Functional characteristics of a novel murine estrogen receptor- β isoform, estrogen receptor- β 2

B Lu, E Leygue, H Dotzlaw, L J Murphy¹ and L C Murphy

Department of Biochemistry and Molecular Biology, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, R3E 0W3, Canada

¹Department of Physiology, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, R3E 0W3, Canada

(Requests for offprints should be addressed to L C Murphy; Email: lcmurph@cc.umanitoba.ca)

ABSTRACT

We have isolated a highly expressed splice variant mRNA of murine estrogen receptor- β (ER β), mER β 2, containing an in-frame 54 nucleotide insertion between exons 5 and 6 of wild-type mER β 1. The predicted ER β 2 protein contains 18 amino acids inserted in the ligand binding domain of mER β 1. Recombinant protein generated by *in vitro* transcription/translation showed that mER β 2 had markedly reduced ligand binding ($K_D = 17.7 \pm 4.7$ nM, mean $\pm$ S.E.M., $n=3$) compared with mER β 1-bound ³H-estradiol ($K_D = 0.56 \pm 0.19$ nM, mean $\pm$ S.E.M., $n=3$). Both receptors bound similarly to palindromic estrogen responsive elements (EREs) *in vitro* and *in vivo*, and similarly bent DNA. Transcriptional activity was assessed using transient transfection analysis into a homologous murine cell line, NIH 3T3 cells. mER β 1 transactivated *ERE-tk-CAT* reporter genes similarly to mER α , whereas mER β 2 had little activity except at high ligand concentrations. However, under conditions in which mER β 2 is unlikely to be ligand saturated, co-transfected mER β 2 inhibited activity of mER α and possibly mER β 1 on *ERE-tk-*

CAT genes. Using a 'novel raloxifene responsive' gene reporter system (TGF- β 3-CAT), we found the ability of estradiol and LY117018 to activate both mER α and mER β 1 on this promoter was identical, and mER β 2 activity in the presence of either estradiol or LY117018 was only slightly less than that observed with either mER β 1 or mER α . Both mER β 1 and mER β 2 when liganded with LY117018 inhibited transcription at a classical ERE-regulated promoter under these transfection conditions, which was in marked contrast to their stimulatory effect at the transforming growth factor- β 3 promoter. These data suggest that responsiveness of gene expression to a relatively highly expressed variant murine ER β isoform, mER β 2, is both ligand and promoter specific. Determination of the relative level of expression of mER β 1 mRNA and mER β 2 mRNA in mouse tissues indicated predominance of mER β 2 mRNA in some but not all tissues. These data suggest that the mER β 2 may have some tissue-specific and promoter-specific modulatory effects.

Journal of Molecular Endocrinology (2000) **25**, 229–242

INTRODUCTION

Estrogen has critical roles in a broad range of both physiological and pathophysiological activities. Estrogen signal transduction is now known to be mediated by at least two forms of receptors, estrogen receptor- α (ER α) and estrogen receptor- β (ER β) (Katzenellenbogen & Korach 1997, Giguere *et al.* 1998). Both these receptors are ligand-regulated transcription factors that classically modulate target gene transcription by binding as homo- or heterodimers, or both, to estrogen-

responsive sequences in target gene promoters (Giguere *et al.* 1998). However, several splice variants for both ER α and ER β have been characterized (Chu & Fuller 1997, Murphy *et al.* 1997, Moore *et al.* 1998, Vladusic *et al.* 1998) and the proteins encoded by these variant mRNAs could also affect estrogen and antiestrogen action. Recently, a highly abundant splice variant of rat ER β (rER β 2) was cloned and characterized (Chu & Fuller 1997, Petersen *et al.* 1998). In addition, we have recently isolated and characterized several splice variants of the murine ER β , including murine

ER β 2, which contains an in-frame 54 nucleotide insertion between exon 5 and exon 6 sequences of the wild-type murine ER, now referred to as ER β 1 (mER β 1) (Lu *et al.* 1998). The murine ER β 2 (mER β 2) is analogous to the rat ER β 2 (rER β 2), although two amino acids within the inserted sequence differ between the two species (Lu *et al.* 1998). Exon-deleted splice variants of ER β were detected in both human and murine tissues, but the human homolog of mER β 2 was not detected in any human tissues analyzed (Lu *et al.* 1998), although, recently, the possibility has arisen that a human homolog of the rodent ER β 2 might be expressed in some human ovarian cancer cell lines in culture (Hanstein *et al.* 1999). The data suggest that both species-specific alternative splicing mechanisms and possibly a function of some ER β variants could exist. Previous studies in which the putative functions of rER β 2 have been characterized have used a heterologous system in which the rat isoforms were evaluated in a human cell line (Petersen *et al.* 1998). Furthermore, the putative functions of the previously cloned mER β 1 have also been determined in either human or monkey cell lines (Tremblay *et al.* 1997). Given the possibility that species-specific expression and therefore functional differences in ER variants may exist, we have characterized the putative functions of the mER β 1 and mER β 2 using a homologous system—mouse NIH 3T3 cells—and have investigated putative function at a so-called ‘non-classical’ estrogen-responsive promoter (Yang *et al.* 1996).

