Cloning of oestrogen receptor beta from Old and New World primates: identification of splice variants and functional analysis

Jayne E Sierens, Graeme A Scobie, Julie Wilson and Philippa T K Saunders

MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK

(Requests for offprints should be addressed to P Saunders; Email: p.saunders@ed.ac.uk)

Abstract

Oestrogens have a major impact on reproductive function in both males and females. Two oestrogen receptor genes known as ER α (*ESR1NR3A1*) and ER β (*ESR2NR3A2*) have been cloned. Splice variant isoforms of the ER β gene have been identified in human, bovine and rodents and it has been suggested that the existence of these forms can influence oestrogen responsiveness. In the human, splicing of an alternative eighth exon results in the formation of a C-terminal variant called hER β cx, or hER β 2, but this isoform has not been identified in other species. The aim of the present study was to clone ER β cDNAs from primates so as to determine how closely they resembled the ER β isoforms found in the human. The two species studied were the stump-tailed macaque (*Macaca arctoides*), an Old World primate, and the common marmoset (*Callithrix jacchus jacchus*), a New World primate.

Full length ER β (wild type, ER β 1) cDNAs were cloned from macaque and marmoset; they encoded proteins of similar size to those found in human (59 and 54 kDa, long and short forms respectively) and shared significant sequence homology (97.5% in macaque and 93.8% in marmoset) with the human peptide sequence. Full length cDNAs homologous to the hER β 2 variant were identified in both primates. Marmoset ER β 2 was slightly shorter than that of human ER β 2 (54 kDa compared with 55 kDa) and did not contain the peptide sequence used to raise an anti-hER β 2 antibody. All the macaque ER β 2 cDNAs contained 56 bp of intronic sequence which included an in-frame stop codon resulting in translation of a truncated protein (~35 kDa). In all three species, truncated, alternatively spliced mRNAs lacking exon 5 were isolated on multiple occasions from all tissue extracts. In transient transfection assays, ER β 2-containing constructs were unable to induce transcription of an oestrogen response element (ERE) reporter plasmid in the presence of oestradiol. ER β 1 from human, macaque and marmoset exhibited minor differences in their ability to induce transcription of the ERE reporter when incubated with different ligands (oestradiol, PPT, DPN, 5- α -androstane-3- β , 17 β -diol (3 β Adiol), genistein) and this may be due to amino acid substitutions within their ligand binding domains.

In conclusion, we have identified and cloned wild type ER β (ER β 1) from macaque and marmoset and demonstrated that splice variant mRNAs homologous to hER β 2 are formed in both species. The marmoset monkey, therefore, provides a suitable animal model in which to investigate the impact of ER β variant expression on tissue responsiveness to oestrogens.

Journal of Molecular Endocrinology (2004) **32**, 703–718

Introduction

Oestrogen action is mediated via specific receptors (ER). The first oestrogen receptor was cloned from a human breast cancer cell line in 1986 (Green *et al.* 1986). In 1996, a second ER was cloned from rat (Kuiper *et al.* 1996) and human (Mosselman *et al.*

1996) tissues. The two receptors are known as oestrogen receptor alpha (ER α , *ESR1NR3A1*) and oestrogen receptor beta (ER β , *ESR2NR3A2*) respectively. Evolutionary studies have suggested that the vertebrate steroid receptors evolved from an ancestral receptor with oestrogen receptor-like function by serial genome expansion (Thornton

2001, Thornton *et al.* 2003). Human (h) ERα and hERβ contain significant sequence homology and are classified as subgroup A of the nuclear receptor subfamily 3 (Nuclear Receptors Nomenclature Committee 1999). They are the products of two genes located on different chromosomes (Enmark *et al.* 1997) and phylogenetic analysis suggests that they arose from a single duplication event at least 450 million years ago (Kelley & Thackray 1999).

ERα and ERβ both bind to a range of oestrogenic ligands although they exhibit different affinities for some ligands notably genistein, raloxifene and tamoxifen (Kuiper *et al.* 1997, Barkhem *et al.* 1998). Key domains within ERα and ERβ include the DNA binding domain (C) and two transactivation domains (AF-1 and AF-2) located in the N (A/B domain) and C terminal (E/F domain) portions of the protein respectively (see reviews by Saunders 1998 and Nilsson *et al.* 2001). The crystal structures of the ligand binding domains of ERα and ERβ have been determined and their overall arrangement shown to be similar, both containing 12 helices (Pike *et al.* 1999). *In vitro* studies have demonstrated that homodimers (ERα-ERα or ERβ-ERβ), or heterodimers (ERα-ERβ), can be formed when both isoforms are expressed in the same cell (Cowley *et al.* 1997, Pettersson *et al.* 1997). Results obtained from *in vitro* and *in vivo* studies have suggested that one role played by ERβ may be to modulate ERα-mediated transcriptional activity (Hall & McDonnell 1999, Weihua *et al.* 2000).

Splice variant isoforms of the ERβ gene have been identified in a number of species including the human (Lu *et al.* 1998, Moore *et al.* 1998, Ogawa *et al.* 1998b, Inoue *et al.* 2000), the bovine (Walther *et al.* 1999) and rodents (Chu & Fuller 1997, Lu *et al.* 1998, 2000). One striking feature revealed by sequencing of these products is that the variant most commonly found in rodent tissues, formed by an insertion of 18 amino acids within the ligand binding domain (Chu & Fuller 1997, Pettersson *et al.* 1997), has not been detected in human tissue extracts (Chu *et al.* 2000; authors' unpublished observations). Furthermore, the extensive splicing of alternative exons at the 3' end of the human ERβ gene which results in formation of a number of C-terminal variants (Moore *et al.* 1998) has not been reported for other species.

We have previously examined the expression of ERβ proteins using a polyclonal antibody directed

against the hinge (D) domain of hERβ as well as monoclonal antibodies specific for hERβ wild type protein (ERβ1) and two of the hERβ C-terminal splice variants (ERβ2, also known as ERβcx, and ERβ4 (Moore *et al.* 1998, Ogawa *et al.* 1998b)). In human tissues immunopositive staining of cell nuclei has been reported using all these antibodies (Saunders *et al.* 2000, 2001, 2002a, Critchley *et al.* 2002, Scobie *et al.* 2002). In addition using the polyclonal antibody, ERβ protein has also been detected in tissues from a wide range of species including primates and rodents (Saunders *et al.* 2000, 2001, Nie *et al.* 2002, Zhou *et al.* 2002). Western blot analysis has shown that this antibody will bind to both hERβ1 and hERβ2 recombinant proteins. Immunohistochemistry using the monoclonal antibody specific for the C-terminus of hERβ1 results in detection of ERβ protein in granulosa cells of the primate ovary (Saunders *et al.* 2000) but we have not previously investigated whether an ERβ2 variant protein is expressed in the primates.

