn*I, *Pst*I or *Sac*I restriction enzymes in 50 µl reactions and run in a 0.7% agarose gel (Appendix II) together with the λDNA/*Eco*RI+*Hind*III molecular weight marker 3.

3.2.7.2. DNA transfer and hybridizations

Following electrophoresis the gel was depurinated in 250 mM HCl, denatured for 15 min, neutralized for 15 min and equilibrated in 20x SSC with agitation (same solutions used in library screening, Appendix I). The gel was then placed in a capillary blotting apparatus (Figure 3.1), DNA was transferred to a nylon membrane (Hybond-XL, Amersham) ON in 20x SSC and was then fixed to the membrane by baking for 2h at 80ºC.

Hybridizations were carried out ON at 68 ºC in PerfectHyb Plus solution (Sigma-Aldrich) with [^{32}P]-α-dCTP- labelled (section 2.11.) sbER cDNA probes: clone 21 (sbERβb), the full-length sbERβa clone (Socorro et al. 2000) and a 1000 bp PCR fragment of ERα (Socorro et al. 2000). Stringency washes were performed at 68 ºC as follows: 2x 20 min with 1xSSC / 0.1% SDS and 10 min with 0.1xSSC / 0.1% SDS, followed by film exposure. These ER probes, utilised for hybridisation in both Southern and Northern blot (see below), were specific for their respective receptor and did not cross-hybridise with the other receptors, as shown by the lack of hybridisation with membranes to which 0.5 µl miniprep of each ER cDNA had been applied.

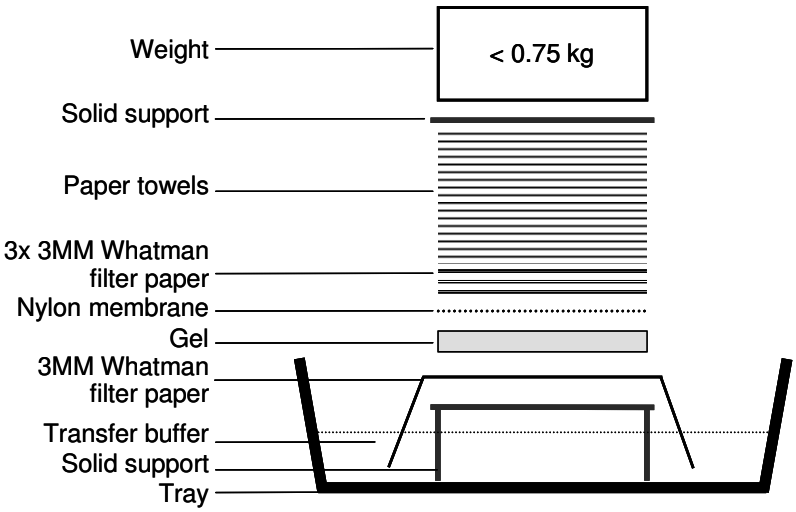

Figure 3.1. Blotting apparatus used for DNA (Southern blot) or RNA (Northern blot) transfer from gel to nylon membranes.

3.2.8. Northern blot

For Northern Blot, because of the limited sensitivity and cost of this method, the analysis was restricted mainly to female tissues in which relatively high ER expression might be expected. Poly(A)$^+$ RNAs (5 µg) from each of several tissues of a female adult sea bream and from the testis of a male sea bream were size fractionated on a denaturing agarose gel (1.5%) with formaldehyde (Appendix II.3) and transferred to a nitrocellulose membrane

(Hybond-XL, Amersham) as indicated above for Southern blot. The membrane was hybridised sequentially with the sbER cDNA probes and with a 200 bp PCR fragment of elongation factor-1α (EF-1α) (Nowell *et al.*, 2000), which was used to give a measure of the relative quantities of mRNA available for each tissue. Hybridisation was carried out at 58ºC and stringency washes were 3 x 15 min with 1xSSC / 0.1% SDS and 5 min with 0.1xSSC / 0.1% SDS at 58ºC. Stripping of membranes between hybridisations with the different probes was carried out by washing in 0.1xSSC / 0.1% SDS for 1 hour at 65 ºC and the efficiency of the process confirmed by film exposure. Transcript size was determined by comparison with the migration of a 0.24-9.5 kb RNA ladder (Invitrogen).

3.2.9. Tissue distribution of sbERs by RT-PCR

The tissues analysed by RT-PCR were those implicated in reproduction (pituitary, brain, liver and gonads) in both male and female, and other tissues from female fish in which estrogen has been observed/proposed to have a function. Semi-quantitative RT-PCR was performed in standard conditions (section 2.12) using specific primers for each sbER (Appendix V) and cDNAs from each of several tissues from the same fish used for Northern blot. The primers were specific for each receptor and did not cross-hybridize with the other ERs or with sbERα transcript variants (chapter 4), as shown by the lack of amplification using miniprep dilutions of each ER form / variant as template. Optimized annealing temperatures and cycle number were as follows: ERα 57ºC-30 cycles, ERβa 59ºC-28 cycles and ERβb 54ºC-32 cycles. 59ºC and 18 cycles were used for the internal control gene 18S.

3.3. Results

3.3.1. Cloning of three sbERβb cDNAs

Using RT-PCR with degenerate primers located in conserved regions of ERs (DNA- and ligand-binding domains), one PCR product of 320 bp was amplified from ovary. This fragment, which shared high homology to other fish ERβ, was used as a probe to screen a sea bream pituitary cDNA library, and yielded two positive clones, 21 and 32, of 2410 and 2181 bp, respectively. Clone 32 had a shorter 5'-untranslated region (UTR) (Figure 3.2) than clone 21 but both ER clones contained a 2007 bp open reading frame (ORF) encoding a protein of 668 amino acids with a deduced molecular weight of 74.4 kDa. The ORF of both clones was followed by a short 3'-UTR lacking a typical polyadenylation signal but containing a poly (A) tail. The longer 5'UTR of clone 21 was characterized by the presence of a short ORF of 34 amino acids, in a different frame and upstream of the main ORF.

Screening of the sea bream pituitary library with the sea bass heterologous probe identified a third ER cDNA (clone 54), of 3223 bp. Clone 54 was truncated at the 5' end of the coding region and was composed of an incomplete ORF of 1629 bp, followed by a long 3'-UTR of 1594 bp which lacked a typical polyadenylation signal although a poly(A) tail was present. Eight putative destabilizing AU-rich motifs with the consensus AUUUA were present in the 3'-UTR (Figure 3.2). Comparison of the 3 isolated cDNAs identified eleven nucleotide substitutions in the coding region (Figure 3.2), three of which caused an amino acid change, Q →E (amino acid 628), S → T (658) and T → S (662).

These cDNA sequences were deposited in the EMBL nucleotide sequence database under the accession numbers AJ580048 (clone 21), AJ580049 (clone 32) and AJ580050 (clone 54).

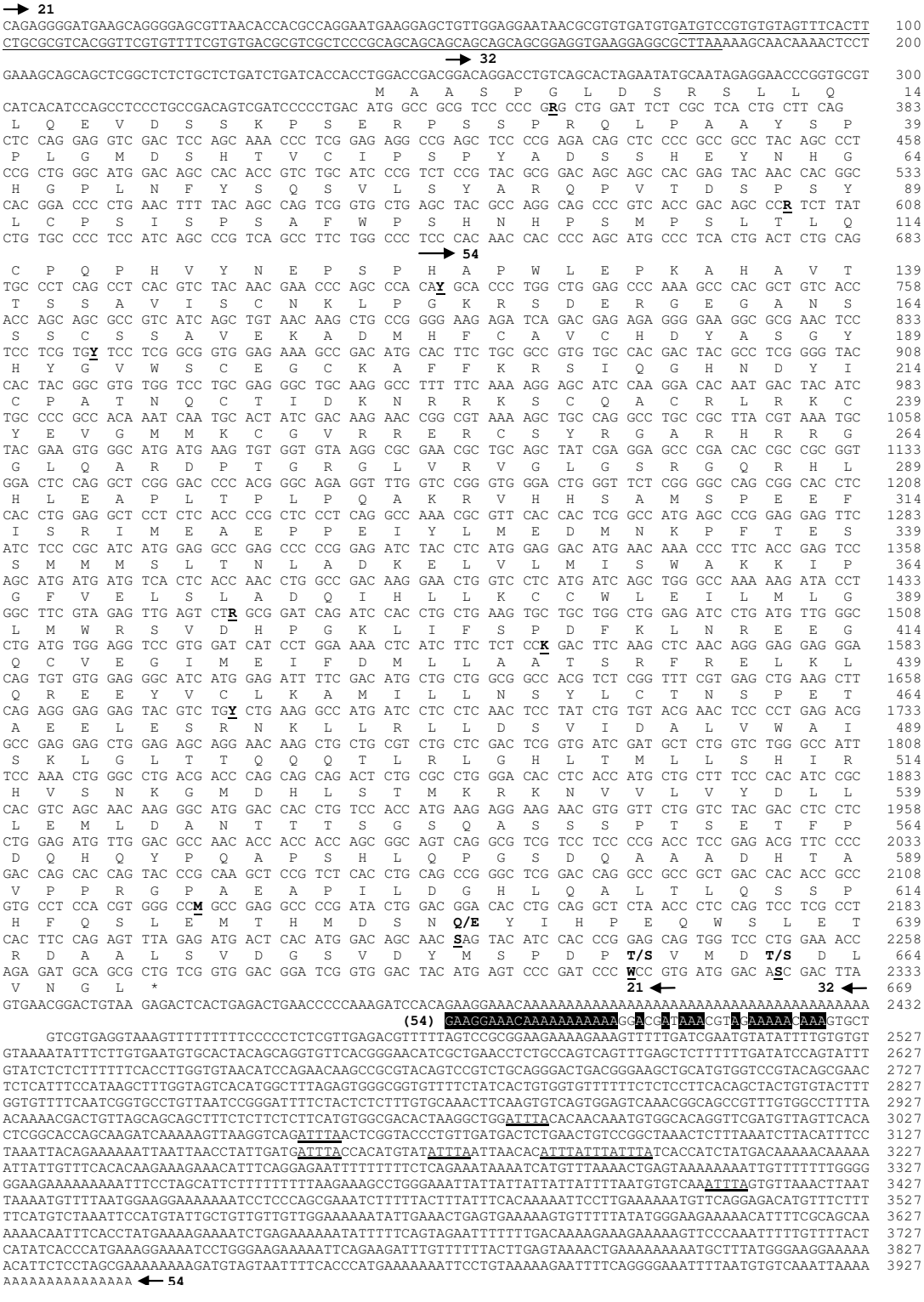

Figure 3.2. The nucleotide sequence and above it the deduced amino acid sequence of the three sea bream ERβb cDNA clones. Arrows indicate the beginning and end of each clone (numbered relative to clone 21). In the region where clones 21 and 32 diverge from clone 54 (poly adenylation tail of 21/32), an alignment is shown, with similar residues highlighted in reverse type. Where the clones overlap nucleotide differences are underlined and annotated as: R=A/G, Y=C/T, K=G/T, M=A/C, S=C/G and W=A/T. Amino acid changes are indicated separated by /, with the amino acid of clone 32 used for expression in *E. coli* and binding assays shown first. The small ORF in the 5'-UTR of clone 21 and eight putative mRNA destabilizing motifs AUUUA in the 3'-UTR of clone 54 are underlined.

3.3.2. Phylogenetic and structural analysis

The deduced amino acid sequence of the isolated ERβ clones gave the highest sequence identity (83%) to Atlantic croaker (ac)ERβb, 47% identity to the previously cloned sbERβ (here designated sbERβa- see discussion) and 33% to sbERα. The newly isolated sbERβ cDNA (named sbERβb) differed from sbERβa over the entire coding and non-coding regions, indicating that the two forms of sbERβ are likely to be generated by distinct genes, rather than by alternative splicing of a single gene.

The *in silico* identification of one ERα and two ERβ putative genes in several fish genomes (*Fugu rubripes, Tetraodon nigroviridis, Danio rerio*) favours the proceeding hypothesis, which was confirmed by the different hybridization pattern obtained with the three sbER probes in a Southern blot of sea bream genomic DNA (Figure 3.3).

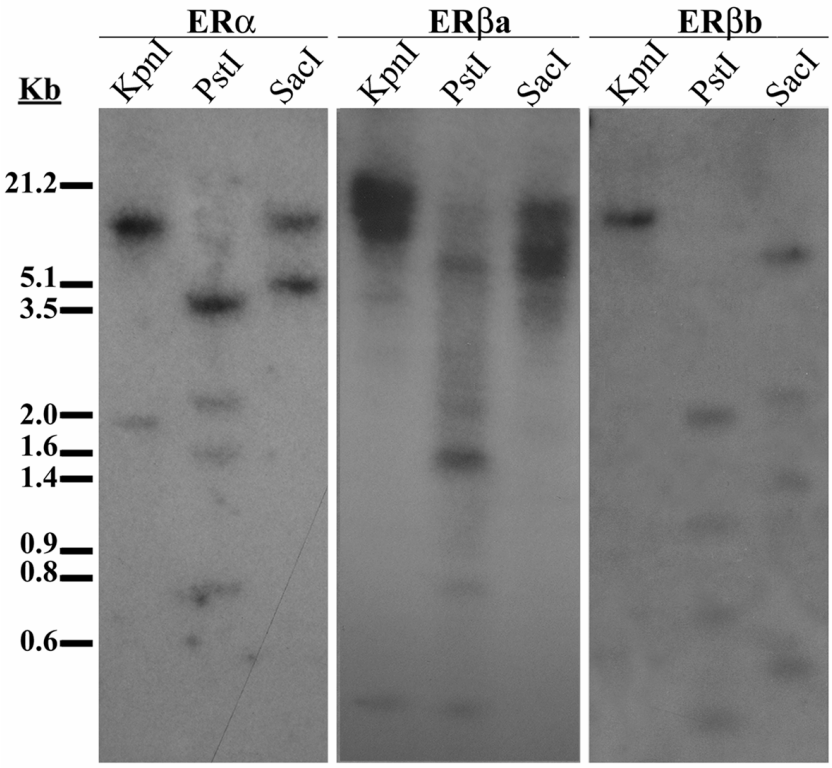

Figure 3.3. Southern blot of sea bream genomic DNA digested with *Kpn*I, *Pst*I or *Sac*I, hybridised with [^{32}P]-α-dCTP- labelled cDNA probes which were specific for sbERα or sbERβa or sbERβb. Molecular size marker bands (EcoRI/HindIII digested λ DNA) are indicated on the left in kilobases (Kb).

The phylogenetic tree of available ERs, including the predicted *Fugu* ERs (chapter 4), contained two major clades (Figure 3.4), which corresponded, respectively, to ERα and ERβ. Analysis of the clades revealed that the tetrapods and cartilaginous fish ERs were clearly separated from teleost ERs. The ERβ clade in teleost fish was composed of two subclades, one containing sequences with the highest similarity to sbERβa and the other with sequences having the highest similarity to sbERβb.

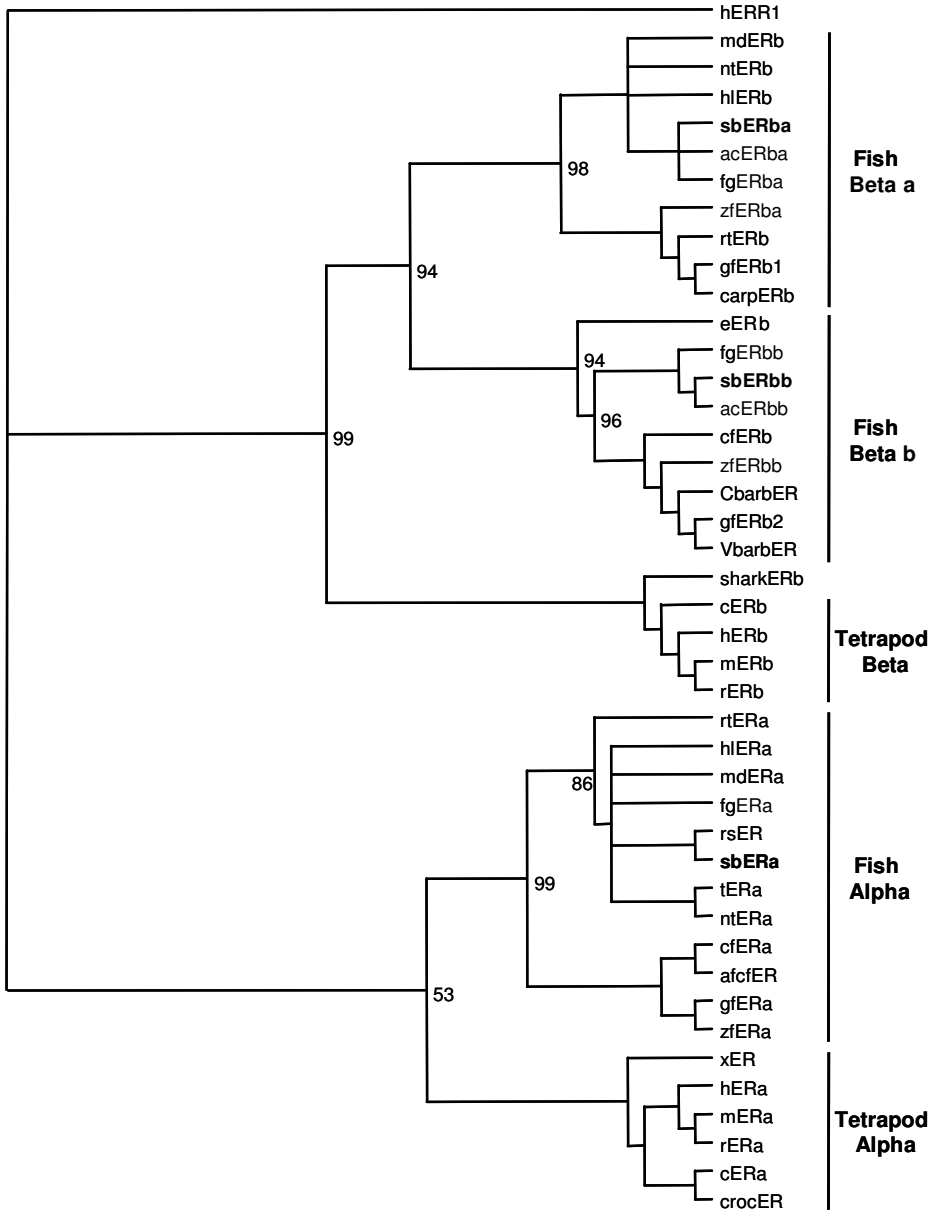

Figure 3.4. Phylogenetic rooted tree of 42 ER sequences constructed using the maximum parsimony method within PAUP and with hERR used as an outgroup (see Methods for abbreviations). Bootstrap support values for each node are shown (percentage of bootstrap trees supporting the node, out of 1000 trees).

As expected, the amino acid sequence identity of sbERβb with other ERs was highest in the DNA-binding domain (DBD or C domain, over 85%) and in the ligand-binding domain (LBD or E domain, between 63 and 94%). The length of domains A/B and F were found to be the most variable. sbERβb and acERβb had the longest F domains of all the ER sequences used in the phylogenetic analysis (120 and 119 amino acids, respectively, against 20-80 in other ERs).

In the A/B domain, all fish ERβb and most ERβa share one or two potential MAPK phosphorylation sites (Figure 3.5). This motif is conserved in all fish ERα and is absent from sbERβa. In the DBD the characteristic two zinc finger modules involved in receptor dimerization and specific DNA binding are 100% conserved and in the LBD, amino acid residues identified in mammals to be important for ligand interaction and receptor transactivation (Figure 3.5) are highly conserved. One residue shown to form the dimer interface in both ERα and ERβ homodimers (hERβ A456) is conserved in all ERs except in sbERβb and acERβb (Figure 3.5). Amino acid conservation contrasts using all ERs included in the phylogenetic analysis identified nine ERβb-diagnostic amino acids and fourteen ERβa-diagnostic amino acids (reverse type columns in Figure 3.5). The analysis of fish ERβs alone allowed the identification of eight additional amino acids conserved in ERβbs but absent in ERβas and ten amino acids conserved in ERβas but absent in ERβbs (shaded columns in Figure 3.5).

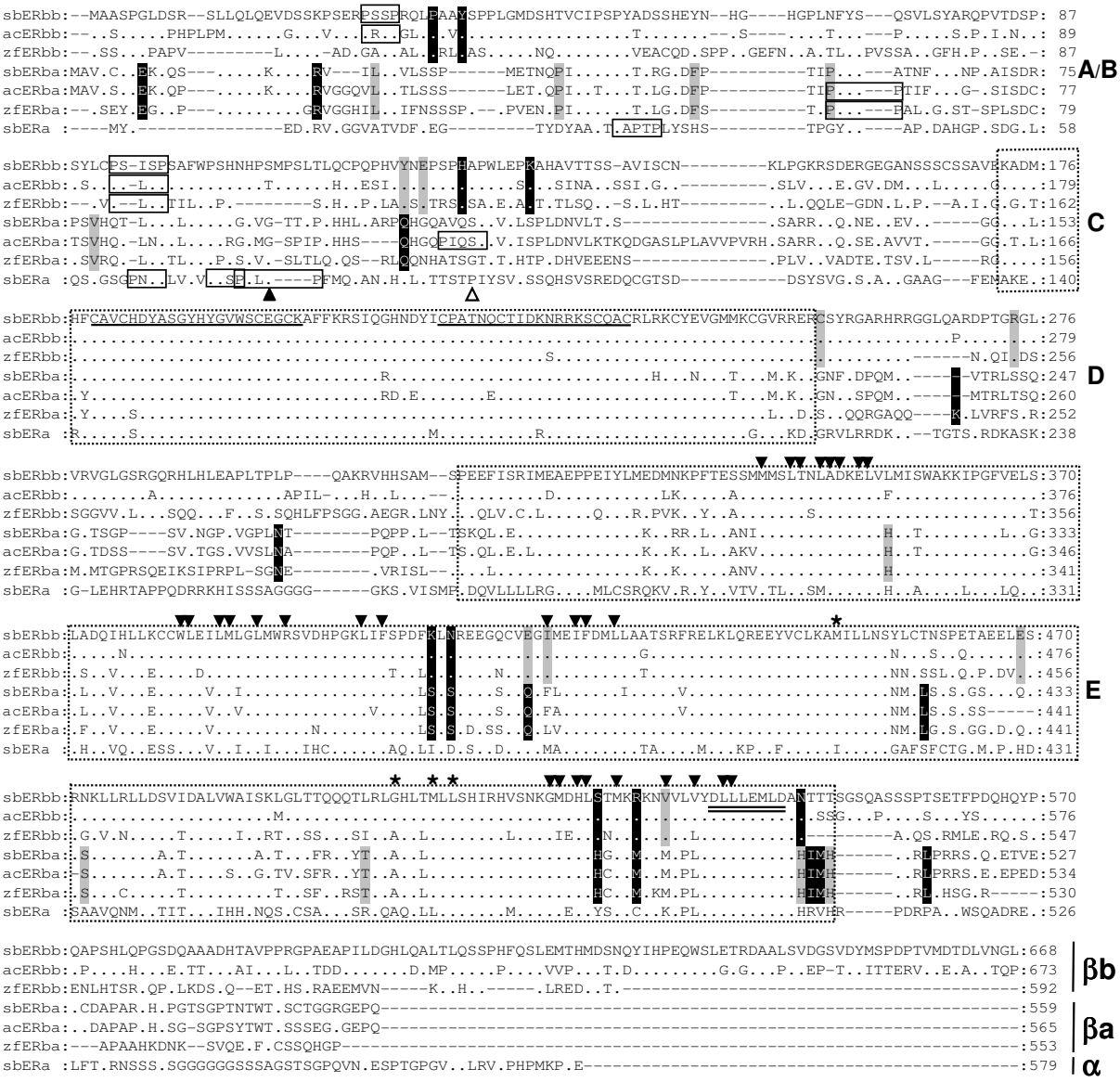

Figure 3.5. Alignment of sea bream ERs with ERβs from Atlantic croaker and zebrafish. Sequences were grouped as ERα, ERβa or ERβb (classification shown in lower panel) according to their position in the phylogenetic tree. Diagnostic residues for each fish ERβ group are in reverse type; additional residues conserved in each fish ERβ group but absent in the other (which may be present in other groups outside fish ERβs) are shaded. Motifs shown: putative phosphorylation sites for the MAPK pathway in the A/B domain (boxed), which include the amino acid positions recognized to be phosphorylated by MAP kinase in mammalian ERα (black arrow) and ERβ (white arrow); two conserved zinc-finger motifs in the C domain (underlined); amino acids recognized to be involved in dimerization (*), ligand interaction (arrows) and transactivation (double underlined) in E domain. ER domains are indicated on the right (C and E boxed in broken line)

3.3.3. *In vitro* phosphorylation

For functional characterization, each recombinant sbER fusion protein with a histidine-rich N-terminal tag (his-sbERs) was expressed in *E. coli* BL21 (chapter 5). The estimated MW of each protein, detected by Coomassie Blue stained SDS-PAGE and Western blot with specific antibodies for each sbER or for the His-tag, was similar to that predicted for the his-sbER construct sequences (approx. 73 kDa for sbERα and sbERβa, 85 kDa for sbERβb- Figures 5.3 and 5.4).

In an *in vitro* phosphorylation assay the sbERα was strongly phosphorylated by MAP kinase 2 (band with approx. 85 kDa), while a weaker phosphorylation of sbERβb occurred and generated two principal proteins of approx. 100 and 85kDa (Figure 3.6), which may correspond to the main and secondary translation products of sbERβb, respectively (Figure 5.4A). No specific phosphorylation was detected for the sbERβa protein but one weakly phosphorylated protein appeared at approximately 110 kDa, which could be a dimer. Differences in MW compared to unphosphorylated ER proteins (see above) are probably due to mobility shift caused by phosphorylation. In the positive control gel (not shown), a strong phosphorylated smear running at approx. 16-24 kDa was detected, corresponding to the expected sizes for MBP at different phosphorylated states.

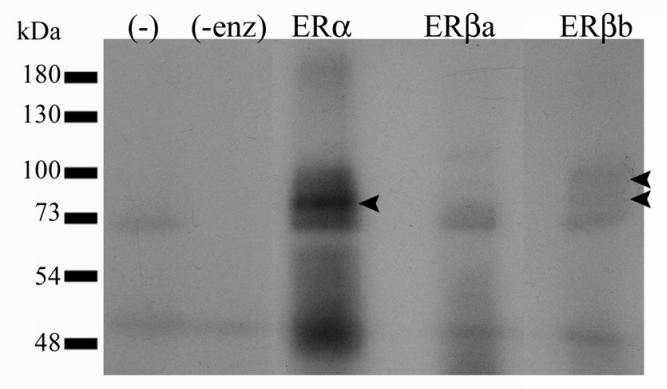

Figure 3.6. *In vitro* phosphorylation of his-sbERα, sbERβa or sbERβb proteins with MAP kinase 2/ERK2, visualised by autoradiography of SDS-PAGE gel. (-): Phosphorylation reaction using the pre-induced control for sbERα, where no proteins had been detected by anti-ERα antibodies, controls for the presence of other ERK2 substractes in the the *E. coli* extract. (-enz): Phosphorylation of sbERα in the absence of ERK2, controls for intrinsic kinase activity in the *E. coli* extract. Specific sbERα and sbERβb phosphorylated bands are indicated with arrows.

3.3.4. Ligand-binding assays

The recombinant his-sbERβb protein bound [^{3}H]E_2 in a saturable manner (Bmax= 34.4 ± 2.0 pM) and with high affinity (*K*d = 1.0 ± 0.19 nM) (Figure 3.7A). A Scatchard plot of the transformed data gave a linear regression confirming the presence of a single binding site. All estrogenic or anti-estrogenic compounds tested were able to displace [^{3}H]E_2 from sbERβb (Figure 3.7B) while testosterone showed negligible competitive ability (not shown) which was significantly different ($P<0.001$) to the strong competitor group formed by diethylstilbestrol (DES) and ethynylestradiol (EE_2) (IC_{50} not significantly different from E_2) and the group formed by the weak competitors ICI 182,780 (ICI) and estrone (E_1) (significantly different from the strong competitors, $P<0.01$). The relative binding affinity (RBA), calculated as the percent ratio of the mean IC_{50} of each test substance relative to E_2 was, in decreasing order of affinity, EE_2 (180%), DES (111%), E_2 (100%), ICI (12%) and E_1 (6%).

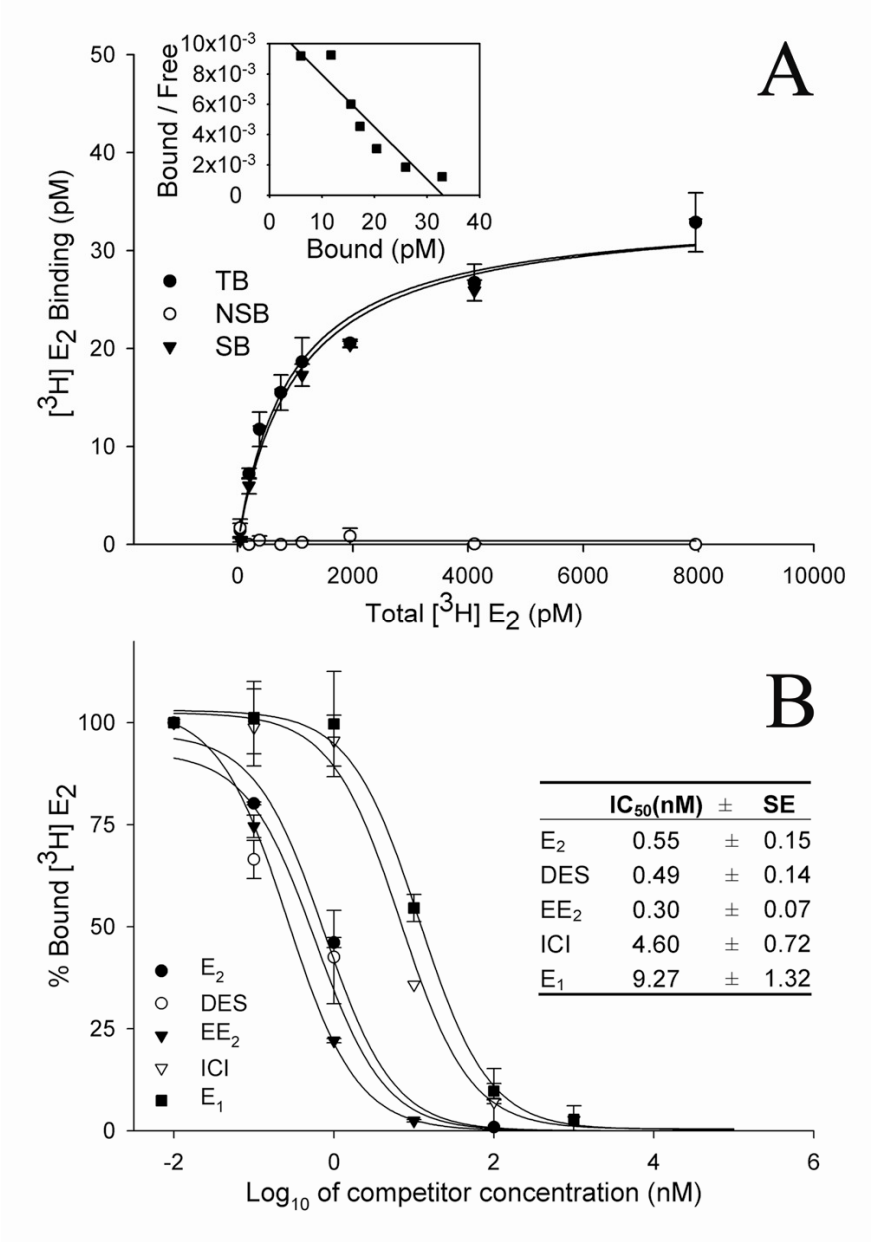

Figure 3.7. A) Saturable binding of [^{3}H]E_2 to the his-sbERβb recombinant protein, expressed in *E. coli* BL-21. Each point is the mean ± SE of three independent experiments. TB, total binding; NSB, non-specific binding; SB, specific binding. Inset: Scatchard plot of the transformed data. The calculated *K*d was 1.0 ± 0.19 nM. B) Representative competitive binding assay of unlabelled steroids, ER agonists and antagonists in relation to 1nM [^{3}H]E_2. E_2, estradiol; E_1, estrone; T, testosterone; DES, diethylstilbestrol; EE_2, ethynylestradiol; ICI, ICI 182,780. Inset: calculated IC_{50} values for each compound (mean ± SE of four independent experiments).

3.3.5. Tissue distribution

Two prominent sbERβa transcripts of approx. 6.1 and 2.4-kb were detected by Northern blot in most tissues of female sea bream and in the only tissue analysed in male sea bream, the testis. A further transcript of 1.1-kb which was relatively abundant was also present in the testis (Figure 3.8). A 3.5-kb transcript was also detected which was expressed at low abundance in female liver, ovary, kidney, intestine and male testis when longer film exposure was carried out (not shown). One prominent sbERβb transcript of approx. 6.6-kb was detected in female liver, intestine, kidney and male testis, while transcripts of lower size were detected in ovary (3.3-kb) and testis (3.3 and 1.9-kb). No sbERβb transcript could be detected in female brain, white muscle or heart. sbERα was expressed mainly in liver and heart (approx. 6.1 and 4.2-kb transcripts), but also in testis, brain and intestine (3.5-kb), and ovary (2.3-kb). No ER or EF-1α transcripts were detected in the pituitary gland most probably because insufficient RNA was available (total pituitary RNA instead of poly(A)+RNA was used due to the limited amount of tissue available).

The results of semi quantitative RT-PCR (Figure 3.9) generally coincided with those obtained by Northern blot. The presence of all the receptors in the pituitary gland of both female and male sea bream was established. sbERα was most strongly expressed in pituitary, liver, white muscle and heart of female and in male pituitary, liver and testis. sbERβa was expressed at highest levels in gonads and pituitary of both sexes, and in female skin, white muscle and male kidney. sbERβb was most abundant in pituitary, liver and intestine of both sexes and in male testis and kidney.

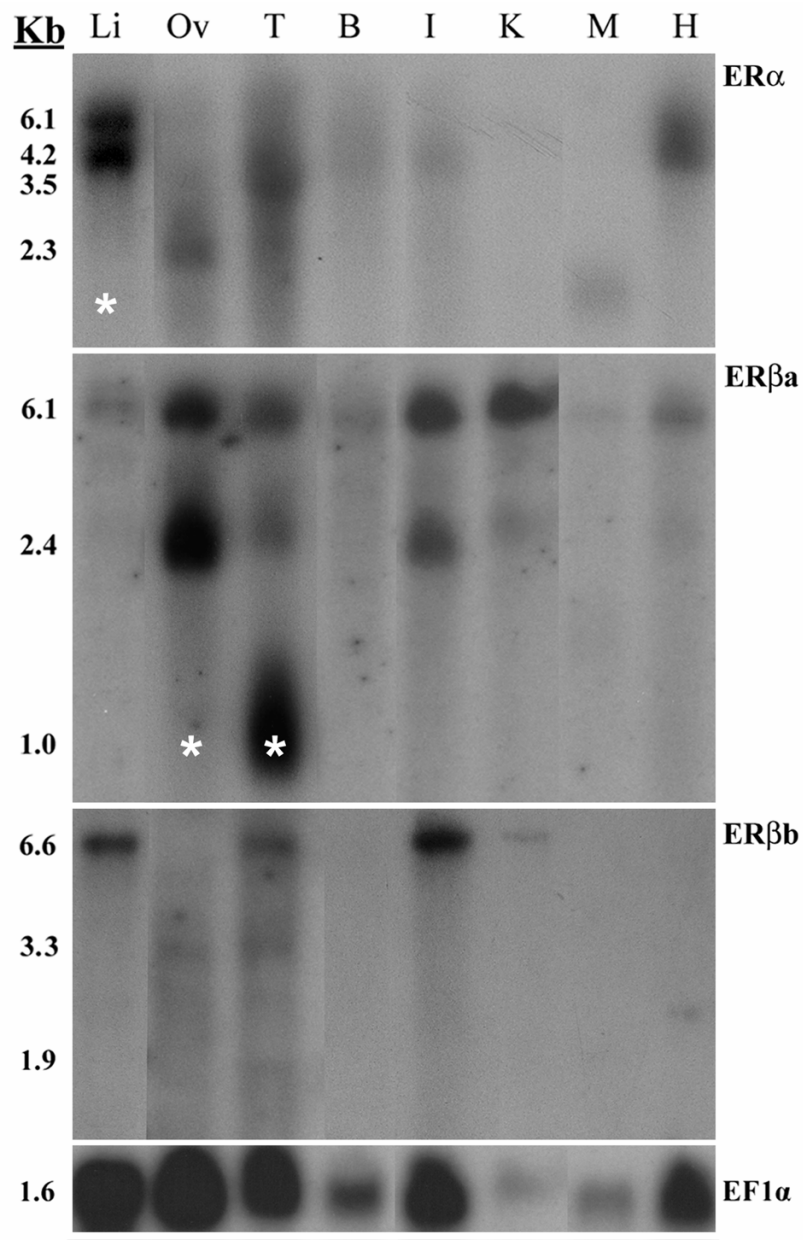

Figure 3.8. Northern Blot of sbERα, sbERβa and sbERβb with the estimated size (Kb) of transcripts indicated on the left. Hybridization to EF-1α was used to give a measure of relative quantities of mRNA loaded for each tissue. Film exposure times for each probe were identical with the exception of liver with sbERα and ovary and testis with sbERβa in which exposure was half the time of other tissues (indicated with *). Li, liver; Ov, ovary; T, testis; B, brain; I, intestine, K, kidney; M, white muscle; H, heart.

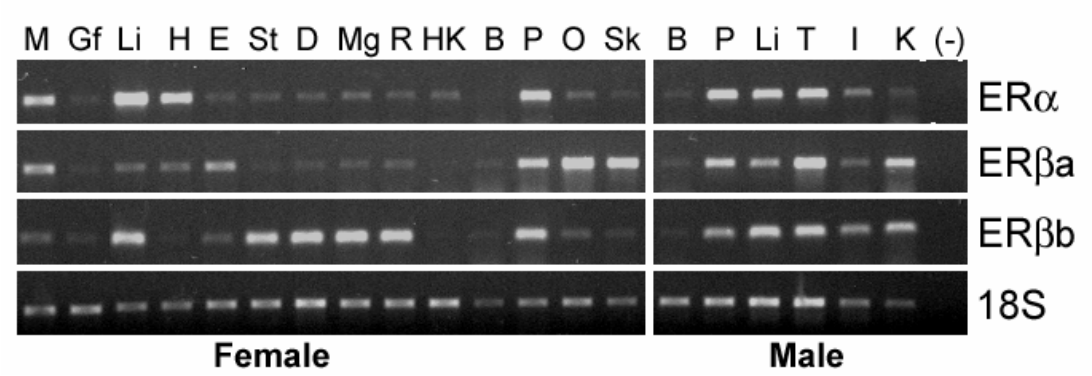

Figure 3.9. RT-PCR of the tissue distribution of sbERα, sbERβa and sbERβb in tissue of mature female and male sea bream, using 18S ribosomal subunit as a control for the relative quantity of cDNA used in PCR reactions. M, white muscle; Gf, gill filaments; Li, liver; H, heart; E, esophagus; St, stomach; D, duodenum; Mg, mid-gut; R, rectum; HK, head kidney; B, brain; P, pituitary; O, ovary; Sk, skin;T, testis; I, total intestine; K, kidney; (-), non-reverse transcribed RNA. The results presented are representative of sbER distribution detected in several RT-PCRs performed with mRNA from different fish.

3.4. Discussion

This study demonstrates the presence in sea bream of two ERβ subtypes encoded by different genes. This evidence is based on the low sequence identity of two ERβ isolated cDNAs and their different hybridization pattern in a genomic DNA Southern blot.

The two genes are active, as demonstrated by their mRNA expression and by sequence and functional analysis (conservation of important functional motifs and E_2 binding to his-sbERβb, this study, and to yeast-produced sbERβa, A. L. Passos, unpublished results). Furthermore, the presence of two putative ERβ genes in the genomes of the distantly related model species, *Fugu rubripes* and zebrafish, *Danio rerio,* and the phylogenetic analysis of available cDNA sequences, strongly suggests that the two genes may be shared by all teleosts, but not the cartilaginous fishes.

The recent discovery of additional ERβ forms in fish has also meant that receptor nomenclature is not consensual. Recently, Hawkins and Thomas (2004) proposed to adopt the zebrafish official nomenclature that designates the two fish paralogues as ERβa and ERβb (or ESR2a and ESR2b, respectively) (Sprague et al. 2003). The present study

follows the proposed nomenclature and thus we named the novel ERβ as ERβb and renamed the previously cloned form as ERβa. In the ensuing discussion fish estrogen receptors will receive the βa or βb designation according to their position in the phylogenetic tree in Figure 3.4.