The aim of this study was to determine whether ERβ splice variant isoforms which resemble those of the human exist in closely related mammalian species. We therefore cloned ERβ cDNAs from two primates, namely the stump-tailed macaque (*Macaca arctoides*), an Old World primate, and the common marmoset (*Callithrix jacchus jacchus*), a New World primate, estimated to have diverged from the human line ~25–27 and ~30–35 million years ago respectively.

Materials and methods

Human and primate tissues

Marmoset tissues were collected from adult captive bred animals ($n=5$) maintained in a colony that has been closed since 1973. Testes with epididymes attached were also obtained from three adult stump-tailed macaques that were being surgically castrated under general anaesthesia for colony management purposes. These samples were the same as those used in previous studies (Saunders *et al.* 2001). Additional blocks of fixed tissues were from an archive held at the Human Reproductive Sciences Unit. Sections of human endometrium were a gift from Professor Hilary Critchley (University of Edinburgh).

Table 1 Primers sequences used for the isolation and sequencing of cDNAs containing the entire open reading frame of hERβ1 and ERβ2

Name	5'-sense	3'-antisense	Size (bp)
ERβ1L	CCACGAATCTTTGAGAACATTAT	GTGGGCCAGTTCACCTCAGG	1698
ERβ2L	CCACGAATCTTTGAGAACATTAT	GTGAAAATGTTACCCAAGAT	1316
GAPDH	GAACGGGAAGCTCACTGGCAT	GTCCACCACCCTGTTGCTGTAG	301
100AS		GCTATAGAATGTCATGGCTGGA	seq
Seq1	ATGAATTACAGCATTCCCAGCA		seq
Seq2	TATGGAGTCTGGTCGTGTGA		seq
Seq3	GTGAAGTGTGGCTCCCGGAG		seq
Seq4	TTTGTGGAGCTCAGCCTGTT		seq

Immunohistochemistry

Antibodies directed against C-terminal peptides specific for hERβ1 and hERβ2 (hERβcx) were raised as described previously (Saunders *et al.* 2000, 2002a). The specificity of these antibodies has been validated using Western blotting with recombinant proteins and tissue extracts (Critchley *et al.* 2002, Saunders *et al.* 2002a). Immunohistochemistry was performed according to standard procedures described previously in detail (Saunders *et al.* 2000, 2001, 2002a). Briefly, all sections were subjected to heat-induced antigen retrieval. Anti-hERβ1 (clone PPG5/10, code MCA1974S; Serotec, Oxford, UK) was diluted 1:100 and anti-ERβ2 (clone 57/3) was diluted 1:40 in NRS/TBS/BSA. Sections were incubated with the primary antibody overnight at 4 °C, washed with TBS and incubated with the secondary antibody (rabbit anti-mouse, Dako, Ely, Cambs, UK) diluted 1:500 in NRS/TBS/BSA for 1 h at room temperature. Following washes in TBS, sections were incubated with avidin-biotin-horseradish peroxidase linked complex (Dako,) and bound antibodies were visualised using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako). Images were captured using an Olympus Provis microscope (Olympus Optical Co, London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co., Rochester, NY, USA) and assembled using Photoshop 6 (Adobe, Mountain View, CA, USA).

Synthesis of complementary cDNAs

Frozen tissue samples (ovary, testis or epididymis) were ground up under liquid nitrogen using a pestle and mortar, divided into approximately

50 mg samples, and stored at -70 °C. RNA was extracted using Tri Reagent (Sigma, Poole, Dorset, UK) according to the manufacturer's instructions, dissolved in RNase-free water, and 5 µg total RNA were subjected to reverse transcription using Expand Reverse Transcriptase (Roche, Lewes, East Sussex, UK) and an oligo dT primer. cDNAs were purified by heating to 100 °C for 5 min, followed by RNase treatment at 37 °C for 15 min and finally by passing through a High Pure PCR Purification column (Roche) and were quantified on a Pharmacia Biotech Genequant (St Albans, Herts, UK). All samples were adjusted to a final concentration of 5 ng/µl in TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA).

Screening of cDNAs using primers specific for the C terminus of ERβ1 and ERβ2

Complementary DNAs prepared from human, macaque and marmoset tissues were used in PCR reactions containing AGS Gold Taq (Hybaid, Ashford, Middlesex, UK). The sense primer which was located in exon 7 (hERβs; 5'-GGCATCTTCCTCCCAGCAGCA) annealed to both hERβ1 (AB006590 starting at position 1432) and hERβcx/2 (AB006589 starting at position 2608). This primer was used in conjunction with one of two antisense primers which were specific for hERβ1 (antisense strand, 5'-CACTGAGACTGTGGGTCTCTGGGA, ends at 1691, expected size 259 bp) and hERβ2 (antisense strand, 5'-CACTGCTCCATCGTTGCTTC, ends at 2762, expected size 155 bp). Control reactions to ensure that intact cDNA was present contained

primers directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). PCR conditions were as follows: 1 cycle of 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, with a final cycle of 72 °C for 10 min.

Amplification of full length ER β 1 and ER β 2 cDNAs

Full length cDNAs were amplified from cDNA pools using primers homologous to human ER β sequences 5' of the ATG start (sense) and 3' of the TGA stop (antisense). Primer pairs (designated as ER β 1L and ER β 2L) are given in Table 1. PCR conditions were the same as those used above except that the annealing temperature was reduced to 55 °C and the extension time increased to 1 min 30 s. PCR products were purified through a Clontech 400 column (BD Biosciences, Cowley, Oxon, UK) and directly sequenced using the primers used for PCR amplification as well as internal sequencing primers homologous to hER β (Table 1). To control for introduction of sequence changes from PCR amplification, the reactions were conducted several times on cDNAs prepared from more than one tissue on more than one occasion; a consensus sequence was determined by multiple alignment.

Preparation of ER β 1/ER β 2 expression constructs

ER β 1 and ER β 2 cDNAs generated from all three species using the 'long' primer pair were cloned into the TA cloning site of the eukaryotic expression vector pCDNA3.1/V5-His (Invitrogen, Paisley, Strathclyde, UK) according to the manufacturer's instructions. This plasmid was chosen because it contains a T7 RNA polymerase site upstream of the insertion site and can therefore be used for *in vitro* synthesis of proteins. The plasmids were sequenced fully and purified using caesium chloride according to standard methods.