Our results are consistent with the recent analysis of nuclear receptor evolution and with the view that the ERβ gene duplicated prior to the divergence of teleosts, including the eel (Anguilliformes), whereas no duplication of the ERα gene appeared to have occurred (Robinson-Rechavi et al. 2001). The inclusion of the only ER isolated in shark in the clade containing tetrapods ERβ, suggests that in chondrichthyes a single ERβ is present and that ERβ gene duplication could be a teleost specific feature (Robinson-Rechavi et al. 2001) or occurred just prior to teleost divergence.

sbERβb had similar domain structure to known ERs with a highly variable A/B domain, possibly indicating species- or subtype-specific mechanisms of transactivation. One or two potential MAPK kinase phosphorylation sites are present in all fish ERα and ERβb. In the present study, sbERα was strongly phosphorylated by MAP kinase 2 *in vitro*, while only a weak phosphorylation of sbERβb apparently occurred (Figure 3.6). Although the phosphorylation of fish ERs had not been demonstrated before, several studies demonstrated that mammalian ERα or β are phosphorylated in response to the MAPK pathway, in specific residues of the A/B domain corresponding to positions located near or within the potential MAPK motifs of fish ERs (Figure 3.5), a mechanism that is able to enhance ER AF-1 transcriptional activity in a ligand-independent manner (Kato et al. 2000; Tremblay and Giguere 2001; Lannigan 2003). The present results suggest that at least sbERα may be a phosphorylation target for the MAPK/ERK pathway, and maybe also sbERβb. In contrast, no MAPK motif nor clear specific *in vitro* phosphorylation were

identified for sbERβa, suggesting that sbERβa and sbERβb are activated by different mechanisms. However, these results require confirmation.

In the E domain, the amino acids identified to be important for ligand binding and ligand-dependent transactivation (AF-2 region) (Danielian et al. 1992; Pike et al. 1999) are almost 100% conserved between all ERα, ERβa and ERβb. Indeed, sbERβb had high affinity for E_2 and other estrogens with a *K*d (1 nM) of the same order of magnitude to that reported for mammalian ERs (Matthews et al. 2000) and other fish ERβb (0.21-0.75, Xia et al. 2000; Menuet et al. 2001; Hawkins and Thomas 2004).

However, some ERβb specific residues were identified in positions or regions considered to be important for ligand binding or transactivation (Figure 3.5). These substitutions may be of functional significance, since isoform-specific or species-specific amino acid substitutions within the LBD have been considered responsible for differences in binding preferences for different compounds (Pike et al. 1999; Matthews et al. 2000; Hawkins and Thomas 2004). For example, catfish ERα binds E_2 with an order of magnitude lower affinity than cfERβb (Xia et al. 2000) and alignment of the two receptors identifies 3 amino acid differences in positions known to be important for ligand binding. One of these is a substitution of L328 in cfERβb (corresponding to hERα L349, conserved in tetrapods ERα and in all ERβs) by M354 in cfERα (and present in all teleost ERαs). Reciprocal mutagenesis between hERα L349 and rtERα M317 caused a temperature-dependent lowering of *K*d for the rainbow trout ERα (Matthews et al. 2001). This suggests that the latter substitution may contribute to the generally lower affinity of teleost ERαs for E_2.

The specificity relationships ($EE_2 = DES = E_2 > ICI = E_1$) of sbERβb were comparable to those of cfERβb ($DES = EE_2 = E_2 > E_1$) (Xia et al. 2000), but different from acERβb ($ICI > DES > E_2 > E_1$) and more similar to acERβa ($E_2 \geq DES > ICI > E_1$) (Hawkins and

Thomas 2004). Mutation of acERβa Phe^{396} to the corresponding acERβb residue Ile have been shown to shift DES affinity towards the relative binding affinity of acERβb, showing that this residue is important for the interaction between ERs and DES (Hawkins and Thomas 2004). Given that there are only 13 amino acid substitutions between the LBDs of sbERβb and acERβb (Figure 3.5) and that sbERβb has an Ile residue at this position (I420) but has a pattern of specificity more similar to that of acERβa, we suggest that to account for the observed differences in ligand preference more than one of these amino acid residues cooperate with the residues already identified as important for ligand interaction. Alternatively, the differences could be a consequence of the different experimental conditions used in the binding assays.

Teleost ERs in general, and sea bream and Atlantic croaker ERβb in particular, have the longest F-domains of all ERs deposited in the databases (Socorro et al. 2000). The function of this domain is not well understood but it could be involved in interactions with coregulators required for ER/Sp1 action (Kim et al. 2003), in the agonist/antagonist discrimination and modulation of the magnitude of the transcriptional activity/repression of each type of compound in a cell-context dependent manner (Montano et al. 1995; Nichols et al. 1998; Schwartz et al. 2002) and in the inhibition of dimerization through interaction with the E-domain (Peters and Khan 1999). Curiously, the E domain residue A456 in hERβ, which has been shown to form the dimer interface in both ERα and ERβ homodimers (Pike et al. 1999), is conserved in all ERs except in sea bream and Atlantic croaker ERβb, in which it is substituted by G504 and G510, respectively. How the length of the F-domain and these mutations may influence ERβb homo or heterodimerization requires investigation.

The three sbERβb cDNAs isolated from pituitary differed mainly in the length of their UTRs (Figure 3.2). Clone 21 contained a small upstream ORF (suORF), also reported for

sbERα (Muñoz-Cueto et al. 1999), but not for sbERβa (Socorro et al. 2000). This may indicate possible differences in regulation among receptors, since in human and mouse ERαs the suORFs present in multiple transcripts with different 5'-UTRs are involved in the regulation of the levels of translation of the ER protein (Kos et al. 2002). The presence of several sbERβb clones which differ in the length of the 3'-UTR and lack of typical polyadenylation signals seems to be feature of teleost ERs (Tan et al. 1996a; Hawkins et al. 2000; Socorro et al. 2000). The sbERβb cDNA clones also showed nucleotide sequence polymorphisms, as also demonstrated in other fish ERs (Tchoudakova et al. 1999; Socorro et al. 2000), which may be the result of polymorphisms among individuals used to prepare the pituitary library (n=30). The residue changes observed in the F domain do not appear to be in key functional positions, although the possibility that they could confer slight functional differences cannot be ruled out.

Transcripts of variable sizes, including small transcripts with sizes that cannot generate the entire coding sequence were detected for sbERβb as well as for sbERα and sbERβa (Figure 3.8). The latter confirms earlier observations for ERα and ERβ in teleost (Tan et al. 1996b; Pakdel et al. 2000; Patiño et al. 2000; Socorro et al. 2000) and mammalian species (Hirata et al. 2003 and references therein) by Northern Blot and cloning of numerous alternative transcripts, shown to be generated mainly by alternative splicing or use of alternative promoters, transcription start sites or polyadenylation signals (Tan et al. 1996a; Pakdel et al. 2000; Hirata et al. 2003). However, the biological functions of the variant proteins are just starting to be elucidated.

The tissue distribution of the three sbERs transcripts agreed broadly with what has been observed in goldfish, zebrafish and sea bass (Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004). Highest mRNA receptor abundance was detected in known E_2 target tissues related to the reproductive function, such as the pituitary, liver and gonads. Of

particular interest, considering the newly found functions of estrogen in male reproduction (Couse and Korach 1999; Carreau et al. 2003; Hess 2003), is the high abundance of the three ER subtypes in testis. The target tissue could be the germinal epithelium where catfish ERα and ERβb appear to be co-expressed (Wu et al. 2001). However, discrepant localization results have been reported in testis in other species (Miura et al. 1999; Legler et al. 2000; Bouma and Nagler 2001; Andreassen et al. 2003), and cellular localization of the three sbERs in testis needs further investigation to clarify their relative roles. The presence of abundant sbERβb transcripts in all regions of the intestine could indicate its involvement in the modulation of calcium transport, through an as yet unexplained mechanism (Guerreiro et al. 2002).

In conclusion, sea bream expresses ERα and two ERβ subtypes which have a different, but partially overlapping pattern of tissue distribution. The comparative analysis of amino acid conservation contrasts, patterns of tissue distribution, estrogen regulation, binding affinities and transactivation and *in vitro* phosphorylation properties of the ERβ subtypes (Bardet et al. 2002; Menuet et al. 2002; Choi and Habibi 2003; Hawkins and Thomas 2004; Menuet et al. 2004) suggest they may have evolved different functions, but it is also possible that the functions of the original ERβ have been divided between the duplicates. The existence of two ERβ subtypes provides an additional variant in the complexity of the mechanisms involved in estrogen actions.

3.5. Acknowledgements of practical work

P. Pinto acknowledges the contribution of R. S. Martins for carrying out the Southern blot experiment.

CHAPTER 4

Cloning of alternative sea bream (*Sparus auratus*) estrogen receptor transcripts

Co-Authors: Helena R. Teodósio, Sílvia Socorro, Deborah M. Power and Adelino V.M. Canário

4.1. Introduction

Estrogen actions are mediated by two functionally distinct but complementary estrogen receptor subtypes (ERα and ERβ), which may modulate the transcription of responsive genes as homo or heterodimers, normally by interacting with estrogen-response elements (ERE) in their promoters (Matthews and Gustafsson 2003). While in higher vertebrates a single ERβ gene appears to be present, in some teleost fish species two ERβs encoded by different genes have recently been isolated, which appear to have arisen by duplication of an ancestral ERβ gene (Robinson-Rechavi et al. 2001).

A comparison between characterized ER genes reveals a highly conserved genomic organization, with eight or nine coding exons and well conserved intron-exon boundaries (Ponglikitmongkol et al. 1988; Le Roux et al. 1993; Tan et al. 1996; Enmark et al. 1997; Lassiter et al. 2002; Swope et al. 2002). A A/B transactivation domain is encoded by exon1 and a portion of exon2, while the two zinc fingers comprising the C (DNA-binding) domain are located separately in exons 2 and 3, respectively. Exon4 encodes the nuclear localization signals (NLS) of the D domain, while exons 4-8 encode the E (ligand-binding, dimerization and transactivation) and F domains. An additional intron divides the D and E domains in fish ERα genes (Le Roux et al. 1993; Tan et al. 1996), which is absent in human or mouse ERα (Ponglikitmongkol et al. 1988; Swope et al. 2002).

Outside the coding region the organization is much more complex and multiple promoters, 5'untranslated exons, intronic exons, alternative coding exons or alternative polyadenylation signals have been identified in different ER genes, which allow the generation of a wide range of ER transcript variants from each gene, expressed in a cell-context specific manner (Tan et al. 1996; Pakdel et al. 2000; Tiffoche et al. 2001; Hirata et al. 2003). Many of these variants differ only in their 5' or 3' untranslated (UTR) regions, which may regulate their

translation levels or RNA stability (e.g. Tan et al. 1996; Kos et al. 2002), while others have alterations that affect the coding region.

The most commonly reported ER variants result from alternative mRNA splicing with the deletion of one or more exons, encoding protein variants with internal deletions or C-terminal truncations in which the activity related to the missing domains of the modular ER protein is generally affected (Bollig and Miksicek 2000; Perlman et al. 2005). Some variant ER proteins have been detected *in vivo* in mammals (e.g. Fuqua et al. 1999; Tiffoche et al. 2001; Li et al. 2003), the biological functions of which have only started to be elucidated. While some variant proteins differ functionally from the wild-type (wt) receptor or mediate non-classical estrogen actions (Bollig and Miksicek 2000; Li et al. 2003; Poola et al. 2005), others may stimulate (dominant positive) or inhibit (dominant negative) the transcriptional activities of the wt ERα and/or ERβ proteins, depending on the cell context, the wt ER subtype and the variant / wt protein ratio (Bollig and Miksicek 2000; Flouriot et al. 2000; Lin et al. 2003; Peng et al. 2003).

In fish, long and short ERα protein isoforms differing in the length of the A/B domain and in their estrogen dependencies have been detected *in vivo* and *in vitro* in rainbow trout. These are encoded by transcripts with different 5'-UTRs as a consequence of alternative splicing and promoter usage and are expressed in a tissue-specific manner (Pakdel et al. 2000; Menuet et al. 2001). Transcripts encoding putative ERα with N-terminal truncations, internal insertions or deletions and an antisense mRNA have been identified in channel catfish (Patiño et al. 2000), and ERα transcripts with different 5' or 3'UTRs in tilapia (Tan et al. 1995). Multiple ER transcripts of different length have been detected by Northern blot of different fish species, some of them too small to contain the entire coding region (Socorro et al. 2000 and references therein; Sabo-Attwood et al. 2004), but their structure and function remains unknown.

The first objective of the present study was to isolate mRNA variants for the three sea bream ER genes, as multiple transcripts of sbERα, βa and βb have previously been detected by Northern blot but have not been further characterised (Socorro et al. 2000 and chapter 3 of this thesis). The approach taken was to identify *in silico* ER genes in the *Fugu rubripes* genome, predict its genomic organization and transfer this information to predict the exon boundaries in sbER cDNAs by sequence comparison. Specific primers were then designed which flanked different exons of each sbER and used to search for single-exon skipping mRNA splice variants for all three sbERs. *Fugu* and sea bream are both diploid marine teleosts belonging to the series *Percomorpha*, and the gene organization and cDNA sequences of a number of different genes have been shown in several studies to be highly similar (Ingleton 2002; Power et al. 2002). The second objective was to compare the patterns of tissue distribution of the identified variants and wild-type ERs and their hepatic expression in control, estrogen- and estrogen antagonist-treated sea bream.

4.2. Specific methods

4.2.1. *In silico* identification and genomic organization of *Fugu rubripes* ER genes

The scaffolds from the *Fugu rubripes* genome (http://fugu.biology.qmul.ac.uk/Analysis/) giving the most significant hit by tBLASTn analysis (Altschul et al. 1997) with each of the sbER translated cDNA sequences (sbERα, accession no. AJ006039; sbERβa, AF136980; sbERβb, AJ580048) were retrieved. *Fugu* ER intron-exon boundaries and the presumptive cDNA coding sequences were predicted using the HGMP NIX Interface (http://www.rfcgr.mrc.ac.uk/Registered/Webapp/nix/). Pairwise alignment of sbER cDNA sequences with the selected *Fugu* scaffolds using Spidey mRNA-to-genome software (Wheelan et al. 2001) permitted identification of the putative intron positions in the sbER genes and was also used to confirm genomic organization, splice sites and coding sequence frame for each putative Fugu ER (fgERα, fgERβa and fgERβb).

4.2.2. Primer design for alternative transcripts of sbERs

Several pairs of primers were designed to flank two introns and one exon in each sbER cDNA (example in Figure 4.1), so that both wild-type and putative alternative transcripts lacking each exon could be amplified with predicted sizes. Primers were selected to have the highest specificity for individual ER subtypes.

4.2.3. Cloning of sbERα exon2-deletion variants

All primer pairs were tested by RT-PCR with 2 µl of cDNA from (1) the liver of an E_2-stimulated immature sea bream (50 g body weight, 72h treatment with coconut oil implants containing 10 mg/kg E_2, S. Socorro, unpublished); (2) the testis from a 370 g male and (3) the ovary from a 3 kg female sea bream. A standard 50 µl PCR reaction (section 2.7) with 1.5 mM $MgCl_2$ was run for 30 cycles at an appropriate annealing temperature for each primer pair. Of all primer pairs tested, only era_1a and era_1as (Appendix V), designed to flank exon2 of sbERα, produced alternative PCR products in addition to the wt ER band, which were gel-purified, cloned into pGEM-T Easy and sequenced (sections 2.8-2.10). Sequencing was repeated at least three times for confirmation.

4.2.4. Cloning of sbERα N-terminal truncated variants

Alternative bands with a lower size than that expected for wt sbERα were amplified from 5 µl of testis cDNA from one male sea bream (approx. 450 g), when testing a pair of primers designed to amplify part of the sbERα coding region (sbERacodF1 and E4, Appendix V). The 50 µl PCR reaction was performed in standard conditions (section 2.7) with 2 mM $MgCl_2$ and 50 pmol each primer. Cycling conditions were 45 cycles of 30 sec at 94ºC, 45 sec at 58ºC and 1 min at 72ºC, followed by 5 min at 72ºC. Alternative bands were gel-purified, cloned into pGEM-T easy and sequenced at least three times each.

4.2.5. Multiple sequence alignments

All sequence alignments, nucleotide or amino acid, were performed with ClustalX using the Blosum62 Symbol comparison Table with an increased gap-opening penalty of 20 and a reduced gap extension penalty of 0.05. ERα internal variants were aligned with the two full-length "wt" ERα cDNAs available in sequence databases, which have a slight difference in amino acid residues at the exon1-exon2 boundary: sbERα_Mc (Muñoz-Cueto et al. 1999, Accession no. AJ006039) and sbERα_Soc (truncated cDNA first cloned by Socorro et al. 2000, Ac.no.AF136979, whose coding sequence was completed in this study- section 5.2.1).

4.2.6. Tissue distribution of sbERα variants by RT-PCR

4.2.6.1. Primer design and specificity tests

For the specific amplification of each exon2-deletion variant in different sea bream tissues (section 4.2.6.2) or in male liver in response to hormonal treatments (section 4.2.7), primers targeting the alternative splice junctions present in each variant were designed (Figure 4.4), and then used in conjunction with general primers that may hybridize to both wt and variant ERα sequences (Appendix V). F1CF1 was used with era_2as for F1C amplification and era_1a with F1BR2 for F1B amplification, respectively (Figure 4.4).

To amplify both wt sbERα cDNAs but not the truncated variants, one reverse primer was designed within exon2 (MCERar673, Appendix V), which is absent in all isolated truncated forms (Figures 4.4 and 4.5). This primer was used with the general sbERα primer MCERaf286, designed so that the amplicon contained one site for the restriction enzyme *Bsr*FI in the sbERα_Mc transcript but not in sbERα_Soc (Figure 4.4), in order to distinguish between the two wt forms by *Bsr*FI digestion of RT-PCR products (section 4.2.6.3). This strategy was used as the size difference between the two amplified products would be too small (6 bp) to allow product discrimination using standard analytical conditions.

Cross-reaction tests with plasmid dilutions of F1B, F1C and wt sbERα_Soc cDNAs showed that while F1C and wt ERα directed primers were specific for their respective gene targets, F1B directed primers hybridised weakly with wt sbERα_Soc (but not with F1C). As a consequence of the less exclusive character of F1B primers, two products were amplified from cDNA, which were 202 bp and 429bp and corresponded to the product of amplification of F1B and wt ERα templates, respectively. As primers and all other reagents for PCR reactions were present in excess it was assumed that during amplification no competition occurred between the two templates.

To amplify both wt ERα and N-terminal truncated variants A22/A43, the primers ERalt22F1 and ERalt22R1 (Appendix V) were designed and are represented in Figure 4.5.

4.2.6.2. RT-PCR on sea bream tissues

All sbERα variant primer pairs indicated above were used for standard semi-quantitative RT-PCR (section 2.12) with 1 µl cDNA from several tissues of female and male sea bream (same used in wt sbER tissue distribution, section 3.2.9). Optimized annealing temperatures and cycle number for detection in the exponential phase of amplification were as follows: F1B 55 ºC-36 cycles, F1C 55 ºC-36 cycles, ERα_Mc/ERα_Soc 53 ºC-31 cycles, N-terminal truncated variants 61ºC-36 cycles and internal control 18S 59ºC-18 cycles. Pearson correlations of expression levels between different forms were calculated as described in section 2.12.

4.2.6.3. *BsrFI* digestion of wt sbERα amplicon

In order to distinguish between wt sbERα_Mc and sbERα_Soc transcripts in the different tissues assayed, 5 µl of PCR reaction product (MCERar673 / MCERaf286 primers, see above) was digested with 5 U of *Bsr*FI (New England Biolabs, Hitchin, UK) in 10 µl of 1x NEBuffer *Bsr*FI. Reactions were allowed to proceed for 1.5h at 37ºC and reaction products

were analysed by 1.5% agarose gel electrophoresis (Appendix II). As a positive control for digestion, a 354 bp PCR product for exons 7-8 of sbERβa, which contained two predicted *Bsr*FI restriction sites, was digested and analysed in the same conditions. This was used in alternative to the positive control using sbERα_Mc, since we did not have this clone.

4.2.7. Expression in liver in response to E_2 and/or antagonist ICI 182,780 treatments

The expression of F1C and F1B ERα exon-deletion variants was investigated by RT-PCR with variant-specific primers (section 4.2.6.1) using liver cDNA prepared from young male sea bream exposed to different combinations of E_2 and antiestrogen ICI 182,780 (experiment 2, section 6.2.1). Standard semi-quantitative RT-PCR conditions were used with an annealing temperature of 55ºC and 36 cycles for both primer pairs and 59ºC and 15 cycles for 18S amplification. The relative expression values for each sample were calculated and differences between treatments evaluated as described on section 2.12. The results were compared with the response of wt ERα for the same experiment (section 6.2.4) using the era_2a and era_2as primers (Figure 4.4) that had been previously shown to specifically amplify wt ERα but not variant cDNAs (section 3.2.9). Pearson correlations between different ERα forms were calculated as described in section 2.12.

4.3. Results

4.3.1. *In silico* identification of ER genes in *Fugu rubripes*

Three ER genes were identified *in silico* in scaffolds M000491, M003095 and M000720 of the *Fugu rubripes* genome (http://fugu.biology.qmul.ac.uk/, October 2001 data freeze set), which were homologues to sea bream (sb) ERα, ERβa and ERβb, respectively. Bioinformatic analysis predicted the genomic organisation of each of the genes in *Fugu* and sea bream (Figure 4.1). Only genomic regions comprising the coding sequences were analysed, due to the high complexity and low conservation in the ER non-coding exons observed in other organisms. The *Fugu* ERβb gene, located at the frontier of scaffold M000720, was incomplete and lacked part of exon1 (A/B domain). The predicted *Fugu* ERα, ERβa and ERβb coding sequences were 1869, 1695 and 1665 bp, and the deduced amino acid sequences 622, 564 and 554 residues, respectively.

4.3.2. Genomic organization of *Fugu* ER genes

The *Fugu* ERβa and ERβb genes have a conserved genomic structure of eight coding exons (Figure 4.1), in common with most other ER genes described, which were numbered 1 to 8 according to mammalian ER genes. In contrast, the *Fugu* ERα is similar to other fish ER genes in that it possesses one additional intron dividing the D (hinge) and the ligand-binding (E or LBD) domains. The resultant exons of *Fugu* ERα, corresponding to exon 4 in all ERβ and in mammalian ERα genes, were here designated as exons 4 and 4a. Consensus sequences at donor and acceptor splice sites (GT-AG) were observed for all the exon-intron junctions (not shown), and intron positions were highly conserved in all ER genes, between ER subtypes and between different organisms (as exemplified by the comparison between human and *Fugu* ERs in Figure 4.2).

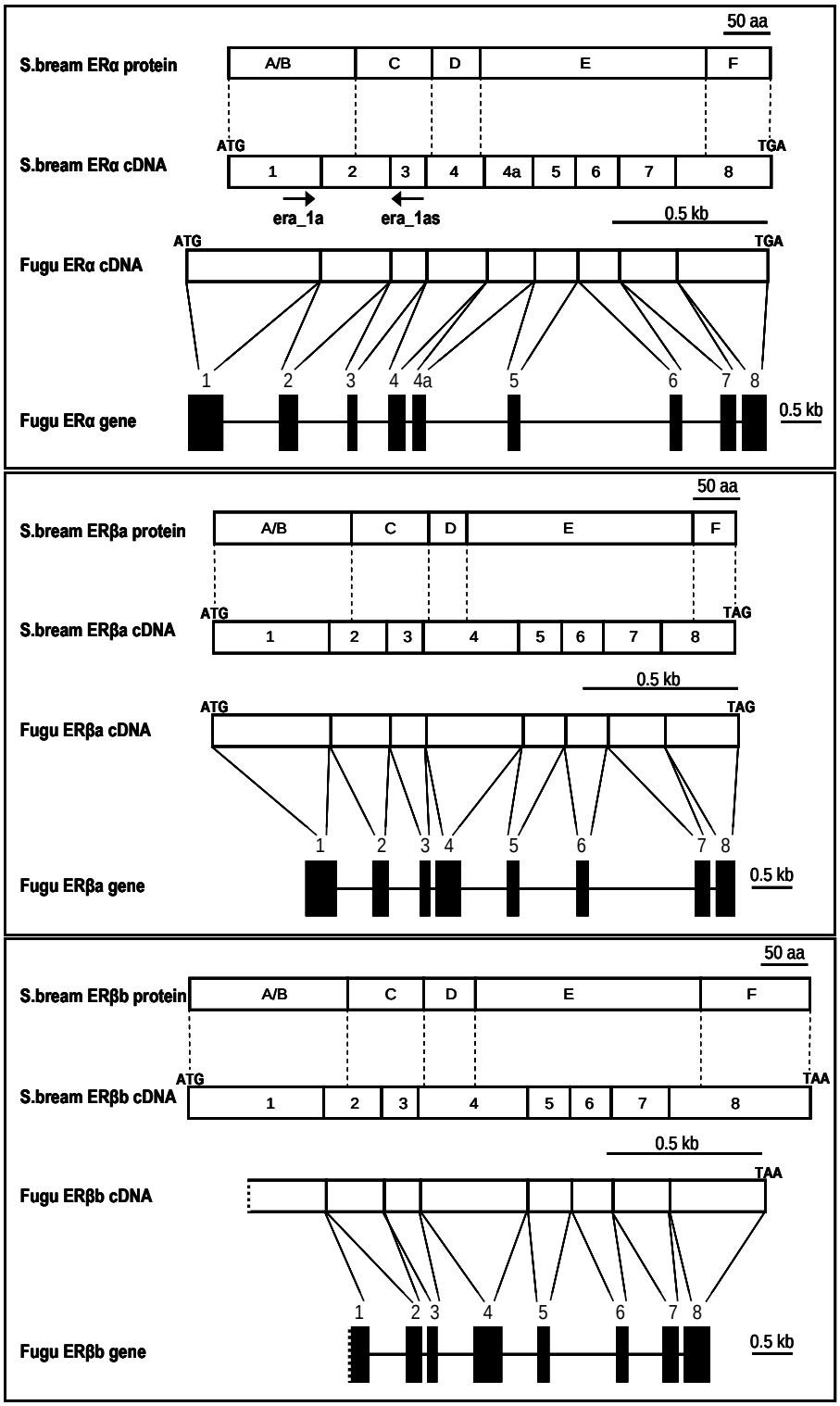

Figure 4.1. Schematic representation of the *in silico* predicted genomic structure of *Fugu* ERα, βa and βb genes and corresponding cDNAs. The length of represented blocks (exons) and lines connecting blocks (introns) is proportional to their length in the sequence. A vertical dashed line in *Fugu* ERβb gene and cDNA indicates that exon1 was incomplete. Predicted exon boundaries in sea bream ER cDNAs, obtained by sequence alignment with *Fugu* genomic and cDNA sequences, are represented by vertical bars dividing exons 1-8, and the corresponding positions in sea bream ER proteins are indicated for orientation relative to domains A-F. Primers designed to clone ERα alternative transcripts lacking exon2 are represented by arrows in panel A (era_1a and era_1as) and are representative of other primers designed to isolate exon-deleted variants for the three sbER cDNAs.

Figure 4.2 - Comparison of intron positions in human and predicted *Fugu* ER genes, represented by vertical lines in the alignment between human (h), *Fugu* (fg) and sea bream (sb) ER deduced protein sequences. Exon numbers are indicated at each exon border. For orientation, the DNA (dashed) and the ligand (solid) -binding domains are boxed.

4.3.3. Prediction of exon boundaries in sea bream cDNAs and PCR strategy to clone exon-skipping alternative transcripts

The alignment of sea bream ER cDNAs with the corresponding *Fugu* genomic and cDNA sequences permitted the intron positions to be predicted for the three sbER cDNAs. Several pairs of primers were designed flanking two introns and one exon, so that both the "wild-type" full-length form, normally the most abundant, and putative alternative transcripts lacking each exon could be amplified and distinguishable by size (Figure 4.1). All primer pairs were tested by RT-PCR on E_2-treated liver of immature sea bream and in the testis or ovary of mature male or female sea bream, respectively. However, it was only possible to amplify and isolate sbERα variants, since no alternative amplification products were obtained with any of the ERβa or ERβb primer pairs on the cDNAs analysed.

4.3.4. Cloning of sbERα variants

Using the era_1a and era_1as pair of primers, designed to clone putative internal variants lacking sbERα exon2 (Figure 4.1), two alternative bands were amplified from E_2-treated liver and one from testis (Figure 4.3A), in addition to the 513 bp band corresponding to the full-length wt ERα.

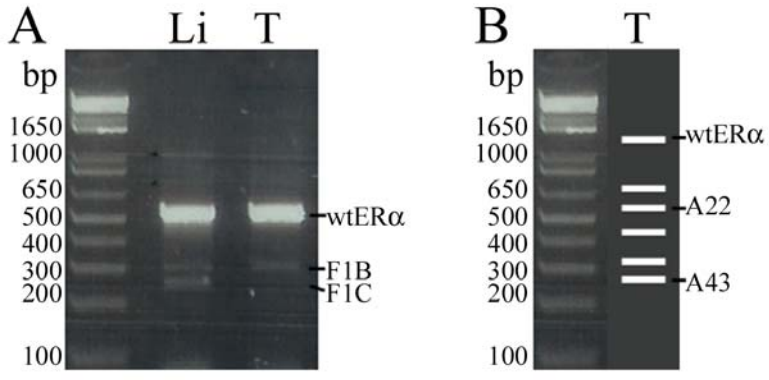

Figure 4.3. A- Amplification of wild-type (wt) and sbERα variants in E_2-treated liver (Li) or in testis (T) using primers flanking exon2. Panel B represents the amplification products from testis cDNA using one pair of primers designed to amplify sbERα from exon1 to 8 (sbERacodF1 and E4); it was not possible to present the electrophoretic analysis of the amplification products due to a technical problem with the agarose gel. In each panel, band sizes (bp) from a molecular weight DNA ladder are indicated on the right, and the names of the isolated variants indicated on the left.

Sequence analysis of the cloned alternative bands indicated that the upper band (F1B, 293 bp) corresponded to an ERα variant with a precise deletion of exon2 (221 bp) between the predicted exon boundaries (Figure 4.4), while the lower band F1C (244 bp) lacked exon2 plus an additional fragment of 49 bp from exon3. An (AG) putative acceptor splice site motif was found at the end of the deleted sequence from F1C, suggesting that this transcript may have been generated by alternative slicing using a splice acceptor site within exon3. Also represented in Figure 4.4 are the two wt ERα cDNAs available in sequence databases, sbERα_Mc and sbERα_Soc. These cDNAs differ by three point mutations at the end of exon1 and the presence of six additional nucleotides at the beginning of exon2 in sbERα_Soc, which result respectively in three amino acid substitutions and two extra amino acids in sbERα_Soc. An (AG) motif was also found associated with the additional 6 nt of sbERα_Soc, suggesting that it may be utilised as an alternative splice acceptor site by the sbERα_Mc transcript.

Deletion of exon2 causes a frame-shift mutation in the F1B variant with generation of a premature stop codon in the deduced protein sequence (Figure 4.6) and, assuming that the remainder of the transcript which was not PCR-amplified is similar in sequence to the wt cDNA, it is predicted to encode a C-terminally truncated ERα protein with only 101 amino acids, comprising part of the A/B domain. Using sequence analysis, similar truncated proteins were predicted from the putative exon2-deleted cDNAs for the human, mouse, tilapia, rainbow trout and *Fugu* ERα genes (not shown), and a consensus polyadenylation signal was found in the intron following exon3 in the *Fugu* gene. In contrast, in F1C the ER reading frame is not disrupted, and this transcript is predicted to encode a protein with an internal deletion of 90 amino acids (Figure 4.6) comprising the last 37 amino acids of the A/B domain and the first zinc finger and a segment of the second one in the DNA-binding domain (DBD).

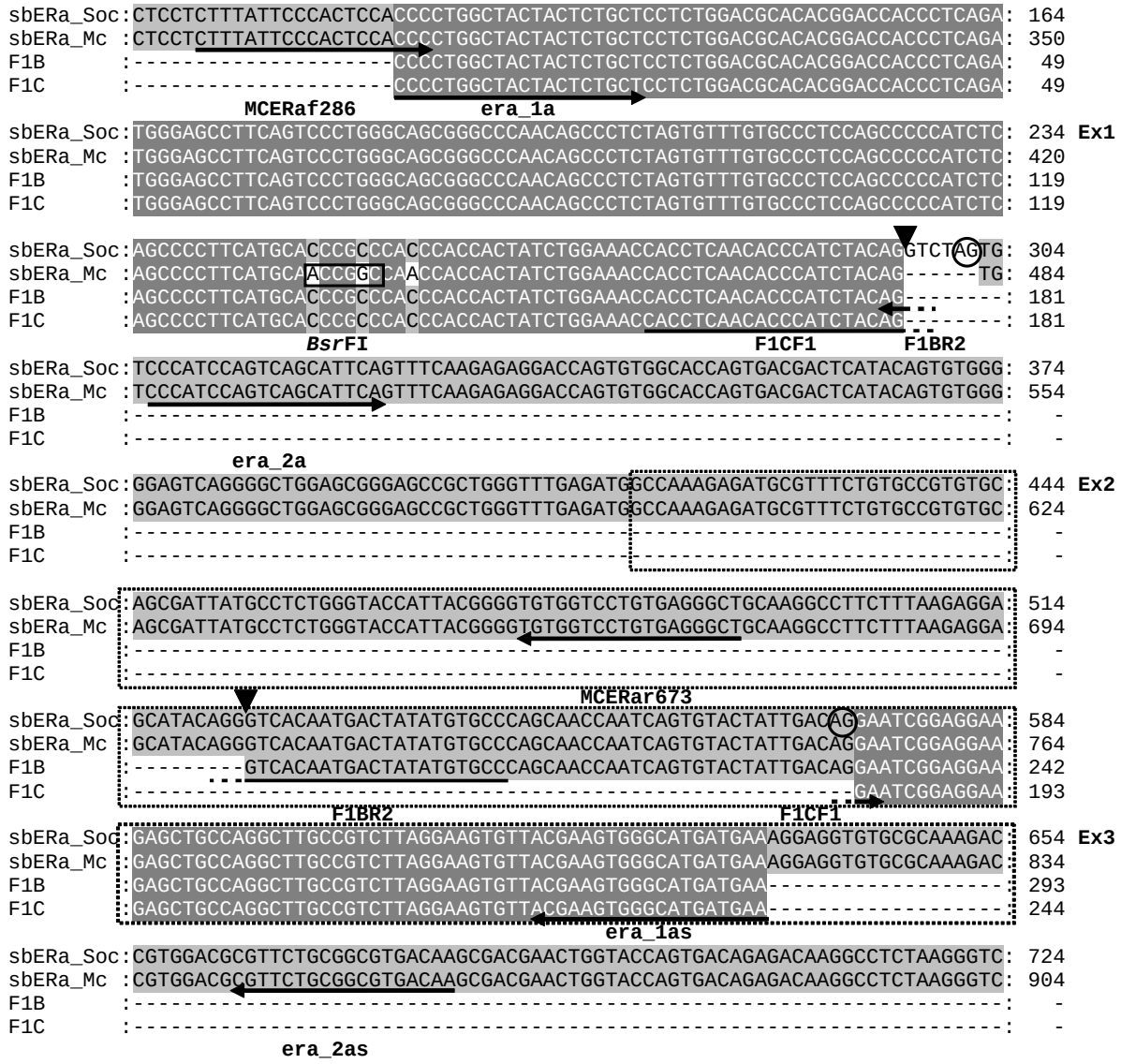

Figure 4.4. Alignment of wt sbERα cDNAs (sbERα_Mc and sbERα_Soc, see section 4.2.5) with the exon2 internal variants isolated in this study using era_1a and era_1as primers (indicated by arrows). Predicted exon boundaries from the comparison with the *Fugu* ERα genomic organization are indicated by triangles with exon numbers on the right, while circles indicate alternative acceptor splice sites that may have been used in sbERa_Mc and F1C transcripts. The DNA-binding domain is indicated within dashed line boxes. Primers used to analyse the tissue distribution of wt or each variant ERα transcripts (see section 4.2.6.1) are represented by arrows. The *Bsr*FI restriction site used to distinguish sbERα_Mc from sbERα_Soc PCR products is boxed in exon1.

When using primers located within exons 1 and 8 (Figure 4.5) to amplify part of the sbERα coding sequence, five variant lower bands were amplified (Figure 4.3B), while only two out of these could be successfully isolated and cloned. The first 66 bp of the 569 bp variant (A22) are identical in sequence to part of the wt ERα_Mc 5'UTR, while its remaining 503

bp align with positions 1135-1637 of the wt ERα transcript (exons 5-7, Figure 4.5). The 244 bp variant (A43) has a 74 bp 5'UTR identical to wt ERα followed by a 1293 bp deletion and 170 bp that are identical to positions 1468-1637 of the wt ERα cDNA (part of exon 7). The break points between alternative forms and wt ERα are not located at predicted exon boundaries and do not conform to the consensus donor/acceptor splice site motifs GT/AG, except for the putative splice acceptor site of A43 (Figure 4.5).

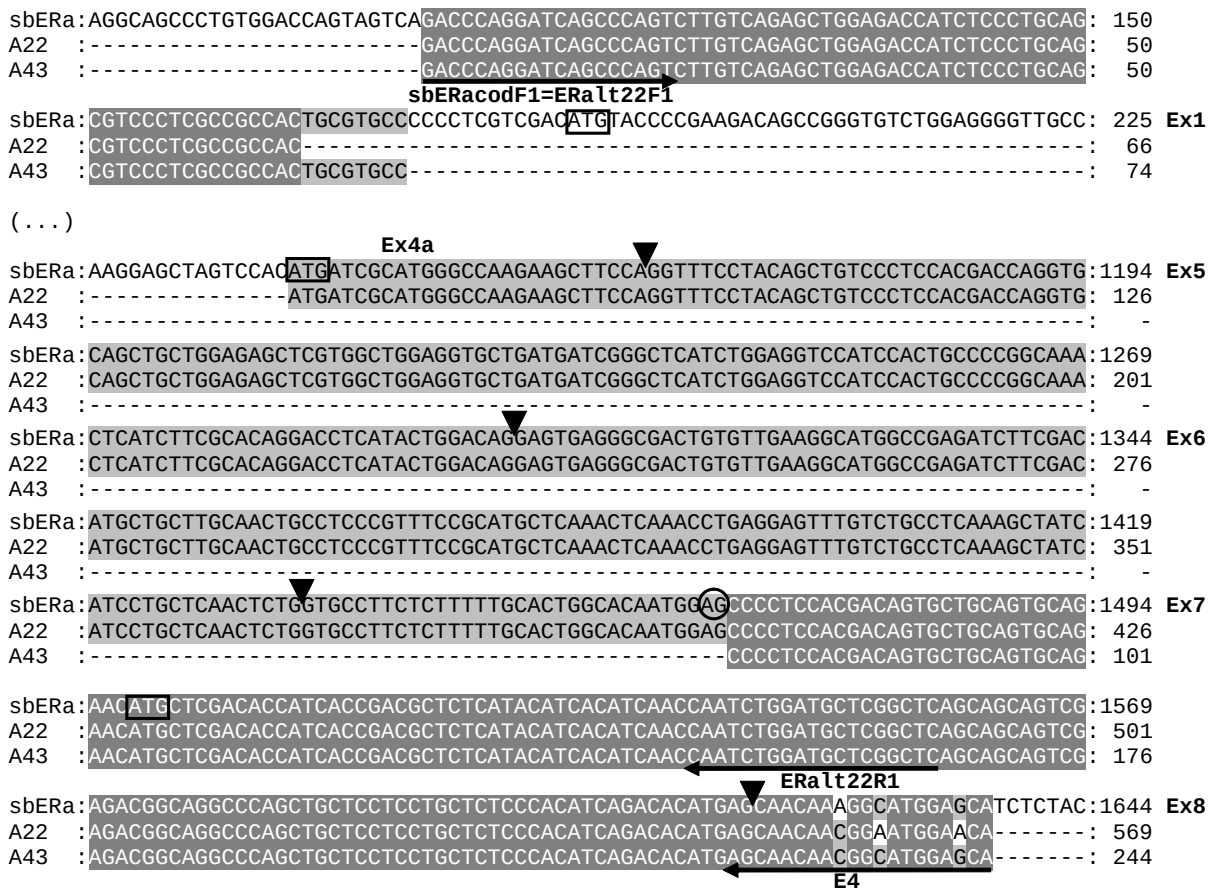

Figure 4.5. Alignment of the wt sbERα_Mc cDNA (AJ006039) with the N-terminally truncated variants isolated in this study using sbERacodF1 primer and the degenerated primer E4 (indicated by arrows). Only some parts of the alignment are shown: part of exon1 with the 5'UTR region and the ATG initiation codon (boxed), and then part of the ligand binding domain (exons 4a-8 indicated on the right with predicted intron-exon boundaries indicated by triangles). The in frame ATG initiation codons in variants A22/A43 are boxed. To analyse the tissue distribution of both wt and variant cDNAs, sbERα specific primers ERalt22F1 and ERalt22R1 were used and are indicated by arrows. A circle indicates a putative alternative acceptor splice site that may have been used in the A43 transcript.

Both A22 and A43 variants lack the region containing the ATG initiation codon at exon1, but each contain an in-frame ATG codon after the deleted region that could open a reading frame identical to the wt ERα protein in that region (Figures 4.5 and 4.6). Thus, these variants consist of the 5'UTR of the ERα gene followed by exons 5-8 (A22 variant) or exons 7-8 (A43 variant), and are predicted to encode N-terminally truncated proteins formed by most of the E domain (A22) or a small part of the E domain (A43) (Figure 4.6).