Transcription/translation analysis of cloned cDNAs (TNT)

Purified pCDNA 3.1 plasmids (1 μ g each) were added to separate tubes containing 50 μ l T7 coupled reticulocyte lysate (TNT, Promega,

Southampton, UK) in the presence of 20 μ Ci ³⁵S-methionine (Amersham, Little Chalfont, Bucks, UK). Transcription coupled translation was allowed to proceed for 90 min at 30 °C. Expressed proteins (2.5 μ l of each lysate) were separated on a 4–20% denaturing gradient gel (Invitrogen) and visualised by direct exposure of the gel to X-ray film (XLS, Kodak).

Oestrogenic ligands

17 β -Oestradiol (E-8875), 5- α -androsterone-3- β , 17 β -diol (Adiol; A-7630) and 4',5,7-trihydroxyisoflavone (Genistein; G-6649) were all purchased from Sigma. DPN and PPT were purchased from Tocris Cookson Ltd (Avonmouth, Bristol, UK). DPN (2,3-bis(4-hydroxyphenyl)-propionitrile) is marketed as an ER β agonist with a 70-fold selectivity over ER α (Meyers *et al.* 2001). PPT (4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol) is marketed as an ER α agonist and is reported to exhibit 410-fold selectivity for ER α over ER β (Stauffer *et al.* 2000). All ligands were reconstituted in ethanol (100% v/v) apart from genistein which was dissolved in DMSO; all stocks were stored at –20 °C.

Transient transfections

Hep G2 cells (human hepatocellular carcinoma, ECACC; Health Protection Agency, Porton Down, UK) were maintained in DMEM phenol red-free medium (Sigma) supplemented with 10% charcoal-stripped fetal bovine serum (GIBCO, Paisley, Strathclyde, UK), D-glucose (Sigma), L-glutamate (Sigma), non-essential amino acids (Ambion, Abingdon, Oxon, UK), fungizone (GIBCO) and penicillin-streptomycin (Sigma). Cells were plated out into 12-well plates (Corning, Fisher Scientific, Loughborough, Leics, UK) at a density of 1×10^5 cells per ml media 24 h prior to transfection. Human ER α was obtained as a full length cDNA in a PIC OH plasmid (ATCC, LGC Promochem, Teddington, Middx, UK) and subcloned into pCDNA3.1 using BamHI and EcoRV restriction sites. ER β 1 and ER β 2 expression vectors were prepared as above. A reporter construct containing 3 $\times$ vitellogenin oestrogen response element (ERE)-linked to TK-luciferase was a kind gift from S C Nagel and D P McDonnell (Hall & McDonnell 1999). In each well, cells were co-transfected with

1 μ g ER, 0.5 μ g 3 $\times$ ERE-luciferase reporter and 100 ng *Renilla* cytomegalovirus (CMV) internal control; to introduce the DNA into the cells they were incubated for 4 h with JetPEI transfection reagent (Cambridge Biosciences, Cambridge, Cambs, UK) and the DNA mix. Ligands or vehicle (ethanol) were added and cells were incubated for a further 24 h before the medium was replaced with fresh medium containing the appropriate ligand for a further 24 h. Cells were harvested and assayed using a luciferase assay kit from Promega. Results were normalised against the *Renilla* internal control, and the amount of luciferase activity obtained in the absence of ligand (ethanol alone) was designated as 1; all results were expressed as a fold increase relative to the value obtained in the ligand-free control present in each experiment. All treatments were performed in duplicate wells and experiments repeated at least 3 times. Results are presented as the mean $\pm$ S.D. of the fold changes seen in the individual experiments.

Results

Detection of ER β 1 and ER β 2 mRNAs in samples from multiple human and primate tissues

RT-PCR analysis using hER β 1- and hER β 2-specific primer pairs resulted in amplification of DNA from ovaries, testes and epididymes from marmoset and from testes and epididymes from macaque (Fig. 1). The sizes of the amplified DNAs were identical to those from human ovaries and testes which were used as positive controls (Fig. 1); no reaction product was seen in negative control samples lacking cDNA. In a more extensive comparison of tissues from human and marmoset the pattern of expression of mRNAs was identical in both species: ER β 1 and ER β 2 (heart, ovary, testis), ER β 1 alone (brain, liver, placenta), neither transcript (kidney). Preliminary sequence analysis confirmed that the DNA fragments from marmoset and macaque were homologous to hER β 1 and hER β 2 (not shown).

Immunohistochemistry using hER β 1- and hER β 2-specific monoclonal antibodies

Consistent with the identification of ER β 1 mRNAs in primate tissue extracts and with previous

Figure 1 Detection of ER β 1 and ER β 2 mRNAs in cDNAs prepared from human, marmoset and macaque tissues. RT-PCR analysis of reproductive tissues from human (hum), marmoset (marm) and macaque (maca). Using sequence-specific primers DNA fragments of the size predicted for ER β 1 (top panel, 259 bp) and ER β 2 (middle panel, 155 bp) were identified in macaque testes (te) and epididymes (ep) as well as in ovaries (ov), testes (te) and epididymes (ep) from the marmoset. The fragments were of an identical size to those in control human tissues (ovary, testes). All samples were positive for the GAPDH control (lower panel, 301 bp). m, molecular weight marker.

immunohistochemical data (Saunders *et al.* 2000), ER β 1 protein was immunolocalised to endometrium (Fig. 2a–c) and testes (Fig. 2g–i) from human, macaque and marmoset using a monoclonal antibody specific for the C-terminus of hER β 1. However, when staining reactions were undertaken on the same tissue panel using an hER β 2-specific antibody, staining was only detected in human tissues (Fig. 2d, j) and no immunopositive reaction was detected in any of the primate tissues examined (Fig. 2e, f, k, i, and unpublished observations).

Sequence analysis of full length cDNAs encoding ER β 1 and ER β 2 variants in macaque and marmoset

Double stranded DNA of the expected sizes were amplified from macaque and marmoset cDNA pools using primers located upstream of the ATG in human ER β and downstream of the stop site

Figure 2 Immunohistochemical evaluation of ER β 1 and ER β 2 expression in endometrium and testes from human, macaque and marmoset using variant-specific monoclonal antibodies. Sections from human (a, d, g, j), macaque (b, e, h, k) and marmoset (c, f, i, l) were stained with hER β 1- or hER β 2-specific monoclonal antibodies. Positive staining of cell nuclei was observed for ER β 1 in endometrium (a–c) and testes (g–i) from all three species. However when the same tissues were stained for ER β 2 only human endometrium (d) and testis (j) were immunopositive.

in either hER β 1 or hER β 2/ β cx (accession no. NM_001437). The nucleotide sequences obtained have been lodged in Genbank (accession nos Y09372, AF393816, AF393815).