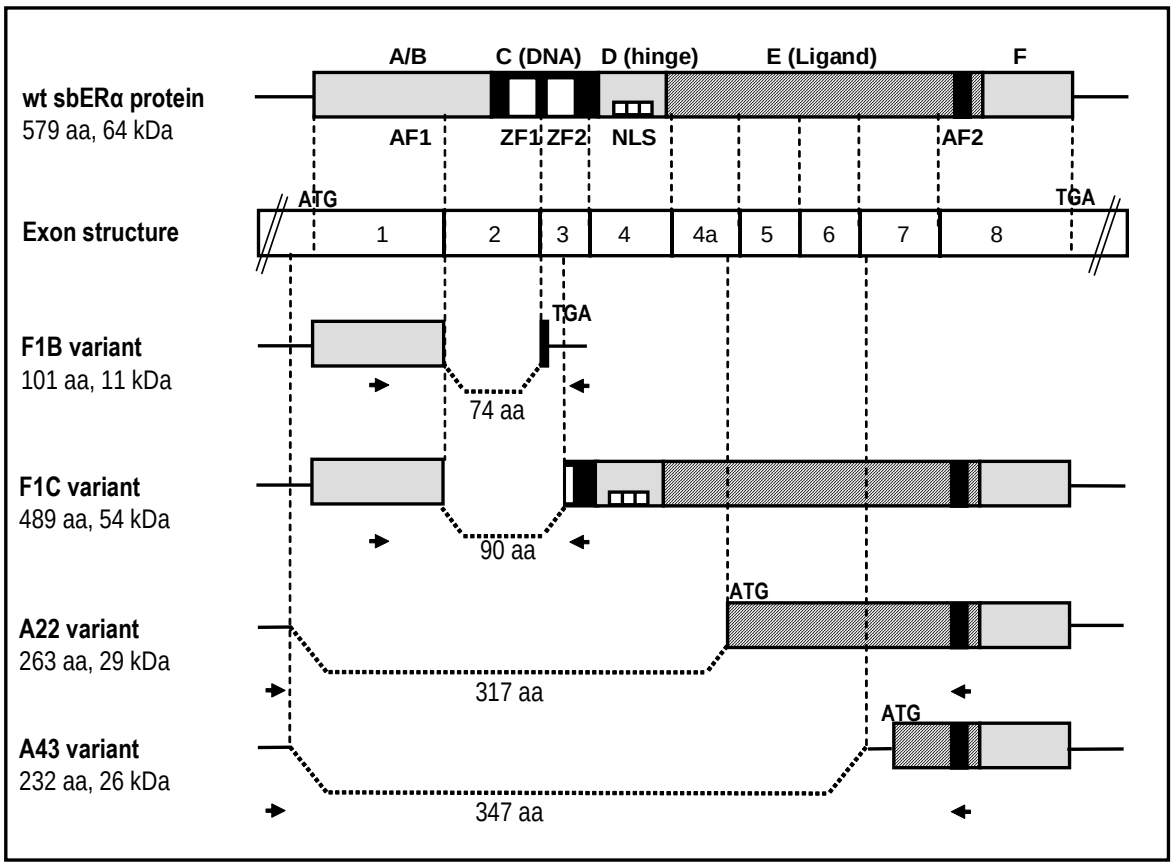

Figure 4.6. Representation of wt ERα protein structure and predicted structure of sbERα variants isolated in this study. The size, molecular weight and functional domains A-F of the wt ERα protein are represented in the first panel. Motifs represented: the AF-1 activation function in the A/B domain; the two zinc fingers (ZF, white boxes) in the C or DNA-binding domain (DBD, black box); the nuclear localization signals (NLS, white boxes) in the D domain and the AF-2 activation function (black box) in the E or ligand-binding domain (LBD, hatched box). The predicted coding-exons in the sbERα cDNA are represented in the second panel, with the initiation (ATG) and termination (TGA) codons indicated. The predicted structure, size and molecular weight of each variant isolated are represented below, based on the translational reading frame of each sequenced cDNA. Regions of the sbERα open-reading frame missing in each variant are represented by a dashed line and the number of amino acids missing is indicated. Premature stop codons generated in some variants are indicated by TGA and in-frame initiation codons in others by ATG. Only sequences between primers (arrows) were PCR-amplified and sequenced, regions outside this region are hypothetical and assume a similar cDNA sequence to the wt ERα cDNA.

4.3.5. Tissue distribution of sbERα variants

Pairs of primers specific for wt ERα and each of the sbERα exon2-deleted variants were designed (Figure 4.4) and their specificity demonstrated by cross-reactivity tests, in which F1B directed primers showed a slight cross reactivity with the wt ERα (section 4.2.6.1).

The pattern of tissue distribution of wt ERα in female and male sea bream tissues (Figure 4.7) generally coincided (Pearson correlation coefficient 0.85, P=0) with the results obtained in chapter 3, section 3.3.5 using the wt-specific era_2a and era_2as pair of primers (represented in Figure 4.4). Highest expression was confirmed in the pituitary, liver, white muscle and heart of females and in the pituitary, liver and testis of males.

The expression of F1C was very low and only detectable in female liver, heart and pituitary but its relative expression levels positively correlated with those of the wt ERα (coefficient 0.69, $P<0.05$). In contrast, F1B expression was not correlated with wt ERα. It was most highly expressed in female pituitary, liver, white muscle and heart and in male pituitary, liver and testis but was also found with relatively high abundance in female brain and male kidney. Although variant and wt ERα are co-expressed in several tissues, the relative expression levels are probably much lower for variant transcripts in most of the tissues, as suggested by the higher amplification cycles necessary to produce a visible reaction product (section 4.2.6.2.) and the relative levels of variant and wt PCR products in E_2-treated liver and testis, obtained by co-amplification using primers flanking exon2 (Figure 4.3A).

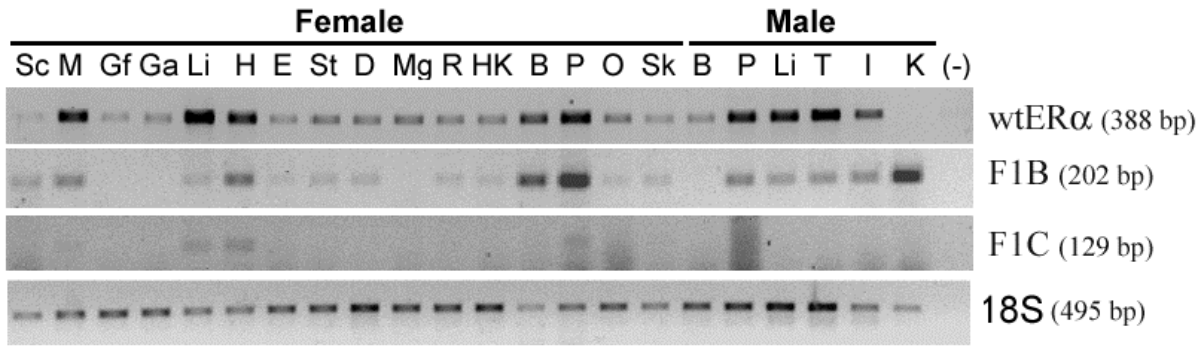

Figure 4.7. RT-PCR of the tissue distribution of wt sbERα and the exon2 variant cDNAs F1B and F1C in tissues of mature female and male sea bream, using 18S ribosomal subunit as internal control. Amplicon sizes are indicated on the right for each target, in base pairs (bp). Sc, scales; M, white muscle; Gf, gill filaments; Ga, gill arches; Li, liver; H, heart; E, esophagus; St, stomach; D, duodenum; Mg, mid-gut; R, rectum; HK, head kidney; B, brain; P, pituitary; O, ovary; Sk, skin; T, testis; I, total intestine; K, kidney; (–), non-reverse transcribed RNA.

To distinguish between the two sbERα wt transcripts, the wt ERα-specific PCR products (MCERaf286 and MCERar673 primers) for each tissue analysed were digested with the restriction enzyme *Bsr*FI. This enzyme should generate two restriction fragments of 150 and 238 bp from the amplified 388 bp product corresponding to sbERα_Mc (Figure 4.4) while the absence of a restriction site for *Bsr*FI in the sbERα_Soc amplicon means it should be unchanged after restriction digests. No digestion product was observed for any of the tissues analysed (data not shown), suggesting that the sbERα_Soc is most abundant and that sbERα_Mc is expressed at only vestigial levels or in particular physiological conditions. Since sbERα_Soc was cloned from E_2-treated liver, the wt ERα expression was also investigated in the same way in numerous liver cDNAs from animals of different ages and exposed to different E_2 doses (experiments 1 and 2, chapter 6, and other experiments previously carried out by the MCE group); no digestion product was observed.

As a positive control to confirm the restriction enzyme was functional a 354 bp PCR product for exons 7-8 of sbERβa was amplified from ovary cDNA and digestion fragments with the expected size (129, 190 and 35 bp) were observed in an agarose gel (not shown).

The tissue distribution of N-terminal variants A22/A43 could not be established, since it was not possible to detect amplified product for variant or wt ERα in any of the tissue utilised or in liver cDNAs from animals of different ages and exposed to different E_2 doses, using the ERalt22F1 and ERalt22R1 pair of primers (Figure 4.5). The results suggest that these transcripts are very rare, as positive control reactions with the plasmids containing the A22/A43 cDNAs amplified well in the PCR reaction.

4.3.6. Regulation of sbERα exon2-internal variants by E_2 / ICI 182,780

The abundance of the transcripts F1B and F1C appeared to be differentially affected by hormone treatment (Figure 4.8). The expression of the transcript F1B was down regulated by the antiestrogen ICI 182,780 (ICI) but not significantly affected by E_2 or combined E_2/ICI treatments. In contrast, F1C was up regulated by both doses of E_2 used to treat the fish, 1 and 0.1 mg/kg body weight E_2, while no significant alteration in F1C expression levels was obtained in the ICI-treated group. Combined E_2/ICI treatments caused a significant up regulation in F1C levels, although not significantly different from that obtained with 1 mg/kg E_2 alone. A strong positive correlation (Pearson coefficient 0.629, $P<0.001$) was found between the response of F1C and the response of the wt ERα transcript, analysed in chapter 6 using the wt ERα-specific primers era_2a and era_2as primers (see Figure 4.4) and here represented for comparison (Figure 4.8). wt ERα was significantly up regulated by both E_2 doses, ICI and simultaneous E_2/ICI treatment, although it was not up regulated in the group (IE3d) pre-treated with ICI. No correlation was found between the expression of F1B and F1C or wt ERα.

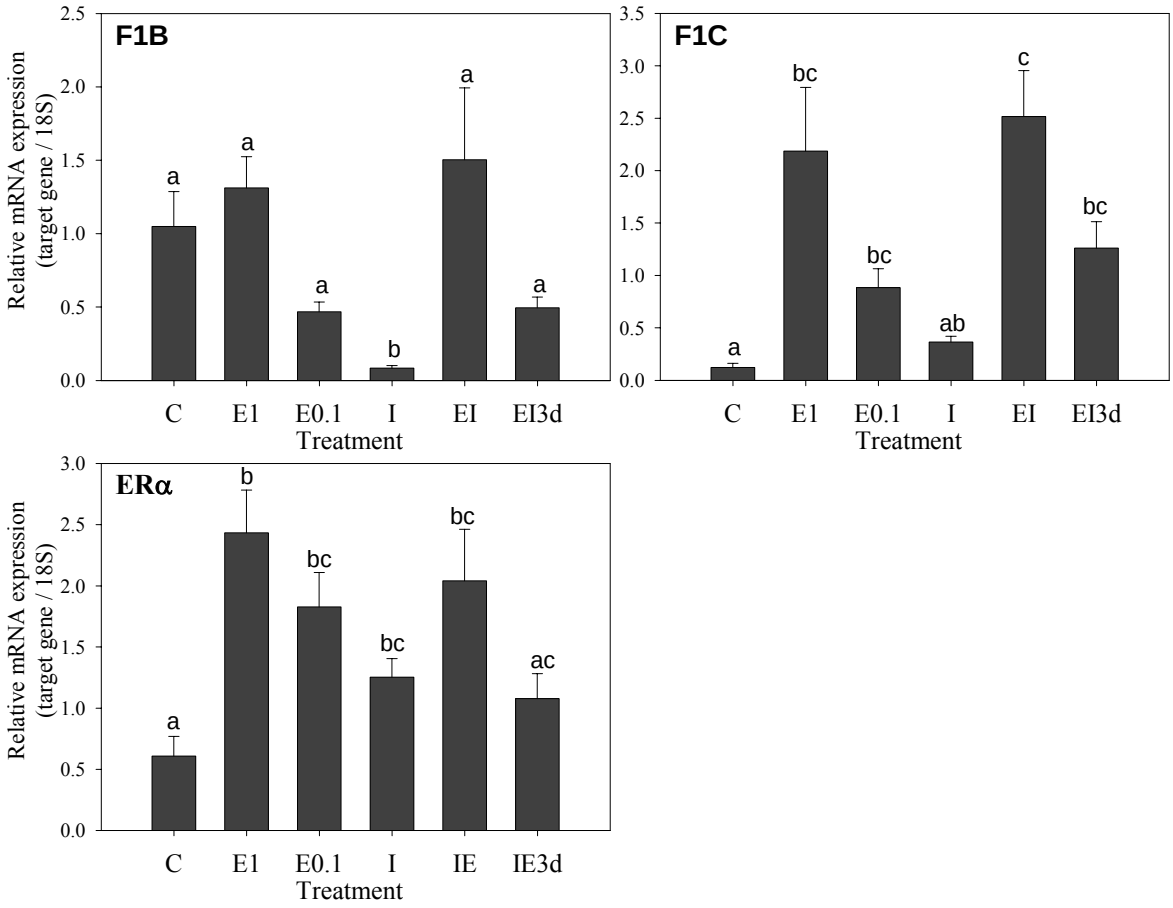

Figure 4.8. Expression of exon2 internal ERα variants F1B/F1C in male sea bream treated with estradiol and/or the antiestrogen ICI 182,780 for 48h. Each bar is the mean ± S.E.M. of the the relative expression values (ratio between target gene and 18S) of eight fish. C= Control group, coconut oil only; E1= 1 mg/kg body weight E_2; E0.1= 0.1 mg/kg E_2. I= 4 mg/kg ICI; EI= 4 mg/kg ICI + 10 mg/kg E_2; EI3d= 10 mg/kg E_2 3 days after injection with 4 mg/kg ICI. For comparison, the expression of the wt sbERα form (chapter 6) is also shown. Different letters indicate statistically significant differences between treatments (general linear model using $\log_{10}$ of expression ratios relative to 18S, $P<0.05$).

4.4. Discussion

In this study, the genomic organization of *Fugu* and sea bream ER genes was predicted by bioinformatics analysis. A well-conserved genomic structure of eight exons and conserved intron positions was demonstrated between the *Fugu* ER genes and characterized mammalian and fish ER genes (Ponglikitmongkol et al. 1988; Enmark et al. 1997; Lassiter et al. 2002; Swope et al. 2002), revealing high conservation throughout evolution. Like other fish ERα genes (Le Roux et al. 1993; Tan et al. 1996), *Fugu* ERα constitutes an exception to this rule in that it possesses an additional intron dividing exon 4 between the hinge domain and the LBD, being formed by nine coding exons. The presence of an intron in a similar position in some thyroid hormone receptor genes from *Xenopus* to human supported the hypothesis of the probable loss of this intron in higher vertebrate ER genes, which was retained in fish ERα (Tan et al. 1996 and references therein).

It is now recognized that a great diversity of transcripts may be generated from the ER genes by multiple mechanisms affecting transcription or transcript processing, although their biological significance have only started to be elucidated (Tan et al. 1996; Pakdel et al. 2000; Hirata et al. 2003). In this study, four different internal variant transcripts for the sea bream ERα gene were identified by RT-PCR, using single-exon spanning primers or primers located at distal exons.

The F1B variant appears to encode a C-terminally truncated receptor due to the precise deletion of exon2 and generation of a premature termination codon, and it was here demonstrated by sequence analysis that a similar consequence would occur in the case of exon2 splicing out in all the other characterized ERα genes (Ponglikitmongkol et al. 1988; Le Roux et al. 1993; Tan et al. 1996; Swope et al. 2002). An exon2-deleted human ERα (ERαΔ2) reconstructed cDNA was shown to produce a 17 KDa truncated protein in Cos7 cells, which was unable to bind ligand or a DNA estrogen-response element (ERE), to

translocate to the nucleus or to activate transcription of a consensus ERE-driven reporter gene (Bollig and Miksicek 2000). This is consistent with its predicted structure (similar to that of F1B, Figure 4.6), lacking both the DBD and LBD and the nuclear localization signals. Nevertheless, the mRNA expression of this and other single exon-deleted variants demonstrated to generate apparently non-functional proteins (Δ4, Δ6, Δ7) (Bollig and Miksicek 2000) has been described in normal and tumorous human tissues (Gotteland et al. 1995; Poola et al. 2000; Yang et al. 2000; Ye et al. 2000; Perlman et al. 2005), but its biological significance, if any, is unknown and its translation into the corresponding variant proteins has never been detected *in vivo*. These alternatively spliced variants may be potential targets for nonsense-mediated decay (NMD), an mRNA surveillance mechanism that selectively degrades transcripts containing premature termination codons as a mean to avoid the production of potentially deleterious proteins from abnormal mRNAs (Lejeune and Maquat 2005). NMD has been identified in all eukaryotes tested so far, while in humans a large proportion of naturally occurring alternative splicing mRNAs are NMD targets (Lewis et al. 2003; Hillman et al. 2004) and it has been recently proposed that mammalian cells routinely use regulated (unproductive) alternative splicing coupled to NMD to achieve proper levels of gene expression. Furthermore, efficient splicing was recently demonstrated to enhance NMD (Gudikote et al. 2005), and this model could explain the *in vivo* expression of ERαΔ2 and other non-productive ERα variants.

In contrast to F1B, the F1C variant contains an in-frame deletion of exon2 and part of exon3 and would encode a variant protein lacking the DBD first and part of the second zinc fingers, whose tetrahedrical structure is most probably disrupted, therefore affecting ERE-recognition and binding and ER dimerization (Ruff et al. 2000). This transcript was possibly generated by the use of an alternative acceptor splice site within exon3 (Figure 4.4), a common mechanism of alternative splicing (Black 2003) that may also have been used to

generate a second full-length wt ERα transcript (Muñoz-Cueto et al. 1999) differing in some amino acids from the one spliced at the predicted exon1-exon2 junction (Socorro 2001).

ERα transcripts with a in-frame deletion at the DBD have also been detected *in vivo* in mammals (Gotteland et al. 1995; Poola et al. 2000; Varayoud et al. 2005), which include Δ2,3 or Δ3, lacking both exon2 and 3 or exon3 alone, respectively. The *in vitro* produced ERαΔ3 protein was unable to bind DNA and activate transcription at a consensus ERE, but could bind ligand, translocate to the nucleus and act as a dominant negative of wt ERα at ERE promoters, negatively affecting its transcriptional activation through the formation of Δ3-wt ERα heterodimers with reduced DNA-binding ability (Bollig and Miksicek 2000). Moreover, the Δ3 variant was able to support transcriptional activation at an AP-1 element-regulated promoter, like wt ERα, despite the lack of an intact DBD (Bollig and Miksicek 2000). This is an alternative mechanism through which ERs mediate transcription of target genes by interaction with other transcription factors (Kushner et al. 2000), and the Δ3 biological role could be to redirect transcription from the classical, ERE-containing promoters, to non-classical response elements (Bollig and Miksicek 2000).

However, it is also possible that the F1B and F1C variants differ from the wt ERα cDNA in the remainder of the transcript sequence that was not PCR-amplified, since in mammals a large number of ER transcripts are also expressed which are generated by the deletion of multiple exons, consecutive or not (Poola et al. 2000; Ye et al. 2000; Perlman et al. 2005). It would be necessary to isolate the full-length F1C/F1B transcripts, by cDNA library screening or RT-PCR with primers spanning the entire coding sequence, before performing their functional characterization.

The alternative transcripts A22/A43 contain deletions of several coding exons between 1 to 7 that are followed by in-frame initiation codons, and are predicted to encode N-terminally truncated variants retaining only part of the LBD and the F domain. One transcript predicted

to encode a short-ERα variant formed by the last 192 amino acids of the receptor was isolated from channel catfish liver (Patiño et al. 2000), while variants formed by an unique 5'UTR encoded by an intronic exon followed by exons 4-8 or 5-8 (similar to A22) have been identified in human testis or rat pituitary, respectively (Tiffoche et al. 2001; Hirata et al. 2002). The rat truncated ERα protein (TERP-1) may act either as a dominant negative of wt ERα (and ERβ) at consensus ERE-containing promoters (at high TERP:ERα ratios) by formation of heterodimers, or as an ERα dominant positive (low TERP:ERα ratios) by titration of ERα repressor proteins (Resnick et al. 2000; Lin et al. 2003).

Although the divergence points between the A22/A43 variant and wt transcripts are located within exons and do not conform to the consensus donor/acceptor splice site motifs GT/AG (except for one of the A43 splice sites), these characteristics are also found in some ERα variant transcripts identified in human cancer cell lines and in the catfish short-ERα transcript (Anandappa et al. 2000; Patiño et al. 2000; Ye et al. 2000). However, it was not possible to detect the expression of these variants nor wt ERα in any of the tissues analysed using primers located in exons 1 and 8. Since in rainbow trout two transcripts with different 5'UTRs are transcribed from the ERα gene by alternative splicing and promoter usage in a tissue specific-manner (Pakdel et al. 2000; Menuet et al. 2001), it is possible that the same occurs for sbERα. One possible explanation for the lack of amplification is that the sbERα_Mc transcript containing the 5'UTR targeted by the ERalt22F1 primer (Figure 4.5) is low expressed in the tissues analysed. The expression of A22/A43 requires confirmation using different primers, before considering performing their functional characterization.

The expression of alternative transcripts for the fish ERβ genes is also expected, based on the previous detection of multiple transcripts of different sizes for ERβa or ERβb by Northern blot in sea bream and other fish species (Todo et al. 1996; Tchoudakova et al. 1999; Socorro et al. 2000; Sabo-Attwood et al. 2004 and chapter 3 of this thesis), the

cloning of numerous alternative transcripts for mammalian ERβ (reviewed in Hirata et al. 2003) and of two goldfish ERβa internal variants (Tchoudakova et al. 1999). However, the presence of sbERβa/βb variants was not detected in this study, which may be due to the limited number of cDNA samples analysed, the low sensitivity of the RT-PCR approach used (including the co-amplification of exon-deleted variants with the wt transcripts, usually present in large excess) and / or the fact that only single-exon skipping variants were investigated. An alternative RT-PCR method that has been used in mammals to specifically amplify ER internal variants uses primers directed against alternative splice junctions created by the deletion of each exon, together with primers to one of the extremities of the coding region, eliminating wt transcript co-amplification and allowing the specific amplification of both single and multiple exon-deleted variants (Williams et al. 1999; Poola et al. 2000; Poola et al. 2002; Perlman et al. 2005). However, in this study this "splice targeted primer approach" was not used since the splice junctions were only predicted. Future studies may include using different combinations of the exon-spanning primers to investigate multiple-exon deletions or using 3' or 5' RACE PCR (rapid amplification of cDNA ends) to investigate the presence of transcripts with different untranslated regions, for all the three sbER genes.

As a step to understand the functional significance of identified sbERα variants, the patterns of tissue distribution and the hormonal regulation in liver were established between the two exon2-deleted variants F1B and F1C and the wt ERα. F1C was expressed at very low levels in a restricted number of female sea bream tissues but was highly inducible in liver by E_2 with similar patterns to that of the wt ERα (discussed in chapter 6), which is consistent with its putative role in modulating the wt ERα cellular responses. In contrast, F1B showed a differential pattern of tissue distribution and hormonal regulation comparing to the wt ERα and F1C, suggesting different functions between the two variants. Differential patterns of

estrogen regulation have also been described between wt ERα and the mRNAs coding for several ER variant proteins with distinct functional properties (including the above referred ERαΔ3 and TERP-1 and some ERβ variants), which was considered an important regulatory mechanism for the self-tuning of the estrogen responsiveness in the tissues analysed (Tena-Sempere et al. 2004; Varayoud et al. 2005).

Although some tissues were shown to co-express both variants and the wt ERα, the expression of each receptor subtype may localize to different cell types and/or sub-cellular compartments, as observed for some mammalian variants (Pasqualini et al. 2001; Saunders et al. 2002; Cammarata et al. 2005; Saunders 2005), and further studies are required to localize the expression of the different sbERα forms within these tissues. In addition, we have used restriction enzyme analysis and RT-PCR to distinguish between the two full-length "wt" ERα transcripts and concluded that the sbERα_Soc is the major form expressed in al the tissues analyzed, while sbERα_Mc is probably expressed only at vestigial levels or in particular physiological conditions.

In summary, the results from this study point for the expression of several ERα variants in sea bream, which seem to fall into two main categories: those encoding putative receptors with internal deletions of exon2 (including C-terminal truncations) and those encoding putative N-terminally truncated receptors. The differential patterns of tissue distribution and hormonal regulation observed for the two exon2-deleted variants suggest they may have different biological significance, and further studies will be required to clarify their cellular localization and potential role.

4.5. Acknowledgements of practical work

P. Pinto acknowledges the contribution of S. Socorro for supplying some of the cDNAs and carrying out the PCR reaction containing the N-terminal sbERα variants A22/A43, and H. R. Teodósio for performing some of the semi-quantitative RT-PCRs.

CHAPTER 5

Immunolocalization and mRNA expression of estrogen receptor subtypes in sea bream (*Sparus auratus*) calcified tissues

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5.1. Introduction

In addition to its role in regulating reproductive functions in both female and male vertebrates, estrogens also affect a number of other physiological systems. For example, in mammals, estrogen exerts protective effects on the skeleton, where it prevents bone resorption by negatively affecting osteoclast lifespan and activity and exerts positive effects on bone formation by osteoblasts (Barkhem et al. 2004; Syed and Khosla 2005).

Most of the estrogens biological actions are mediated by specific estrogen receptors (ERs), which classically regulate the transcription of target genes by binding as dimers to their promoters (Nilsson et al. 2001). While most vertebrates have two ER subtypes (ERα and ERβ), fish have two ERβ receptors in addition to ERα, designated ERβa and ERβb (see chapter 3), although their biological significance is still unclear.

In mammals, ERα and ERβ appear to be functionally distinct but complementary, and the analysis of their patterns of mRNA and protein tissue distribution and cellular localization have greatly contributed to clarify their specific roles in many estrogen target tissues (reviewed by Matthews and Gustafsson 2003). Although both subtypes are widely distributed throughout the body and co-expressed in many tissues, including the ovary, uterus, mammary gland, testis, brain, blood vessels and bone, each ER may localize to different cell-types within a particular tissue or in different physiological states, thus suggesting distinct functions for each ER subtype in these tissues (Couse et al. 1997; Kuiper et al. 1997; Saunders et al. 1997; Koehler et al. 2005). In contrast, many cell-types have been reported to simultaneously express both ERα and ERβ proteins (e.g. osteoblasts or osteocytes in the bone, Shughrue et al. 1998; Bland 2000) and are potential targets for the formation of heterodimers and interplay between the two receptors (Ogawa et al. 1998). Moreover, differential subcellular localization has also been found between ER subtypes (Solakidi et al. 2005).

In fish, the main estrogen functions appear to be the control of reproductive functions, sex differentiation, yolk accumulation in growing oocytes (vitellogenesis) and calcium homeostasis (Lange et al. 2002). In contrast with the anti-resorptive effects in calcium metabolism in mammals, estradiol (E_2) is known to induce an increase in calcium plasma levels in several fish species, associated with periods of increased calcium demand such as vitellogenesis (Bromage et al. 1982; Norberg et al. 1989; Li et al. 1993). Moreover, the effects of E_2 in calcium homeostasis in fish appear to involve the redistribution of calcium between 1) the internal and external pool and 2) the internal pool. In the former situation E_2 increases whole body calcium influx from the environment (Persson et al. 1994; Guerreiro et al. 2002) while in the latter case it promotes calcium mobilisation from internal stores, especially the scales, where it appears to increase osteoclast activity (Mugiya and Watabe 1977; Persson et al. 1995; Armour et al. 1997; Rotllant et al. 2005). The presence of estrogen binding sites and of ERα mRNA in scales (Armour et al. 1997; Persson et al. 2000) suggests that calcium mobilisation is mediated by the direct action of E_2 binding to this receptor in this tissue. However, the contribution, if any, of other calcified tissues and other ER subtypes (βa or βb) in the E_2-induced calcium mobilization is so far unknown, and information about the expression and cellular distribution of the three ER subtypes in fish calcified tissues would contribute to this discussion.

Moreover, the information on the ER distribution and localization is also scarce in other fish tissues. The three ERs have partially overlapping but distinct patterns of mRNA tissue distribution, with co-expression in brain, pituitary, liver and gonads from both sexes (Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004; Hawkins and Thomas 2004), but mRNA localization studies by *in situ hybridization* (ISH) have concentrated mainly on three tissues (brain, ovary and testis) and in most cases only one or two ER subtypes (e.g. Menuet et al. 2001; Wu et al. 2001; e.g. Menuet et al. 2002; Andreassen et

al. 2003). Nevertheless, ISH for the three ERs in the brain of zebrafish and Atlantic croaker demonstrated that, like in mammals, fish ERs show both differential patterns of localization within this tissue (Hawkins et al. 2000; Menuet et al. 2002) and co-expression in the same cells (Pellegrini et al. 2005). However, immunohistochemistry (IHC) has only been carried out for the ERα protein in rainbow trout brain and testis (Anglade et al. 1994; Bouma and Nagler 2001) and in goldfish ultimobranchial gland (Suzuki et al. 2004), and additional localization studies are required to investigate the involvement of each ER subtype in estrogen functions in fish.

The first objective of this study was to produce and characterize specific antibodies for each sea bream ER subtype in order to use in cellular localization studies by IHC. For that, each recombinant sea bream ER protein (sbERα, sbERβa and sbERβb) was produced in *E. coli* and used to test the specificity of the produced anti-sbER antibodies by Western blot. The second objective was to investigate the expression of sbER transcripts in calcified tissues by RT-PCR and to localize sbER proteins in scales by IHC using each ER-subtype specific antibodies, as a first step to understand the functions and mechanisms of action of estrogens in calcium metabolism in fish. Finally, the specific antisera were used to assess the subcellular distribution of sbER proteins in fractioned protein extracts from several tissues by Western blot.

5.2. Specific methods

5.2.1. Cloning of the full-length sbERα coding region

In order to express recombinant sbER proteins, the full-length cDNA clones for sbERβa (Socorro et al. 2000) and sbERβb (clone 21, chapter 3) were used as template to amplify the ER coding regions and clone them into an expression vector (section 5.2.2.2). As no full-length cDNA was available for sbERα, it was first necessary to isolate the full-length coding region from cDNA. This was carried out by RT-PCR, using as template 2 µl of

liver cDNA from a 3kg female sea bream and specific primers (sbERacodF1 and sbERacodR1-Appendix V) designed based on the sbERα full-length sequence (Ac.no. AJ006039). A band of the expected size (1910 bp) was amplified in a 50µl reaction containing 40 pmol each primer, 200 µM dNTPs and 1.2 U of a proofreading polymerase (*Pfu* DNA Polymerase, Promega) in 1x *Pfu* reaction buffer. Cycling conditions were 2 min at 95 ºC, 40 cycles of 40 sec at 95 ºC, 30 sec at 60ºC and 4 min at 72 ºC, followed by 5 min at 72 ºC. The resulting band was gel purified into 40 µl stH_2O and 3 µl of the reaction product were reamplified and gel purified in the same conditions.

In order to insert the sbERα coding fragment into pBlueScript II KS (Appendix III), the purified band was amplified with primers designed to contain a *Bam*HI or an *Xba*I restriction site (era_xps3 and era_xpas respectively, Appendix V). Amplification was performed in the same conditions as above except that annealing temperature was 42ºC. The purified band was digested with *Bam*HI and *Xba*I, cloned in pBlueScriptII KS (Appendix III) with compatible ends and sequenced. The sbERα coding sequence obtained from this clone had, however, slight differences from that of the full-length sbERα cDNA AJ006039 (here designated sbERα_Mc, see section 4.2.5) in the region surrounding the predicted exon1-exon2 boundary, but was identical to that of the partial cDNA sbERα_Soc (AF136979). Moreover, the cloned cDNA contained the first 153 nucleotides of the coding sequence that were missing in sbERα_Soc.

5.2.2. Production of recombinant sbER fusion proteins

5.2.2.1. Expression vector and *E. coli* strain

sbER recombinant proteins for Western blot (section 5.2.6), *in vitro* phosphorylation (section 3.2.5) and sbERβb ligand binding assays (section 3.2.6) were expressed in *E. coli* BL21(DE3)pLysS cells (Appendix IV) under the control of a T7 promoter (pCR T7/NT-TOPO expression vector, Appendix III).

The pCR T7-TOPO expression vectors allow the direct cloning of *Taq* polymerase-amplified PCR products containing the gene of interest (by T/A-cloning, described in section 2.8) into the proper context of transcription/translation regulatory elements required for high yield expression in *E. coli*. To facilitate detection and purification, the pCR T7/NT-TOPO vector is designed to express the recombinant protein fused to an N-terminal tag (35 amino acids, approx. 4.5 kDa) containing the Xpress epitope and a polyhistidine region (His_6), which is cleavable by enterokinase. The BL21(DE3)pLysS strain contains the T7 RNA polymerase gene under the control of the l*ac*UV5 promoter, inducible by isopropyl-beta-D-thiogalactopyranoside (IPTG). This strain also carries the pLysS plasmid, which confers resistance to chloramphenicol and produces T7 lysozyme. This enzyme reduces the basal activity of T7 RNA polymerase and facilitates cell lysis by freeze-thaw cycles after protein production. The pCR T7/NT TOPO TA Expression Kit (Invitrogen) was used, which includes the expression vector, bacterial strains and most reagents needed.

5.2.2.2. Cloning of sbER coding regions in the expression vector

The entire coding regions of the three sbERs were amplified in independent PCR reactions from 1 µl of 1:500 plasmid dilutions containing each target cDNA (section 5.2.1). Specific primers (Appendix V) were designed to generate a product with the coding region in frame with the N-terminal tag (see vector map, Appendix III). Standard PCR conditions (section 2.7) were used with 25 cycles of 45 sec at 94ºC, 30 sec at the optimized annealing temperature (Appendix V) and 1.5 min at 72 ºC, followed by 10 min of extension at 72ºC. PCR products (1 µl) were cloned into the pCR T7/NT-TOPO vector and the ligation products used to transform One Shot TOP10F' competent cells (Appendix IV) following the supplier's instructions. Plasmid DNA was then isolated and sequenced (sections 2.9-2.10) to confirm insert orientation and ensure they were in-frame.

5.2.2.3. Expression of recombinant ER fusion proteins

Plasmid DNA (0.5 µl) from positive constructs for each sbER were used for transformation of One Shot BL21(DE3)pLysS competent cells, which were then selected on LB agar plates with 100µg/ml ampicillin and 34 µg/ml chloramphenicol (Appendix I). One positive transformant for each sbER was grown overnight at 37ºC in 3 ml of LB with ampicillin/chloramphenicol and 300 µl of each pre-culture were inoculated in 15 ml of LB ampicillin/chloramphenicol, grown at 37ºC to Abs_{600} of 0.6 and then split into three 5 ml-cultures. One of these fractions (pre-induced control) was immediately centrifuged for 20 min at 4300xg, 4ºC, and the bacterial pellet frozen at -20ºC. Fusion protein expression was induced in the other recombinant bacterial cultures by adding 0.5mM IPTG and incubating for 6h or 16h (overnight, ON) at 30ºC, after which the cells were harvested as above.

For cell lysis, the bacterial pellets were resuspended in 2 ml of TE buffer, pH 7.5 supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and subjected to 3-4 freeze/thaw cycles in liquid nitrogen followed by incubation in a heating block at 42ºC. DNAse (55U, Amersham Biosciences) and $MgCl_2$ (2.75 mM) were added to the lysate, incubated for 30 min on ice and centrifuged for 1 h at 28000xg, 4ºC. The supernatants were transferred to clean tubes and all pellets resuspended in 1 ml of TE with 1 mM PMSF, except for the pellet from the ON induction of sbERβb, resuspended in 2 ml.

7.5-15 µl of each supernatant or pellet were analysed by standard SDS-PAGE (section 5.2.4) and recombinant fusion proteins were detected by Western blot using anti-His-tag or ER-specific antibodies (section 5.2.6).

For *in vitro* phosphorylation and ligand binding assays (chapter 3), low molecular weight proteins and salts were first removed from a suspension of the recombinant bacterial pellet (ON cultures) by dialysis or ultrafiltration. sbERα and sbERβb recombinant bacterial pellets were dialysed (dialysis cellulose membranes, 12 kDa cut off, Sigma-Aldrich) for 2

h at 4°C against 2 changes of 1.5 L of Tris (10mM pH 7.5). The sbERβa recombinant bacterial pellet was purified and concentrated four times by centrifuging for 2.5 h at 4500xg in a Centricon YM centrifugal filter unit (10 kDa cut off, Millipore, Billerica, USA), with addition of 500 µl of fresh Tris buffer twice.

5.2.3. Subcellular protein fractionation from sea bream tissues

In order to study the subcellular distribution of native sbER proteins, subcellular protein extracts were prepared from sea bream tissues, based on the protocols used by Loomis and Thomas (1999) and Monje and Boland (2001). The sea bream tissues analysed were those in which the highest RT-PCR expression of each sbER mRNA had been observed (chapter 3): female heart (sbERα), ovary (sbERβa) and male kidney or intestine (sbERβb).

In brief, 200-300mg of tissue were homogenized with 1.5 ml of TEDG buffer (Appendix I.10) containing 0.75 µl of 40x proteinase inhibitors cocktail (Sigma-Aldrich), using a glass homogenizer. The homogenate was centrifuged for 20 min at 800xg to produce a nuclear pellet which was resuspended in 500-750 µl of high-salt buffer TEDGK (Appendix I.10), while the supernatant was subject to further centrifugation (see below). This fraction (nuclear pellet, fraction 1) was divided into two volumes: half was stored at -20ºC and the remainder was further extracted by incubation on ice for 2h, mixing vigorously every 15 min. It was then centrifuged (12800xg for 30 min) and the supernatant containing nuclear proteins recovered (extracted nuclear pellet, fraction 2).

The supernatant from the first centrifugation was centrifuged at 10,000xg for 15 min to obtain a mitochondrial-enriched pellet (fraction 3). The resulting supernatant was then centrifuged for 1 h at 100,000xg, producing a pellet containing the microsomal fraction (fraction 4) and the cytosolic extract in the supernatant (fraction 5). All fractions were stored at -20ºC. The protein content of each fraction was quantified by a standard Lowry assay (Lowry et al. 1951) and 100µg of each extract were analysed by 8% SDS-PAGE for

Coomassie blue detection of total protein or for Western blot analysis with anti-sbER antibodies (sections 5.2.4- 5.2.6).

5.2.4. SDS-PAGE

SDS-polyaclylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their size, after denaturation at 100ºC with sodium dodecyl sulphate (SDS) and the reducing agent dithiothreitol (DTT). In this work, the stacking gel was composed of 5% polyacrylamide. The 8% polyacrylamide used in the resolving gel was used for the separation of *E. coli* extracts or sea bream tissue extracts containing the sbER proteins (predicted MW of 63-79 kDa). In chapter 3 (section 3.2.5), 12% polyacrylamide gels were used to run MBP (18-23 kDa). The composition and preparation of stacking and resolving gels and other reagents used in SDS-PAGE are described in Appendix I.

Protein fractions were denatured with 0.5 volumes of 2x SDS sample buffer for 5 min at 100ºC, loaded onto polyacrylamide gels and electrophoresis was carried out using a constant current (30 mA) in 1x Tris-glycine running buffer. Protein bands were detected by Coomassie blue staining and excess dye was removed with destain solution. Alternatively, proteins were transferred to nitrocellulose membranes for Western blotting (section 5.2.6) and detected with specific antibodies. The PageRuler Prestained Protein Ladder (Fermentas, Hanover, USA) was run on all gels as molecular weight marker.

5.2.5. Production of anti-sbER antibodies

Polyclonal antibodies were raised in rabbit against synthetic peptides corresponding to amino acids in the N- or C-terminal regions of each sbER protein (Table 5.1) and were used in Western blot (section 5.2.6) and immunohistochemistry (section 5.2.9). All anti-sbER antibodies were directed to epitopes specific for each ER form (Figure 5.1) in order to achieve high specificity and avoid cross-reaction with the other receptors.

For anti-sbERα and anti-sbERβa, both N- and C-terminal specific antibodies were produced at the University of Sheffield (Socorro 2001). Peptides were synthesised by the Krebs Institute (Sheffield University, UK) and then conjugated to bovine thyroglobulin using carbo-diimide, emulsified in complete Freund's adjuvant and injected in rabbits. A second injection of the conjugate emulsified in incomplete Freund's adjuvant was administrated two months later. The antisera were aliquoted and stored at -20ºC, or lyophilised and stored at 4ºC in a desiccator. The antibody against the N-terminal region of sbERβb (KPS) was produced in AgriSera, Sweden, by injecting one rabbit with the respective synthetic peptide conjugated through the N-terminus to keyhole limpet haemocyanin. The conjugate was injected in the rabbit three times over a 14-week period (approx. 250 μg per immunization). The antiserum was stored at -20ºC or lyophilised and stored at 4ºC in a desiccator. Working solutions for each antisera were prepared in buffers containing sodium azide (0.1%) and kept at 4ºC.