The peptide sequences predicted from the open reading frames of the ER β 1 cDNAs from macaque and marmoset are compared in Fig. 3A. Note that there is a high degree of sequence conservation with 97.5% (517/530) homology between macaque and human and 93.8% (497/530) homology between marmoset and human. In two positions

(amino acids 341 and 478) identical amino acid substitutions were present in macaque and marmoset but all other changes were different in the two species. The sequence homology within the peptide used to raise the ER β 1-specific monoclonal antibody (underlined at the C-terminus of hER β 1) was 18/19 and 17/19 for macaque and marmoset respectively compared with human.

A full length protein of 486 amino acids was predicted from the open reading frame (ORF) of the marmoset ER β 2 cDNA (C-terminal portion

of protein illustrated in Fig. 3B). Comparison with human ER β 2 showed 91.1% amino acid conservation (451/495); however the protein was slightly shorter than the human protein and the peptide sequence used to raise the human ER β 2-specific monoclonal (underlined in Fig. 3B) was not conserved in the marmoset. Although a cDNA of comparable size to hER β 2 was amplified from macaque cDNA pools, sequence analysis revealed that it included an additional 56 bp of sequence which was homologous to sequences at the 5' end of intron 4 in the human ER β gene sequence. The inclusion of this intronic sequence resulted in the introduction of an in-frame stop codon resulting in a predicted ORF of 321 amino acids. Extensive analysis of multiple clones from several macaque tissues did not result in isolation of any cDNAs lacking this intronic sequence.

Analysis of recombinant proteins prepared by *in vitro* translation

Proteins corresponding to both the long (59 kDa, Ogawa *et al.* 1998a) and short (54 kDa, Mosselman *et al.* 1996) forms of hER β 1 were translated from the human cDNAs (Fig. 4 lane 1) consistent with Western data showing that both AUG start sites are functional *in vivo* (Saunders *et al.* 2002b). Plasmids containing ER β 1 cDNAs isolated from marmoset and macaque generated proteins of a similar size to hER β 1 (Fig. 4 lanes 3, 5, 6). Proteins synthesised from hER β 2-containing plasmids were smaller ($\sim$ 55.5 kDa and $\sim$ 50.5 kDa) than those of hER β 1, consistent with the use of alternative AUG starts and the incorporation of an alternative terminal coding exon with a shorter ORF than that of hER β 1. Translation of ER β 2 cDNAs from marmoset and macaque resulted in synthesis of proteins of 54.3 kDa (lane 4) and 35.8 kDa (lanes 7 and 8) respectively. In macaque, a second prominent protein band was detected at 30 kDa and this would be consistent with the use of the alternative AUG resulting in a protein lacking 54 amino acids at the amino terminus.

Reporter gene activation in transient transfection assays

Incubation of cells co-transfected with an ERE reporter and hER α or hER β 1 (wild type, WT) with increasing concentrations of oestradiol (Fig. 5)

resulted in a ligand-dependent induction of reporter gene expression. Consistent with previous reports, ER α was able to induce four- to fivefold higher levels of reporter gene expression than ER β 1 at equivalent ligand concentrations (Table 2). Activation of the reporter gene by ER β 1-containing constructs was similar for human, macaque and marmoset (Fig. 5), although the marmoset ER β 1 was slightly more active than its human homologue at higher concentrations of oestradiol (10^{-7} , 10^{-6} M). Consistent with the absence of a functional AF-2 domain in hER β 2, the protein did not activate ERE-luciferase reporter gene expression in the presence of any of the ligands tested (e.g. oestradiol, Fig. 5 (hER β 2)). When incubated with increasing concentrations of PPT, activation of the ERE reporter by the ER β 1-containing constructs (Fig. 6) was minimal compared with that observed using the hER α -positive control (Fig. 6) and only the macaque ER β 1 showed any activity although this was approximately 12-fold lower than hER α even at 10^{-6} M ligand concentration (Table 2). DPN is marketed as an ER β selective agonist and at concentrations between 10^{-9} and 10^{-7} M ERE reporter activation by human, macaque and marmoset ER β 1-containing constructs was greater than that seen with hER α (Fig. 6). However, at higher concentrations of ligand (i.e. 10^{-6} to 10^{-4} M) DPN was able to act as a potent agonist for hER α . Identical reporter gene activation assays were carried out in the presence of Adiol and genistein and the results obtained are compared with those from incubations with oestradiol, PPT and DPN in Table 2.

Discussion

The aim of the current study was to establish whether wild type and variant ER β proteins in primate species share significant sequence homology with those from the human. We have previously detected ER β protein expression in tissues from these primate species using immunohistochemistry with an antibody directed against the hinge domain of human ER β (Saunders *et al.* 2000, 2001). A comparison of the ER β 1 and ER β 2 proteins identified in this study is presented in Fig. 7. It is notable that in both an Old World and a New World primate not only did the ER β 1 proteins share significant sequence homology with