Table 5.1. Peptides used to generate rabbit anti-sea bream ER antibodies.

Antibody	Notation	Peptide Sequence	Location
Anti-sbERα (N-terminal)	5NTA	RSSVPSSQHSVSREDQC	N-terminal, A/B domain
Anti-sbERα (C-terminal)	3CTA	SHESPTSPGVLQYGGSRSEC	C-terminal, F domain
Anti-sbERβa (N-terminal)	5NTB	CSPEKDQSLLQLQKVDSSR	N-terminal, A/B domain
Anti-sbERβa (C-terminal)	3CTB	SSRLPRRSPQQETVEQC	C-terminal, F domain
Anti-sbERβb (N-terminal)	KPS	KPSERPSSPRQL	N-terminal, A/B domain

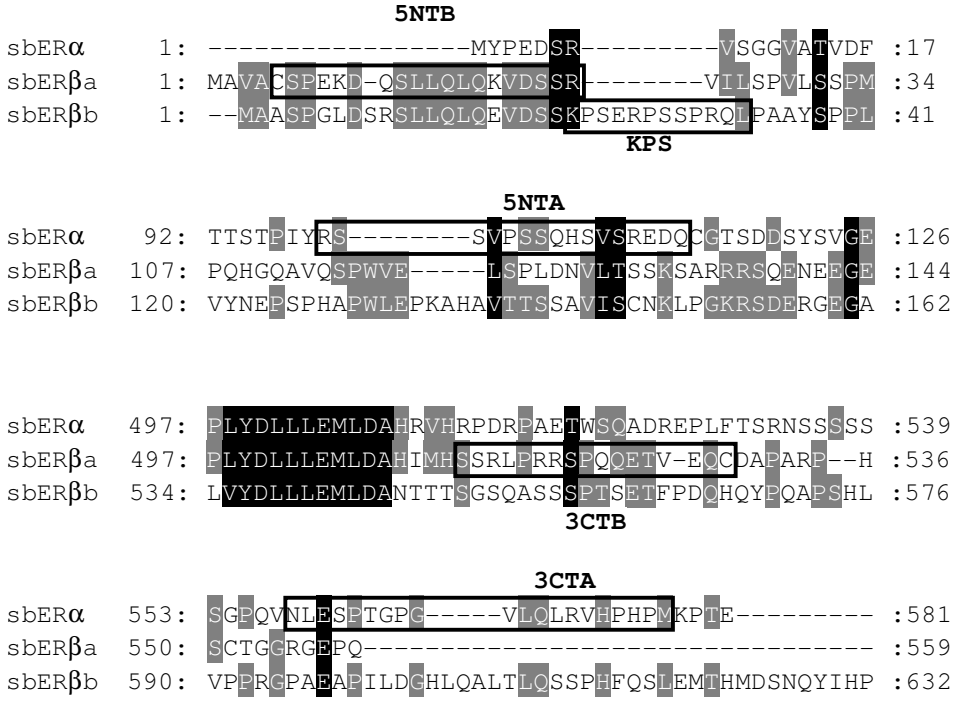

Figure 5.1. Amino acid sequence alignment of the N- and C-terminal regions of the predicted sequence of the three sbER proteins. The positions of synthetic peptides used to produce polyclonal antibodies are boxed.

5.2.6. Western Blot

5.2.6.1. Anti-His-tag Western blot

All solutions used in Western blot are described in Appendix I. To compare the levels of expression of the three sbER fusion proteins induced in *E. coli* for 6h or 16h, bacterial extracts were analysed by Western blot using an anti-His-tag antibody (Amersham). The mouse His-tag monoclonal antibody detects the six histidine-residue tag (His_6), which is present in the N-terminal tag of sbER fusion proteins (his-sbERs) generated in this study.

Pellet or supernatant (7.5µl) from lysed *E. coli* cultures in which the expression of each recombinant ER protein was induced for either 6h or 16h (ON) (section 5.2.2) were separated by SDS-PAGE on duplicate 8% polyacrylamide gels (section 5.2.4). One gel was stained with Coomassie blue to visualize total protein while the other was assembled in a transfer cassette (Figure 5.2) with a nitrocellulose membrane (Hybond ECL, Amersham). Transfer was carried out in a vertical tank electroblotting system (TE 22

Mighty Small Tank transfer, Hoeffer, San Francisco, USA) filled with 1x Transfer buffer, for 1 h at 300 mA with cooling.

The blotted membrane was incubated ON at 4ºC in blocking solution and washed in 1x Tris Buffered Saline (TBS) for 10 min. The blot was then incubated for 1.5 h at room temperature with the anti-His-tag primary antibody, diluted 1:3000 in 1x TBS. Subsequently the primary antisera were removed and the membrane washed 3x 15 min with TBS-Tween (TBST) prior to incubation for 1h at room temperature with the secondary antibody (Anti-mouse IgG, biotinylated, Amersham) diluted 1/1000 in 1x TBS. Membranes were washed 3x 15 min with TBST, incubated for 1h with the streptavidin-horseradish peroxidase conjugate substrate (Amersham) diluted 1/1000 in 1x TBS, and washed 3x 15 min in TBST. For development, the membrane was incubated for 5 to 20 min in the dark in freshly prepared DAB (diaminobenzidine) substrate solution containing nickel chloride for colour intensification. When an appropriate signal was achieved, the reaction was stopped by washing in dH_2O for 10 min and the developed membrane dried and stored in the dark.

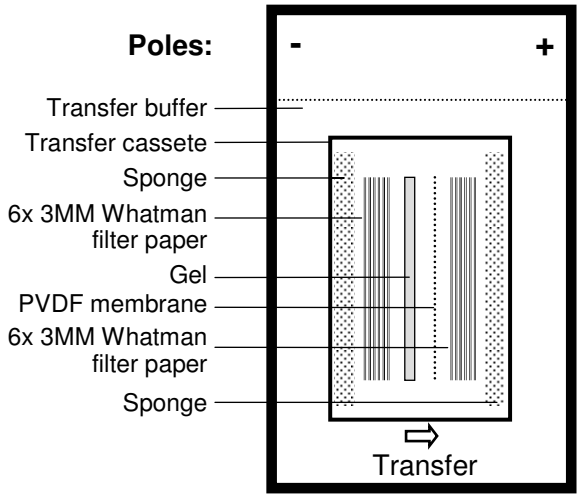

Figure 5.2. Representation of the transfer cassette used for electroblotting of proteins from a polyacrylamide gel to a nitrocellulose membrane, in a vertical tank transfer system.

5.2.7.2. Anti-ER Western blots

Western blot with anti-sbER subtype specific antibodies (section 5.2.5) was performed to characterize their specificity, by incubating with nitrocellulose blots containing the three recombinant sbER proteins expressed in *E. coli* (cross-reaction test). Western blot with the same antibodies was also used to detect and study the cellular localization of native sbER proteins in subcellular protein extracts from sea bream tissues (section 5.2.3).

Before carrying out the Western blot experiments, different antisera concentrations were tested for the detection of 1 µl of *E. coli* pellet containing each ER recombinant protein, spotted on 1x1 cm pieces of Hybond ECL membrane (Dot blot technique). After incubating membranes with different dilutions of each primary antibody (1:50 to 1:3000 in 1xTBS) and the secondary antibody anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich; 1:1000 to 1:5000 in 1xTBS), membranes were developed as described above. Optimised antisera dilutions, which gave the highest signal intensity, were chosen for use in Western blotting: 1:500 for anti-ERα and 1:300 for anti-ERβa, using the secondary antibody at 1:2000, and 1:250 for anti-ERβb using the secondary antibody at 1:1000. In the case of ERα and ERβa, N- and C- terminal antibodies (Table 5.1) were mixed in a 1:1 proportion and then used at the final concentration indicated.

Briefly, for the anti-ER Western blots 7.5 µl-15µl of each lysed pellet from the ON induced cultures of *E. coli* expressing each sbER protein (section 5.2.2) or 100 µg of subcellular protein extracts from sea bream tissues (section 5.2.3) were separated by SDS-PAGE on 8% polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes. ER proteins were detected using the optimized primary and secondary antibody dilutions and developed as described for the anti-His-tag Western blot. No incubation with peroxidase conjugate substrate was required for the anti-rabbit IgG

secondary antibody, since this antibody is already conjugated to peroxidase, the enzyme responsible for the chromogenic reaction involving DAB and hydrogen peroxide.

5.2.7. Sampling of scales

Scales were plucked with forceps from the caudal region of one juvenile sea bream (approx. 50 g) or one adult sea bream (approx. 400 g). Collected scales were immediately fixed overnight at 4ºC in fresh 4% paraformaldehyde (PFA, Appendix I), washed with sterile 1x phosphate buffered saline (1x PBS, Appendix I) and stored in methanol at 4ºC until used for immunohistochemistry (section 5.2.9). Alternatively, after fixation scales were washed with 1x PBS and stored in 70% ethanol at 4ºC until they were used for *in situ* hybridization (5.2.10) or for general histological analysis (5.2.11).

Additionally, scales and skeleton tissues for RT-PCR were snap frozen in liquid nitrogen immediately after sampling and stored at –80 ºC for subsequent RNA extraction, which was performed using TRI Reagent (section 2.4) after mechanical disruption of the tissue in liquid nitrogen.

5.2.8. Tissue distribution of sbERs by RT-PCR

Tissue distribution of each sbER was performed by RT-PCR using 2 µl of cDNA (prepared as described in section 2.6) from different calcified tissues of a 300 g sea bream, scales from three individual 300-400 g fish, scales from a 3 kg female sea bream and testis from a 300g male, which served as positive control for the RT-PCR. Primers and PCR conditions were the same as those used for assessment of the general tissue distribution of sbERs (section 3.2.9), except that 5 pmol of each primer and 100 µM each dNTP were used. Optimized cycle numbers were as follows: ERα 37 cycles, ERβa 33 cycles, ERβb 37 cycles and 18 cycles for the internal control gene 18S.

5.2.9. Whole mount immunohistochemistry (IHC) in scales

Whole mount IHC using anti-ER subtype specific antibodies (section 5.2.5) was carried out on whole scales from adult and juvenile sea bream, fixed in 4% PFA and stored in methanol as described above. Before immunoreaction, endogenous peroxidase was blocked by immersing the scales in a 0.3% hydrogen peroxide solution (in methanol) for at least 1h at room temperature. The scales were rehydrated through a series of 1x PBS solutions containing decreasing concentrations of methanol (75%, 50% and 25%), for 10 min in each solution, and then immersed for 10 min in phosphate-triton buffer (PBST, Appendix I). Scales were incubated for 1h in phosphate-carragenin-triton buffer (PCT, Appendix I) containing 10% (v/v) sheep serum, before incubation with the primary antibody, which was carried out ON at room temperature with slow agitation.

After several preliminary tests to tritate the antisera (concentrations range 1/200 to 1:1000, diluted in PCT), the final concentrations selected for IHC were 1:500 for anti-ERα, 1:1000 for anti-ERβa and 1:750 for anti-ERβb. For ERα and ERβa, N- and C- terminal antibodies (Table 5.1) were mixed in a 1:1 proportion to make the final optimized concentration. Specificity of the antibodies was assessed by incubating the samples without primary antibody (negative control).

After incubation with the primary antibody, scales were washed 3x 15 min in PBST and incubated for 45 minutes at room temperature with the secondary antibody solution (anti-rabbit IgG peroxidase conjugate), diluted 1/50 in PCT buffer. After washing 3x 15 min in PBST, the colour development was carried out by immersing scales in developing solution (1 tablet of DAB and 1 tablet of urea H_2O_2 in 1 ml of dH_2O, Sigma Fast DAB kit) and incubating in the dark until the desired signal was detected. Colour development was stopped by washing samples 2x 10 min in PBST and then for 10 min in dH_2O. Scales were mounted in glycerol gelatine and covered with a clean glass coverslip.

5.2.10. Whole mount *in situ* hybridization (ISH) in scales

In order to confirm if the sea bream scale cells which stained with anti-ER antibodies were osteoclasts, the mRNA expression of tartrate-resistant acid phosphatase (TRACP), an osteoclast marker (Persson et al. 1995), was analysed by ISH using a digoxygenin-labelled antisense RNA probe. In order to avoid RNase contaminations, all solutions used for ISH were prepared with DEPC treated water (Appendix I).

5.2.10.1. DNA template preparation

A partial sequence of the TRACP mRNA coding region (400 bp) was amplified by RT-PCR from adult sea bream scales with primers based on other piscine TRACP cDNA sequences. The amplified fragment was cloned in pGEM-T Easy and sequenced to confirm its identity (B. Redruello, unpublished).

For riboprobe production, the TRACP recombinant vector was linearized with *Sal*I, which cuts downstream from the insert, and *in vitro* transcribed with T7 RNA polymerase, so that the riboprobe obtained was complementary (antisense) to the target mRNA and lacked plasmid sequences. For linearization of the vector, 5 µl of recombinant plasmid was digested for 1.5 h at 37ºC with 10 U of *Sal*I in a 20 µl reaction volume in the appropriate buffer. To purify the linear DNA, the reaction volume was increased to 100 µl with stH_2O and extracted with 100 µl of basic phenol (pH 8) at room temperature for 35 min, with occasional mixing. The mixture was centrifuged for 10 min (10,000 rpm at room temperature) and the upper aqueous layer containing the DNA transferred to a new tube. The extracted DNA was then ethanol precipitated (Appendix II.4), resuspended in 20 µl of RNase and DNase free water (Sigma) and visualised in a 1% agarose gel (Appendix II.1).

5.2.10.2. Synthesis of the digoxygenin-labelled riboprobe

The riboprobe was synthesised by *in vitro* transcription in one reaction containing 20 U of T7 RNA polymerase (Promega), 1µl of digoxygenin RNA labelling mix (Roche-diagnostics, Basel, Switzerland), 4µl of 5x transcription buffer (Promega), 1µl of 100mM DTT, 0.2 µl of 10 mg/ml bovine serum albumin (BSA) and 0.5 µl RNA Guard (Promega). An appropriate volume of linear DNA (approx. 1mg/ml, see below) was added, the reaction volume made up to 20 µl with stH_2O and incubated for 1.5 h at 37ºC. The reaction was stopped with 2 µl of 0.2M EDTA pH 8.0 and incubated 2 min on ice. The synthesized riboprobe was selectively precipitated with 2.5 µl of 4M lithium chloride and 75 µl of 100% ethanol, for 1 h at -80ºC or ON at -20ºC, and centrifuged at 10,000 rpm for 15 min at 4ºC. The resulting pellet was washed twice with cold 70% ethanol, dried and resuspended in 25 µl of RNase and DNase free water. The appropriate volume of linear DNA for the synthesis of labelled riboprobe was initially optimized by carrying out *in vitro* transcription reactions with unlabelled nucleotides (dNTPs, 0.4 mM each), using different DNA concentrations, and comparing the yields of transcription by agarose gel electrophoresis (Appendix II.2).

5.2.10.3. *In situ* hybridization (ISH)

ISH was carried out with whole scales from juvenile sea bream, fixed in 4% PFA and stored in 70% ethanol as described above (section 5.2.7). All procedures conducted at room temperature were carried out in a 96-well plate (one scale per well). Scales were hydrated by immersion in phosphate-tween buffer (PTW, Appendix I) for 5 min and then treated for 5 min at room temperature with 0.01 mg/ml proteinase K (diluted in PTW from a 1 mg/ml stock). Treated scales were washed twice in PTW, re-fixed with 4% PFA and washed again in PTW. Scales were transferred to hybridization solution (Appendix I), pre-hybridized at 65ºC for 2-4 h, and then hybridized ON at 65ºC (high stringency) in

hybridization solution containing approx. 3µg/ml of digoxygenin-labeled TRACP riboprobe. Non-specifically bound probe was removed by performing stringency washes at 65ºC, twice for 10 min with 2x SSC, 10 min with 1xSSC and then 10 min with 0.2x SSC. During the pre-hybridization, hybridization and stringency washes, all at 65ºC, scales were maintained between two glass slides to prevent them curling.

Samples were then washed at room temperature twice in 2x SSC containing 0.12% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), followed by a 1:1 mix of 2x SSC and PTW, and finally in PTW. Scales were covered with 2% blocking solution (Appendix I) containing 10% (v/v) sheep serum and incubated for 3-5 h at room temperature. Detection of the digoxygenin-labeled probe was carried out ON at 4ºC with anti-digoxygenin-AP Fab fragments (Roche), diluted 1/100 to 1/600 in 1% blocking solution, with the scales maintained between two glass slides. Samples were washed twice in a solution containing 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl and then equilibrated for 10 min in developing buffer (Appendix I). Colour development was performed for 2-8 h at 37ºC, in developing buffer containing 0.45% (v/v) of substrate NBT (4-nitroblue tetrazolium chloride, Sigma) and 0.35% of BCIP (5-bromo-4-chloro 3-indolylphosphate, Roche). The reaction was stopped by transferring the scales to 1x PBS, which were then fixed for 15 min at room temperature with 4% PFA. Finally, scales were washed twice for 5 minutes in 1x PBS, twice with dH_2O and then mounted in glycerol gelatine and covered with a clean glass coverslip.

5.2.11. General histology of sea bream scales

The histology of whole scales from juvenile sea bream was characterized by haematoxylin-eosin (H&E) staining, described in section 7.2.7 for tissue sections.

Briefly, whole scales fixed in 4% PFA and stored in 70% ethanol (section 5.2.7) were hydrated by immersing for 5 min in dH_2O. Scales were than stained for 30 sec in Harris

haematoxylin solution (Appendix I), washed in tap water, immersed in a 1% eosin Y solution (Appendix I) for 30 sec and washed in dH_2O. To obtain definitive preparations, scales were dehydrated through an ascending series of ethanol dilutions (70%, 95%, 100%, 5 min each), cleared for 2x 10 min in xylene, and then mounted in glycerol gelatine and covered with a clean glass coverslip.

5.3. Results

5.3.1. Expression and detection of recombinant sbER fusion proteins in *E.coli*

The coding regions for each of the three sbERs were cloned in the correct frame into the expression vector pCR T7/NT TOPO, as confirmed by sequence and restriction analysis, and each sbER protein was expressed in *E. coli* BL-21 as a fusion protein with a histidine-rich N-terminal tag (his-sbERs). The expression of recombinant proteins was carried out at 30ºC for 6 or 16 h (ON) after IPTG induction, rather than the 4-6 h at 37ºC as indicated in the expression kit instructions, to favour proper protein folding.

SDS-PAGE analysis of supernatant and pellet extracts after cell lysis (Figures 5.3A and B) shows that sbERα and sbERβb fusion proteins were expressed at significant levels and were the predominant products of translation visible in both fractions, after 6h or ON (16h) induction, migrating at approx. 73 and 85 kDa, respectively. In contrast, no products of induction for sbERβa could be detected by Comassie staining of SDS-PAGE.

The expression results obtained with pCR T7/NT TOPO system in *E. coli* were consistent with previous results obtained using *in vitro* translation of the three sbERs using *E. coli* extracts or rabbit reticulocyte extracts. The yield of receptor protein obtained by *in vitro* translation was much lower than with the TOPO system (not shown), but the relative level of expression of the three ER proteins was similar and sbERβa was always lower than sbERα or sbERβb. Comparison between the supernatants and pellets obtained from bacterial cell lysates of induced cultures revealed that the majority of the recombinant fusion protein produced for each ER subtype was found in the pellet, while a minor amount of soluble fusion protein was found in the supernatant (Figure 5.3). The results of this analysis indicate that the recombinant proteins produced were either associated with the pelleted bacterial membranes or were present as inclusion bodies.

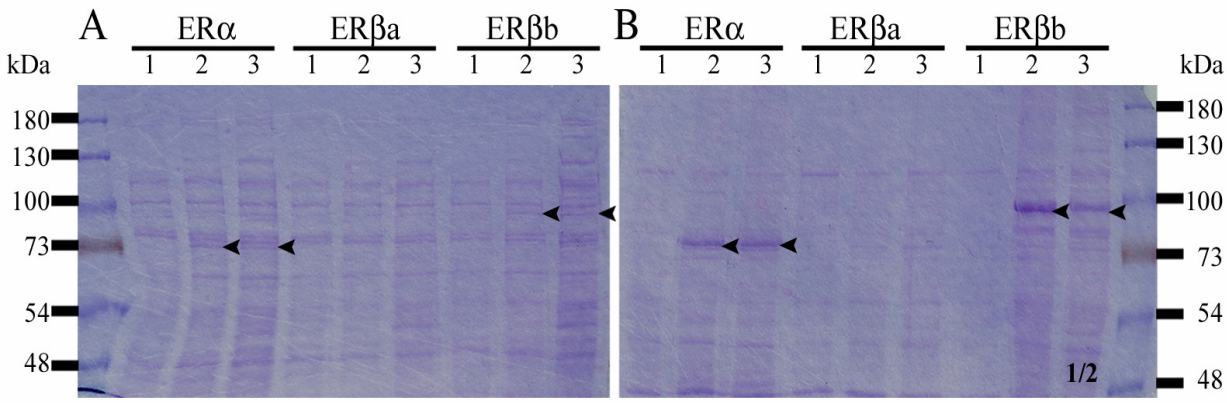

Figure 5.3. Coomassie stained SDS-PAGE of the his-sbER recombinant proteins in the cell lysis supernatants (A) or pellets (B) from *E. coli* BL-21 transformed with each pCR T7/NT TOPO-sbER construct (ERα, ERβa or ERβb). For each ER subtype, cell lysates were obtained from bacterial cultures before IPTG induction (pre-induced control, lane 1), or after 6h (lane 2) or ON induction (lane 3) with IPTG. The ON induced sbERβb pellet was diluted in the double of the volume compared with the other pellets but the same volume was charged on gel (indicated by "1/2", two times less total protein on the gel). Band sizes (kDa) from a molecular weight marker are indicated in each panel. Bands corresponding to each sbER recombinant protein are highlighted by arrows.

Nevertheless, as the fusion protein present in the bacterial cell lysis pellet could be rendered soluble using denaturing condition in SDS-PAGE, it was possible to perform Western blot assays using this fraction to establish the molecular weight (MW) of fusion proteins and to test the cross-reactivity of the sea bream specific ER antisera used for IHC. Initially the fusion proteins were detected in Western blot using the anti-His-tag antisera (Figure 5.4A), in order to establish their MW and to compare their levels of expression for different times of induction. In sbERα cultures, one principal protein of approximately 73kDa was produced, which approximately corresponds to the predicted size for the N-terminal tagged sbERα protein (68 kDa), while a few secondary products were also found.

It was not possible to detect sbERβa, and for sbERβb a principal product of approximately 85kDa was observed, which is within the same range of the predicted his-sbERβb MW (79 kDa). Two smaller secondary products (approx. 75 kDa and 50 kDa) were also obtained.

No significant differences in sbERα expression levels were observed between 6h or ON induction (Figure 5.4A, ERα lanes 2 and 3). For sbERβb, only the 6h induced proteins were completely transferred to the membrane (due to a technical problem with the blotting, Figure 5.4A, ERβb lane 2), but the detected sbERβb band had a strong intensity, indicating that 6h of induction at 30ºC is enough to achieve a significant level of expression of sbER fusion proteins in this system.

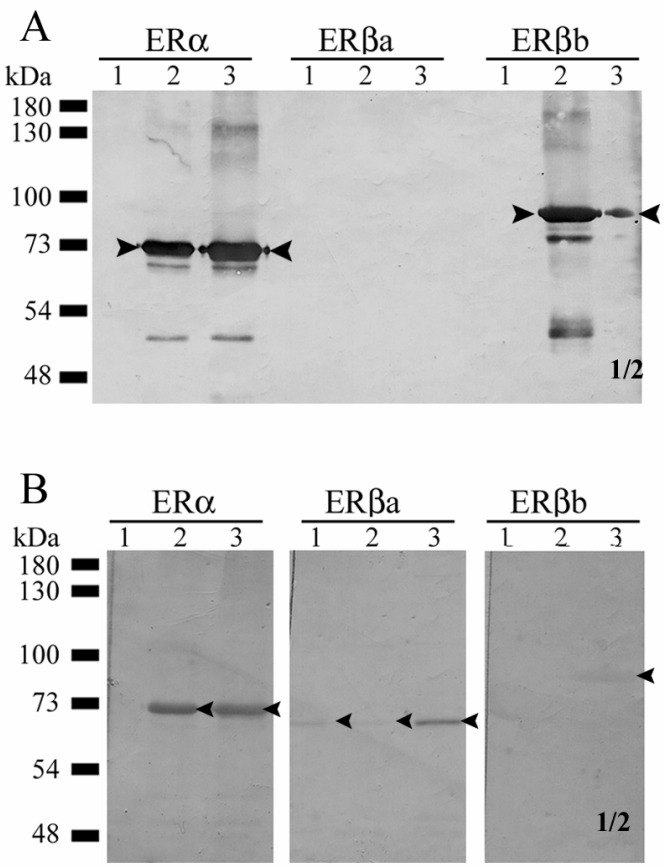

Figure 5.4. Western blot showing the detection of each his-sbER fusion protein (α, βa or βb), using the anti-His-tag antisera (A), which recognizes the histidine-rich region in their N-terminal tag, or using sbER-subtype specific antisera (B) produced in this study (section 5.2.5). For each ER subtype, bacterial cell lysis pellets run on the SDS-PAGE gel and transferred to the nitrocellulose membrane were obtained from each pre-induced control culture (lane 1), or from the 6h (lane 2) or ON IPTG-induced (Lane 3) cultures. The same volumes were charged on both gels A and B. The ON induced sbERβb pellet charged on the gels was two times more diluted than the other pellets (indicated by "1/2"), and in the anti-His Western blot this lane was only partially transferred to the membrane. Band sizes (kDa) from a molecular weight marker are indicated in each panel. Bands corresponding to each sbER recombinant protein are highlighted by arrows.

5.3.2. Characterization of anti-sbER antibodies

The fusion proteins were then detected by Western blotting using anti-sbER antisera (Figure 5.4B), raised in rabbit against synthetic peptides corresponding to amino acids in the N- or C-terminal regions of each sbER protein (see section 5.2.5). Using the anti-sbERα sera, it was possible to detect the principal his-ERα protein product (approx. 73 kDa) in both the 6h or ON induced extracts. Interestingly, although no immunoreactive sbERβa fusion protein was detected with the anti-His-tag sera, a protein of approximately 70kDa (which corresponds approximately to its predicted MW, 67kDa) was detected using the anti-sbERβa sera, suggesting these antisera are very sensitive. Moreover, the sbERβa protein was detected at comparable levels in the pre-induced control and in the 6h induced extracts, with a slightly higher level for the ON induction (Figure 5.4B, ERβa lanes 1-3), indicating that this construct had a basal level of constitutive expression that was not significantly increased by IPTG. In contrast to ERα or ERβa, a very weak signal was obtained with anti-sbERβb, which could detect the 85kDa his-sbERβb protein in the ON induced but not in the 6h induced extract.

To establish the specificity of the different sbER antisera, Western blot was carried out using each fusion protein and determining the presence of cross-reaction with each of the antisera. The sbERα antisera are specific for sbERα fusion protein, as the antisera did not detect either of the sbERβ fusion proteins (Figure 5.5, ERα lane 3 and 4). Anti-sbERβa reacted with its antigen and did not detect sbERα, although a slight cross reaction occurred with ERβb (Figure 5.5, ERβa lane 4). However, this was most likely due to the higher concentrations of sbERβb protein used compared to sbERβa (based on Coomassie staining and anti-His-tag western blot), and at the higher dilutions of anti-sbERβa used in the IHC the cross-reaction is unlikely to be detected.

While sbERa and sbERβb antibodies recognized principally their respective receptor, the anti-sbERβb antibody KPS showed significant cross-reaction for sbERα (Figure 5.5, ERβb lane 2). This polyclonal antibody had been raised against a synthetic peptide corresponding to a specific region in the N-terminal region of sbERβb, designed based on the alignment between the three sbER protein sequences in order to achieve maximum specificity (see Figure 5.1). Given the cross-reactivity detected for the sbERα protein, a new alignment was performed between the KPS peptide sequence and the sbERα protein (Figure 5.6), which identified a region located at the end of the ERα A/B domain with some sequence similarity (two proline residues and one lysine) with the peptide that could explain the cross-reaction. These results indicate that the IHC results obtained using these antisera should be interpreted with caution and compared with the anti-ERα signal before any conclusions about the sbERβb expression can be made.

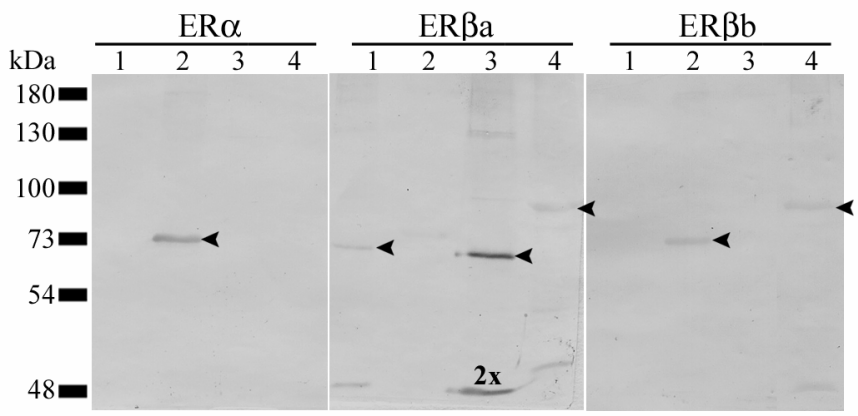

Figure 5.5. Western blot analysis of the cross-reactivity between each sbER subtype-directed antisera (ERα, ERβa and ERβb) against each recombinant fusion protein sbERα (Lane 2 of each panel), sbERβa (Lane 3) or sbERβb (Lane 4), present in bacterial cell lysis pellets from the ON induction of recombinant *E. coli* cultures with IPTG. Lane 1 in each panel is the pellet from the pre-induced control culture for each ER subtype. The volumes charged on gel were the same used in the anti-His or anti-ER Western blots (Figure 5.4), except that the double volume was used for sbERβa (indicated by "2x") and for sbERβb (used at 1x concentration) ON induced extracts. Band sizes (kDa) from a molecular weight marker are indicated on the left and bands detected with each antisera are highlighted by arrows. Some cross-reactivity was detected between anti-sbERβa and the sbERβb protein and between anti-sbERβb and the sbERα protein.

```
KPS     1: --------KPSERPSSPRQL--------------- :12
sbERα  71: FVPSSPHLSPFMQPANHHYLETTSTPIYSVPSSQH :105
```

Figure 5.6. Amino acid sequence alignment between the N-terminal region of the sbERα predicted protein and the sequence of the KPS synthetic peptide, used to produce the anti-sbERβb polyclonal antibody, showing a region with some sequence similarity that could explain the cross-reactivity detected.

5.3.3. Subcellular distribution of native sbER proteins in sea bream tissues

The anti-sbER antisera were used to investigate the expression of native sbER proteins by Western blotting in different subcellular fractions of protein extracts prepared from sea bream tissues previously shown to express each sbER mRNA (Figure 5.7). The detected signals were generally very weak, supporting low levels of expression of ER proteins *in vivo*, and no signal was detected using the anti-sbERβb sera in kidney or intestine extracts (not shown). For sbERα, one band of approx. 54kDa (within the same range as the predicted MW for the native sbERα, 64 kDa) was detected in the heart, mainly in nuclear protein extracts. The same band was detected with low intensity in mitochondrial and cytosolic fractions, while a higher size band with approx. 58kDa was detected in the cytosol, possibly phosphorylated sbERα. Although no band of the predicted size for the sbERβa protein (63kDa) was detected in ovary sub-cellular fractions, two bands with more than 130kDa were detected in the mitochondrial and microsomal fractions, which could represent ER dimers that for some reason were not efficiently denatured by SDS in the gel. Two strong bands migrating at approximately 48 and 50kDa were also detected with anti-sbERβa in the mitochondrial, microsomal and cytosolic extracts, although they may have resulted from unspecific hybridization, since these proteins were present in an extremely high concentration in these ovary extracts as detected by Coomassie staining of SDS-PAGE (not shown).

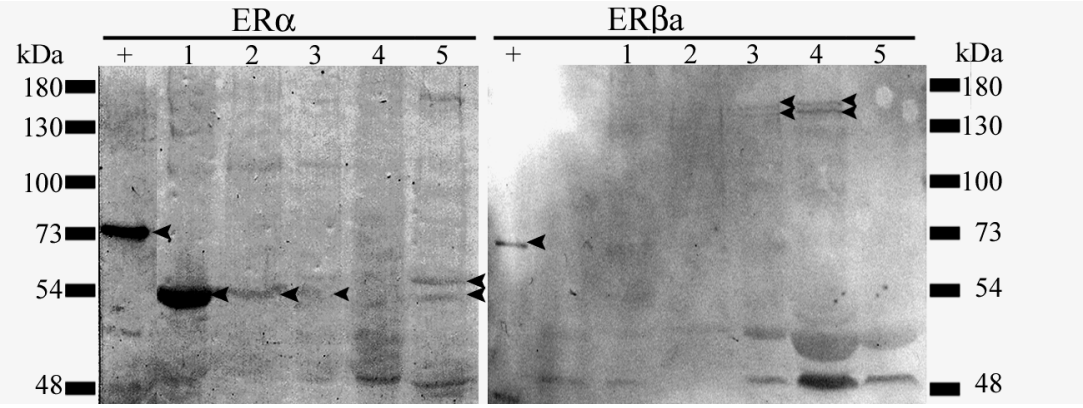

Figure 5.7. Subcellular distribution of sbERα and sbERβa native proteins in protein extracts from sea bream heart or ovary, respectively, detected by Western blot using each sbER subtype-specific antisera. Lane +: *E. coli* cell lysis pellets containing each recombinant sbER protein, as positive control. Lane 1: nuclear fraction. Lane 2: extracted nuclear fraction. Lane 3: mitochondrial fraction. Lane 4: microsomal fraction. Lane 5: cytosolic fraction. Band sizes (kDa) from a molecular weight marker are indicated in each panel. The brightness and contrast of these images had to be manipulated to improve the visualization of putative ER detected bands (indicated by arrows), since all had very weak intensity. No bands could be detected in the sbERβb blot, which was not included here.

5.3.4. ER protein localization in sea bream scales by immunohistochemistry

The cellular localization of sbERs in scales was determined by whole mount immunohistochemistry. The three forms of ER were expressed in scales from juvenile and adult sea bream although the signal localization and intensity varied with the age of animals and the type of receptor (Table 5.2). The sbERα subtype was detected in both juvenile and adult sea bream and in the anterior and posterior regions of the scales. In juvenile sea bream, the signal was mainly found in groups of rounded cells located in the scale posterior region (Figure 5.8A), while in adult sbERα was mainly expressed by isolated cells present in the scale anterior region (Figure 5.8E). sbERβa appears to be weakly expressed in scales, but in juvenile sea bream a few rounded cells in the scale posterior region express this receptor (Figure 5.8B and F). sbERβb was mainly detected in the posterior region of the scales, and in juvenile sea bream it was abundantly expressed in round cells of the scale posterior region (Figure 5.8C), similar to those that also expressed sbERα and sbERβb.

mRNA expression of the osteoclast marker TRACP (tartrate-resistant acid phosphatase) was detected by *in situ* hybridization in the posterior region of juvenile sea bream scales (Figure 5.8D), in cells with a similar morphology (Figure 5.8H) to those co-expressing the three sbER proteins, suggesting these could be putative osteoclasts.

Table 5.2 – Relative levels of immunoactivity of estrogen receptors (α, βa and βb) in scales analysed by whole mount immunohistochemistry. Results are presented for the anterior (Ant.) and posterior (Post.) regions of the scales of juvenile and adult sea bream.

Proteins	**Juvenile sea bream**		**Adult sea bream**	
	Ant.	**Post.**	**Ant.**	**Post**
ERα	+/-	++	++	+/-
ERβa	+/-	+	+/-	+/-
ERβb	-	++	+/-	+

(-) not detected, (+/-) very low abundance, (+) low abundance, (++) high abundance and (+++), very high abundance

5.3.5. ER mRNA distribution in sea bream calcified tissues

RT-PCR was used to analyse the distribution of the three sbER mRNAs in calcified tissues from adult sea bream (Figure 5.9). While the two sbERβ isoforms were expressed in scales and different bone and cartilage structures, the expression of sbERα was generally very low to undetectable in those tissues. Moreover, there was a high variability in expression between different individuals in the scales, especially for sbERβb, with some fishes showing strong expression while in others it was almost undetectable. Considering the number of amplification cycles necessary to produce a visible reaction product, it is evident that the expression of the three sbERs in calcified tissues is lower than in the non-skeletal tissues (compare tissue distribution by RT-PCR in Figures 5.9 and 3.9 and cycle numbers in sections 5.2.8 and 3.2.9).

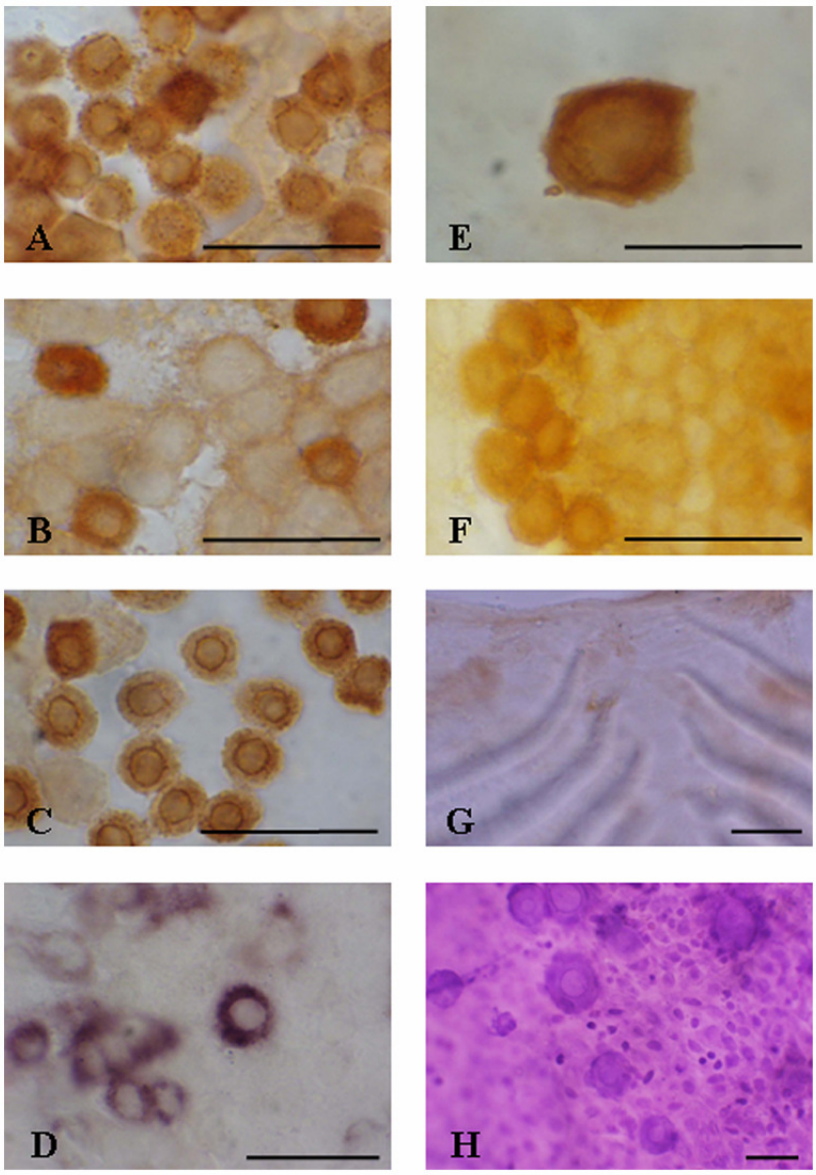

Figure 5.8. Whole mount immunohistochemistry of sea bream ER proteins in scales from adult and juvenile sea bream, using ER subtype-specific antibodies. Expression of sbERα (A), sbERβa (B) and sbERβb proteins (C) was detected in groups of rounded cells in the posterior region of the scale of juvenile sea bream. Expression was also identified for sbERα in isolated cells in the anterior region of adult sea bream scales (E) and for sbERβa in rounded cells located near the scale posterior margin in juvenile sea bream (F). No signal was detected in the negative control (G), carried out without primary antibody.
TRACP mRNA was detected in the posterior region of juvenile sea bream scales by *in situ* hybridization (D), in the same cell type where sbERs were immunodetected (putative osteoclasts). Panel H shows the morphology of these cells in the posterior region of a scale stained by H&E. These pictures were kindly provided by M. D. Estêvão (from Estêvão 2005).

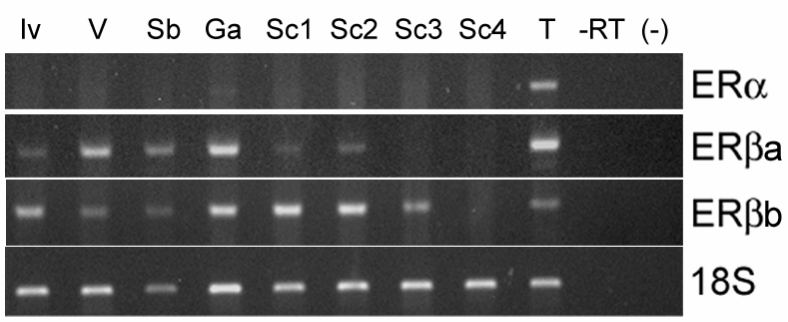

Figure 5.9. Tissue distribution of sbERα, sbERβa and sbERβb detected by RT-PCR in calcified tissues from adult sea bream, using 18S ribosomal subunit as a control for the relative quantity of cDNA used in PCR reactions. Iv, intervertebral disc, V, vertebra, Sb, skull bone and Ga, gill arches from a 300g sea bream; Sc1, scales from a 3 kg female sea bream; Sc2-4, scales from three individual 300-400 g fish; T, testis of a male 300g fish (positive control); –RT, PCR on non-reverse transcribed RNA (to detect genomic contamination) and (-) PCR without DNA.

5.4. Discussion

In the present study, recombinant sea bream ERα, βa and βb proteins were produced in order to use in immunodetection (this chapter) and in *in vitro* phosphorylation and ligand binding assays (chapter 3). Recombinant ERs were expressed as N-terminal tagged fusion proteins in *E. coli*, a prokaryotic expression system widely used to purify and characterize prokaryotic and eukaryotic proteins mainly due to its rapid growth, easy transformation and induction, low cost and high protein yields (Higgins and Hames 1999). Its major drawback is the inability of *E. coli* to perform the post-translational modifications carried out in eukaryotic cells, and the fact that recombinant proteins may accumulate into insoluble aggregates known as inclusion bodies, in most cases due to protein misfolding (Sorensen and Mortensen 2005).

Although some post-translational modifications have been reported for the ERs, including phosphorylation, acetylation, sumoylation or palmitoylation, these appear to influence mainly the ligand-independent transactivation or plasma membrane signalling or to have a modulatory effect on the ligand-induced transcriptional activation of target genes (Fu et al. 2003; Lannigan 2003; Acconcia et al. 2004; Sentis et al. 2005), while no modification

appear to be required for ligand binding or immunodetection. Indeed, other studies have used *E. coli*-expressed ER proteins for ligand or DNA binding assays, mutant analysis or characterization of anti-ER antibodies (Wittliff et al. 1990; Pakdel et al. 1994; Matthews et al. 2000; Su et al. 2000; Matthews et al. 2001; Hawkins and Thomas 2004).

Although in the present study the recombinant ER proteins were mainly found in the cell lysis pellet, suggesting they were insoluble or associated to bacterial membranes, their detection by Western blot (this chapter), and the results from ligand binding and *in vitro* phosphorylation (chapter 3) suggest they were (or could be rendered) soluble and functional. However, low levels of expression were consistently obtained for the sbERβa protein, either in *E. coli* or in different *in vitro* translation systems, and using both the ERβa coding region inserted in the proper context of regulatory elements required for *E. coli* expression or from the full-length cDNA regulated by its native 5'UTR. These results suggest that some factor or factors intrinsic to the ERβa coding sequence may be responsible for its low levels of expression. Further studies will be required to identify them. Nevertheless, enough ERβa protein was obtained for Western blot detection with anti-sbERβa antisera and, after concentration by ultrafiltration, for functional assays.

The Western blot analysis showed that while the anti-sbERα and anti-sbERβa antisera were specific for their respective ER recombinant protein, anti-sbERβb was less specific and cross-reacted with sbERα. Antibodies recognize only small epitopes of an antigen, usually formed by three to eight amino acids residues in peptidic antigens, which may be adjacent in the sequence or after folding, but sometimes they may recognize similar epitopes in related proteins (Owen and Steward 1998). In this study, sequence comparison identified a small region in the A/B domain of the sbERα protein showing some sequence similarity to that of the sbERβb peptide KPS used for rabbit immunization, formed by two conserved prolines and one leucine residue separated by a conserved number of three

other amino acids. Since prolines are often involved in the formation of specific three-dimensional structures in proteins, it is possible that these residues may be part of the anti-sbERβb recognized epitope and could be responsible for the cross-reactivity. Moreover, the higher specificity and apparently higher sensitivity of anti-ERα and anti-ERβa antisera may result from the fact that a mixture of two different polyclonal antibodies raised against the N- and C- terminal regions of each ER proteins were used, while only one polyclonal antibody was used for sbERβb. Future work may require raising additional antibodies against the sbERβb C-terminal region.

Using the anti-ER antisera, the expression of each sbER protein was localized by IHC in the scales of adult and juvenile sea bream. In juvenile sea bream, the three sbERs co-localized in cells of the scale posterior region that also expressed the mRNA for TRACP, a marker for osteoclasts in both mammals and fish (Persson et al. 1995), suggesting they were putative osteoclasts. These results are in agreement with previous studies showing that E_2 treatment induced TRACP (osteoclastic) activity and calcium mobilization from scales in several fish species (Mugiya and Watabe 1977; Persson et al. 1995; Armour et al. 1997; Suzuki et al. 2000; Suzuki and Hattori 2002), and this appears to be one of the mechanisms through which E_2 causes a rise in calcium plasma levels in fish. Other mechanisms appear to be the downregulation of proteins involved in mineralization (e.g. osteonectin, Lehane et al. 1999) or the increase in whole body calcium influx from the environment, demonstrated in sea bream and rainbow trout (Persson et al. 1994; Guerreiro et al. 2002). The recent demonstration of an E_2-induced increase in TRAPC activity in an *in vitro* bioassay with sea bream scales (Rotllant et al. 2005) and the localization of sbERs in putative osteoclasts in this study support that calcium mobilization from scales in response to E_2 may also occur in sea bream. The significance for the hypercalcemic effects of E_2 in fish have been attributed to an increase in calcium demand during vitellogenesis,

with calcium, vitellogenin and E_2 plasma levels rising in parallel during this period (Bromage et al. 1982; Norberg et al. 1989; Li et al. 1993; Yeo and Mugiya 1997).

The expression of the three sbER proteins have also been demonstrated in other regions of the scales from both juvenile and adult sea bream, although the signal localization and intensity varied with the age of animals and the type of receptor, suggesting that each ER subtype may have different roles during the fishes' lifetime. In addition to increasing TRACP osteoclastic activity and calcium mobilization in the scales, E_2 was recently shown to strongly increase the alkaline phosphatase (ALP) osteoblastic activity in regenerating scales, while a lower increase in ALP activity was observed for the ontogenic scales (Yoshikubo et al. 2005). This activity was higher in the early reproductive stage, which suggest that E_2 may affect both osteoblastic (mineralization) and osteoclastic (calcium mobilization) activities in fish, depending on the reproductive stage.

Whether the action of E_2 on scales is direct or indirect has not yet been established. The co-localization of the three ER proteins in putative osteoclasts demonstrated in this study suggests that the three ER subtypes may contribute to a direct calcium mobilization action of E_2 in scales, although the most abundant and probably most important receptors appear to be ERα and ERβb. The stronger immunoactivity obtained with anti-sbERβb in the putative osteoclasts when compared with anti-sbERα (Figure 5.8A and C) suggests that both ERs are expressed and may represent a cumulative signal of the detection of both receptors with anti-sbERβb. Moreover, the different signal intensity and localization patterns obtained for anti-sbERα or anti-sbERβb sera in adult and juvenile sea bream scales (Table 5.2) suggest that no significant cross-reactivity occurred between anti-sbERβb and the sbERα protein at the dilution used in IHC.

The fact that the three ER proteins were co-expressed in the same cell-type in juvenile sea bream opens the possibility of interplay between different ER subtypes in mediating E_2 effects in these cells. The formation of heterodimers between the two mammalian ER subtypes α and β has been demonstrated *in vitro* and *in vivo* (Ogawa et al. 1998), and these were shown to activate different sets of target genes than that of the α or β homodimers in human osteoblastic cell lines that co-express both ERs (Monroe et al. 2005). Moreover, the ERβ dominant negative effects observed on the ERα-mediated gene transcription *in vitro* or *in vivo* in human bone have been hypothesised to occur via the formation of α/β heterodimers (Hall and McDonnell 1999; Lindberg et al. 2003). However, ER heterodimerisation in fish has not been studied.

High variability was also found between the ER mRNA levels of expression in scales, suggesting that the physiological state of the individual may also determine the relative levels of expression between receptors and probably the final E_2 effect. Moreover, different results were obtained between the detection of ER mRNAs by RT-PCR and ER proteins by IHC in sea bream scales, which may reflect the use of animals in different physiological states or the fact that the mRNAs are usually short-lived molecules while the protein expression levels may be cumulative. This was especially evident for the sbERα subtype, which is consistent with the reported short half-life of the ERα mRNA molecules (Keaveney et al. 1993; Tan et al. 1996; Kenealy et al. 2000).

The expression of both sbERβ mRNAs was also demonstrated by RT-PCR in a number of other calcified tissues of sea bream, which suggests that they may also be targets for estrogen action through direct binding to these receptors. Previous studies in other fish species supported a protective role for estrogens in bone, like in mammals. E_2 was shown to decrease bone resorption and increase bone mineral content in rainbow trout (Armour et al. 1997; Persson et al. 1997), while in killifish and goldfish it suppressed calcium

deposition without stimulating bone resorption (Mugiya and Watabe 1977). However, bone resorption could be also induced by E_2 in cases of increased demand, such as starvation or when a large number of scales had been removed (Mugiya and Watabe 1977; Persson et al. 1997). Further studies are required to investigate the localization of sbERs in calcified tissues where ERβ mRNAs have been detected and it will be of interest to investigate if they are mobilized or spared in periods of increased calcium demand and in response to E_2 treatment.

Finally, the ER subtype-specific antibodies were used to localize ER proteins by Western blot in subcellular fractions. However, the signal obtained was weak for the three ER subtypes, and this preliminary assay must be repeated using a more sensitive system of detection. Nevertheless, the sbERα could be localized in heart extracts, mainly in the nuclear fraction, while lower levels of protein were detected in mitochondrial and cytosolic fractions. These results are consistent with localization studies in humans supporting that ERα is primarily nuclear, regardless of ligand interaction, but may also localize to the cytoplasm where it probably interacts with cytoplasmic signalling cascades (Shank and Paschal 2005; Solakidi et al. 2005). The localization of ER proteins in mitochondrial extracts had previously been reported for ERα and ERβ, where they appear to regulate transcription from mitochondrial DNA (Monje and Boland 2001; Chen et al. 2005; Solakidi et al. 2005). Moreover, an increasing number of studies report the localization of ER proteins to the inner face of the plasma membrane, where they exert non-genomic actions (Razandi et al. 2004; Levin 2005), and plasma membrane fractions of sea bream tissues should be included in future studies on the subcellular localization of sbERs. The detection of putative sbERβa dimers in some fractions requires further confirmation.

To our knowledge, this is the first study reporting the generation and characterization of specific antisera against each of the three fish ER subtypes. These powerful tools may allow to study and compare their cellular localization in a number of estrogen target tissues in fish, in order to clarify their involvement and relative roles in a range of biological estrogen functions. In this study, IHC using these antibodies demonstrated the co-localization of the three sbER proteins in putative osteoclasts in juvenile sea bream scales, suggesting that the calcium mobilizing action of E_2 on scales is via a direct action on these cells, while the detection of the mRNAs for sbERβa and sbERβb in a number of calcified tissues suggests they may also be estrogen targets. The subcellular distribution of the sbERα protein was also demonstrated in heart using the specific sbERα antisera.

5.5. Acknowledgements of practical work

M. D. Estêvão provided the results reported in this chapter for sea bream and tilapia scale histology, immunohistochemistry and *in situ* hybridization. P. Pinto, the author of this thesis was responsible for the remainder of the work carried out in this chapter.

CHAPTER 6

Effects of E_2 and the antiestrogen ICI 182,780 on the hepatic and testicular expression of estrogen receptor subtypes and estrogen-responsive genes in sea bream (*Sparus auratus*)

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6.1. Introduction

Estrogens actions are mediated by specific nuclear estrogen receptors, which classically regulate the transcription of estrogen-responsive genes (ERGs) by binding as dimers to specific estrogen-responsive elements (ERE) found in their regulatory regions (Nilsson et al. 2001). Two estrogen receptor subtypes (ERα and ERβ) have been identified in most vertebrates, while two ERβ forms (ERβa and ERβb) encoded by different genes have been identified in some teleost fish species, in addition to ERα (chapter 3).

It is known from mammals that each ER subtype may have different functional properties and distinct (but also overlapping) roles *in vivo* (Matthews and Gustafsson 2003), and one of the molecular determinants of the estrogen action in a given cell is the relative expression level of each ER subtype (McDonnell 2004). The factors regulating the expression of the ER genes are better known for the human ERα gene, which contains a complex promoter whose expression can be affected by progesterone agonists/antagonists, androgens, vitamin D or vitamin D agonists, gonadotropins or gonadotropin releasing hormones, some growth factors and estradiol (E_2) itself, as well as estrogen antagonists and antagonists (reviewed by Pinzone et al. 2004).

The ERα gene self-regulation by estrogens has been reported in several mammalian and non-mammalian species and has been considered as a mechanism to enhance estrogen responsiveness in target tissues (e.g. Varriale and Tata 1990; Ninomiya et al. 1992; MacKay et al. 1996; Zou and Ing 1998; Pinzone et al. 2004). However, ER down regulation by estrogens may also occur and differential regulation has been found between different tissues, cell-types, ER subtypes and among different species (e.g. Zou and Ing 1998; Nephew et al. 2000; Ihionkhan et al. 2002; Sabo-Attwood et al. 2004). Thus, understanding the regulation of expression of each ER gene is an important step to understand the estrogen responses within a given tissue.

In fish, differential estrogen regulation has been demonstrated between the three ER subtypes in largemouth bass and zebrafish liver, with E_2 causing ERα up regulation, while the ERβ genes are either slightly up or down regulated depending on the species and subtype (Menuet et al. 2004; Sabo-Attwood et al. 2004). However, the information on estrogen regulation in non-hepatic tissues in fish is scarce.

In addition to the classical mechanism of estrogen and ER action, there is now increasing evidence for alternative mechanisms. These include the indirect transcriptional activation by ligand-bound ER through interaction with other transcription factors at their response elements (e.g. AP-1 sites, Kushner et al. 2000), the ligand independent activation of ERs in response to intracellular signalling cascades (Driggers and Segars 2002) or non-genomic actions mediated by membrane-localized receptors (Levin 2005). Moreover, some estrogen effects may derive from the indirect action on other tissues, such as the liver or the components of the brain-pituitary-gonads axis, or on the expression of other transcription factors, hormones or hormone receptors (Bentley 1998; O'Lone et al. 2004).

ERs are also known to accept a wide range of ligands, including natural estrogens, synthetic estrogens or antiestrogens, phytoestrogens and a wide variety of xenoestrogens (Pike et al. 1999), and while many behave as estrogen agonists, other compounds may act either as agonists or antagonists depending on the species, tissue, promoter or ER subtype (the selective ER modulators, SERMs) (Frasor et al. 2004). The tissue-selective effects of SERMs have been exploited in mammals to develop new drugs for the treatment of several estrogen-related diseases, although some have shown to have unwanted side effects in part due to its agonist effects in some tissues (Henke and Heyer 2005).

ICI 182,780 (trade names Faslodex, Fluvestrant) belongs to a new class of antiestrogens developed to have no agonistic effects, and besides its therapeutic potential demonstrated in several clinical trials, it has been used as an alternative and efficient model to “knock-

out" ER effects in studying estrogen functions and in investigating the contribution of nuclear ERs in particular estrogen actions (Lee et al. 2000; Singh et al. 2000; Morris and Wakeling 2002; Osborne et al. 2004; Matthews et al. 2005). In mammals, ICI 182,780 appears to act at several levels to block estrogen actions (reviewed by Osborne et al. 2004; Carlson 2005), but little is known about their effects and mechanisms of action in fish.

The first objective of this study was to analyze the *in vivo* estrogen regulation of expression of the three sea bream ER mRNAs (sbERα, sbERβa and sbERβb) in male liver and testis. The liver is one of the most important estrogen target tissues in oviparous animals, where the egg yolk (vitellogenins) and eggshell precursors (choriogenins) are produced in response to E_2 in mature females or in males exposed to estrogenic compounds (Arukwe and Goksøyr 2003). The testis is also an important target organ, where estrogens appear to control normal testis physiology, sex differentiation, sex reversal and endocrine disruptive effects and where the three fish ER subtypes are expressed (see chapter 7). Understanding the regulation of each ER gene in these tissues may contribute to understand their relative roles.

The second objective was to investigate the effects of the antiestrogen ICI 182,780 (ICI) on different types of *in vivo* estrogenic responses in two teleost fish species, the tilapia (*Oreochromis mossambicus*) and the sea bream (*Sparus auratus*), as a step towards understanding the mechanisms of action of ICI and estrogens in fish. A preliminary experiment was carried out in tilapia, a gonochoristic species readily available, easy to breed and maintain, which allowed to analyze the time-course effects of E_2 and ICI on the calcium plasma levels, an indicator of vitellogenesis (Bromage et al. 1982), and to establish the hormone doses causing higher estrogenic response. The short-term effects of E_2 and ICI on the hepatic and testicular gene expression of the three ER subtypes and the

estrogen-responsive genes vitellogenin II and choriogenin L were analysed in sea bream, for which these molecular markers were available (chapter 3 and chapter 7).

6.2. Specific methods

6.2.1. Fish treatments

Adult tilapias were maintained at the University of Algarve (Faro, Portugal) in 150L closed circuit freshwater aquaria with water temperature of 24ºC and 12:12 LD photoperiod. All male sea bream used in the *in vivo* experiments were obtained and maintained as described in section 2.2. Three independent experiments were performed, in which different male tilapia or sea bream were treated with different hormonal treatments and sampled as described in section 2.3.

In June/July, five groups of five mature male tilapia (body weight 45.9 ± 3.6 g) received i.p. implants of coconut oil containing 35 mg/kg body weigtht (bw) ICI (I_{35} and I_{35}E3d groups), 10 mg/kg bw ICI (I_{10}E3d group), or coconut oil alone (C, control group, and E group-see below). Three days after the start of treatment, fish were anesthetised with 2-phenoxyethanol, the blood was collected, centrifuged and the plasma retained (section 2.3). Each individual fish from the E, I_{35}E3d and I_{10}E3d groups were then injected with coconut oil containing 10 mg/kg bw E_2, the control group was injected with coconut oil alone, and the I_{35} group was not injected. Blood was collected 5, 11 and 20 days after the first injection and calcium levels were determined in the resulting plasma samples (section 6.2.2).

In September (sea bream experiment 1), three groups of eight mature male sea bream (body weight 367.1 ± 8.9 g) received i.p. implants of coconut oil containing 10 mg/kg bw E_2 (E group), 10 mg/kg bw ICI (I group) or coconut oil alone (C, control group). Twenty eight hours later, the fish were killed and transverse sections of testis and liver were

collected and stored at -80ºC for subsequent RNA extraction. Analysis of gene expression by RT-PCR (sections 6.2.4 and 7.2.5) and subtractive library construction (section 7.2.2) were conducted using the extracted RNA.

In November (sea bream experiment 2), six groups of eight young male sea bream (body weight 239.2 ± 3.3g) were injected with the vehicle (coconut oil) at the first day of experiment, except for group IE3d which was injected with 4 mg/kg bw ICI in coconut oil. After three days, fish were injected with coconut oil implants containing 1 mg/kg bw E_2 (E_1 group), 0.1 mg/kg bw E_2 ($E_{0.1}$ group), 4 mg/kg bw ICI (I group), 4 mg/kg bw ICI plus 1 mg/kg bw E_2 (IE group) or 1 mg/kg bw E_2 (administrated to the group injected with ICI in the first day, IE3d). The control group (C) was injected with coconut oil only. Forty eight hours later, blood was collected and plasma samples stored at -20ºC. Fish were killed and transverse sections of the liver and testis sampled and stored at -80ºC for RNA extraction and analysis of gene expression by RT-PCR (sections 6.2.4 and 7.2.5). Transverse sections of the testis were collected in Bouin-Holland fixative for histological analysis (section 7.2.7).

6.2.2. Quantification of total calcium in plasma

Total plasma calcium (bound plus free) was measured in duplicate 10 µl plasma samples from individual tilapia and sea bream in experiment 2, using a colorimetric assay (Calcium kit, procedure no. 587, Sigma) and the calcium standards provided with the kit. For the time-course experiment carried out in tilapia, differences between treatments and sampling times were evaluated by two-way repeated-measures ANOVA (SigmaStat v.3.00) using $\log_{10}$ of calcium plasma levels, followed by pairwise multiple comparison by the post-hoc Tukey test. Differences between treatments in sea bream experiment 2 were evaluated by one-way ANOVA with $\log_{10}$ of calcium plasma levels followed by the Tukey test. Statistical significance was established at $P<0.05$.

6.2.3. Estradiol radioimmunoassay

E_2 was quantified in individual plasma samples collected from sea bream in experiment 2 (section 6.2.1) using specific antibodies (Research Diagnostics, USA) and the general radioimmunoassay method described by Scott et al. (1982). Briefly, plasma samples (50 µl) were diluted 1:20 in 0.05 M phosphate buffer with 1g/l gelatine (Appendix I), denatured for 1h at 80ºC, and 100 µl were then mixed with 100µl of an appropriate dilution of the anti-E_2 antibodies (optimized to achieve 50% of total binding) and 1500 cpm of [^{3}H]-E_2 (specific activity 5698 Gbeq/mmol, Amersham). The calibration curve was prepared by mixing known amounts of E_2 (0.5-500 pg) with the antibodies and [^{3}H]-E_2 in the same conditions. All reactions were in duplicate and incubated ON at 4ºC. Bound E_2 was determined after separation with 1.25 volumes of dextran-coated charcoal (Appendix I) and counting the supernatant. Cross-reactivity of the antibody with ICI was evaluated by quantifying several samples with known concentration in ICI, and was approx. 20%. Differences between E_2 levels for each treatment against the control group were analysed by one-way ANOVA with $\log_{10}$ of E_2 plasma levels (SigmaStat 3.00), followed by the post-hoc Tukey test (statistical significance at $P<0.05$).

6.2.4. Semi-quantitative RT-PCR

The mRNA expression of sea bream ERs, vitellogenin II (VgII) and choriogenin L (ChgL) was analyzed by RT-PCR on liver or testis cDNA samples from male sea bream treated with different combinations of E_2 and ICI (experiments 1 and 2, section 6.2.1). Specific primers for each sbER were the same as those used to analyse tissue distribution (section 3.2.9), while specific primers for VgII and ChgL were the same as those used to analyse their E_2 induction (section 7.2.5). Standard semi-quantitative RT-PCR reactions (section 2.12) were performed with 1 µl of liver and testis cDNA prepared for each fish, using 18S

as internal control. Optimized cycle numbers for the detection in the exponential phase of amplification are indicated for each gene in Table 6.1, while annealing temperatures are described in Appendix V. The relative expression values for each sample were calculated and differences between treatments and correlations between genes were evaluated as described on section 2.12.

Table 6.1. Cycle numbers used for the RT-PCR analysis of estrogen receptors and the estrogen-responsive genes vitellogenin II and choriogenin L in liver or testis cDNAs from male sea bream treated with different combinations of E_2 and the antiestrogen ICI 182,780 (sea bream experiments 1 and 2).

	Experiment 1		**Experiment 2**
Gene product:	**Liver**	**Testis**	**Liver**
Estrogen receptor α (ERα)	25	25	25
Estrogen receptor βa (ERβa)	27	23	29
Estrogen receptor βb (ERβb)	25	22	26
Vitellogenin II (VgII)	25	30	18
Choriogenin L (Chg L)	20	25	18
18S ribosomal RNA (18S)	18	18	15

6.3. Results

6.3.1. Effects of estradiol and ICI 182,780 on the time-course changes on plasma calcium levels in tilapia

In tilapia, although there was an increase in calcium levels with E_2 treatment (Figure 6.1, days 5 and 11), they were not statistically different from the initial calcium levels of the E_2-treated group nor from those of the control group at each sampling point. A significant decrease in the plasma calcium levels was observed from day 11 until the end of the experiment (day 20) for the E_2-treated group, as well as for all the other experimental groups. Apart from this, no temporal changes were observed for the control group or the group treated with 35 mg/kg of ICI alone. In contrast, when fish were treated with 10 mg/kg E_2 three days after the pre-treatment with either 35 or 10 mg/kg of ICI, significant increases in calcium plasma levels were induced. The levels obtained after two days of E_2 treatment (Figure 6.1, day 5) were approx. 2-fold higher than the initial levels in those groups and significantly higher than the levels obtained with the treatment of each hormone alone, indicating a synergistic effect between E_2 and ICI. While in the group treated with the higher dose of ICI (I_{35}E3d) calcium levels further increased approx. 1.5 fold for the next six days, they did not change significantly in the lower ICI dose group (I_{10}E3d). At day 11 (Figure 6.1) I_{10}E3d differed significantly from the I_{35}E3d group but not from the E_2-treated group. Nevertheless, since the I_{10}E3d treatment was able to achieve a response of the same magnitude to that of the higher ICI dose after two days of E_2 exposure, the doses used in this treatment (10 mg/kg ICI and 10 mg/kg E_2) were chosen to investigate the short-term effects of these hormones on gene expression in sea bream (next section).

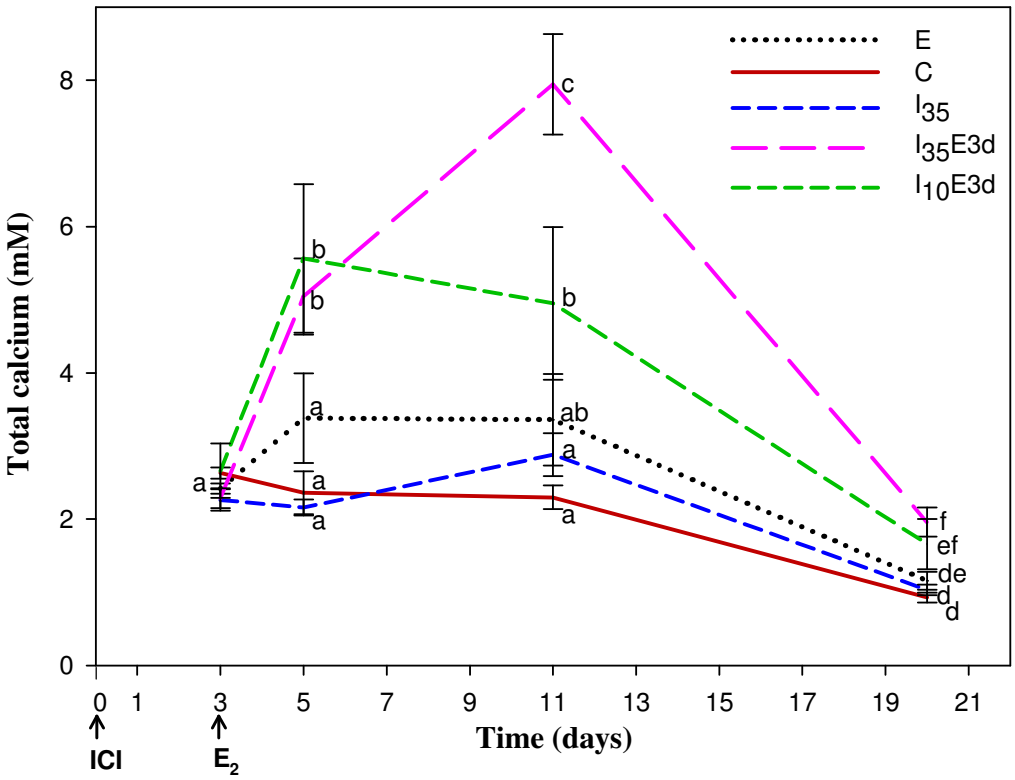

Figure 6.1. Time-course of the calcium plasma levels of adult male tilapia injected with different combinations of estradiol (E_2) and of the antiestrogen ICI 182,780 (ICI). The times of injection of these compounds in the various experimental groups are represented by arrows on the lower panel. C= control group, coconut oil only; E= 10 mg/kg body weight E_2; I_{35}= 35 mg/kg ICI; I_{35}E3d and I_{10}E3d= 10 mg/kg E_2 injected three days after injection with 35 or 10 mg/kg ICI, respectively. Plasma samples were collected at days 3, 5, 11 and 20 after the first injection, calcium levels (mM) were determined and represented as mean ± S.E.M. of 4-5 fish for each group. Different letters indicate statistically significant differences ($P<0.05$) among treatments and sampling times, evaluated by two-way repeated-measures ANOVA using $\log_{10}$ of calcium plasma levels. No significant differences between groups were found at the initial sampling point (day 3), as indicated by a single "a".

6.3.2. Short-term effects (28 h) of estradiol or ICI 182,780 on the hepatic and testicular gene expression of ER subtypes and ERGs in sea bream

As expected, E_2 treatment caused a significant up regulation in ERα (approx. 4-fold), VgII (6-fold) and ChgL (11-fold) mRNAs in liver (Figure 6.2). In contrast the mRNA levels of both ERβ subtypes significantly decreased approx. 10-fold compared to the control group. Interestingly, treatment with ICI also caused a significant up regulation in ERα, VgII and ChgL, with the same magnitude to that of E_2 in the case of ERα but significantly less in

the case of VgII and ChgL (1.8- and 2.5-fold increase relatively to the control, respectively). No effect was observed in the expression of sbERβa or βb. Strong positive Pearson correlations were found between the ERα, VgII and ChL responses to E_2 and ICI in this tissue (Pearson coefficient 0.658-0.939, P<0.01), but not between ERβa and ERβb.

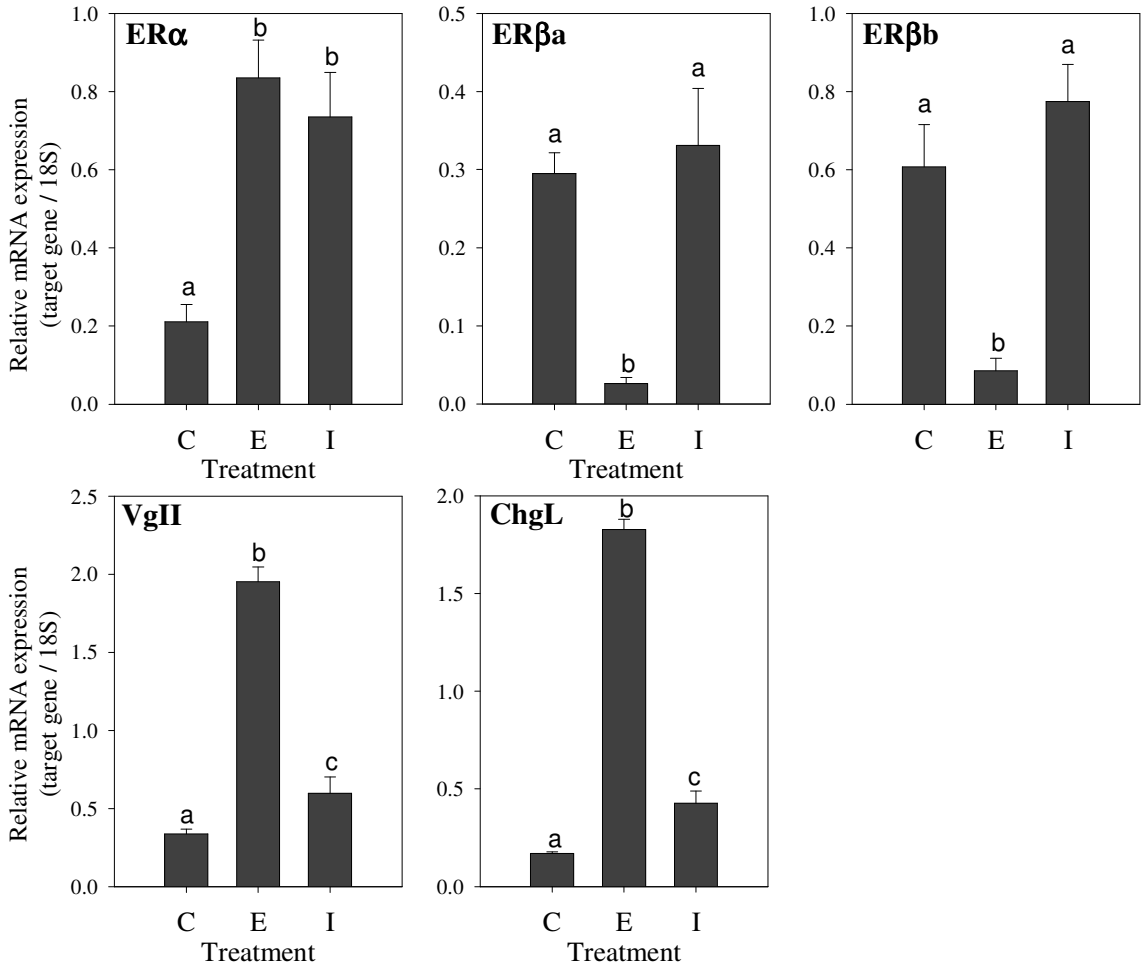

Figure 6.2. Semi-quantitative RT-PCR of estrogen receptors (ERα, ERβa, ERβb) and estrogen-responsive genes, vitellogenin (VgII) and choriogenin (ChgL), in male sea bream liver following administration of 10 mg/kg body weight E_2 (E), 10 mg/kg ICI (I) or coconut oil alone (C) for 28 h. Each bar is the mean ± S.E.M. of the relative expression values (target gene/18S) of eight fish. Different letters indicate statistically significant differences between treatments (general linear model using $\log_{10}$ of the relative expression values, P<0.05).

In the testis, no statistically significant changes in gene expression were obtained for any of the ER subtypes (Figure 6.3). Both VgII and ChgL were up regulated by E_2 (same results presented in chapter 7) but remained unaltered by ICI treatment. In contrast to that observed in the liver, ERα was positively correlated with ERβa (Pearson coefficient 0.640, P<0.05) but not with VgII and Chg, highly correlated between them (0.853, P=0).

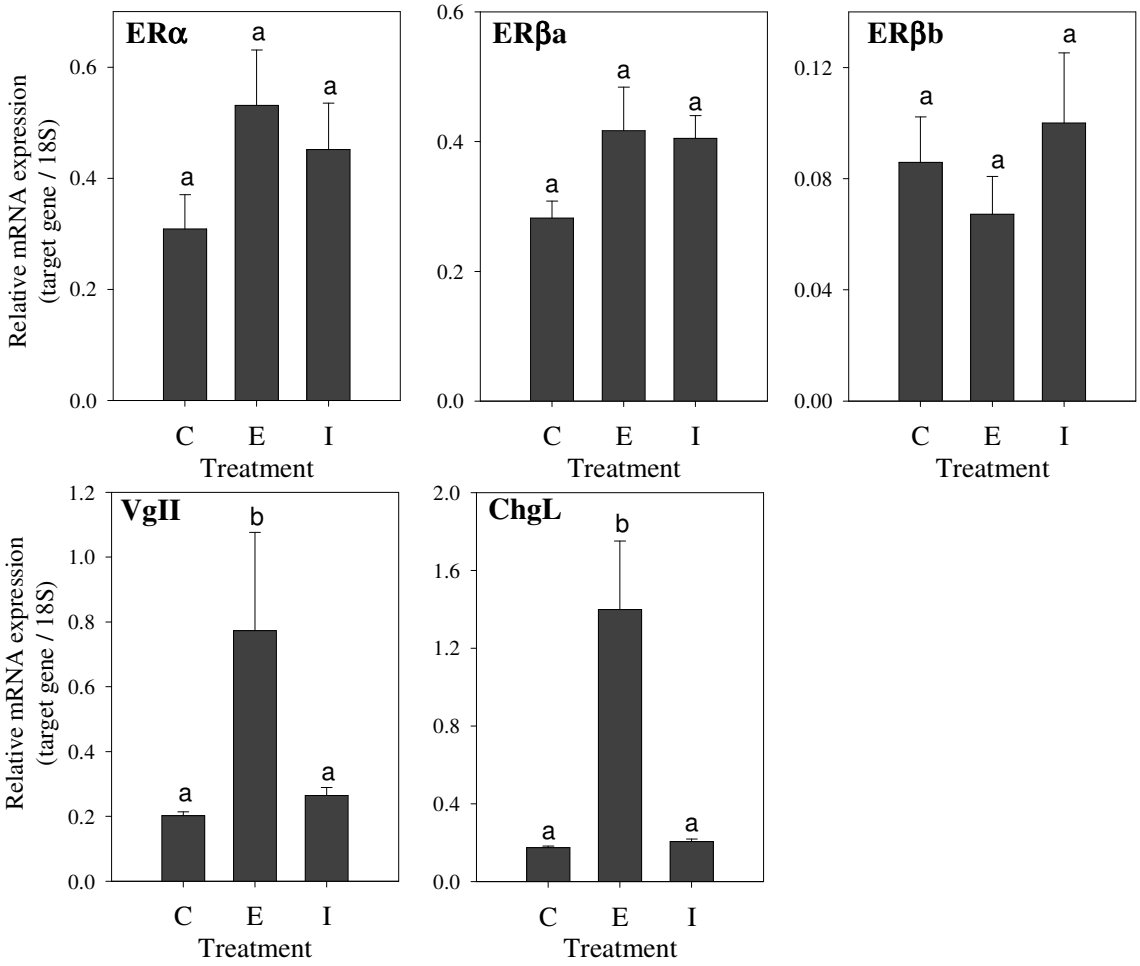

Figure 6.3. Semi-quantitative RT-PCR of estrogen receptors (ERα, ERβa, ERβb) and estrogen-responsive genes, vitellogenin (VgII) and choriogenin (ChgL), in sea bream testis following administration of 10 mg/kg body weight E_2 (E), 10 mg/kg ICI (I) or coconut oil alone (C) for 28 h. Each bar is the mean ± S.E.M. of the relative expression values (target gene/18S) of eight fish. Different letters indicate statistically significant differences (P<0.05) between treatments, evaluated by a general linear model using $\log_{10}$ of the relative expression values.

6.3.3. Effects of combined estradiol and ICI 182,780 treatments (48 h) on plasma calcium levels and hepatic gene expression of ER subtypes and ERGs in sea bream

In order to further investigate and confirm the obtained effects of E_2 and ICI on the different types of estrogenic response, a second experiment was performed with male sea bream in which lower doses of hormones were used (1 or 0.1 mg/kg E_2, 4 mg/kg ICI) in different combinations, including the simultaneous administration or the pre-treatment with ICI before E_2 (section 6.2.1). The time of sampling was 48 h after the E_2 injection, in common with the tilapia experiment, after which plasma calcium levels and mRNA expression of ERs and ERGs in liver were analysed. Expression in testis was not analysed due to the lack of response detected for ERs in experiment 1 and high variability among individuals in the VgII and ChL response.

To confirm the effectiveness of the treatments, E_2 plasma levels were determined for each fish at the end of the experiment (Figure 6.4). Control sea bream had circulating plasma E_2 levels of 0.6 ± 0.2 ng/ml, while in E_2-implant groups these had increased 26-fold to 15.5 ± 4.0 ng/ml (E_1 group) or 7-fold to 4.0 ± 0.6 ng/ml ($E_{0.1}$ group). The E_2 plasma levels in the ICI-implanted groups (see Figure 6.4) were overestimated due to cross-reaction of the Anti-E_2 antibody with ICI (20 %).

No significant changes were detected in the total calcium plasma levels after two days of treatment of male sea bream with none of the E_2 doses, 1 or 0.1 mg/kg (Figure 6.5). However, like in tilapia, the pre-treatment with ICI followed by E_2 three days later (IE3d group) was able to induce a significant increase in the calcium levels, while a slight increase was detected in the group treated simultaneously with both hormones.

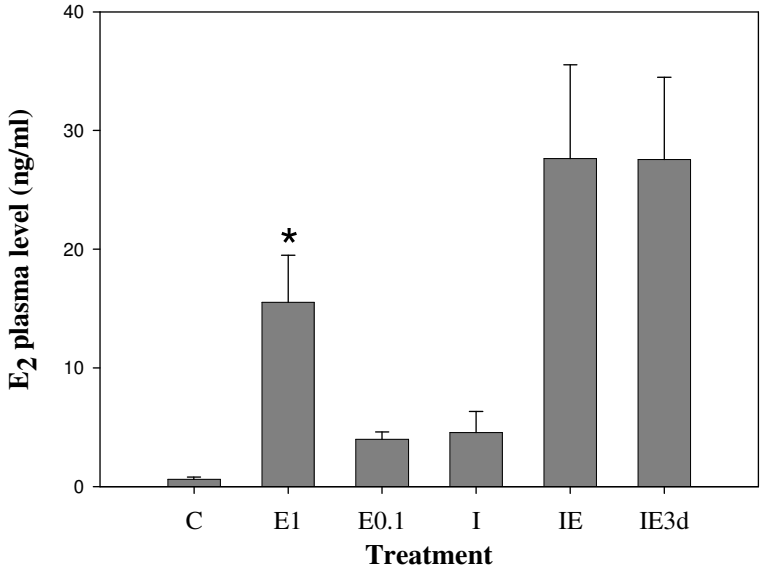

Figure 6.4. Circulating plasma levels of E_2 in male sea bream two days after implantation with coconut oil implants containing 1 mg/kg body weight E_2 (E_1), 0.1 mg/kg E_2 ($E_{0.1}$), 4 mg/kg ICI (I), 4 mg/kg ICI plus 1 mg/kg E_2 (IE), 1 mg/kg E_2 in fish implanted 3 days before with 4 mg/kg ICI (IE3d) or coconut oil alone (C). Each bar is the mean ± S.E.M of E_2 plasma levels (ng/ml) of eight fish. I, IE and IE3d groups include the detection of both E plus irICI, immunoreactive ICI, due to cross-reaction of the anti-E_2 antibody with ICI (20 %). * indicates significant differences from the control ($P<0.05$, evaluated only for the E_1 and $E_{0.1}$ groups, by one-way ANOVA with $\log_{10}$ of E_2 levels).