A		10	20	30	40	50
Contig# 1						
hERbeta 1	MDIKNSPSSLNSPSSYNCSQSILPLEHGSIIYIPSSYVDSHHEYPA					
mac ERbeta 1	MDIKNSPSSLNSPSSYNCSQSILPLEHGSIIYIPSSYVESHHEYPA					
marm ERbeta 1	MDIKNSPSSLNSPSSYNFGQSILPLEHGPYIYIPSSYVESHHEYPA					
		60	70	80	90	100
Contig# 1						
hERbeta 1	YSIPSNVTNLEGGPGRQTTSFNVLPWTPGHLSPVLVHRQLSHLYA					
mac ERbeta 1	YSIPSNVTNLEGGPGRQTASPNVLWPTPGHLSPVLAVHRQLSHLYA					
marm ERbeta 1	YSIPSSVTNLEGGPGRQITSPNMLWSTPGHLSPVLAVHHQLSHLYA					
		120	130	140	150	160
Contig# 1						
hERbeta 1	RSLEHTLPVNRETLKRKVSNGRCASPTVTPGSKRDAHFCVCS					
mac ERbeta 1	RSLEHTLPVNRETLKRKVSNGRCTSPVTPSSSKRDAHFCVCS					
marm ERbeta 1	RSLEHTLPVSRETLKRKVSNGHCASPTVTPSSSKRDAHFCVCS					
		170	180	190	200	210
Contig# 1						
hERbeta 1	CEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKC					
mac ERbeta 1	CEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKC					
marm ERbeta 1	CEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKC					
		230	240	250	260	270
Contig# 1						
hERbeta 1	RERCGYRLVRRQRSADQLHCAGKAKRSGGHAPRVRELLDAL					
mac ERbeta 1	RERCGYRLVRRQRSADQVHCASKAKRSGSHTPLVRELLDAL					
marm ERbeta 1	RERCGYRLVRRQGNAAEQVHCAGKAKRSGGHVPRVRELLDAL					
		280	290	300	310	320
Contig# 1						
hERbeta 1	EPHVLISRPSAPFTEASMMSLTKLADKELVHMISWAKKIPGFV					
mac ERbeta 1	EPHVLISRPSAPFTEASMMSLTKLADKELVHMISWAKKIPGFV					
marm ERbeta 1	EPHVLISRPSVPFTEASMMSLTKLADKELVHMISWAKKIPGFV					
		340	350	360	370	380
Contig# 1						
hERbeta 1	LESCWMEVLMVGLMWRSIDHPGKLIFAPDLVDRDEGKCEGILE					
mac ERbeta 1	LESCWMEVLMVGLMWRSIDHPGKLIFAPDLVDRDEGKCEGILE					
marm ERbeta 1	LESCWLEVLMVGLMWRSIDHPGKLIFAPNLILDRDEGKCEGILE					
		390	400	410	420	430
Contig# 1						
hERbeta 1	RFRELKLQHKEYLCVKAMILLNSMYPLVTATQDADSSRKL					
mac ERbeta 1	RFRELKLQHKEYLCVKAMILLNSMYPLVTATQDADSSRKL					
marm ERbeta 1	RFRELKLQHKEYLCVKAMVLLNSQYDPLVTATQDAESSQL					
		450	460	470	480	490
Contig# 1						
hERbeta 1	IAKSGISSQQSMRLANLLMLLSHVRHASNKGMEHLLNMCKN					
mac ERbeta 1	IAKSGISSQQSMRLANLLMLLSHVRHASNKGMEHLLSMCKN					
marm ERbeta 1	IAKSGFSSQQSVRLANLLMLLSHIRHASNKGMEHLLSMCKN					
		500	510	520		
Contig# 1						
hERbeta 1	NAHVLRGCKSSITGSECSPAEDSKSKEGSQNPQSQ					
mac ERbeta 1	NAHVLRGCKSSIMGSACSSPAEDSKNKEGSQNPQSQ					
marm ERbeta 1	NAHVVRGCKSSITGSECSPAEDSKSKEGSQNPQSP

Figure 3 Comparison of peptide sequences of macaque and marmoset ERβ1 and ERβ2 proteins with that of the human. (A) Peptides sequence alignment of full-length ERβ1 proteins from human (NM_001437), *Macaca arctoides* (stump-tailed macaque; mac) and *Callithrix jacchus jacchus* (common marmoset; marm). (B) Peptide sequence of C-terminal portion (from amino acid 198) of ERβ2 proteins from human (AF051428), macaque and marmoset. Note that the peptides used to raise ERβ1- and ERβ2-specific antibodies have been underlined on the human peptide sequence.

hER β , but variant mRNAs homologous to hER β 2 (also known as hER β cx) were identified. This splice variant isoform has not previously been identified in other species and its existence in primates would suggest that the mechanisms that control the alternative splicing of the eighth coding exon of the ER β gene evolved at, or before, the divergence of the New World primates from the human line approximately 35 million years ago. We have not found any evidence in these, or other unpublished studies, for the existence of a variant equivalent to the rodent ER β 2 variant (Chu & Fuller 1997, Petersen *et al.* 1998) in human or primate tissues, a finding in agreement with other investigators who have also failed to detect hER β 2 homologues in rodents (Chu *et al.* 2000). Further studies are needed to determine if the inclusion of intronic sequences such as those seen in the macaque are

replicated in other species. Interestingly, studies on the organisation of spermatogenesis in primate species have revealed that the marmoset more closely resembles the human than does the macaque (Millar *et al.* 2000, Wistuba *et al.* 2003).

Sequence comparisons between the peptides predicted from the open reading frames of the ER β 1 and ER β 2 cDNAs from the macaque and marmoset with the hER β 1 and hER β 2 proteins solved the apparent conflict between results obtained with RT-PCR and immunohistochemistry. Although sequences homologous to the peptide used to raise the anti-hER β 1 monoclonal were identified in the ER β 1 sequences isolated from both primates, this was not the case when ER β 2 protein sequences were analysed. *In vitro* transcription/translation revealed that in the primate species, as well as in the human, more than

Figure 4 Analysis of proteins expressed *in vitro* from human, macaque and marmoset ER β 1 and ER β 2 cDNAs. Wells contained proteins synthesised from full length cDNAs by T7-coupled transcription/translation as follows: lane 1, hER β 1; lane 2, hER β 2; lane 3, marmoset ER β 1; lane 4, marmoset ER β 2; lane 5, macaque ER β 1; lane 6, macaque ER β 1; lanes 7 and 8, macaque ER β 2. Molecular weight markers were run in a parallel lane on all gels. Note that all proteins showed evidence of use of alternative ATG start sites within the cDNAs (e.g. arrows). Results from two separate gels are shown.

one size of protein was generated from the full length cDNAs and that their sizes were consistent with the use of two different AUG start sites as previously documented (Ogawa *et al.* 1998a, Tremblay *et al.* 1999). We have shown that ER β 1 proteins with sizes identical to long (59 kDa) and short (54 kDa) recombinant hER β can be extracted from human breast cancers (Saunders *et al.* 2002b) consistent with induction of translation at both AUGs *in vivo*.