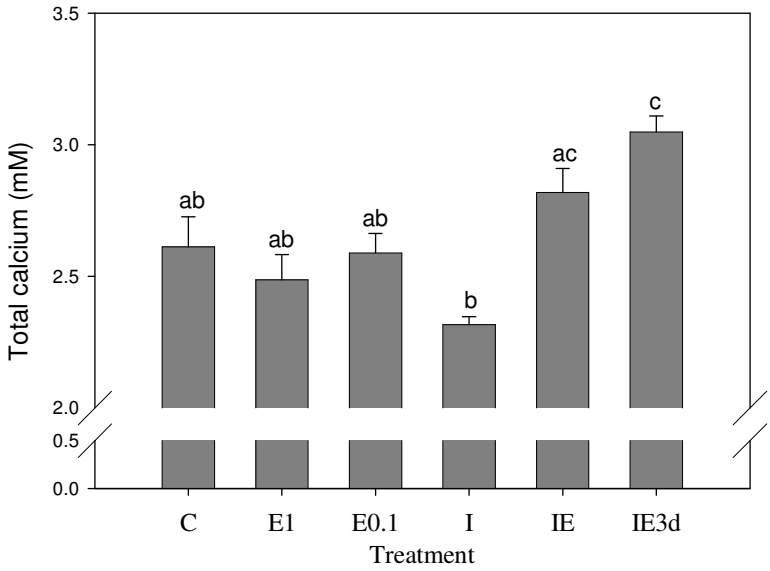

Figure 6.5. Total calcium plasma levels in male sea bream two days after implantation with coconut oil implants containing 1 mg/kg body weight E_2 (E_1), 0.1 mg/kg E_2 ($E_{0.1}$), 4 mg/kg ICI (I), 4 mg/kg ICI plus 1 mg/kg E_2 (IE), 1 mg/kg E_2 in fish implanted 3 days before with 4 mg/kg ICI (IE3d) or coconut oil alone (C). Each bar is the mean ± S.E.M of the calcium plasma levels (mM) of eight fish. Different letters indicate statistically significant differences ($P<0.05$) between treatments, evaluated by one-way ANOVA using $\log_{10}$ of calcium plasma levels.

As in the first experiment, ERα was significantly up regulated by both doses of E_2 and by ICI alone (Figure 6.6). Simultaneous administration of ICI and E_2 (IE group) also caused a significant increase in the ERα expression levels, although not significantly different to that obtained with the same dose of E_2 alone (E_1 group). In contrast, in the group pre-treated with ICI (IE3d group), the ERα levels 48h after E_2 treatment were not significantly different from those of the control but differed from those treated with E_2 alone (E_1), suggesting an inhibition of the E_2-induced ERα up regulation.

For ERβa, no significant differences were detected between the different treatments, although a slight decrease comparing to the control levels is apparent for the lower dose of E_2 ($E_{0.1}$), ICI alone (I) and pre-treatment with ICI followed by E_2 (IE3d) (Figure 6.6). Similarly, ERβb showed a trend for down regulation with both doses of E_2 or with ICI, while the two combined E_2/ICI treatments were able to significantly down regulate ERβb, thus supporting a synergistic effect of ICI and E_2. The expression of both VgII and Chg was significantly up regulated by both doses of E_2 and combined E_2/ICI treatments but no expression could be detected in response to ICI alone. It is possible that some up regulation also occurred with ICI alone, as in the first experiment, but could not be detected at the low cycle numbers (N=18) that had to be used in the RT-PCR to detect in the exponential amplification phase the high levels of VgII/ChgL induced in the E_2-treated groups. Strong positive Pearson correlations were found between the ERα, VgII and ChL responses (Pearson coefficients 0.466-0.981, $P<0.01$) and between ERβa and ERβb (Pearson coefficient 0.679, $P=0.00$), while negative correlations were found between ERβb and both VgII and ChgL (coefficients -0.435 and -0.398, respectively, $P<0.05$).

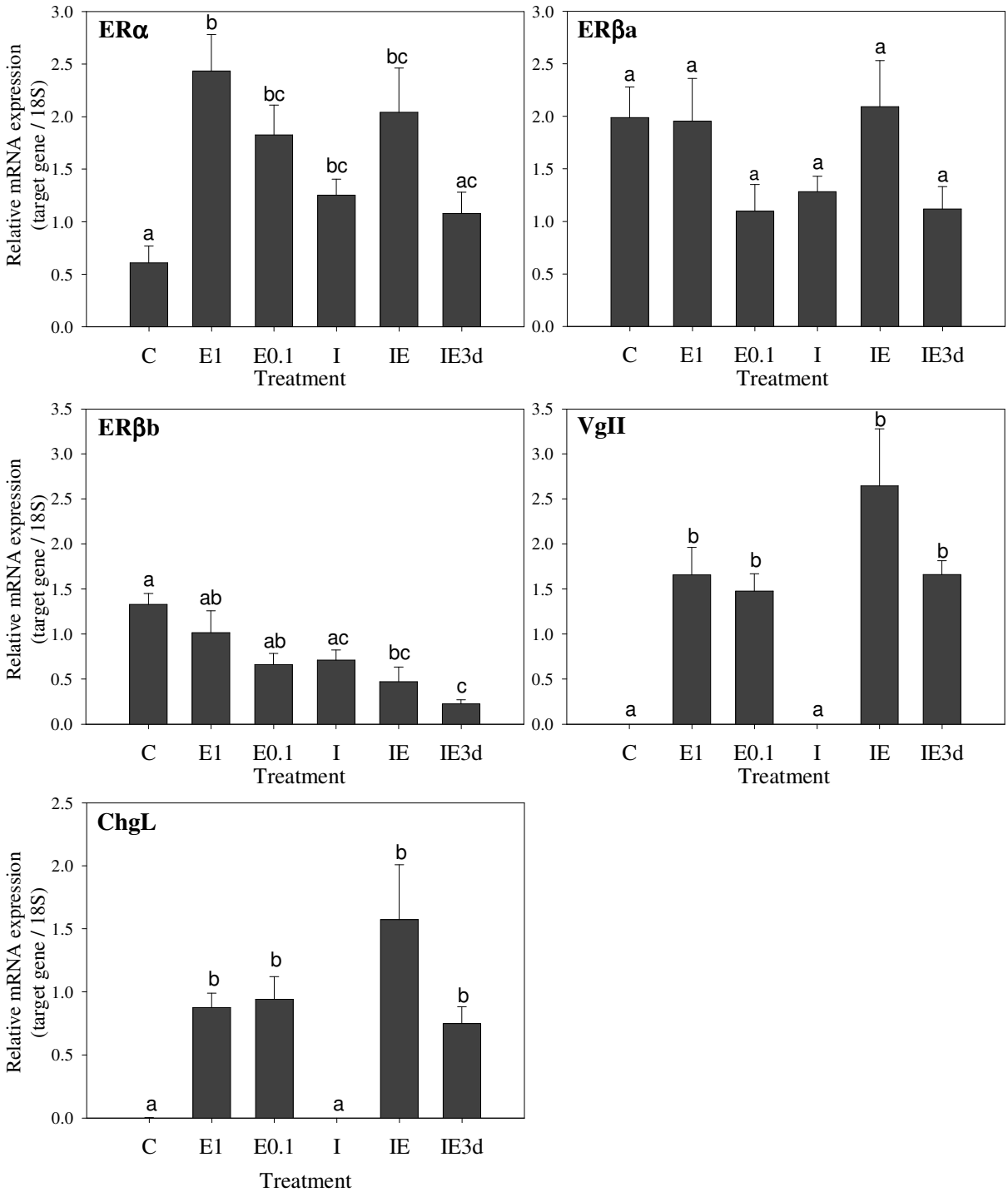

Figure 6.6. Semi-quantitative RT-PCR of estrogen receptors (ERα, ERβa, ERβb) and estrogen-responsive genes, vitellogenin (VgII) and choriogenin (ChgL), in male sea bream liver (experiment 2) 48 h following administration of 1 mg/kg body weight E_2 (E_1), 0.1 mg/kg E_2 ($E_{0.1}$), 4 mg/kg ICI (I), 4 mg/kg ICI plus 1 mg/kg E_2 (IE), 1 mg/kg E_2 in fish implanted 3 days before with 4 mg/kg ICI (IE3d) or coconut oil alone (C). Each bar is the mean ± S.E.M. of the relative expression values (target gene/18S) of eight fish. Different letters indicate statistically significant differences between treatments (general linear model using $\log_{10}$ of the relative expression values, $P<0.05$).

6.4. Discussion

In this study, the estrogen regulation of the three sea bream ER mRNAs was analysed by RT-PCR in male liver and testis. As expected, a strong up regulation was detected for sbERα in the liver. The autoregulation of the ERα gene in liver is a common characteristic of oviparous animals (e.g. Hayward et al. 1982; Evans et al. 1987; MacKay et al. 1996; Custodia-Lora et al. 2004) that has been attributed to its involvement in the production of proteins required for reproduction, such as vitellogenins and choriogenins (Arukwe and Goksøyr 2003). This role has been supported in many fish species by the strong correlation found between the increases in hepatic ERα, Vg and Chg mRNA levels, hepatic E_2-binding activity and plasma Vg and Chg protein levels during the vitellogenic stage in females, coincident with the elevation of E_2 in plasma (e.g. Sabo-Attwood et al. 2004; Fujita et al. 2005), or to their dose-dependent increase in embryos, immature fish or adult males treated with exogenous E_2 (e.g. Pakdel et al. 1991; MacKay et al. 1996; Salbert et al. 1998; Celius et al. 2000; Bowman et al. 2002; Brown et al. 2004; Andreassen et al. 2005). The sbERα liver up regulation detected in this study, which correlated with that of VgII and ChgL (Figures 6.2 and 6.6), support that this also occurs in sea bream. The fact that the sbERα mRNA up regulation was detected in the liver of both mature male (sea bream experiments 1 and 2) and immature sea bream (Socorro 2001), and that the sbERα protein levels increased in the liver of adult females in response to E_2 (Mosconi et al. 2002), confirms that, like in other fish species, this response is inducible in both sexes and throughout the fishes' lifetime.

The ERα up regulation and resultant increase in liver cellular responsiveness to E_2 appears to occur first and to be required for the full induction of vitellogenin synthesis in oviparous animals, since the ERα mRNA and ERα protein have been shown to accumulate faster and in response to lower E_2 doses than that of different Vg mRNAs, *in vivo* and *in*

vitro (Barton and Shapiro 1988; Flouriot et al. 1997; Bowman et al. 2002; Yamaguchi et al. 2005). Furthermore, the E_2-induced Vg gene expression was found to be directly proportional to the amount of synthesised ERα protein in hepatocytes of male trout or *Xenopus*, as expected for an estrogen-responsive gene (Corthesy et al. 1990; Pakdel et al. 1991; Flouriot et al. 1997). Confirmation that these relationships also exist in sea bream will require the characterization and comparison of the E_2 dose-response and early time-course patterns of the hepatic induction of sbERα, VgII and ChgL, detected in this study for all doses and induction times tested (0.1-10 mg/kg E_2, 28 or 48h), as well as for the VgI and ChgH forms (chapter 7).

In contrast to ERα, the estrogen regulation of the fish ERβ genes has been poorly investigated and appears to be much more variable. While in zebrafish liver the expression of ERβb was markedly reduced by E_2 and ERβa remained unchanged (Menuet et al. 2004), significant up regulation was detected for the ERβa mRNA in the liver of juvenile goldfish, in parallel with the increase in plasma Vg levels, which was considered as an evidence for a role of ERβa in mediating the E_2 effects on vitellogenesis (Soverchia et al. 2005). Similarly, a slight up regulation of the largemouth bass ERβa mRNA was detected in female livers, concomitant with the rise of ERα and Vg mRNAs in liver and E_2 plasma levels that occurred in spring, and in male livers in response to E_2, although the magnitude of the induction was much lower than that of ERα (Sabo-Attwood et al. 2004). In this study, both sbERβ subtypes were strongly down regulated in liver in the first sea bream experiment, but only slightly down regulated in the second sea bream experiment in which lower doses and younger fish were used (Figures 6.2 and 6.6, respectively). These results probably resulted from their regulation being dose-dependent (and less sensitive to E_2 than that of the sbERα gene) and influenced by the life-stage of the fish. This is supported by

the fact that no changes in expression were detected for sbERβa in the liver of immature sea bream (Socorro 2001).

These results indicate that fish ERα and ERβ genes have a differential estrogen regulation in liver and support the hypothesis that the role of ERβ, if any, in the transcriptional regulation of genes associated with reproduction, such as Vgs, in liver, is probably less important than that of the ERα subtype and may depend on the life stage of the fish and / or the species.

The differential regulation between ER subtypes may result from the presence of different regulatory elements in their promoters, since only ERE half-sites have been identified in the promoter of the zebrafish ERβa gene, considered as probable explanation for its lack of response to E_2 (Lassiter et al. 2002), while one imperfect ERE plus one ERE half-site were found to act synergistically to autoregulate the transcription of the rainbow trout ERα gene (Le Drean et al. 1995; Petit et al. 1999). This discussion awaits the characterization of the ERβb promoter, and of ERα, βa or βb promoters in other fish species.

Different from the liver, a slight up regulation of both sbERα and sbERβb but not ERβb was detected in the testis (Figure 6.3), which suggests that the regulation of ER subtypes varies among tissues. In agreement with this, a reduction in ERα mRNA expression had been previously detected in sea bream ovarian tissue in response to E_2 (Socorro 2001), and tissue-specific ER regulation has also been described for other fish, avian and mammalian species (Ninomiya et al. 1992; Salbert et al. 1993; Zou and Ing 1998; Sabo-Attwood et al. 2004). The up regulation of VgII and ChgL in testis confirms its identification as ERGs in this tissue, as discussed in chapter 7.

Interestingly, while ICI alone did not change the total calcium plasma levels, the pre-treatment with ICI potentiated the hypercalcemic effect of E_2 in both sea bream and tilapia. Total calcium correlates with Vg protein and E_2 plasma levels, in females during

vitellogenesis and in males in response to E_2, and is thus used as a vitellogenesis marker (Bromage et al. 1982; Persson et al. 1994; Guerreiro et al. 2002). The fact that no significant increase in calcium was detected in sea bream with E_2 treatment alone (Figure 6.5) may be due to the low doses and/or exposure time used (48h, 0.1-1 mg/kg E_2) when compared to those used in tilapia (>48h, 10 mg/kg E_2) or previous experiments with sea bream (>4 days, 10 mg/kg E_2, Guerreiro 2002).

ICI mimicked the E_2 effects in up regulating ERα, VgII and ChgL in sea bream liver, but not in the testis nor in down regulating the two ERβ subtypes in liver, supporting tissue- and gene-specific effects as indicated above for E_2. The simultaneous administration of ICI with E_2 did not block the E_2 effects on the expression of any of the genes, neither did the ICI pre-treatment in the E_2-induced up regulation of ChgL and VgII, suggesting that ICI did not act as an antagonist. However, ICI pre-treatment potentiated the E_2-down regulation of the sbERβb gene, while it appeared to have an inhibitory effect on the E_2-up regulation of sbERα (Figure 6.6), at least in the time-frame and doses analysed. Since ICI, E_2 and ICI plus E_2 all caused an increase in ERα mRNA levels in liver, this effect probably reflects a mechanism of negative control decreasing ERα levels after the initial increase by ICI, but this pattern needs to be confirmed by analysing the earlier time-course changes in this response.

These results contrasts with ICI being considered a pure antagonist, reported to block the effects of E_2 and some partial agonists (e.g. tamoxifen) with no detected agonistic activities in several *in vivo* and *in vitro* models of estrogen action in different mammalian species (reviewed by Howell et al. 2000; Osborne et al. 2004). However, some studies have also reported some agonistic or partial agonistic activities for ICI in some species (Jones et al. 1999; Robertson et al. 2001; Wu et al. 2004; Wu et al. 2005). It appears that ICI actions may depend on the species, the tissue, the ER subtype and the promoter, like

other SERMs. However, the mechanisms responsible for the agonistic effects are not known. Most antiestrogens act through competitive binding to ERs and induction of an inactive conformation of the ER ligand-dependent transactivation function 2 (AF-2), and their context-specific agonistic activities have been mainly attributed to a tissue- or promoter-specific activation of the ligand-independent function AF-1 or to the induction of a partially active AF-2 conformation (Nettles and Greene 2005). However, ICI appears to act at several levels to completely block ER-mediated actions, including the competitive inhibition of agonist binding to ERs, the inhibition of ER dimerization, nuclear translocation and transcription activation through both AF-1 and AF-2, and the increased ER protein degradation (reviewed by Osborne et al. 2004; Carlson 2005). While estrogens are known to rapidly down regulate the ERα and ERβ protein levels in several mammalian cell types but up regulate its mRNA levels (Pinzone et al. 2004), ICI has been shown to cause ERα protein degradation without affecting the ERα mRNA levels (Osborne et al. 2004), thereby leading to an effective reduction of the ER protein levels.

One of possible explanation given for the partial agonism of ICI is the inhibition of ERα protein down regulation, as observed in cells of the sheep uterus or in human breast cancer cells in which ICI behaved as agonist (Robertson et al. 2001; Wu et al. 2005). They have also been attributed to species-specific differences in the N- or C-terminal regions of ERs, which could influence ligand discrimination, while some actions were shown to be induced by ICI via non-classical mechanisms such as non-genomic actions, activation of intracellular signalling cascades or gene activation at alternative response elements (e.g. AP-1) trough ERβ activation (Jones et al. 1999; Wu et al. 2004; Wu et al. 2005).

However, it is not known if the same mechanisms occur in fish. ICI was also unable to block the *in vitro* Vg protein production in hepatocyte cultures from trout, but not from

surgeon (Latonnelle et al. 2002), but inhibition of E_2 effects (antagonism) has also been reported (Celius et al. 1999; Monteverdi and Di Giulio 1999; Bouma et al. 2003).

Different from mammals, in fish estrogens have been shown to increase both ERα mRNA, through increased transcription and enhanced stability, and ER protein levels in liver (Pakdel et al. 1991; Flouriot et al. 1996; MacKay et al. 1996; Andreassen et al. 2005). The ICI up regulation of ERα in liver detected in the present study may contribute to the observed agonistic effects, and the potentiation effects observed for the ICI pre-treatment may be due to an increased responsiveness of the tissue at the time of E_2 administration, through ERα up regulation by ICI. Whether the ICI up regulation of ERα mRNA is followed by an increase in ERα protein levels in liver, like for E_2, must be investigated in future studies.

The lack of ICI inhibition in the up regulation of ERα, VgII and ChgL by E_2 in liver (Figure 6.6) could be interpreted as evidence for an ER-independent mechanism, as suggested for other ICI-insensitive actions (e.g. Singh et al. 2000). However, this appears not to be the case, since ICI alone was able to up regulate these genes (Figure 6.2) and because the E_2-induced transcriptional activation of both ERα and VgII genes have been demonstrated to involve binding of the ER proteins to specific response-elements in their promoters (Le Drean et al. 1995; Teo et al. 1998; Petit et al. 1999; Menuet et al. 2004; Bouter et al. 2005). Moreover, ERα and Vg mRNA stabilization appear to occur via the increase in production of an unidentified gene product, mediated by E_2/ER complexes (Flouriot et al. 1996). The dependence on ERα has also been demonstrated in some studies reporting ICI agonism in mammals, using ER-specific siRNA (Wu et al. 2004).

In summary, in this study we have identified the differential regulation of sbERs by E_2 and ICI, which appear to depend on the ER subtype and the tissue. The strong up regulation of ERα in correlation with Vg and ChgL and the down regulation of both sbERβs suggest

that ERα is probably the most important ER subtype controlling liver gene expression in response to E_2. The agonistic effects identified for ICI or combined ICI/E_2 treatments on several estrogenic actions and in two different fish species question the use of ICI as a pure antiestrogen to "knock-out" estrogen functions in fish, at least until their effects and mechanisms of action are better characterized. It would be also interesting to investigate the effects of other "pure antagonists" such as ICI 164,384 and RU 58668 on the estrogenic actions analyzed in this study.

6.5. Acknowledgements of practical work

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CHAPTER 7

Identification of estrogen-responsive genes in the testis of sea bream (*Sparus auratus*) using suppression subtractive hybridization

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7.1. Introduction

Estrogens exert their widespread actions in vertebrate physiology through the regulation of transcription of numerous estrogen-responsive genes (ERGs) in target cells, mediated by the interaction with specific nuclear receptors, the estrogen receptors (ER), or with plasma membrane localized receptors (Nilsson et al. 2001; Razandi et al. 2004).

Although estrogens have long been considered as "female hormones", stimulating in oviparous vertebrates the liver production of yolk proteins, in particular vitellogenin (Vg), and egg shell proteins (Pakdel et al. 1991; Flouriot et al. 1996; Celius et al. 2000), it is now established that low estrogen levels are important for normal male physiology (O'Donnell et al. 2001; Carreau et al. 2003; Hess 2003). Testicular synthesis of estradiol-17β (E_2) has been reported in several vertebrate species including fish, amphibian and mammals (Varriale et al. 1986; Fostier et al. 1987; Loomis and Thomas 1999; Carreau et al. 2003). Moreover, two nuclear ER forms in mammals (O'Donnell et al. 2001) and three in fish (Hawkins et al. 2000; Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004; Pinto et al. 2005) have been found to be expressed in testis. Membrane ER-mediated actions of E_2 have also been proposed to occur in fish testis (Loomis and Thomas 2000).

In mammals, the disruption of the ERα, either in the knockout mouse or by treatment with antiestrogen, resulted in failure of fluid resorption regulation in efferent ductules, with eventual decrease in fertility (Couse and Korach 1999; Hess 2003). Additional studies also support a function in the control of testis development and spermatogenesis (O'Donnell et al. 2001; Carreau et al. 2003; Hess 2003; Saunders 2005) and in fish, E_2 promoted stem cell renewal in testis of huchen, *Hucho perryi* (Amer et al. 2001) and Japanese eel, *Anguilla japonica* (Miura et al. 1999).

However, it is well established that exposure to estrogenic contaminants released in the environment (xenoestrogens) cause deleterious effects in male reproductive functions in

wildlife and possibly in humans also (O'Donnell et al. 2001). For example, xenoestrogen exposure has been suggested to cause, among others, demasculinization and hermaphroditism in amphibians (Hayes et al. 2002), Vg accumulation, testicular regression and hermaphroditism in fishes (e.g. Jobling et al. 1996; Jobling 1998; Allen et al. 1999) and proposed as the possible cause for decreased sperm counts and increase in male reproductive disorders in the last decades in humans (Sharpe 1993).

However, the genes involved in both normal testis physiology and endocrine disruption are not well known in most organisms, including fishes. Few E_2 target genes are known in fish, and only recently have some systematic studies to identify ERGs by differential display, suppression subtractive hybridization and macroarrays been reported, all targeting the liver (Denslow et al. 2001; Bowman et al. 2002; Larkin et al. 2002; Larkin et al. 2003a; Brown et al. 2004). The genes identified encoded mainly proteins involved in reproduction, such as vitellogenins, egg envelope proteins and ERα.

The main objective of this study was to identify estrogen-responsive genes in the testis of the gilthead sea bream (*Sparus auratus*). The sea bream is a protandrous hermaphrodite teleost fish, which develops functional testis during the first reproductive cycle but may undergo sex reversal to female in one of the subsequent spawning seasons (Zohar et al. 1978). A role for E_2 in sex reversal has been recognized since E_2 treatment can induce testicular regression and development of functional ovaries (Happe and Zohar 1988; Condeca and Canario 1999). In this study, in order to obtain a gene transcription profile following E_2 exposure, which would allow to probe the mechanisms of E_2 actions in normal testis physiology and during endocrine disruption, suppression subtractive hybridization (SSH) (Diatchenko et al. 1996) was used to isolate differentially expressed cDNAs between E_2-treated and control sea bream testis. Differential expression was confirmed by semi-quantitative RT-PCR in testis and liver.

7.2. Specific methods

7.2.1. Animal treatments

Two groups of eight mature male sea bream (body weight 359.1 ± 8.1g, sea bream experiment 1, described on section 6.2.1) were treated for 28 h with i.p. injections of 10 mg/kg body weight (bw) E_2 in coconut oil (E group) or coconut oil alone (C, control group). The same E_2 dose and exposure time have previously been shown in our laboratory to modify ERα and ERβ expression in sea bream liver and testis (Socorro 2001). At the time of sampling, fish were anaesthetized, killed and visual inspection of the gonads performed to assure that all fish were male, as described on sections 2.2 and 2.3. Liver and testis transverse sections were collected and stored at -80ºC for RNA extraction, which was used for subtractive library construction (section 7.2.2) and for RT-PCR expression analysis (section 7.2.5).

Differential expression of ERGs was also confirmed using testis RNA from three groups of eight young male sea bream (body weight 233.8 ± 4.4g, sea bream experiment 2, section 6.2.1) treated for 48h with 1 mg/kg bw E_2 (E_1 group), 0.1 mg/kg bw E_2 ($E_{0.1}$ group) or coconut oil only (C group). Transverse sections of the testis were collected for histology in Bouin's fixative and stored at room temperature (section 7.2.7), or for RNA extraction and stored at -80º C.

7.2.2. Suppression subtractive hybridization (SSH)

cDNA subtractive hybridization is a technique that allows the identification of differentially expressed cDNAs between two different mRNA populations without prior knowledge of the mechanisms or genes involved. cDNA subtraction methods generally involve hybridization of cDNA from one population (tester) to excess of cDNA from another population (driver, the reference cDNA) and then separation of hybridized (common) sequences from the unhybridized fraction. The unhybridized fraction is thus

enriched in genes that are expressed in the tester, but are absent or expressed at low levels in the driver mRNA.

Suppression subtractive hybridization (SSH) is a PCR-based subtraction technique in which differentially expressed sequences are selectively amplified with adaptor-specific primers, with simultaneous normalization between high and low abundance transcripts by suppression PCR, thus increasing the probability of identifying differential expression of low abundance transcripts (Diatchenko et al. 1996).

The PCR-Select^{TM} cDNA Subtraction kit (Clontech, Palo Alto, USA) was used in this study to construct a subtractive library between E_2-treated (tester) and control testis (driver). First, total RNA was extracted with TRI reagent (section 2.4) from frozen testis sections from fish from experiment 1 (control or E_2-treated, n=8 each group) and poly(A)$^+$ RNA was purified from the two total RNA pools passing twice through Oligo-dT columns (section 2.5). Library construction followed the manufacturer's instructions. Briefly, both poly(A)$^+$ RNA populations (2 μg) were converted into cDNA, digested with *Rsa*I and specific adaptors were ligated to the tester cDNA. Tester and driver were hybridized to exclude common sequences and the resultant cDNA was subjected to two rounds of PCR for the selective amplification of differentially expressed sequences (up regulated with E_2), using adaptor specific primers. A portion of the adaptor ligated tester cDNA was not hybridized, to serve as unsubtracted control. A reverse subtraction using control testis as tester and E_2-treated as driver was also performed to identify mRNAs down regulated with E_2, but as a consequence of technical problems which resulted in the loss of mRNA it was not possible to complete the experiment.

7.2.3. Subtractive library cloning

10 µl of the subtracted and unsubtracted secondary PCR products were run on a 2% agarose gel to observe the differential display pattern. Discernible bands in the subtracted lane were gel extracted, reamplified, cloned into pGEM-T vector and plasmid DNA isolated with the Wizard Plus SV Miniprep DNA purification system (sections 2.8 and 2.9). To isolate less abundant PCR products, 0.5 µl of the subtracted secondary PCR product were directly cloned into pGEM-T and plasmid DNA was isolated from 168 randomly selected colonies as above.

7.2.4. Sequencing and homology searches

Cloned inserts from both band extraction and direct cloning were sequenced with M13F or M13R primers on an automated ABI3100 DNA Sequencer (Molecular Biology Unit, Biosciences Department, Cardiff University, UK). After removal of vector and adaptor sequences, identity searches were carried out using BlastX and BlastN against non-redundant public databases (Altschul et al. 1997). BlastN searches against the dbEST database were performed for clones whose identity could not be determined with the first two methods. Only matches with expect values (E) < 10^{-5} were considered significant. Overlapping redundant sequences were clustered using the BlastClust tool available at http://www.ncbi.nlm.nih.gov/. Non-overlapping redundant sequences matching different zones of the same gene were identified by blasting against the *Fugu rubripes* and *Tetraodon nigroviridis* genomes at http://fugu.biology.qmul.ac.uk/blast/ and http://www.ensembl.org/Multi/blastview, respectively. Once orthologs were established, genes were classified by biological process according to the Gene Ontology consortium rules (Ashburner et al. 2000), through searches in the GeneCards webpage (http://www.genecards.org/). Genes were annotated as putative housekeeping genes by searching in lists of genes showing constitutive expression in a large number of tissues

using microarrays (Hsiao et al. 2001; Eisenberg and Levanon 2003). Searches in the ERG database (http://research.i2r.a-star.edu.sg/promoter/Ergdb-v11/index.htm) allowed the identification of genes previously shown to be estrogen responsive in other tissues and/or organisms.

7.2.5. RT-PCR analysis of selected candidate genes

RT-PCR using specific primers designed for selected genes isolated from the library (Appendix V) was carried out to verify differential expression on cDNAs of each individual fish from both experiment 1 and 2. Primers for the vitellogenin receptor were based on the sequence with accession no. AY970973, itself obtained by RT-PCR with primers designed from sequence CX734947 and other teleost vitellogenin receptor sequences in GeneBank.

All reactions were performed with liver or testis cDNA (1 µl) using standard semi-quantitative RT-PCR conditions (section 2.12), with 18S as internal control. Optimized cycle numbers for the detection in the exponential phase of amplification are indicated for each gene in Table 7.1, while annealing temperatures for each primer pair are listed on Appendix V. No products were detectable in cross-amplification tests between clones of the same family (choriogenins, vitellogenins, fibrinogens) nor in the reverse transcriptase-minus reactions used to control for genomic DNA contamination. Primers for choriogenin H were shown to amplify choriogenin H minor by sequence analysis of different bands amplified from genomic DNA. No primers were designed for the putative egg envelope component ZPB because the cloned fragment was located in a conserved region and could not be discriminated from other genes of the same family.

Table 7.1. Cycle numbers used for the RT-PCR analysis of selected ERGs obtained from SSH, used in the confirmation of differential expression in liver or testis cDNAs from male sea bream treated with different concentrations of E_2 (experiments 1 and 2). Gene name abbreviation and accession number of the clones for which the primers were designed are indicated.

	Experiment 1		Experiment 2
Gene product:	**Liver**	**Testis**	**Testis**
Choriogenin L (ChgL, CX734876)	25	30	31
Choriogenin H (ChgH, CX734959)	24	28	31
Vitellogenin I (VgI, CX734983)	23	31	34
Vitellogenin II (VgII, CX734956)	20	25	27
Apolipoprotein A-I (ApoaI, AF013120)	21	33	36
Fibrinogen beta (FgB, CX734890)	19	29	33
Fibrinogen gamma (FgG, CX734892)	20	29	33
Complement C3 (C3, CX734936)	18	25	-
ER-binding fragment- associated gene 9 (Ebag9, CX734885)	21	24	-
Transferrin (Trf, CX734865)	23	31	-
Cysteine proteinase (CysP, CX734950)	29	25	-
Thyroid receptor interacting protein 4 (Trip4, CX734900)	35	30	-
Vitellogenin Receptor (VgR, AY970973)	33	24	-
ZPAX (ZPAX, CX735020)	36	21	-
18S ribosomal RNA (18S)	20	18	18

7.2.6. Quantification and statistical analysis

Relative expression values for each gene were determinate by densitometry analysis of RT-PCRs performed for both experiments, and differences between treatments and correlations between genes evaluated with SYSTAT, as described on section 2.12. Relative expression values for the second experiment were represented as box-plots using SigmaPlot. Covariance analysis was performed with the general linear model (GLM) module of SYSTAT, using the relative expression values from each gene as dependent variable, the treatment as independent variable and fish weight and length, E_2 plasma levels, ovarian proportion and total area in the gonad section as covariates.

7.2.7. Histology

In order to estimate the proportion of ovary in the testes of each fish from experiment 2, testicular tissue sections were prepared for general histological analysis using haematoxylin-eosin staining. This is a basic procedure to allow the morphological identification of cells and tissues, in which the negatively charged nuclei stain purple and the basic cytoplasm stains pink.

Briefly, testis sections were fixed in Bouin-Holland fixative (Sigma) for 1 day at room temperature and then washed for 5 days in 70% ethanol, which was substituted with fresh 70% ethanol each day. Tissue sections were then dehydrated through an ascending series of ethanol dilutions (70%, 95%, 100%), cleared in xylene and xylene:paraffin (1:1) and embedded in paraffin wax, using an automatic tissue processor (Leica TP 1020). Transverse sections (5 μm) of paraffin embedded tissue were cut using a microtome (Leica RM 2125 RT) with disposable stainless steel blades, mounted in albumin:glicerol (1:1) coated slides and dried for 2 days at 37ºC.

Before staining, slides were dewaxed by immersion 2x 10min in xylene and then rehydrated in several ethanol baths of decreasing concentration (100%, 95% and 70%) for 5 min each, followed by immersion for 5 min in dH_2O. Slides were immersed for 30 sec in Harris haematoxylin solution (Appendix I), washed in tap water, immersed in a 1% eosin Y solution (Appendix I) for 30 sec and then washed in dH_2O. For definitive preparations, tissue sections were dehydrated through an ascending series of ethanol dilutions (70%, 95%, 100%, 5 min each), cleared for 2 x 10 min in xylene, mounted in DPX (Fluka) and covered with a clean glass coverslip. Sections were analysed using a microscope (Olympus BH2) coupled to a digital camera (Olympus DP 11) and the proportion of ovary relative to the total area of the tissue section was determined using the ImageTool software (UTHSCSA, Texas, available at http://ddsdx.uthscsa.edu/dig/itdesc.html).

7.3. Results

7.3.1. Cloning of E_2-regulated genes

SSH was used to isolate mRNA species up regulated by E_2. The secondary PCR from the subtracted cDNA (E_2-treated testis subtractive library) showed a different pattern from that of the unsubtracted control, which indicated a successful subtraction (Figure 7.1).

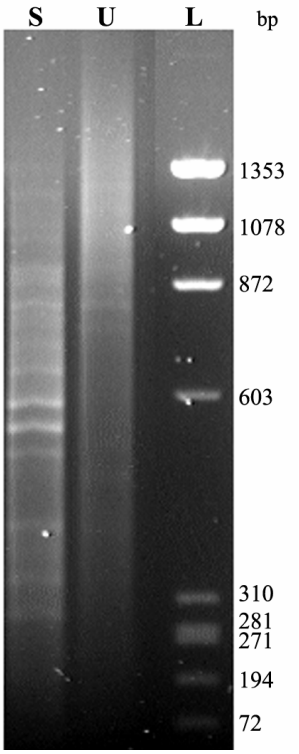

Figure 7.1. Secondary PCR from the subtraction between E_2-treated and control sea bream testis (E_2-treated subtractive library, lane S) and its unsubtracted control (U). L= φX174 DNA/ *Hae* III digest molecular weight DNA ladder.

A total of 183 sequences were obtained from both band isolation from the gel and cloning of randomly selected clones from the secondary PCR, with an average length of 415 bp, ranging between 86 and 1265 bp. All sequences were submitted to the expressed sequence tag database (dbEST) at NCBI with consecutive accession numbers from CX734847 to CX735033. Of these, 129 sequences (70%) were identified as orthologous to known genes or ESTs (Table 7.2), while 54 (30%) could not be identified by similarity searches in the databases. A total of 31 redundant sequences were found, including seven non-

overlapping redundant ESTs matching different regions of the apolipoprotein A-I (*ApoaI*), vitellogenin II (*VgII*), choriogenin L (*ChgL*) or choriogenin H (*ChgH*) genes, identified by Blast searches against the *Fugu rubripes* and *Tetraodon nigroviridis* genomes. All ApoaI, VgII and ChgL sequences matched unique genes, while ChgH sequences matched three different genes, choriogenin H (*ChgH*), choriogenin H minor (*ChgHm*) and egg envelope component ZPB (*zpb*), which were named by sequence comparison with the medaka egg envelope protein genes (Kanamori et al. 2003).

Of the remaining sequences, 9 were identified as housekeeping genes, 36 matched proteins or ESTs for which no annotation is yet available, while 53 matched known gene products. From these, only three had previously been cloned in sea bream: *ChgL*, *ApoaI* and *VgII*. *ApoaI*, *VgII*, *ChgL* and *ChgH* had the highest frequency among randomly picked clones (Table 7.3), providing strong positive evidence for their induction by E_2 in testis.

Table 7.2. Summary of the results from Blast searches with the 183 clones isolated from the E_2-treated testis subtractive library. Values are the number (N) or percentage (%) of sequences within each group.

Blast results	N	**%**
Unidentified ESTs	54	**30**
Identified	129	**70**
Unamed proteins/ESTs	36	**19**
Redundant	31	**17**
Housekeeping genes	9	**5**
Known genes	53	**29**

Clones matching known genes were classified by biological process according to the Gene Ontology Consortium rules (Table 7.3). Table 7.4 shows that, as expected, a significant proportion of these genes are involved in sexual reproduction (14%), cell proliferation, including positive regulators of cell proliferation, oncogenes and tumor suppressing genes (13%), lipid metabolism (11%), signal transduction (10%) and transport (10%), categories where estrogens are normally involved. Indeed, several genes belonging to these categories had previously been identified as ERG in other tissues / organisms (Table 7.3).

Table 7.3- Classification of identified genes according to gene ontology terms.
Gene identity determined through Blast searches; b- Best BlastX protein sequence match (lowest E value) or, if no match, best BlastN (* indicates nucleotide sequence match); c- E (expect) value; d- number of clones sampled for each gene (frequency); e- "Y" indicates genes previously identified as ERG, based on the ERG database.

Accession Numbers	Identity[a]	Best match[b]	E[c]	F[d]	ERG[e]
1- Development- Sexual reproduction [GO:0019953]					
CX734849, CX734852, CX734878, CX734955, CX734956, CX734964, CX734975, CX735032	Vitellogenin II (VgII)	BAB79591	1E-56	8	Y
CX734983, CX734994	Vitellogenin I (VgI)	BAC20186	5E-45	2	Y
CX734862, CX734876, CX734911, CX734952, CX734987, CX734996, CX734999	Choriogenin L (ChgL)	CAA63709	1E-103	7	Y
CX734959, CX734963	Choriogenin H (ChgH)	BAA13994	5E-06	2	Y
CX734907, CX734970	Choriogenin Hminor (ChgHm)	CAA04220	2E-65	2	
CX734850	Egg envelope component ZPB (ZPB)	AAM91820	3E-24	1	
CX735020, CX735021	Egg envelope component ZPAX (ZPAX)	AAD38904	4E-97	1	
CX734947	Vitellogenin Receptor (VgR)	AAO92396	3E-06	1	
CX734851	Sperm associated antigen 1 (Spag1)/ infertility-related sperm protein	NP_003105	6E-33	1	
2- Lipid metabolism [GO:0006629]					
CX734859, CX734867, CX734899, CX734914, CX734953, CX734954, CX734957, CX734979, CX734997, CX735008, CX735017, CX735019, CX735026	Apolipoprotein A-I (ApoaI)	O42175	2E-59	13	Y
CX734856	Mitochondrial carnitine-acylcarnitine translocase (Slc25a20)	NP_065266	3E-53	1	
CX734897	Long-chain-fatty-acid-CoA ligase 1 (acsl1)	Q9JID6	9E-42	1	
CX734946	Long-chain-fatty-acid-CoA ligase 5 (acsl5)	Q8JZR0	2E-37	1	
CX734968	Oxysterol-binding protein-like protein 7 (Osbpl7)	AAL40659	1E-18	1	
CX735004	Delta-9-desaturase 2 (Ech1) *	CN981038	6E-10	1	
CX734998	Long-chain L-2-hydroxy acid oxidase (Hao2)	NP_057611	1E-06	1	
3- Protein metabolism [GO:0019538]					
	Protein biosynthesis [GO:0006412]				
CX734853	Ribosomal protein L13a (Rpl13a)	NP_997949	6E-12	1	
CX734903	40S ribosomal protein S11 (Rps11)	AAG22825	2E-20	1	
CX734958	Ribosomal protein L17 (Rpl17)	AAF61071	2E-15	1	
CX734960	Seryl-aminoacyl-tRNA synthetase 1 (Sars1)	AAH08612	3E-34	1	
	Protein catabolism [GO:0030163]				
CX734922	Trypsinogen-like serine protease *	AF134323	2E-36	1	
CX734909	Proteasome subunit beta type 8 (Psmb8) / Low molecular weight polypeptide 7	AAL37206	4E-24	1	Y
CX734937	Proteasome subunit beta type 6 (Psmb6) *	AY190669	1E-71	1	
CX734950	Cysteine proteinase (CysP)	AAB82743	4E-78	1	
	Protein modification [GO:0006464]				
CX734924	Protein arginine N-methyltransferase 3 (Hrmt1l3)	NP_005779	1E-30	1	
CX734991	Serine/threonine-protein kinase / Vaccinia-related kinase 1 (Vrk1)	NP_998550	8E-41	1	
CX735033	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (Dyrk1a) *	NM_001396	4E-24	1	
	Protein folding [GO:0006457]				
CX734923	Cyclophilin A / peptidylprolyl isomerase A (Ppia)	AAR11779	6E-43	1	Y

Accession Numbers	Identity[a]	Best match[b]	E[c]	F[d]	ERG[e]
4- Nucleic acid metabolism [GO:0006139] and mRNA processing (GO:0006397)					
CX734895	WD repeat protein Gemin5	AAL38980	6E-46	1	
CX734984	Nuclear cap binding protein subunit 2 (ncbp2)	NP_775356	4E-53	1	
CX734973	IMP cyclohydrolase (Atic)	NP_112276	7E-59	1	
5- Transport [GO:0006810]					
CX734966	Translocating chain-associating membrane protein (Tram)	NP_705955	1E-66	1	
CX734945	Signal recognition particle 9kDa (Srp9)	NP_957390	1E-12	1	
CX734910	Ran-binding protein 7 / Importin7 (Ipo7) *	AY286403	8E-11	1	Y
CX734865, CX734969	Transferrin (Trf)	AAQ63949	6E-49	2	Y
CX734943	Copine III (Cpne3)	NP_956461	1E-97	1	
CX734990	Amine oxidase, flavin containing 1(Aof1)	XP_173173	5E-28	1	Y
6- Cell communication- Signal transduction [GO:0007165]					
CX734858	SPRY domain-containing SOCS box protein (Ssb1)	NP_079382	1E-16	1	
CX734904	Serine/threonine kinase receptor associated protein (Strap)	NP_956598	6E-15	1	Y
CX734935	Protein PM1 (C17orf35)	P17152	6E-09	1	
CX735011	Annexin 6 (Anxa6) *	AY178800	5E-21	1	
CX734981	Phospholipase A2 (Pla2) *	AB050633	2E-20	1	
CX735003, CX735009	Cofilin 1 (Cfl1)	NP_005498	9E-27	2	
7- Cell proliferation [GO:0008283]					
CX734891	Proliferating cell nuclear antigen (Pcna)	AAS67694	4E-25	1	Y
CX734868	Mitochondrial DNA polymerase accessory subunit (Polg2)	AAC51321	2E-50	1	
CX734890	Fibrinogen beta (FgB)	AAA52429	2E-22	1	Y
CX734892	Fibrinogen gamma (FgG)	AAK19752	1E-44	1	
CX735002	Proteoglycan 4 / Megakaryocyte stimulating factor (Prg4)	NP_997918	2E-07	1	
CX734885	Estrogen receptor-binding fragment-associated antigene 9 (Ebag9)	NP_957388	9E-08	1	Y
CX734889, CX734893	RET II oncogene / Golgi autoantigen, golgin subfamilyA member 5 (Golgin-84)	Q8TBA6	4E-09	2	Y
CX734900	Thyroid receptor interacting protein 4 (Trip4)	Q15650	1E-27	1	Y
8- Immune response [GO:0006955					
CX734936	Complement component C3 (C3)	BAA92285	8E-31	1	Y
9-Energy pathways					
CX734971	Vacuolar ATP synthase subunit H (Atp6v0e)	NP_775377	1E-51	1	
CX735006	Mitochondrial ATP synthase, O subunit (Atp5O)	NP_620238	3E-84	1	
CX734989	ATP synthase mitochondrialF1 complex assembly factor 2 (Atpaf2)	CAG04462	4E-76	1	
CX734929, CX734930	NADH dehydrogenase (ubiquinone) Fe-S protein 1 (Ndufs1)	NP_777245	1E-72	1	Y
CX734976	Aldolase C (Aldoc) *	AF041454	2E-18	1	
10-Structural proteins					
CX734928	Golgi peripheral membrane protein p65 (Gorasp1)	NP_062258	9E-40	1	
CX734855	Lamin B3 (LmB3)	BAB32977	5E-22	1	Y
11- Unknow process					
CX734883	Envelope protein, endogenous retrovirus	AAM34209	7E-02	1	
CX734871	Warm-temperature-acclimation-related-65kDa-protein-like-protein (Wap65-2)	BAD18110	4E-74	1	
CX734879, CX734880	Nuclear RNA helicase, DECD variant of DEAD box family (Ddx39)	NP_998142	1E-84	1	

Table 7.4. Distribution of identified genes by biological process categories. Values are the number or percentage (%) of genes within each group, out of the 62 genes for which an ortholog was established.

Biological Process	No. genes	%
Protein metabolism	12	19
Sexual reproduction	9	14
Cell proliferation	8	13
Lipid metabolism	7	11
Signal transduction	6	10
Transport	6	10
Energy pathways	5	8
Nucleic acid metabolism	3	5
Unknown process	3	5
Structural proteins	2	3
Immune response	1	2
	62	100

7.3.2. Confirmation of differential expression by RT-PCR

RT-PCR with gene specific primers was used to validate the differential expression in testis of the more frequently isolated genes, of those previously identified as ERG in other studies and of some genes belonging to the categories reproduction, cell proliferation and lipid metabolism. Since many of these genes had been identified as ERG in liver, RT-PCR of liver cDNA provided a positive control (Figure 7.2).

As expected, choriogenins and vitellogenins were up regulated in liver, while *ApoaI*, transferrin (*Trf*) and fibrinogens (*FgB* and *FgG*) were down regulated (Figure 7.2A). Moreover, there was significant down regulation of complement C3 (*C3*), estrogen receptor-binding fragment-associated antigene 9 (*Ebag9*), cysteine proteinase (*CysP*) and vitellogenin receptor (*VgR*). In testis, there was a high variability in gene expression among individuals, with some fish showing a high response to E_2 while others showed little or no response (Figure 7.2B). Despite the high individual variability, statistically significant up regulation by E_2 in testis was found for *ChgL*, *ChgH*, *VgI*, *VgII*, *ApoaI*, *FgB*, *FgG* and thyroid receptor interacting protein 4 (*Trip4*). Notably, low levels of expression of some of the genes were also detected in testis from control fish at the cycle number

tested (*VgI*, *ApoaI*, *FgB*, *FgG* and *Trip4*) or at higher cycle number (*ChgL*, *ChgH* and *VgII*, not shown). No changes in gene expression in liver or testis with E_2 treatment was detected for the egg envelope component ZPAX (Figure 7.2), annexin 6, oxysterol-binding protein-like protein 7, proliferating cell nuclear antigene, mitochondrial carnitine-acylcarnitine translocase and translocating chain-associating membrane protein (not shown). This suggests that either they were false positives or that the level of induction was too low to detect by RT-PCR.

Since in the first experiment a relatively high dose of E_2 was used (10 mg/kg), the expression of the same up regulated genes was analysed in testis after stimulation of sea bream with lower doses of E_2 (0.1 and 1 mg/kg). Histological analysis confirmed that all fish had active testes. The gonads had a dominant ventral testis, with cysts containing predominantly spermatids and spermatozoa, with few spermatogonia. Most fish had the sperm duct full of spermatozoa and released sperm when the abdomen was slightly pressed. A small vestigial ovary was present in the dorsal part of the gonad, containing oocytes at the chromatin-nucleolus and perinucleolus stages. In cross-section, the surface area occupied by the ovary was $2 \pm 0.7\%$, with a maximum of 10%.

In common with the first experiment, significant up regulation was detected for *ChgL*, *VgI* and *VgII* at both E_2 doses (Figure 7.3). Although the induction for the other genes did not show statistical significance, the plots show a trend for up regulation with at least some fish appearing to show response to E_2 (increased range of response). Again, high variability among individuals was observed. In order to try to account for the cause for this variability, analysis of covariance was performed using as covariates the proportion of ovary present, the total area of the gonad section (as a measure of gonadal development), plasma E_2 level and the weigh and length of each fish. However, no significant covariation

could be found between these variables and the gene expression levels, indicating that other factors influenced the response to E_2 treatment.

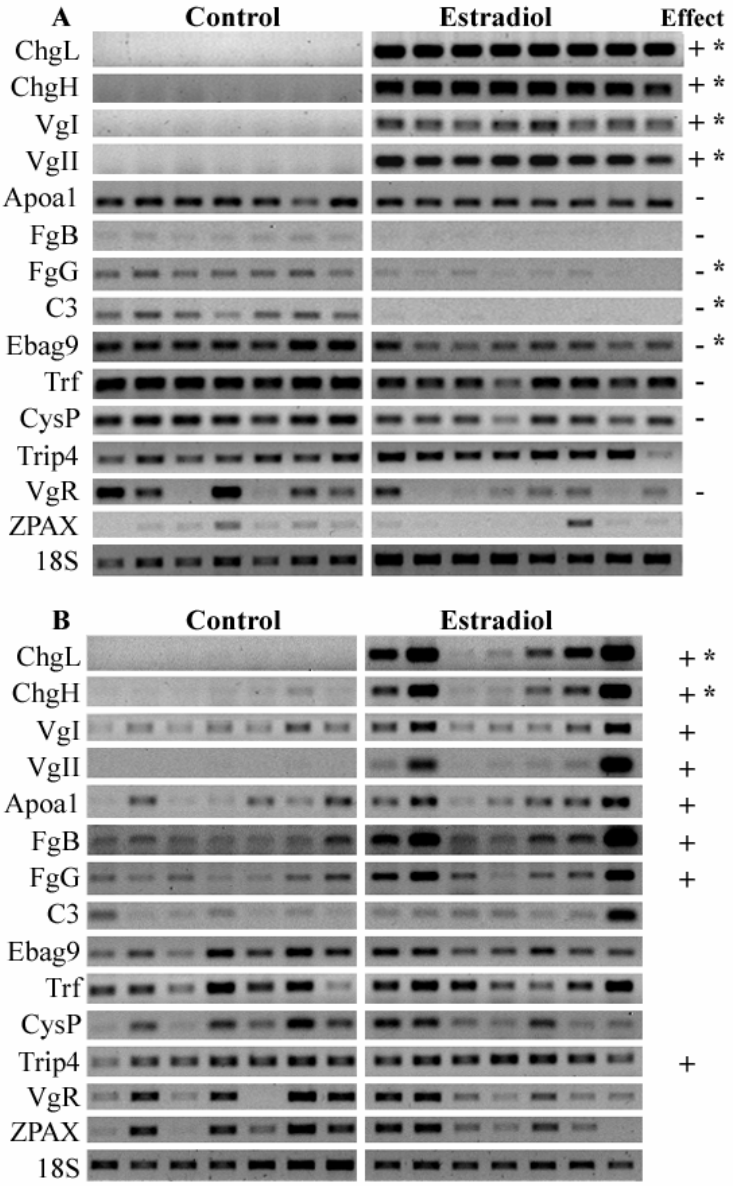

Figure 7.2. RT-PCR for confirmation of differential expression of selected genes between estradiol-treated and untreated (control) cDNAs from liver (A) or testis (B). cDNAs belonged to individual fish used in the E_2-treated testis subtractive library (experiment 1, 10 mg/kg E_2 for 28h). Gene name abbreviations (left-hand column): ChgL/ChgH: choriogenin L or H; VgI/VgII: vitellogenin I or II; ApoaI: apolipoprotein A-I; FgB/FgG: fibrinogen β or γ; C3: complement C3; Ebag9: estrogen receptor-binding fragment-associated antigene 9; Trf: transferrin; CysP: cysteine proteinase; Trip4: thyroid receptor interacting protein 4; VgR: vitellogenin receptor; ZPAX: Egg envelope component ZPAX. In the right-hand column, "+" or "-" indicate significant up or down regulation with E_2, respectively ($P<0.05$, evaluated by a general linear model using logarithm of expression ratios relative to 18S; * indicates $P<0.001$).

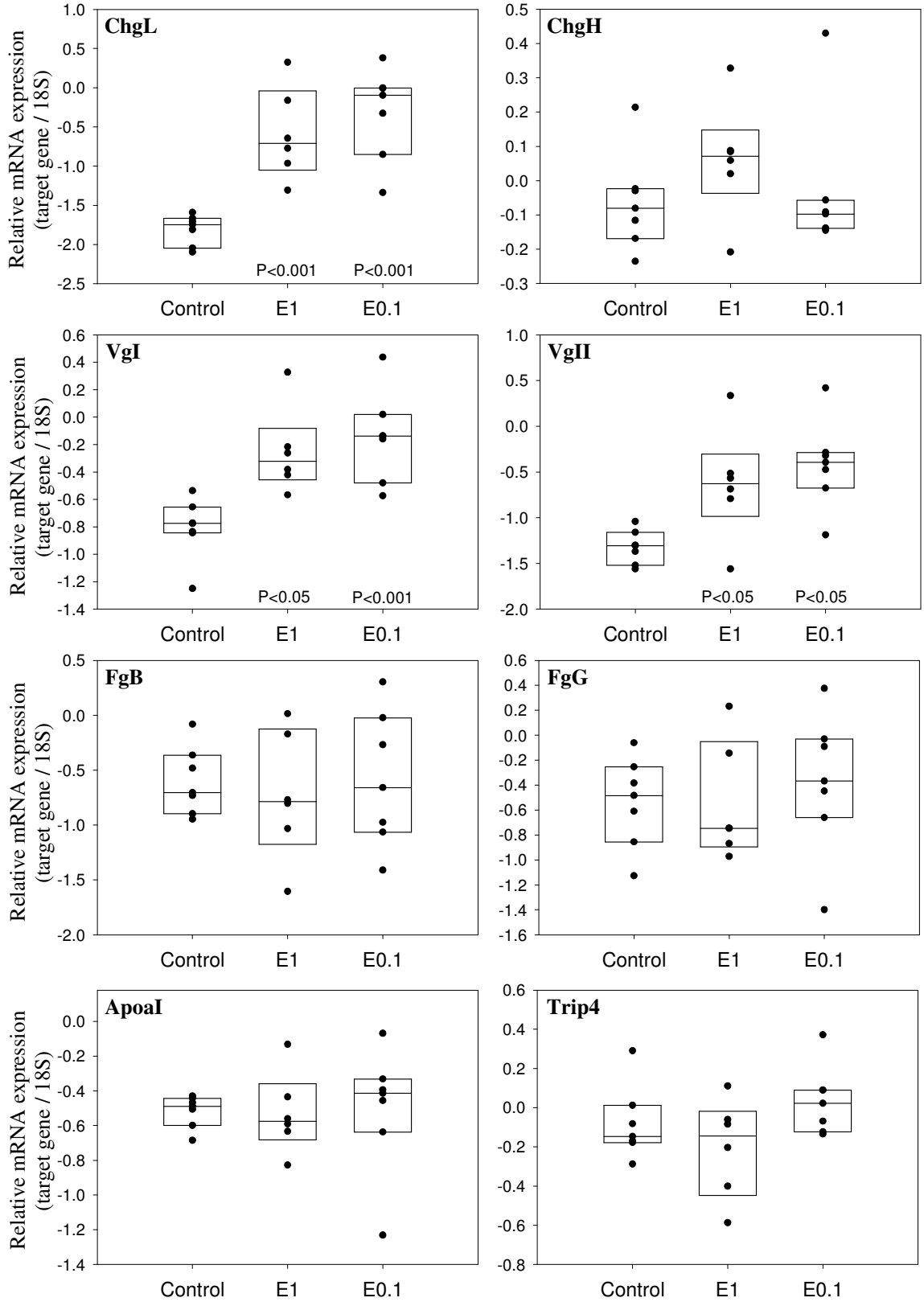

Figure 7.3. Box plot of RT-PCR for selected genes, using testis cDNAs from experiment 2 (E_1 = fish treated with 1 mg/kg bw E_2; $E_{0.1}$ = 0.1 mg/kg, 48 h exposure). Relative expression values for each fish are indicated by dots. Middle horizontal line inside each box indicates median; bottom and top lines are the 25th and 75th percentiles, respectively. P<0.05 / 0.001 indicates significant difference between treatment and control.

In experiment 1, strong positive correlations were found between *ChgL*, *ChgH*, *VgI*, *VgII* in testis and in liver (Table 7.5). The same genes were positively correlated with *FgB* and *FgG* in testis but inversely correlated in liver. In testis, *ApoaI* was also positively correlated to *VgI* and *FgB*, while *FgB* and *FgG* were positively correlated in both testis and liver. In experiment 2, strong positive correlations were held in testis (liver was not assayed) betweenVgs and Chgs and between *FgB* and *FgG* and *ApoaI*.

Table 7.5. Pearson correlations of the level of gene expression within testis (lower triangle) and within liver (upper triangle) for experiment 1. Statistically significant correlations ($p<0.05$) are shown in bold; underlined are the correlations that held significant in experiment 2 (in which only testis was assayed). In all analyses, control and treatment were included.

		Liver							
		ChgL	ChgH	VgI	VgII	FgB	FgG	ApoaI	Trip4
Testis	ChgL		**0.987**	**0.975**	**0.980**	**-0.848**	**-0.859**	-0.676	-0.178
	ChgH	**0.981**		**0.986**	**0.996**	**-0.864**	**-0.858**	-0.621	-0.099
	VgI	**0.776**	**0.841**		**0.989**	**-0.873**	**-0.849**	-0.576	-0.081
	VgII	**0.866**	**0.921**	**0.861**		**-0.876**	**-0.868**	-0.603	-0.075
	FgB	**0.882**	**0.894**	**0.840**	**0.857**		**0.952**	0.595	0.238
	FgG	**0.820**	**0.845**	0.747	**0.826**	**0.896**		0.593	0.217
	ApoaI	0.705	0.733	**0.775**	0.699	**0.862**	0.626		0.159
	Trip4	0.337	0.359	0.468	0.283	0.256	0.105	0.440	

Testis: n=14 for experiment 1 and n=20 for experiment 2; liver: n=15.

7.4. Discussion

SSH is a powerful global, open-ended gene expression technology which has recently been employed to investigate various aspects of fish biology (reviewed by Larkin et al. 2003b). This study reports for the first time in a teleost fish the use of SSH to identify genes up regulated in testis upon E_2 treatment. This approach allowed the isolation of 152 different candidate ERGs from the sea bream testis, many related to reproduction and other E_2 controlled processes, as well as proportion of unidentified gene products which may also be important effectors of estrogen actions.

The subtractive testes library was particularly enriched in a small subset of gene products previously known to be responsive to E_2 in liver: the egg yolk precursor VgII, the egg envelope components ChgL and H, and ApoaI, the major protein component of the serum high-density lipoprotein (HDL). The E_2 up regulation of the above referred genes as well as VgI, the coactivator Trip4, FgB and FgG was confirmed by RT-PCR in the testis of individual male sea bream treated with 10 mg/kg E_2. The induction of VgI, VgII and ChgL was further confirmed in male sea bream with lower doses of E_2 (1 or 0.1 mg/kg). Overall, the pattern of testicular gene responsiveness was the same although some genes did not show a significant response. This was most likely a result of their induction being dose-dependent and could be influenced by the stage in the reproductive cycle (the fish would expectedly be functional males in their second and first reproductive seasons, respectively in the first and second experiments).

To our knowledge, this is the first time that the E_2 up regulation of choriogenin and vitellogenin genes in testis is described. Vgs are the main source of nutrients for developing embryos in oviparous species, and Chgs are glycoproteins that form an external layer around vertebrate oocytes and are responsible for egg protection and proper fertilization (Arukwe and Goksøyr 2003). These proteins are normally synthesized in

response to E_2 in the liver of mature oviparous females, secreted in the plasma and transported to the ovary, where they are incorporated into the growing oocyte (Arukwe and Goksøyr 2003). When males are exposed to exogenous estrogen or estrogen mimics, Vg and Chg mRNA and protein expression in liver and protein secretion into the plasma are rapidly induced, making them current accepted biomarkers for endocrine disruption (Sumpter and Jobling 1995; Celius et al. 2000; Funkenstein et al. 2000). However, there is little information on the relationship between their induction in liver and the formation of reproductive dysfunctions in male fish exposed to endocrine disrupting compounds (EDCs) (Islinger et al. 2002). The demonstration that Vgs and Chgs are induced by E_2, not only in liver but also in testis, suggest that they could be implicated in mediating the adverse effects of EDCs in testis, which include the induction of ovotestis, sex reversal, permanent disruption of male gonad ducts and inhibition of testicular growth (Jobling et al. 1996; Rodgers-Gray et al. 2001; Matthiessen et al. 2002). Future studies of EDC regulation of Vg and Chg in testis will determine their potential as biomarkers for endocrine disruption in testis.

There have been few reports of Vg gene or protein expression in non-hepatic tissues: in the testis of male sturgeon where it was slightly downregulated by E_2 (Bidwell and Carlson 1995), in the mucus of mouth brooding male tilapia, *Sarotherodon melanotheron* and *Oreochromis mossambicus* (Kishida and Specker 1994; Kishida and Specker 2000), in the mucus of E_2-treated male and female cichlid fish *Cichlasoma dimerus* (Moncaut et al. 2003) and in the mucus of garter snakes (Garstka and Crews 1981), where it was suggested to function as a pheromone. The fact that both Vgs and VgR are expressed in untreated sea bream testis suggests that locally expressed Vg may have a role in normal testis physiology. Whether this role could be, as hypothesized for sturgeon, in nutrient

transport, uptake of hormones, vitamins and other biomolecules for spermatocytes (Bidwell and Carlson 1995) requires investigation.

While in most vertebrates egg envelope proteins are only transcribed in the developing oocytes, teleost fishes and birds possess genes coding for oocyte-specific (classified as the zona pellucida protein families ZP-A, -AX, -B, -C or -D) and liver specific forms (choriogenin L, belonging to the ZPC family, and choriogenin H and Hminor, from the ZPB family), which are synthesized in response to E_2 (Kanamori et al. 2003). The sea bream testicular ZPB, ZPAX, Chg L, H and Hminor were expressed at low levels in control testis and ChgL and ChgH were induced by E_2. Immature eel testes also expressed ZPB and ZPC which were down regulated by gonadotropin and 11-ketotestosterone and it was hypothesized that they could play a role in the prevention of initiation of spermatogenesis (Miura et al. 1998). Whether these proteins mediate the E_2-induced inhibition of testis development in sea bream (Condeca and Canario 1999) and other fishes (e.g. Jobling et al. 1996; Allen et al. 1999) needs investigation.

FgB and FgG, which were both up-regulated in the testis, are important factors for haemostasis but are also known as positive regulators of cell proliferation in wound healing and in tumors (Staton et al. 2003). It is possible that these proteins are involved in the proliferation of stem cells observed during spermatogenesis in agreement with the observation that E_2 is able to stimulate spermatogonial proliferation in huchen (Amer et al. 2001), and the renewal of spermatogonial stem cells in Japanese eel (Miura et al. 1999). Apolipoprotein A-I up regulation may be related to its function in the selective uptake of HDL-cholesteryl esters required for steroidogenesis (Plump et al. 1996).

In liver, which was used as a positive control with reference to published studies, the up regulation of ChgL, ChgH, VgI and VgII, and down regulation of ApoaI, FgB, FgG and Trf were confirmed, as previously observed for sea bream and other species (Keyong and

Ing 1998; Funkenstein et al. 2000; Bowman et al. 2002; Larkin et al. 2002; Larkin et al. 2003a). Nevertheless, this study describes for the first time the down regulation in liver of the immune complement component C3, reported as ERG in rat uterus (Diel et al. 2004); of Ebag9, a prognostic marker for tumors isolated as ERG from a human breast cancer cell line (Watanabe et al. 1998); and of VgR and cysteine proteinase.

Interestingly, ApoaI, FgB, FgG and Trip4 showed a differential response to E_2 in liver and testis. This tissue-specific regulation, which has also been described for synthetic estrogens and environmental estrogens, has been the base for research of new selective estrogen receptor modulators (SERM) for the treatment of some types of cancers (Diel 2002). The exact mechanisms of the differential regulation are not fully understood but are believed to result from a different cell context, containing different proportions of ER subtypes, other transcription factors, coactivators and corepressors, which may lead alternative signaling pathways, resulting in up or down regulation of specific genes (Diel 2002; McDonnell 2004). Trip4, here identified to be up regulated by E_2 in testis but not in liver, may be one of the genes involved in mediating the different pathways, since it has been reported as transcription coactivator of nuclear receptors (Kim et al. 1999).

Another difference between the two tissues was the variability of the response among individuals found in testis, but not in liver, which may reflect slight differences in developmental stages of the testes (although all fish were in active spermatogenesis, not all were spermiating). No correlation could be found between ERG induction and the vestigial proportion of ovary always present in the hermaphrodite gonad, suggesting that the induction occurred only or mainly in the testicular part of the gonad.

In conclusion, these results show that E_2 is able to modify the expression in testis of a number of genes that are normally associated with liver. Although the confirmation of differential expression was limited to a small subset of genes, it is likely that many other genes are up regulated. Low levels of mRNA expression of all E_2-regulated genes could be detected in non-stimulated testis and are likely to have a function in normal testis physiology. Further studies will be required to clarify their cellular localization and potential role. From a practical point of view, it can be envisaged that non-lethal biopsies from testis can be taken from natural populations to access endocrine disruption based on the expression of the above genes identified as highly responsive to E_2.

CHAPTER 8

General discussion and perspectives

In this thesis, the presence of two ERβ subtypes encoded by different genes was demonstrated in a teleost fish, the sea bream. Phylogenetic analysis and *in silico* identification of ERβ genes from available fish genomes strongly supported the view that the two genes arose by duplication of the ERβ gene in an ancestor common to all teleosts (Robinson-Rechavi et al. 2001). The existence of three ERs in teleosts (ERα, ERβa and ERβb) instead of two (ERα and ERβ) as identified in other groups of vertebrates, raises a number of questions about the persistence of two ERβ forms in the genome and their relative role in ER-mediated estrogen functions in fish. Are they both functional? Do they have the same or different functions? Are they expressed in the same tissues/cell-types? Are they regulated by the same factors?

This work supports that both sea bream ERβ genes are active and encode functional proteins, as demonstrated by their mRNA and protein expression and functional analysis.

A comparative analysis between the two ERβ subtypes from several teleost fish, including sea bream, suggests that they may have evolved different functions, based on their different, although partially overlapping, patterns of tissue distribution and cellular localization; different patterns of amino acid conservation; different binding and transactivation properties and different regulation during development, during the reproductive cycle or in response to estrogen treatment (Hawkins et al. 2000; Bardet et al. 2002; Halm et al. 2002; Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004; Hawkins and Thomas 2004; Menuet et al. 2004; Sabo-Attwood et al. 2004; Tingaud-Sequeira et al. 2004; Filby and Tyler 2005; Hawkins et al. 2005; Pellegrini et al. 2005). Indeed, a difference in the E_2-induced upregulation of the ERα gene was recently demonstrated for the zebrafish ERβ genes, with the ERβa protein, but not ERβb, being able to activate the expression of a reporter gene driven by the zebrafish ERα promoter (Menuet et al. 2004). Moreover, motif sequence analysis and the *in vitro* phosphorylation

assay performed in the present study, demonstrating for the first time in fish the phosphorylation of the ERα protein, suggest a differential activation of the two sbERβ subtypes by MAP kinase 2, which in mammals is involved in the ligand-independent activation of ERs. Further experiments are required to confirm these results and to better characterize the activation of sbERs in response to this signalling pathway. It is also possible that some of the functions of the original ERβ have been divided between the two duplicates, or that they have redundant or complementary functions in the tissues (e.g. pituitary, liver, testis and kidney) or cell-types (e.g. putative osteoclasts in juvenile sea bream scales) where they co-express.

The sbERβa appears to be the predominant ER mRNA expressed in the ovary, as previously observed for other fish species (Hawkins et al. 2000; Choi and Habibi 2003), suggesting a major role in the regulation of ovarian functions by estrogens. Consistent with this, the sbERβa protein, but not sbERα, was detected in the nucleus of developing oocytes and laminar epithelium of the ovary (Socorro 2001), but no information is available on the localization of the sbERβb protein.

In the testis, the high expression detected for all three ER mRNAs (Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004 and this thesis) suggests they all may have a role in mediating the reported estrogen functions on sex differentiation, sex change, early stages of spermatogenesis and endocrine disruption in fish testis (Sumpter 1995; Devlin and Nagahama 2002; Miura and Miura 2003). However, ER localization studies have been restricted to only one or two ER subtypes and their results have not been consensual, possibly reflecting species and / or seasonal differences (Miura et al. 1999; Legler et al. 2000; Bouma and Nagler 2001; Socorro 2001; Wu et al. 2001; Andreassen et al. 2003). The sbER-subtype specific antisera generated in this study makes it possible to study and compare the cellular localization of the three fish ER proteins in several target tissues,

including the ovary and testis, and the clarification of their relative roles, with particular interest on the comparison between the two ERβs.

The expression of all sbERs in the pituitary of both sexes agrees with that observed for other fish species (Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004), supporting the known effects of estrogens on gonadotrophin synthesis and release, although the specific functions of different ER subtypes still need to be determined (Kah et al. 1997; Melamed et al. 1998; Weltzien et al. 2004).

One of the unexpected results obtained in this study was the undetectable levels of sbERs in the brain of both male and female sea bream, contrasting with RT-PCR results from other fish species (Menuet et al. 2002; Choi and Habibi 2003; Halm et al. 2004). In the zebrafish and croaker brain, the three ERs showed different but overlapping patterns of localization, mainly in neuroendocrine regions involved in regulating pituitary functions (Hawkins et al. 2000; Menuet et al. 2002; Hawkins et al. 2005). It is possible that sbERs are expressed at low levels and/or within particular regions of the brain, rendering its detection difficult when analysing the whole tissue. Experiments to localize the sbER proteins (by IHC) and mRNAs (by ISH) in the sea bream brain and pituitary are in progress to investigate their relative roles in these tissues.

Although all sbERs were expressed in the liver, the strong upregulation of ERα in correlation with VgII and ChgL and the downregulation of both sbERβs agree with the idea that the ERα subtype is the most important ER mediating the E_2-induced expression of genes involved in female reproduction in this tissue (Menuet et al. 2004; Sabo-Attwood et al. 2004). The contribution of ERβs, if any, is probably less important and may depend on the life stage of the fish and/or the species. The differential regulation between ER subtypes probably results from the presence of different regulatory elements in their promoters, as suggested by the comparison between the rainbow trout ERα and zebrafish

ERβa promoters (Le Drean et al. 1995; Petit et al. 1999; Lassiter et al. 2002), but no information is yet available regarding the promoter of any ERβb gene. This discussion would beneficiate from future promoter analyses, which would include *in silico* analysis and the isolation and characterization of the three ER promoters.

The sbERβ mRNAs are expressed in the kidney, intestine, different bone and cartilage structures and scales, which suggest their involvement in fish calcium homeostasis, while the ERα mRNA expression is in general lower. Estrogen hypercalcemic effects in fish are apparently achieved by two main processes, the calcium mobilization from internal stores and the increase in calcium influx from the environment (e.g. Persson et al. 1995; Guerreiro et al. 2002), but whether these actions are indirect or directly mediated by ERs expressed in tissues related to calcium metabolism has not been established.

The co-localization of the three sbER proteins in putative osteoclasts of the juvenile sea bream scales demonstrated in this thesis strongly supports that E_2 acts directly on these cells to exert its calcium mobilizing effects, in agreement with the recent demonstration that E_2 induced TRACP (osteoclastic) activity in sea bream scales (Rotllant et al. 2005). The detection of sbERβa and sbERβb mRNAs in other calcified tissues suggests they may also be estrogen targets, but additional studies are required to investigate which are the estrogen effects on these tissues, the estrogen target cells and ER subtypes involved.

The expression of sbERβa and sbERβb mRNAs in the kidney and intestine suggests their involvement in modulating the calcium transport mechanisms between internal and external pools, but the higher abundance of sbERβb in all regions of the intestine suggests a specific role for this subtype. However, the discrepancies found between the sbER relative mRNA and protein levels in adult sea bream scales highlight the importance of confirming this comparison at the protein level. Expression of both ERβs have also been detected in the intestine of other fish species (Menuet et al. 2002; Choi and Habibi 2003;

Filby and Tyler 2005) but its significance was never investigated. Future studies will include the ER protein localization in tissues involved in calcium transport in fish, including the kidney, different regions of the intestine and the gills, where only low levels of ER have been detected, to identify which cell types and ERs are involved in the E_2 effects on these tissues.

Moreover, the mechanisms through which E_2 increases calcium influx or mobilization from the scales remain unexplored, although it is probable that they may involve the interplay with other hypercalcemic factors as suggested in birds (Fairbrother 2000 and references therein). This is supported in sea bream by an E_2-induced increase in the circulating levels of parathyroid hormone-related protein (PTHrP), occurring before the increase in plasma calcium levels (Guerreiro 2002). PTHrP is a hypercalcemic factor, shown to increase whole body calcium influx and scale osteoclastic activity in sea bream (Guerreiro et al. 2001; Rotllant et al. 2005). In order to test the hypothesis that E_2 hypercalcemic effects are mediated by PTHrP, future experiments should analyze the effects of E_2 treatment on the expression of PTH/PTHrP and PTH receptors in the calcium regulatory organs which were here identified as estrogen targets, and in the pituitary, suggested to be a major source of circulating PTHrP in fish (Rotllant et al. 2003). It may also be of interests to analyse if PTHrP modifies ER expression.

Unexpectedly, a synergistic effect on the increase in calcium plasma levels was detected between ICI 182,780, when applied as a pre-treatment, and E_2 in both sea bream and tilapia. ICI agonistic effects were identified in the expression of some genes in the liver (sbERα, VgII and ChgL) but not in others (sbERβa) and not for any gene in the testis, while ICI pretreatment acted synergistically with E_2 to downregulate sbERβb in liver. This confirms reports in fish and mammals (Jones et al. 1999; Robertson et al. 2001; Latonnelle et al. 2002; Wu et al. 2004), which have shown that ICI, like other SERMs, appears to

have agonistic or partial agonistic effects in some estrogenic responses depending on the gene and tissue analyzed. These findings question its designation and use as a "pure ER antagonist", while raising new questions concerning the mechanisms involved in ICI agonism. The liver ERα mRNA upregulation by ICI demonstrated in this study suggests that this could be one of the factors contributing for an increased responsiveness to E_2, although this needs confirmation at the protein level. Moreover, other variables potentially affecting the cellular response to ICI need to be investigated. To better understand the mechanisms involved in the synergism between ICI and E_2 on the hypercalcemic effects, it would be also interesting to analyze the effects of ICI or combined E_2/ICI treatments on the expression of PTH/PTHrP and PTH receptors.

A significant contribution from this thesis was done into the field of the diversity of ERs in fish. Although multiple ER transcripts of different length have been previously detected by Northern blot in different fish species including sea bream (e.g. Socorro et al. 2000; Sabo-Attwood et al. 2004), few have been sequenced and characterized. In this study, several sbER variant transcripts have been identified in sea bream, including ERβb transcripts differing in their UTRs, transcripts encoding putative N-terminally truncated ERα proteins and ERα transcripts with internal deletions of exon 2, encoding putative receptors with internal deletions or C-terminal truncations. These results now open the opportunity to investigate their biological significance. While transcripts with different 5' or 3' UTRs are predicted to differ in their translation levels or RNA stability, those differing in the coding region could produce variant proteins with different properties than that of the wtER, as demonstrated for several ER variants in mammals (reviewed by Hirata et al. 2003). The differential pattern of tissue distribution and hormonal regulation observed for the two exon 2-deleted ERα variants suggests they may have different biological significance. In order to clarify their potential role, further studies are needed to

establish their cellular localization and functional properties. Their functional characterization could follow the same procedure used by (Bollig and Miksicek 2000) to characterize all the single exon-deleted variants of human ERα, which included *in vitro* translation from reconstructed cDNAs and characterization of their DNA and ligand-binding properties, transactivation at consensus EREs or at alternative response elements (e.g. AP-1), cellular localization, interaction with coactivators, dimerization with wtERs and the effects (e.g. dominant negative or dominant positive) on the transcriptional activity of wtERs. In addition, Northern blot results (Socorro et al. 2000 and chapter 3 of ths thesis) point for the existence of multiple transcripts for both sbERβs, some of them smaller than the complete coding region, but their identity remains to be established.

The construction of an E_2-treated testis subtractive library allowed the isolation of 152 different candidate ERGs from the sea bream testis. Using RT-PCR for confirmation of differential expression, the testicular E_2-upregulation of seven genes that are normally associated with liver, including different forms of vitellogenins and choriogenins, was first reported. Since the hepatic induction of Vgs and Chgs are commonly used biomarkers of exposure to endocrine disrupting compounds (EDCs) (Sumpter and Jobling 1995; Celius et al. 2000), their induction by E_2 in testis suggests a possible role in mediating the adverse effects of EDCs in testis (e.g. Matthiessen et al. 2002). This opens the way for future studies on the EDC regulation of Vg and Chg in testis and its correlation with testicular dysfunctions, in order to determine their potential as biomarkers for endocrine disruption in testis. Another interesting finding was the expression of both Vgs and Vg receptor in untreated sea bream testis. This suggests a role for locally expressed Vg in normal testis physiology, which needs further investigation by cellular localization and functional studies.

The nature of discovery by subtractive hybridization dictates that this task was only the

beginning. Besides the functional characterization of identified ERGs, the E_2-induction of other candidate genes must be confirmed using RT-PCR or differential screening, and more genes may be isolated from the library. The further characterization of the E_2-treated testis subtractive library may, from a fundamental point of view, help to elucidate the mechanisms of estrogen action in sea bream testis physiology and, from a practical point, identify a number of biomarkers for testicular endocrine disruption. Analysis of their expression by quantitative PCR or through the construction of a customized macro/microarray could be used in the future to detect endocrine disruption effects, using non-lethal testis biopsies taken from natural populations.

Other aspects of the estrogen mechanisms of action remain unexplored in fish but have not been investigated in the present study. For instance, the heterodimerization between fish ER subtypes has never been studied but its possibility was suggested in this study by the co-localization of the three ER subtypes in the putative osteoclasts of juvenile sea bream scales. In mammals, the heterodimerization between ERα and ERβ subtypes was demonstrated *in vitro* and *in vivo* and appears to allow the (negative) modulation of the transcriptional activity between ER subtypes or the activation of different sets of target genes than that of the homodimers (Hall and McDonnell 1999; Lindberg et al. 2003; Monroe et al. 2005). The presence of an additional ER subtype in fish may allow the potential formation of more types of heterodimers, and it would be interesting to investigate whether they occur *in vitro* and *in vivo*, as well as their potential consequences.

During the course of this thesis, many new reports appeared on the identification of rapid, non-genomic estrogen actions initiated at the plasma membrane, and their mechanisms have started to unravel. However, it is still not clear if these actions are mediated by a subpopulation of the nuclear ERs that localize to the plasma membrane, by novel membrane receptors unrelated to nuclear ERs, or, most probably, by both (Razandi et al.

2004; Thomas et al. 2005). It would be very interesting to investigate the receptors and mechanisms involved in rapid estrogen actions in fish, such as androgen production in the testis and vasodilatation in the heart (Loomis and Thomas 2000; Agnisola et al. 2004). For example, in the present study sbERα protein was mainly localized to nuclear fractions of the sea bream heart but the localization in membrane fractions was not assessed.

In conclusion, the present thesis provided evidence for the expression of three ER genes and several transcript variants in sea bream, with a different but partially overlapping pattern of tissue distribution and regulation by estrogen and ICI 182,780. Like in mammals, the estrogenic actions within a given fish cell most probably depend on the relative expression levels of ER subtypes and variants in the cell, their subcellular localization, their functional properties and the possibility of interplay among them or with other transcription factors, as well as on the cell-type specific array of intracellular signaling molecules and coregulator proteins, but the presence of two ER subtypes in fish provides an additional variant in this complex picture. The findings and tools obtained (ER subtype/variant cDNAs, ER recombinant proteins, ER subtype-specific antibodies) pave the way for future research on sea bream ER functional characterization, cellular and sub-cellular localization and interaction studies, which are required to understand the relative roles and mechanisms of action of each ER subtype/variant in a number of estrogen-regulated processes in fish. This study started to unravel some mechanisms involved in the poorly understood E_2 effects on calcium metabolism in fish, but many aspects remain to be investigated. Moreover, a new and previously unexplored field of investigation was opened that concerns the identification of ERGs in fish testis, and further characterization of the constructed subtractive library may contribute to understand the mechanisms involved in estrogen actions in testis physiology and to identify biomarkers for assessment of endocrine disruption.

CHAPTER 9

References

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APPENDICES

Appendix I- List of buffers, stock solutions and media

Table of contents:

I.1. General buffers and stock solutions

DEPC-treated water
Add 100 µl of diethyl pyrocarbonate (DEPC) for each litre of dH_2O. Incubate ON at room temperature for the inactivation of RNAses and autoclave to inactivate DEPC.

0.5 M EDTA pH 8.0
Dissolve 186.12 g of disodiumethylenediaminetetra acetic acid (EDTA-Na_2x2H_2O) in 800 ml of dH_2O. Adjust pH to 8.0 with 5M sodium hydroxide (NaOH), check if dissolution is complete and autoclave.

3M NaAc pH 5.2
Dissolve 24.6 g of sodium acetate (NaAc) in 100 ml of dH_2O. Adjust pH to 5.2 with glacial acetic acid and autoclave.