Inoue *et al.* (2000) cloned and sequenced a splice variant isoform of hER β that lacked exon 5 and showed that this was able to act as a dominant negative inhibitor of ER α - and ER β 1-induced gene activation. We (Critchley *et al.* 2002, Scobie *et al.* 2002; present study) and others (Speirs *et al.* 2000, Poola *et al.* 2002a) have reported the expression of exon 5-deleted mRNAs in normal and malignant tissues and cell lines. It is notable that the cDNA for the macaque ER β 2 contained a portion of intron 4 consistent with the activation of a cryptic splice site, suggesting that the exon 4/intron 4 splice site is particularly susceptible to alternative splice site selection. Although multiple cDNA pools were analysed from several tissues we never identified a macaque ER β 2 cDNA that lacked this intron-derived sequence. The macaque ER β 2 proteins

generated *in vitro* from the cDNA clones migrated with apparent molecular masses of ~35 and 30 kDa on acrylamide gels and it is notable that in previous studies using protein extracts from macaque tissues, proteins of similar size were identified on Western blots (see Fig. 4 in Saunders *et al.* 2001). Because the antibodies directed against hER β 2 did not cross-react with the ER β 2 proteins identified in either of the primates, a direct comparison between the pattern of expression of hER β 2 and primate ER β 2 proteins using Western analysis or immunohistochemistry will require development of new antibody reagents.

Functional studies were carried out using transient co-transfection of ER-containing constructs and a luciferase reporter gene driven by a promoter made up of three copies of the vitellogenin ERE. This reporter has been used extensively to investigate the ability of oestrogens to activate ER α and ER β and is strongly induced by ER α in the presence of a range of oestrogens and xenoestrogens (Hall & McDonnell 1999, Hall & Korach 2002). Consistent with previous reports (Ogawa *et al.* 1998b, Peng *et al.* 2003) ER β 2-containing constructs were unable to activate reporter gene expression in the presence of oestradiol. All the ER β 2 proteins identified lacked the core AF-2 domain (see Fig. 7), which is located in helix 12 of ER α and ER β 1 (Tanenbaum *et al.* 1998), but contain sequences essential for dimerisation and DNA binding (Lees *et al.* 1990).

The functional significance of the expression of ER β 2 and other ER β splice variants in human cell lines and tissues has been investigated in a number of ways. Using gel-shift analysis, Moore *et al.* (1998) demonstrated that ER β 1 (WT) and ER β 2 could form homo- and heterodimers. From transient co-transfection studies, there is evidence that hER β 2 can act as a dominant negative suppressor of oestrogen-activated hER α -mediated gene activation *in vitro* (Ogawa *et al.* 1998b, Peng *et al.* 2003). ER β variant mRNAs have been detected in a range of malignant tissues including breast cancers (Poola *et al.* 2002b), and in support of the idea that ER β 2 may act as a dominant negative 'receptor' it has been reported that co-expression of ER α and ER β 2 is associated with reduced expression of progesterone receptor in breast cancer specimens (Saji *et al.* 2002). Our studies showing that the marmoset contains an ER β splice variant with close homology to hER β 2 as well as an exon 5-deleted

Figure 5 Impact of oestradiol on the ability of ER expression constructs to activate an ERE-luciferase reporter. Panels show human ER α (note different vertical scale), hER β 1, hER β 2, macaque ER β 1 (mcER β 1) and marmoset ER β 1 (maER β 1). Oestradiol was added to wells in the concentration range 10^{-12} to 10^{-6} M. All changes in induction of the ERE-luciferase reporter construct were corrected using an internal *Renilla* control. Fold differences are related to the level of reporter gene expression in the absence of ligand (0).

variant mRNA means this species may provide a suitable animal model in which to assess the impact of the expression of ER β variants on tissue responsiveness to oestrogens.

Detailed structural analysis of ER proteins bound to agonists and antagonists has provided clues as to the way(s) in which these ligands influence co-factor recruitment by altering the position adopted by

helix 12 (Brzozowski *et al.* 1997, Shiau *et al.* 1998). Although amino acids corresponding to helix 12 in human ER β (488 to 499) (Pike *et al.* 1999) are fully conserved in macaque ER β 1, in the marmoset leucine 495 is changed to methionine. This amino acid is homologous to leucine 548 in mouse ER α ; mutation of this amino acid to alanine was shown by Mahfoudi *et al.* (1995) to alter the responsiveness

Table 2 Results of transient transfection studies using a 3 $\times$ ERE-luc reporter construct. Summary of fold induction of ERE-luciferase construct observed in transient transfection experiments with ER β 1 constructs. hER α is shown for comparison. Results are the mean of at least 3 experiments

Ligand	Human		Macaque		Marmoset		hER α	
	10 ⁻⁷	10 ⁻⁶	10 ⁻⁷	10 ⁻⁶	10 ⁻⁷	10 ⁻⁶	10 ⁻⁷	10 ⁻⁶
Oestradiol	11.7	15.3	12.1	14.0	17.1	18.9	59.4	68.8
Adiol	5.4	10.4	12.2	19.1	5.5	22.3	67.6	415.8
Genistein	4.9	7.9	2.8	5.3	4.8	7.3	3.7	3.9
PPT	1.2	1.4	7.1	14.4	1.2	6.4	169.1	176.2
DPN	7.8	9.6	9.1	11.7	20.1	27.5	5.3	66.3

of the mutant receptor to agonists and antagonists. Both leucine and methionine are hydrophobic and this substitution may therefore have a minimal impact on the orientation of helix 12 following ligand binding. It is notable that the marmoset ER β 1 was able to induce transcription of the ERE-luciferase reporter gene *in vitro* and we therefore assume a receptor–ligand dimer was formed and was able to recruit endogenous co-activator proteins efficiently.

Crystallographic studies have identified the amino acids within the ligand binding domains of ER α and ER β that line the binding cavity and contribute to ligand-specific binding (Brzozowski *et al.* 1997, Tanenbaum *et al.* 1998, Pike *et al.* 1999). These are located in helices 3, 6, 8, 11 and 12 (Pike *et al.* 1999). Comparison of the ER β 1 protein sequences from human, macaque and marmoset revealed strong sequence homology and although some amino acids within the ligand binding domain were changed, most of these were not amino acids which interact directly with ligand. In several cases the amino acid encoded by the primate sequence was changed from that seen in the human sequence to the amino acid found in rat or mouse ER β . Examples of this type of change in the macaque are V239, S243, T252, V341, N520 and in the marmoset S261, L325, V341, E421. Of more interest are changes that either result in substitution of an amino acid to one identical to hER α and/or a change in an amino acid which is conserved between hER α and hER β (Pike *et al.* 1999). For example, in the macaque, the amino acid at position 478 is a serine, in hER β an asparagine is present in this position but hER α has a serine (527). However, this substitution appears unlikely to explain why macaque ER β 1 was

activated by the ER α selective ligand PPT slightly more efficiently than hER β 1, as both rat (Pike *et al.* 1999) and marmoset ER β 1 have a serine in this position and in the present study marmoset ER β 1 did not induce reporter gene activity in the presence of PPT any more efficiently than did hER β 1. Also of interest is the observation that in the marmoset a leucine is present at position 336 (changed from methionine in hER β 1); the equivalent amino acid in hER α is L384. In hER α L384 is one of the amino acids implicated in determining the ligand binding specificity of the ligand binding pocket (Tanenbaum *et al.* 1998) but more detailed analysis would be needed to determine whether this affects the ability of marmoset ER β to bind ligands.