20x SSC (Saline-Sodium Citrate) buffer
175.3 g of NaCl
88.2 g of sodium citrate
Dissolve in 800 ml dH_2O. Adjust pH to 7.0 with a few drops of 10N NaOH. Add dH_2O to 1 L and autoclave.

TE (Tris-EDTA) buffer pH 7.4 / 7.5 / 8.0
10 mM Tris-HCl pH 7.4 / 7.5 / 8.0 (see below), 1 mM EDTA pH 8.0 (see above)

1 M Tris-HCl
For 1 L of 1 M Tris-HCl at a given pH, combine the following amounts of Trizma-Base and Trizma-HCl (Sigma):

	Trizma base (g)	Trizma HCl (g)
1 M Tris-HCl pH 7.4	19.4	132.2
1 M Tris-HCl pH 7.5	23.6	127.0
1 M Tris-HCl pH 8.0	53.0	88.8

Add dH_2O to 1 L, check pH and autoclave.

I.2. Solutions used in agarose gel electrophoresis

6x DNA loading buffer
40 % (w/v) sacarose, 0.25% (w/v) bromophenol blue in stH_2O. Store at 4ºC.

10x MOPS
20.9 g of 3-(N-Morpholino)propanesulfonic acid (MOPS)
2.05 g of sodium acetate
10 ml of 0.5 M EDTA pH 8.0
Add DEPC water to 500 ml, mix, check the pH and adjust to 7.0 with NaOH pellets. Store at room temperature in the dark.

6x RNA loading buffer
40 % (w/v) sacarose, 0.25% (w/v) bromophenol blue in DEPC water. Store at 4ºC.

10x TBE (Tris-Borate-EDTA) buffer
108 g of Trizma base
55 g of boric acid
40 ml of 0.5 M EDTA pH 8.0
Add dH_2O to 800 ml, mix until it dissolves, check the pH and if necessary adjust to 8.3 with boric acid. Add dH_2O to 1 L and autoclave.

I.3. Solutions used in plasmid DNA purification by Alkaline Lysis

GTE (Glucose-Tris-EDTA) buffer
50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0. Autoclave.

3M KAc
60 ml of 5M potassium acetate (KAc)
11.5 ml of glacial acetic acid
Add dH_2O to a final volume of 100 ml and autoclave.

I.4. Solutions used for cDNA library screening

Church-Gilbert hybridization solution
2 ml of 0.5 M EDTA pH 8.0
500 ml of 0.5M sodium phosphate buffer (see below)
350 ml of 20% SDS
Add dH_2O to1 L.

Denaturation solution
1.5 M NaCl, 0.5 M NaOH

1 M $MgSO_4$
Dissolve 246.4 g of $MgSO_4x7H_2O$ in 1 L of dH_2O and autoclave.

Neutralisation solution
1.5 M NaCl, 0.5 M Tris pH 8.0

SM buffer
5.8 g of NaCl
2.0 g of $MgSO_4x7H_2O$
50 ml of 1 M Tris-HCl pH 7.5
5 ml of 2% (w/v) gelatine
Add dH_2O to1 L and autoclave.

0.5 M Sodium phosphate buffer
Dissolve 71 g of Na_2HPO_4 in 800 ml dH_2O. Add 4 ml of H_3PO_4 85% and check if pH is between 7-8. Add dH_2O to a final volume of 1 L and autoclave.

I.5. Solutions used for ligand binding assays and radioimmunoassays

Dextran-coated charcoal
1.5 % (w/v) charcoal and 0.15% (w/v) dextran in phosphate-gelatine buffer (see below). Mix for 5 min and store at 4ºC until use. Add 1.25 volumes to the reaction mixture, incubate 12 min on ice and centrifuge for 12 min at 2000 rpm, 4ºC. Count the supernatant in a scintillation counter.

0.5 M Phosphate buffer pH 7.6
5.75 g of Na_2HPO_4
14.8 g of $NaH_2PO_4x2H_2O$
Add dH_2O to 1 L, correct pH to 7.6 and autoclave. Make 50 ml aliquots and store at -20ºC.

0.05 M Phosphate buffer with gelatine
Dissolve 0.5 g of gelatine in 100 ml dH_2O in the microwave, add 50 ml of 0.5 M Phosphate buffer (see above) and dH_2O to a final volume of 500 ml. Store at 4ºC.

I.6. Solutions used for SDS-PAGE and Western-blot

Blocking solution for Western blot
1x TBS, 10% (w/v) skimmed milk powder, 0.1% (v/v) Tween-20.

Coomassie blue staining solution
0.25% (w/v) Coomassie brilliant blue, 10% (v/v) glacial acetic acid, 45%(v/v) methanol.

DAB substract solution
1x TBS, 0.04% (w/v) 3,3-diaminobenzidine (DAB, Sigma), 0.02 %(w/v) nickel chloride, 0.0025%(v/v) hydrogen peroxide.

Destain solution
10% (v/v) glacial acetic acid, 10%(v/v) methanol.

2x SDS sample buffer
100 mM Tris-HCl pH 6.8, 200 mM DDT, 4%(w/v) SDS, 0.2%(w/v) bromophenol blue, 20%(v/v) glycerol.

Stacking and resolving gel for SDS-PAGE

Reagents	Stacking Gel (Total volume = 4 mL)	Resolving Gel (Total volume = 10 mL)	
Polyacrylamide percentage	**5%**	**8%**	**12%**
ddH_2O	2.92 ml	5.3 ml	4.3 ml
40%Acrylamide:Bisacrylamide*	0.5 ml	2 ml	3 ml
Tris-HCl buffer	0.5 ml of 1M Tris-HCl pH6.8	2.5 ml of 1.5M pH8.8	2.5 ml of 1.5M pH8.8
10% SDS	0.04 ml	0.1 ml	0.1 ml
10% Ammonium Persulfate (APS)	0.04 ml	0.1 ml	0.1 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	4 µl	7 µl	7 µl

* PlusOne ReadySol IEF acrylamide solution with 3% bisacrylamide, Amersham

For the resolving gel: combine water, acrylamide, Tris-HCl buffer and SDS. Add freshly prepared 10% (w/v) APS and TEMED, mix well and pour solution into the gel sandwich (plates assembled with 0.75-1.5 mm spacers), leaving sufficient space at the top for the stacking gel. Gently overlay with dH_2O and allow to polymerize for 30 min.
Pour off aqueous layer, prepare stacking gel as above and pour into the gel sandwich. Immediately insert comb and allow to polymerise for 30 min.

1.5 M Tris, pH 8.8
Dissolve 36.2 g of Tris-base (Merck) in 150 ml ddH_2O, adjust pH to 8.8 with concentrated HCl and add ddH_2O to 200 ml.

1 M Tris, pH 6.8
Dissolve 12.1 g of Tris-base (Merck) in 75 ml ddH_2O, adjust pH to 6.8 with concentrated HCl and add ddH_2O to 100 ml.

10x TBS (Tris Buffered Saline)
24.2 g of Trizma base (Sigma)
80 g of NaCl
Add ddH_2O to 1 L and adjust pH to 7.6 with concentrated HCl.
To make a 1x TBS dilution, dilute 1:10 with ddH_2O.

TBST (TBS-Tween)
TBS 1x with 0.05% (v/v) Tween-20

5x Tris-Glycine running buffer
Dissolve 15.1g of Tris base (Merck) and 94g of glycine in 900ml ddH_2O. Add 50 ml of 10%(w/v) SDS and ddH_2O to a final volume of 1L.
To make 1x running buffer, dilute 1:5 with ddH_2O.

Western Transfer buffer
Dissolve 3.03g of Tris base (Merck) and 28.8 g of glycine in 800 ml ddH_2O. Add 100 ml of methanol, 5 ml of 20% SDS and ddH_2O to 1 L. Store at 4ºC until use.

I.7. Solutions used for immunohistochemistry (IHC)

10x PBS (Phosphate Buffered Saline)
7.60 g of NaCl
1.25 g of $Na_2HPO_4x2H_2O$
0.48 g of $NaH_2PO_4x2H_2O$
Dissolve in 60 ml ddH_2O, correct pH to 7.0 and add ddH_2O to 100 ml. Autoclave.
To make 1xPBS, dilute 1:10 in stH_2O or with DEPC water.

PCT (Phosphate-Carragenin-Triton) buffer
50 ml of 1x PBS
0.7 g of carragenin
500 µl Triton-X
Dissolve overnight at 4°C with gentle agitation, add 1x PBS to 100 ml and store at 4ºC.

PBST (Phosphate-Triton) buffer
PBS 1x with 0.1% (v/v) Triton-X

I.8. Solutions used for *in situ* hybridisation (ISH)

2% or 1% Blocking solution
Dissolve 2g or 1g of Blocking reagent (Boehringer-Mannheim, Germany) in 100ml of maleic acid buffer (see below) at 65ºC, mixing occasionally. Cool to room temperature, aliquot and store at -20ºC.

50x Denhardt's solution
1g of bovine serum albumin (BSA)
1g ficoll
1g polyvinilpirrolidone (PVP)
Add dH_2O to 100 ml, dissolve overnight at 4ºC, filter, aliquot and store at -20ºC.

Developing buffer
100 mM Tris-HCl pH 9.5 (see below), 100 mM NaCl, 50 mM $MgCl_2$

***In situ* hybridization solution**
50 ml of deionized formamide (Sigma)
20 ml of sterile 20x SSC
2 ml of sterile 50x Denhardt's solution (see above)
2ml of torula RNA yeast (50mg/ml, see below)
2ml of 2% (w/v) CHAPS (3-[3-cholamidpropyl dimethylammonio]-1-propanesulfonate, dissolved in DEPC water)
1ml of heparin (10 mg/ml)
Dissolve all the reagents in 80 ml of sterile DEPC water, adjust pH to 6.0 with 2M citric acid solution and make a final volume of 100 ml with sterile DEPC water. Aliquot and store at -20ºC.

Maleic acid buffer
1.161 g of maleic acid
0.8775 g of NaCl
Dissolve in 90 ml dH_2O and adjust pH to 7.5. Add dH_2O to 100 ml, autoclave, allow to cool to room temperature and add 100µl of Tween-20. Store at room temperature.
Final composition is 100mM maleic acid, 150mM NaCl, 0.1% tween-20, pH 7.5.

4% PFA (paraformaldehyde) for ISH
Dilute 10.8 ml of 37% (v/v) paraformaldehyde solution (Sigma) in 89.2 ml of PTW (see below) and use immediately.

PTW (Phosphate-Tween buffer)
PBS 1x with 0.1% (v/v) Tween-20.

Torula RNA yeast (50mg/ml)
Dissolve 0.5g of torula RNA yeast powder (Roche-diagnostics) in 9 ml dH_2O, add 50µl of 5M NaOH and warm to 60ºC until it is completely dissolved. Allow to cool to room temperature, aliquot and store at -20ºC.

1 M Tris-HCl pH 9.5
Dissolve 12.1 g of Tris-base (Merck) in 60 ml ddH_2O, adjust pH to 9.5 with concentrated NaOH and add ddH_2O to 100 ml.

I.9. Solutions used for general tissue processing and histology

Eosin Y 1% aqueous solution
Dissolve 1g of eosin Y (Sigma) in 100ml of ddH_2O and store until use.

Harris haematoxylin solution
Dissolve 1 g of haematoxylin (Merck) in 5ml of absolute ethanol.
Dissolve 10 g of aluminium potassium sulphate x 12 H_2O in 100ml of warmed dH_2O.
Combine the two solutions, boil for 4 minutes, remove from heat and add 0.25 g of mercuric oxide (Merck). Mix well and boil for 1 min or until the dye becomes a dark purple colour.
Cool the solution rapidly under running water and add 4 ml of glacial acetic acid. Filter. Immediately prior to use dilute 50:50 in absolute ethanol and filter the resulting solution. Store the stock solution at room temperature in the dark.

4% PFA for tissue fixation
Dissolve 4 g of paraformaldehyde (PFA) in 90 ml stH_2O, add 10 µl of 5M NaOH and warm to 65ºC with agitation until it clears, in a fume cupboard. Allow to cool to room temperature, add 10 ml of sterile 1M Phosphate buffer pH 7.4 (see below) and correct pH to 7.4 with NaOH, if necessary.
Stable for 1 week at 4ºC.

1M Phosphate buffer, pH 7.4, for the preparation of PFA solutions
31.15 g of $Na_2HPO_4x2H_2O$
11.70g of $NaH_2PO_4x2H_2O$
Dissolve in 200 ml dH_2O, correct pH to 7.4, add dH_2O to 250 ml and autoclave.

I.10. Other buffers

ADBI buffer, for *in vitro* phosphorylation
20mM MOPS, pH 7.2
25mM β-glycerol phosphate
5mM Ethyleneglycol-bis(2-aminoethoxy)-tetraacetic acid (EGTA)
1mM sodium orthovanadate
1mM dithiothreitol (DTT)

TB buffer, for competent cells preparation
10 mM 1,4-Piperazinediethanesulfonic acid (PIPES, Sigma)
55 mM manganese chloride ($MnCl_2$)
15 mM calcium chloride ($CaCl_2$)
250 mM potassium chloride (KCl)
Mix all the components in dH_2O except the $MnCl_2$. Ajust pH to 6.7 with potassium hydroxide, add $MnCl_2$ and filter sterilize the solution (0.45 μm filter). Store at 4º.

Extraction buffer, for DNA extraction
50 mM Tris-HCl pH 8.0
100 mM EDTA pH 8.0
100 mM NaCl
1% (w/v) SDS

TEDG buffer, for protein extraction
50 mM Tris-HCl pH 7.4
1.5 mM EDTA
1 mM DTT
20%(v/v) glycerol

TEDGK buffer, for nuclear protein extraction
TEDG buffer containing 0.6M KCl

I.11. Culture media

LB (Luria-Bertani) broth
Dissolve 1 LB broth tablet (Sigma) / 50 ml dH_2O and autoclave. Final composition for 1 liter: 5 g NaCl, 10 g tryptone, 5 g yeast extract. For LB broth with antibiotics, add antibiotic to the desired concentration (see below) after autoclaving and store at 4ºC.

LB agar plates
Dissolve 1 LB agar tablet (Sigma) / 50 ml dH_2O, autoclave, allow to cool to 55ºC and pour into Petri dishes. Final composition for 1 litre: 5 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar. For selective agar plates, add antibiotic, IPTG or X-Gal to the desired concentration before pouring into petri dishes.

Antibiotics, IPTG and X-Gal:

Ampicillin	Stock 50 mg/ml	Used at 80-100 μg/ml
Chloramphenicol	Stock 34 mg/ml	Used at 34 μg/ml
Tetracycline	Stock 15 mg/ml	Used at 12.5 μg/ml
Kanamycin	Stock 50 mg/ml	Used at 50 μg/ml
IPTG	Stock 0.5M	Used at 0.5 mM
X-Gal	Stock 80 mg/ml	Used at 80 μg/ml

NZY broth

5 g NaCl
2 g $MgSO_4x7H_2O$
5 g yeast extract
10 g NZ-amine (casein hydrolysate)
Add dH2O to a final volume of 1 L. Ajust pH to 7.5 with NaOH and autoclave.

NZY agar plates

Prepare 1 L of NZY broth. Add 15 g agar, adjust pH to 7.5 with NaOH and autoclave. Pour into square 150 mm Petri dishes.

NZY Top agar

Prepare 1 L of NZY broth. Add 0.7% (w/v) agarose and autoclave.

SOB liquid medium, for competent cells preparation

2% (w/v) tryptone
0.5% (w/v) yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$

Appendix II- Basic molecular biology methods

Table of contents:

II.1. Agarose gel electrophoresis to analyse DNA

Agarose gels of different percentages are used to separate DNA fragments according to their sizes:
-0.7% agarose (w/v) to separate fragments > 2000 bp;
-1% agarose for fragments between 250 and 2000 bp;
-1.5% agarose for fragments < 250 bp.

Dissolve agarose in 1x TBE (Appendix I), melt at 100ºC in a microwave oven and cool to 55ºC before addition of ethidium bromide to a final concentration of 0.5 µg/ml. Pour this solution into a gel cast with combs assembled, allow to solidify at room temperature for 30-40 min and transfer to an electrophoresis tank filled with 1x TBE buffer.
Choose an appropriate DNA molecular weight marker to estimate the size of fragments in the sample. Markers used in this project were the 1 kb Plus DNA ladder (Invitrogen) or the λDNA/*Eco*RI+*Hind*III marker 3 (Fermentas), used for larger fragments.
Mix samples or marker with 0.2 volumes of 6x DNA sample buffer (Appendix I), load the gel and run at 4-5V/cm for an appropriate time. Visualize the gel on an UV light trans-illuminator and photograph.

II.2. Agarose gel electrophoresis to analyse total RNA

Prepare 1x TBE buffer by diluting the 10x TBE stock in DEPC water.
Use this buffer to prepare a 1% agarose gel as indicated above and fill an electrophoresis tank, previously washed in 1% SDS for 1 h followed by DEPC water. Mix samples with 0.2 volumes of 6x RNA sample buffer (Appendix I), load the gel, run at 4-5V/cm and visualize on an UV light transiluminator.

II.3. Denaturing agarose-formaldehyde gel electrophoresis to analyse poly(A)$^+$ RNA

Denaturing agarose-formaldehyde gels are used to separate RNAs according to their size, which is estimated by comparison with the migration of an RNA molecular weight marker. The 0.24-9.5Kb RNA ladder (Invitrogen) was used in this project.
For 200ml of 1.5% gel: melt 3 g of agarose in 150 ml of DEPC water, allow to cool to 55ºC, mix with 20 ml of 10x MOPS buffer (Appendix I) and 30 ml of formaldehyde.
Pour into the gel cast with combs assembled and allow to solidify for 1h. Pre-run for 0.5 h at 3-4V/cm.

Add to the poly(A)$^+$ RNA samples and to the marker 3 volumes of denaturation solution containing 2.4 M formaldehyde, 75% (w/v) deionised formamide (Amresco, Ohio, USA), 1.3x MOPS and 67 µg/ml ethidium bromide. Denature for 5 min at 65ºC, cool on ice and add 0.2 volumes µl of 6x RNA sample buffer (Appendix I). Load the pre-run gel, run at 3-4V/cm for an appropriate time and visualize on an UV light transiluminator.

II.4. Concentration of DNA/RNA solutions by ethanol precipitation

Estimate the volume (V) of DNA solution. Add 2.5V of cold 100% ethanol and 0.1V of 3 M NaAc pH 5.2 (Appendix I), mix well and incubate for at least 1h at -80ºC or ON at -20ºC.
Pellet DNA by centrifugation for 15 min in a microcentrifuge at 4ºC and maximum speed. Wash the pellet with 0.5-1 ml of cold 75% ethanol for 5 min at 4ºC and maximum speed. Repeat the washing step, air dry the pellet and resuspend in an appropriate volume of stH_2O or TE buffer (Appendix I).
To concentrate a RNA solution, proceed as above except that washing steps are done with 75% ethanol prepared in DEPC water, and the pellet is resuspended in DEPC water.

II.5. Preparation of XL1-Blue MRF' *E. coli* competent cells [1]

Streak out a glycerol of XL1-B MRF' *E. coli* (Stratagene, Appendix IV) on an LB agar plate containing 80 µg/ml ampicillin (Appendix I) and incubate ON at 37ºC.
Isolate one single colony, inoculate in 20 ml of LB broth and grow ON at 37ºC with orbital shaking. Use 50 µl of this ON culture to inoculate 250 ml of SOC medium (Appendix I) in a 2-liter flask and grow at 18ºC with vigorous agitation (200-250 rpm) until Abs_{600} reaches 0.6.
Incubate the culture for 10 min on ice, transfer to chilled 50 ml Falcon tubes and pellet cells for 10 min at 2500xg, 4ºC. Gently resuspend the pellets in a total of 80 ml ice cold TB (Appendix I), incubate on ice 10 min and spin down as above. Resuspend cells in a total of 20 ml of ice cold TB, add DMSO (dimethylsulphoxide) to a final concentration of 7% (v/v) and incubate on ice for 10 min. Dispense 100 µl aliquots into sterile 1.5 ml microcentrifuge tubes, snap freeze in liquid nitrogen and store at -80ºC until use (stable for at least 1 month).

II.6. Restriction enzyme digestions

General restriction enzyme reaction (analytic):

PCR or plasmid DNA - 0.5-2 µg
10x restriction enzyme buffer- 1 µl
Restriction enzyme - 5-6 U
stH_2O up to - 10 µl

Incubate for 1.5 h at 37ºC, add 2 µl of 6x DNA sample buffer and run on a 0.7-1.5% agarose gel. When higher amounts of digestion product were required (preparative digestion), all volumes were increased in the same proportion (2-5 times). All restriction enzymes and respective buffers were purchased from Promega or Amersham Biosciences.

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[1] As described in Inoue, H., Nojima, H. et al. (1990). "High efficiency transformation of *Escherichia coli* with plasmids." Gene 96(1): 23-8.

Appendix III- Vectors used in this study

Vector name	Supplier	Application	Selection	Promoters
pGEM-T	Promega	Cloning of PCR products	Amp+ B/W	T7 / SP6
pGEM-T Easy	Promega	Cloning of PCR products	Amp+ B/W	T7 / SP6
pBlueScript SK-	Stratagene	Isolation and sequencing of cDNA clones from cDNA libraries	Amp+ B/W	T7 / T3
pBlueScript II KS	Stratagene	Subcloning	Amp+ B/W	T7 / T3
pCRT7 NT TOPO	Invitrogen	Expression in *E. coli* or coupled *in vitro* transcription and translation	Amp	T7

Amp = ampicillin selection of cells containing the plasmid
B/W = Blue/white selection color selection of plasmids containing an insert (see section 2.8)

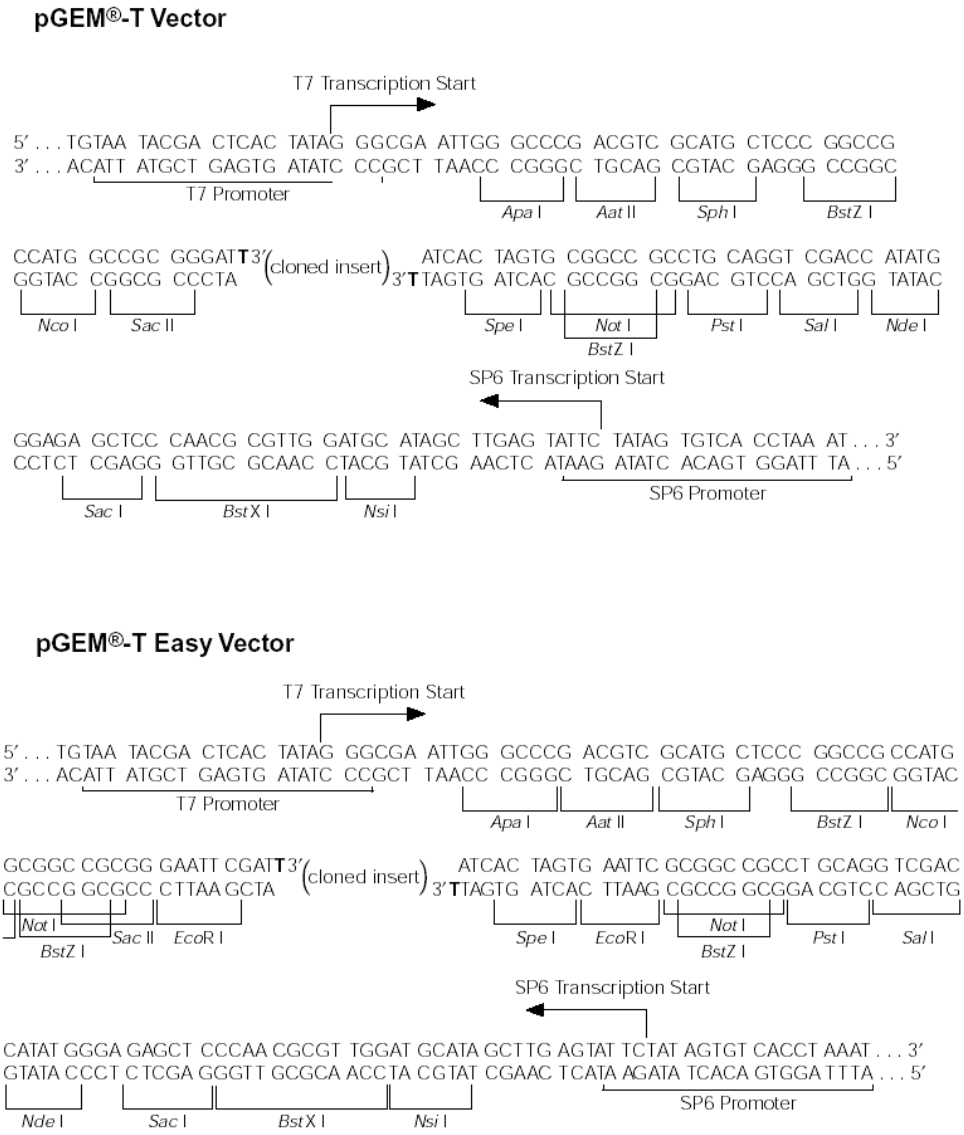

Figure III.1. The promoter and multiple cloning region of the pGEM-T and pGEM-T Easy Vectors (from pGEM-T and pGEM-T Easy vector systems Technical manual, Promega).

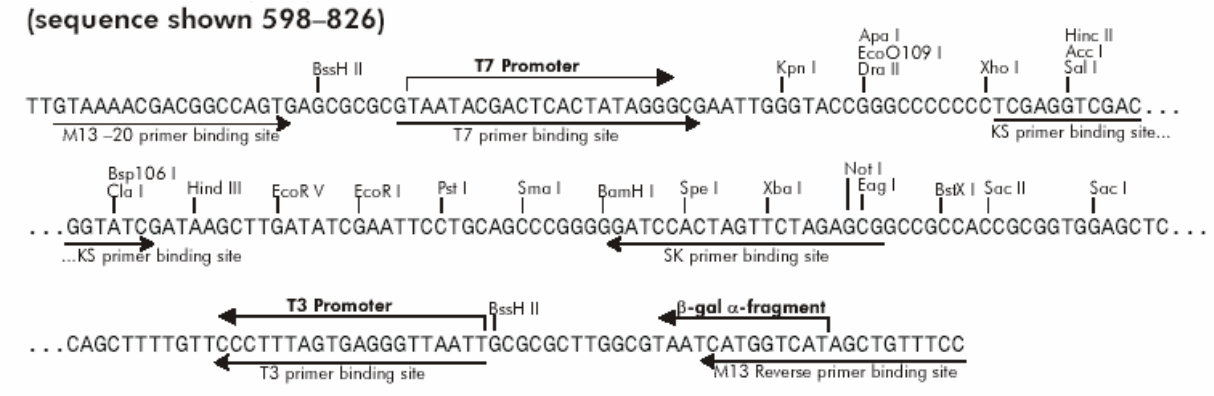

Figure III.2. The promoter and multiple cloning region of pBlueScript SK(+/-) phagemid (from UNI-ZAP XR cDNA cloning kit instructions manual, Stratagene)

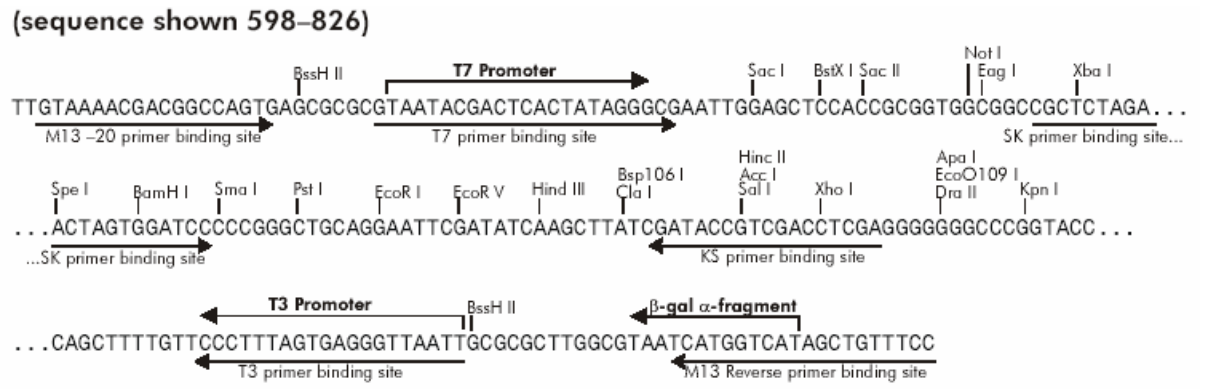

Figure III.3. The promoter and multiple cloning region of pBlueScript II KS (+/-) phagemid (from pBluescript II Phagemid Vectors instructions manual, Stratagene).

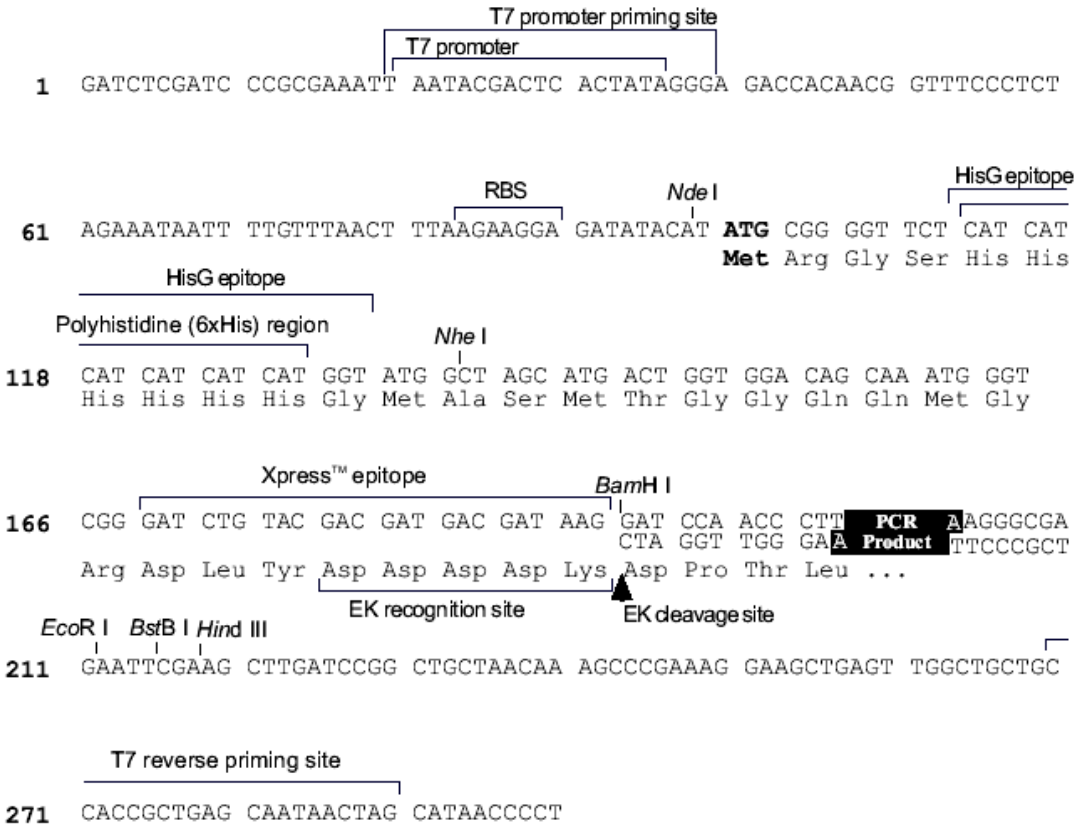

Figure III.4. Cloning site for the pCR T7/NT-TOPO expression vector. Indicated are the T7 promoter binding site, ribosome-binding site (RBS) and N-terminal tag coding region (from pCR®T7 TOPO® TA Expression Kits instructions manual, Invitrogen).

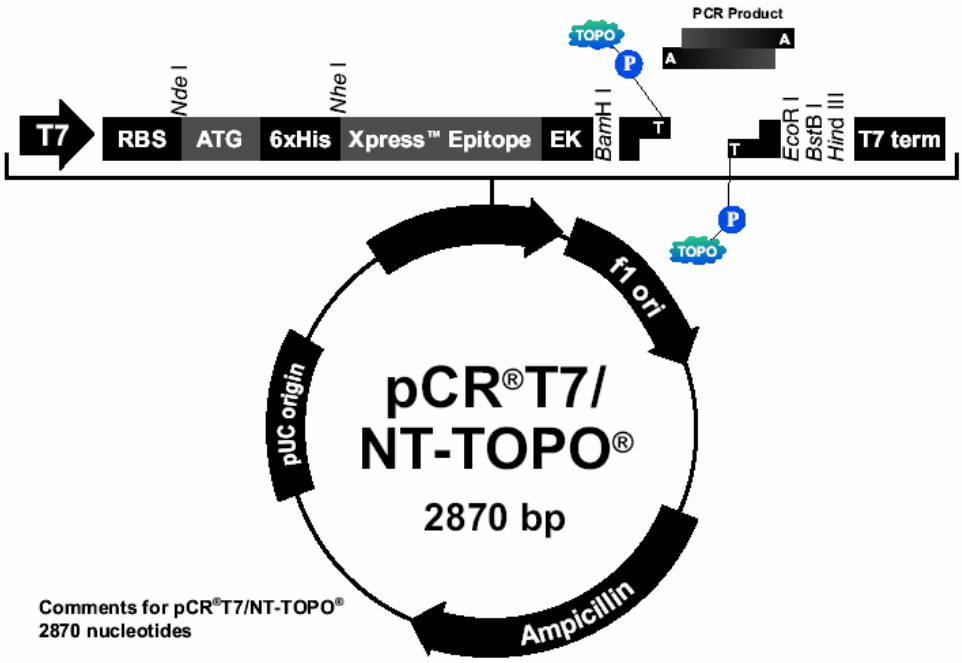

Figure III.5. Circular map of the pCR T7/NT-TOPO expression vector (from pCR®T7 TOPO® TA Expression Kits instructions manual, Invitrogen).

Appendix IV- Bacterial strains (*E. coli*) used in this study

Strain	Supplier	Application	Selection
XL1 Blue MRF'	Stratagene	Library screening Preparation of competent cells (DNA cloning)	Tetracycline
SOLR	Stratagene	Excision of cDNA library clones in pBluescript from Uni-ZAP XR	Kanamycin
TOP10F´	Invitrogen	Cloning of PCR products	Tetracycline
BL21(DE3)pLysS	Invitrogen	Expression of recombinant proteins	Chloramphenicol

IV.1. Preparation of XL1-Blue MRF' *E. coli* host cells for library phage plating

Inoculate an appropriate volume of LB broth medium (Appendix I) with a single colony of XL1-Blue MRF' *E. coli* cells isolated on tetracycline LB agar plates (12.5 μg/ml). Grow cells ON at 30 ºC with shaking, spin at 500xg for 10 min and discard the supernatant. Gently resuspend the pellet in half of the original volume with sterile 10 mM $MgSO_4$ (Appendix I).

IV.2. Preparation of SOLR *E. coli* host cells for in vitro excision of phagemids

Inoculate an appropriate volume of LB broth medium with a single colony of SOLR *E. coli* cells isolated on kanamycin LB agar plates (50μg/ml). Grow cells ON at 30 ºC with shaking, spin at 500xg for 10 min and discard the supernatant. Gently resuspend the pellet in half of the original volume with sterile 10 mM $MgSO_4$.

Appendix V- List of primers used in this project

F or R indicate forward or reverse primer, both represented in the 5'-3' sense. Annealing temperature used for each pair of primers (Ta, ºC) and product size (bp) are shown. In some cases, the target gene/variant name is indicated in blankets after each primer name.
Code for degenerate oligos is N=A/G/C/T, R=A/G, Y=C/T, M=A/C, W=A/T, S=G/C and K=G/T.

Primers used to clone the sbERβb probe:		**F/R**	**Ta (ºC)**	**Product (bp)**
ERf1	CGCAAATGCTACGA**M**GT**K**G	F	51	320
ERr1	ATCATCATGTTGGC**Y**TC**N**G	R		

Primers used to clone sbERα alternative transcripts:				
era_1a	CCCCTGGCTACTACTCTGCT	F	55	514 (wt ERα)
era_1as	TTCATCATGCCCACTTCGT	R		293 (F1B) 244 (F1C)
sbERacodF1	GACCCAGGATCAGCCCAGT	F	58	1537(wt ERα)
E4	TG**Y**TCCAT**K**CC**K**TT**R**TT**R**CT	R		569 (A22) 244(A43)

Primers used to clone the full-length sbERα coding region:				
sbERacodF1	GACCCAGGATCAGCCCAGT	F	60	1910
sbERacodR1	TCTCCCTAAACAACTACAGCTACAAA	R		
era_xps3	GGATCCCAACATGGACCCCGAAG	F	42	1838
era_xpas	ATGTCTCTCTAGACAACTACA	R		

Primers used to clone the sbER coding regions in the pCR T7/NT expression vector:				
ERacodf1 (ERα)	ATGTACCCCGAAGACAGCCG	F	58	1742
ERacodr1 (ERα)	TTTCATTCTGTAGGTTTCATAGGAT	R		
ERb1codf2(ERβa)	ATGGCCGTTGCCTGCTC	F	58	1704
ERb1codr1 (ERβa)	CATTGCATCTGAATTCTGATCCG	R		
ERb2codf1 (ERβb)	GCGTCCCCCGGGCTGGATT	F	63	2009
ERb2codr1 (ERβb)	TGAGTCTCTTACAGTCCGTTCACTA	R		

Primers for semi-quantitative RT-PCR

Internal control (18S rRNA):				
RT18SFw	TCAAGAACGAAAGTCGGAGG	F	59	495
RT18SRv	GGACATCTAAGGGCATCACA	R		

Primers for sbERs:				
era_2a (ERα)	CCCATCCAGTCAGCATTCA	F	57	374
era_2as (ERα)	TTGTCACGCCGCAGAACG	R		
erb1_5a (ERβa)	GCTGATGATCGGACTGATGTG	F	59	348
erb1_5as (ERβa)	GGTGTACTGTTGGCGGAAAG	R		
erb2_4a (ERβb)	TGATGATGTCACTCACCAACC	F	54	291
erb2_4as (ERβb)	TTCAGCTCACGAAACCGA	R		

Primers for semi-quantitative RT-PCR (continuation)

Primers for sbERα variants:		F/R	Ta (ºC)	Product (bp)
MCERaf286(wtERα)	CTTTATTCCCACTCCACCC	F	53	388
MCERar673(wtERα)	AGCCCTCACAGGACCACA	F		
F1CF1 (F1C)	CACCTCAACACCCATCTACAGGA	F	55	129
era_2as (F1C)	TTGTCACGCCGCAGAACG	R		
era_1a (F1B)	CCCCTGGCTACTACTCTGCT	F	55	202
F1BR2 (F1B)	GGCACATATAGTCATTGTGACCT	R		
PP_ERalt22F1	GACCCAGGATCAGCCCAGT	F	61	1458(wtERα)
PP_ERalt22R1	CAATCTGGATGCTCGGCTC	R		490 (A22)
				165 (A43)

Primers for estrogen-responsive genes:				
cor F (ChgL)	AGAGGGATGCTGTCGTAG	F	56	290
cor R (ChgL)	GTGATGCCTTTGGTAGTG	R		
6B F (ChgH)	GCCCTTGTCCTGACTCTT	F	53	166
6B R (ChgH)	AGTGACCTGGGAACTCCA	R		
Vtg1 F (VgI)	GGCTTTGCATCTTCCCTAC	F	54	204
Vtg1 R (VgI)	TCTTGTGCCAGAACTTGGT	R		
Vtg2 F1 (VgII)	CACTTGGCATTGGTCTCCC	F	58	130
Vtg2 R2 (VgII)	ATGGTGCACTCAGCTGCATG	R		
ApoF2 (ApoaI)	TTCTGCTGGCTGTCGGCTCT	F	53	432
Apo R (ApoaI)	TTTCCTCATCGTGCTTGG	R		
Betafib F (FgB)	ACTGCTGGTAACTGTTTCTTGG	F	56	148
Betafibr R (FgB)	CCTTGGAGCACTGTTTGGA	R		
GamafibF (FgG)	ATCACTCAGGAGCAGCAAAT	F	55	250
Gamafib R (FgG)	TCTCACAATAGACCAGGAACG	R		
87f1 (C3)	GACGGCTTTCTGCCTCATT	F	57	191
87r1 (C3)	GGATCTCCCGGTTCAGTTTG	R		
37 F (Ebag9)	ATGGAGCAGCAAAGGAAG	F	53	249
37 R (Ebag9)	GTTGGTGGAAACAAGGTG	R		
TransF (Trf)	GGTGTCTTGTTGAGGGAGC	F	54	154
TransR (Trf)	TCACTGACTGGCACTGGACT	R		
B1.1 F (CysP)	TGACACCAGTGAAGGACCAG	F	53	156
B1.1 R (CysP)	AGCCGTTATTGCCGAAAC	R		
52f (Trip4)	ACCGACTGCCTGTCTCAG	F	53	266
52r (Trip4)	AAATAATCCTCCGTGTTCC	R		
vtgrf1 (VgR)	CTGCCGATGCTAATATGTCT	F	56	271
vtgrr2 (VgR)	TCTCGTCAGTCCCATCCTC	R		
zpaxf1 (ZPAX)	GCCAGACTTGACTTGCTTC	F	53	373
zpaxr1 (ZPAX)	GGATATGGTTTGCCTGTAAT	R		