Two of the amino acid substitutions in the marmoset ER β 1 sequence are of particular note when considering ligand interactions. Both changes result in substitution of a valine (V376, V404) in place of an isoleucine which is conserved in hER α , hER β , rat ER β and macaque ER β (Pike *et al.* 1999). Although both isoleucine and valine are classed as hydrophobic amino acids, valine has a shorter side chain than isoleucine. In hER α isoleucine 424 (equivalent to I376 in hER β) has been implicated in contributing to the hydrophobic binding site of the receptors (Brzozowski *et al.* 1997). Studies examining binding of genistein to hER β concluded that I376 was one of those making contact with the bound ligand (Pike *et al.* 1999). In the current series of transient transfections maximal fold activation of the 3 $\times$ vitellogenin ERE containing reporter by marmoset ER β 1 in the presence of selected ligands was as good (e.g. with genistein, oestradiol) or better (e.g. Adiol, DPN) than the equivalent result obtained with hER β 1,

Figure 6 Activation of ER β 1 from human, macaque and marmoset following incubation with PPT or DPN. Upper panels, PPT (10⁻¹² to 10⁻⁶ M); lower panels, DPN 10⁻¹⁰ to 10⁻⁴ M. Cells were transfected with plasmids containing hER α (control) or ER β 1 from human (hER β), macaque (mcER β) and marmoset (maER β). Note that PPT is a potent activator of ER α -induced reporter gene expression but does not activate ER β 1 protein from human or marmoset. DPN is marketed as an ER β 1 selective agonist but at concentrations of 10⁻⁶ M or greater it acted as an ER α agonist.

Figure 7 Summary of the structure of the ERβ1 and ERβ2 proteins identified in human, stump-tailed macaque and common marmoset. Note that ERβ1 proteins in all 3 species contain a functional AF-2 domain (486–500 in human) whereas this is absent from the full length ERβ2 proteins (human, marmoset). The cDNA for the macaque contained a fragment of intronic sequence which included a stop codon (x), resulting in the synthesis of a truncated protein. Sizes given are those equivalent to the protein synthesised from the first ATG in the open reading frame.

suggesting that this substitution might have influenced receptor activity. However, further studies would be required before we can be sure that the ligand binding pocket has been changed in the marmoset protein.

In conclusion, we have cloned and sequenced ERβ cDNAs from both Old World (*M. arctoides*) and New World (*C. jacchus jacchus*) primates and have demonstrated not only that the ERβ1 sequences are largely conserved but also that the alternative splicing of the eighth exon of the ERβ2 gene occurs in these species as well as in the human.

References

- Anon 1999 A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97** 161–163.
- Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J & Nilsson S 1998 Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen receptor agonists/antagonists. *Molecular Pharmacology* **54** 105–112.
- Brzozowski AM, Pike ACW, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson J-A & Carlquist M 1997 Molecular basis of agonism and antagonism in the estrogen receptor. *Nature* **389** 753–758.
- Chu S & Fuller PJ 1997 Identification of a splice variant of the rat estrogen receptor beta gene. *Molecular and Cellular Endocrinology* **132** 195–199.
- Chu S, Mamers M, Burger HG & Fuller PJ 2000 Estrogen receptor isoform gene expression in ovarian stromal and epithelial tumors. *Journal of Clinical Endocrinology and Metabolism* **85** 1200–1205.
- Cowley SM, Hoare S, Mosselman S & Parker SG 1997 Estrogen receptors alpha and beta form heterodimers on DNA. *Journal of Biological Chemistry* **272** 19858–19862.
- Critchley HOD, Henderson TA, Kelly RW, Scobie GS, Evans LR, Groome NP & Saunders PTK 2002 Wild type estrogen receptor, ERβ1 and the splice variant (ERβcx/β2) are both expressed throughout the normal menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **87** 5265–5273.
- Enmark E, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M & Gustafsson J-A 1997 Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **82** 4258–4265.
- Green S, Walter P, Kumar V, Krust A, Bornert J-M, Argos P & Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320** 134–139.
- Hall JM & McDonnell DP 1999 The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140** 5566–5578.
- Hall MJ & Korach KS 2002 Analysis of the molecular mechanisms of human estrogen receptors α and β reveals differential specificity in target promoter regulation by xenoestrogens. *Journal of Biological Chemistry* **277** 44455–44461.
- Inoue S, Ogawa S, Horie K, Hoshino S, Goto W, Hosoi T, Tsutsumi O, Muramatsu M & Ouchi Y 2000 An estrogen receptor beta isoform that lacks exon 5 has dominant negative activity on both ERα and ERβ. *Biochemical and Biophysical Research Communications* **279** 814–819.
- Kelley ST & Thackray VG 1999 Phylogenetic analyses reveal ancient duplication of estrogen receptor isoforms. *Journal of Molecular Evolution* **49** 609–614.
- Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S & Gustafsson J-A 1996 Cloning of a novel estrogen receptor expressed in rat prostate. *PNAS* **93** 5925–5930.
- Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson J-A 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen

- receptors alpha and beta. *Endocrinology* **138** 863–870.
- Lees JA, Fawell SE, White R & Parker MG 1990 A 22-amino acid peptide restores DNA-binding activity to dimerization-defective mutants of the estrogen receptor. *Molecular and Cellular Biology* **10** 5529–5531.
- Lu B, Leygue E, Dotzlaw H, Murphy LC & Watson PH 1998 Estrogen receptor- β mRNA variants in human and murine tissues. *Molecular and Cellular Endocrinology* **138** 199–203.
- Lu B, Leygue E, Dotzlaw H, Murphy LJ & Murphy LC 2000 Functional characteristics of a novel murine estrogen receptor-beta isoform, estrogen receptor-beta 2. *Journal of Molecular Endocrinology* **25** 229–242.
- Mahfoudi A, Roulet E, Dauvois S, Parker MG & Wahli W 1995 Specific mutations in the estrogen receptor change the properties of antiestrogens to full agonists. *Proceedings of the National Academy of Sciences of the USA* **92** 4206–4210.
- Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS & Katzenellenbogen JA 2001 Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *Journal of Medicinal Chemistry* **44** 4230–4251.
- Millar MR, Sharpe RM, Weinbauer GF, Fraser HM & Saunders PTK 2000 Marmoset spermatogenesis: organisational similarities to the human. *International Journal of Andrology* **23** 266–277.
- Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Klier SA, Lehmann JM & Willson TM 1998 Cloning and characterisation of human estrogen receptor beta isoforms. *Biochemical and Biophysical Research Communications* **247** 75–78.
- Mosselman S, Polman J & Dijkema R 1996 ERbeta: identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392** 49–53.
- Nie R, Zhou Q, Jassim E, Saunders PTK & Hess RA 2002 Differential expression of estrogen receptors α and β in reproductive tracts of the adult male dog and cat. *Biology of Reproduction* **66** 1161–1168.
- Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M & Gustafsson JA 2001 Mechanisms of estrogen action. *Physiological Reviews* **81** 1535–1565.
- Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998a The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha *in vivo* and *in vitro*. *Biochemical and Biophysical Research Communications* **243** 122–126.
- Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y & Muramatsu M 1998b Molecular cloning and characterization of human estrogen receptor β cx: a potential inhibitor of estrogen action in human. *Nucleic Acids Research* **26** 3505–3512.
- Peng B, Lu B, Leygue E & Murphy LC 2003 Putative functional characteristics of human estrogen receptor-beta isoforms. *Journal of Molecular Endocrinology* **30** 13–29.
- Petersen DN, Tkalecic GT, Koza-Taylor PH, Turi TG & Brown TA 1998 Identification of estrogen receptor β 2, a functional variant of estrogen receptor β expressed in normal rat tissues. *Endocrinology* **139** 1082–1092.
- Pettersson K, Grandien K, Kuiper GGJM & Gustafsson J-A 1997 Mouse estrogen receptor β forms estrogen receptor response element-binding heterodimers with estrogen receptor α . *Molecular Endocrinology* **11** 1486–1496.
- Pike ACW, Brzozowski AM, Hubbard RE, Bonn T, Thorsell A-G, Engstrom O, Ljunggren J, Gustafsson J-A & Carlquist M 1999 Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO Journal* **18** 4608–4618.
- Poola I, Abraham J & Baldwin K 2002a Identification of ten exon deleted ERbeta mRNAs in human ovary, breast, uterus and bone tissues: alternate splicing pattern of estrogen receptor beta mRNA is distinct from that of estrogen receptor alpha. *FEBS Letters* **516** 133–138.
- Poola I, Abraham J & Liu A 2002b Estrogen receptor beta splice variant mRNAs are differentially altered during breast carcinogenesis. *Journal of Steroid Biochemistry and Molecular Biology* **82** 169–179.
- Saji S, Omoto Y, Shimizu C, Horiguchi S, Watanabe T, Funata N, Hayash S, Gustafsson JA & Yoi M 2002 Clinical impact of assay of estrogen receptor beta cx in breast cancer. *Breast Cancer* **9** 303–307.
- Saunders PTK 1998 Oestrogen receptor beta (ER β). *Reviews of Reproduction* **3** 164–171.
- Saunders PTK, Millar MR, Williams K, Macpherson S, Harkiss D, Anderson RA, Orr B, Groome NP, Scobie G & Fraser HM 2000 Differential expression of estrogen receptor-alpha and -beta and androgen receptor in the ovaries of marmoset and human. *Biology of Reproduction* **63** 1098–1105.
- Saunders PTK, Sharpe RM, Williams K, Macpherson S, Urquhart H, Irvine DS & Millar MR 2001 Differential expression of oestrogen receptor alpha and beta proteins in the testes and male reproductive system of human and non-human primates. *Molecular Human Reproduction* **7** 227–236.
- Saunders PTK, Millar MR, Macpherson S, Irvine DS, Groome NP, Evans LR, Sharpe RM & Scobie GA 2002a Estrogen receptor beta (ER β 1), and the estrogen receptor beta 2 splice variant (ER β cx/2), are expressed in distinct cell populations in the adult human testis. *Journal of Clinical Endocrinology and Metabolism* **87** 2706–2715.
- Saunders PTK, Millar MR, Williams K, Macpherson S, Bayne C, O'Sullivan C, Anderson TJ, Groome NP & Miller WR 2002b Expression of oestrogen receptor beta (ERbeta1) protein in human breast cancer biopsies. *British Journal of Cancer* **86** 250–256.
- Scobie GS, Macpherson S, Millar MR, Groome NP, Romana PG & Saunders PTK 2002 Human estrogen receptors: differential expression of ERalpha and beta and the identification of ERbeta variants. *Steroids* **67** 985–992.
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA & Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95** 927–937.
- Speirs V, Adams IP, Walton DS & Atkin SL 2000 Identification of wild-type and exon 5 deletion variants of estrogen receptor beta in normal human mammary gland. *Journal of Clinical Endocrinology and Metabolism* **85** 1601–1605.
- Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson KE, Sun J, Katzenellenbogen BS & Katzenellenbogen JA 2000 Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *Journal of Medicinal Chemistry* **43** 4934–4947.
- Tanenbaum DM, Wang Y, Williams SP & Sigler PB 1998 Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *PNAS* **95** 5998–6003.
- Thornton JW 2001 Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *PNAS* **98** 5671–5676.
- Thornton JW, Need E & Crews D 2003 Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. *Science* **301** 1714–1717.
- Tremblay GB, Tremblay A, Labrie F & Giguere V 1999 Dominant activity of activation function (AF-1) and differential stoichiometric requirements for AF-1 and -2 in the estrogen receptor alpha-beta heterodimeric complex. *Molecular Cell Biology* **19** 1919–1927.
- Walther N, Lioutas C, Tillmann G & Ivell R 1999 Cloning of bovine estrogen receptor beta (ERbeta): expression of novel deleted isoforms in reproductive tissues. *Molecular and Cellular Endocrinology* **152** 37–45.

- Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson J-A 2000 Estrogen receptor (ER) β , a modulator of ER α in the uterus. *PNAS* **97** 5936–5941.
- Wistuba J, Schrod A, Greve B, Hodges JK, Aslam H, Weinbauer GF & Luetjens CM 2003 Organization of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny and mating system. *Biology of Reproduction* **69** 582–591.
- Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS & Hess RA 2002 Localization of androgen and estrogen receptors in

adult male mouse reproductive tract. *Journal of Andrology* **23** 870–881.

Received 12 December 2003

Accepted 24 March 2004

Made available online as an

Accepted Preprint 30 March 2004