MATERIALS AND METHODS

Materials

We obtained 17 β -estradiol (E₂), diethylstilbestrol (DES), 4-hydroxytamoxifen (4-OH-Tam) and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) from Sigma Chemical Co. (St Louis, MO, USA). ICI182,780 was a gift from Dr A E Wakeling (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK). LY117018 was a gift from Eli Lilly and Company (Indianapolis, IN, USA). [³H]17 β -Estradiol ([³H]E₂), [¹⁴C]chloramphenicol, and [³⁵S]methionine were from New England Nuclear (Boston, MA, USA). [α -³²P]dCTP, [γ -³²P]ATP, and [³⁵S]ATP were from ICN Pharmaceuticals (Irvine, CA, USA). All cell culture reagents were obtained from Gibco Brl (Burlington, Ontario, Canada). All other cell culture ingredients were purchased from Flow Laboratories (Mississauga, Ontario, Canada). ER α H222 antibody, which recognizes an epitope in the E-domain of ER α , was

a gift from Dr G Greene (University of Chicago, Chicago, IL, USA). ER β antibody PAI-310 raised to a synthetic peptide corresponding to rER β C-terminal amino acids 467–485 (as determined from the rER β sequence quoted by Kuiper *et al.* (1996)) was from Affinity BioReagents (Golden, CO, USA). The mER α expression plasmid (pcDNA3/wild-type vector) (Couse *et al.* 1995) was a gift from Dr K Korach (NIEHS, Research Triangle, NC, USA) and the mER β 1 expression plasmid (pCMX-mER β 1) (Tremblay *et al.* 1997) was a gift from Dr V Giguere (McGill University, Montreal, Quebec, Canada). This expression vector represents cDNA that will encode the originally described N-terminally truncated 485 amino acid murine ER β 1. The murine ER β 2 expression plasmid (pCMX-mER β 2) was constructed by replacement of the murine ER β 1 cDNA sequences spanning nucleotides 737–1054 (Tremblay *et al.* 1997), which are encompassed within a SphI–PstI restriction fragment, with the same fragment from the corresponding region of the murine ER β 2 generated by RT-PCR, which includes the 54 nucleotide insertion (Lu *et al.* 1998). The resulting expression plasmid was then sequenced. This vector will therefore encode an N-terminally truncated 503 (i.e. 485 $\pm$ 18) amino acid murine ER β 2 protein. *In vitro* transcription/translation studies using these expression vectors identified proteins of the expected molecular mass as determined by SDS-PAGE (data not shown). The ERE-tk-CAT reporter plasmid, containing one xenopus vitellogenin B1 ERE (5'-AGTCACTGTGACC-3'), was a gift from Dr W Wahli (Seiler-Tuyns *et al.* 1986). The ERE₄₅₂-delta-TCO plasmid containing two vitellogenin A2 (–333/–288, 5'-GGTCACAGTGACC-3') EREs upstream of a chloramphenicol acetyltransferase (CAT) reporter was a gift from Dr P Webb (Webb *et al.* 1992). CMV-(ERE)₂-CAT (Reese & Katzenellenbogen 1992) was a gift from Dr B Katzenellenbogen (University of Illinois, Urbana, IL, USA), respectively. The TGF- β 3-CAT reporter (Yang *et al.* 1996) was a gift from Dr N Yang (Lilly Research Labs, Indianapolis, IN, USA). pCH110 (constitutive β -galactosidase expression vector) was from Amersham/Pharmacia (Oakville, Ontario, Canada).

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI, USA). Reactions were performed according to the manufacturer's instructions.

Tissues, RNA extraction and RT-PCR

Mouse tissues were obtained from female mice aged 8–9 weeks, and mammary tissues were obtained from adult lactating mice. Total RNA was extracted using Trizol reagent (Gibco Brl) according to the manufacturer's instructions. Integrity of RNA was confirmed by denaturing gel electrophoresis, as previously described (Dotzlaw *et al.* 1990). Total RNA (1–2 μ g per reaction) was reverse-transcribed as previously described (Lu *et al.* 1998). One microliter of this reaction was amplified by PCR incorporating [32 P]dCTP in a final volume of 10 μ l. Four microliters of this reaction were separated on 6% polyacrylamide gels containing 7 M urea, and autoradiographed as previously described (Dotzlaw *et al.* 1997). The primers used to detect both murine ER β 1 and ER β 2 at the same time are located in exons 5 and 6 respectively, defined by analogy to the human ER β gene (Enmark *et al.* 1997). Bands corresponding to ER β 2 and ER β 1 PCR products were quantified by scanning and the relative ratio of ER β 2 to ER β 1 calculated from these signals. (It should be noted that the number expressed in Fig. 1 reflects the relative ratio of the products and not the absolute initial mRNA levels.) Validation of this approach as a method for determining relative expression has been published previously (Daffada *et al.* 1994, 1995, Leygue *et al.* 1996). Primer set sequences are: murine ER β (sense) 5'-GCTGATGGTGGGGCTGATGT-3' (priming site in exon 5, nucleotides 890–909, as numbered in reference (Tremblay *et al.* 1997)); murine ER β (antisense) 5'-ATGCCAAAGATTTCCAG AAT-3' (priming site in exon 6, nucleotides 993–1012). The PCR conditions were 35 cycles of 1 min at 94 °C, and 30 s at 60 °C. Polyadenylated RNA was enriched using Dynabeads (oligo-dT25 attached to magnetic beads) according to the manufacturer's instructions (DynaL, Oslo, Norway).

Scatchard analysis

Murine ER α (pcDNA3/wild-type vector), murine ER β 1 (pCMX-mER β 1), and murine ER β 2 (pCMX-mER β 2) proteins were synthesized by *in vitro* transcription/translation as described above. Ligand binding studies were conducted as described previously (Tremblay *et al.* 1997). *In vitro*-generated receptor was diluted tenfold in buffer (10 mM Tris chloride, pH 7.5, 1.5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred microliters of the diluted protein were used in each binding reaction, which contained varying concentrations of [3 H]E $_2$ (0.01–100 nM), followed by overnight incubation at 4 °C. Non-

specific binding was determined by parallel incubations containing a 200-fold excess of unlabeled E $_2$. Unbound steroid was removed by addition of 500 μ l 0.5% charcoal–0.05% dextran in the above dilution buffer for 30 min at 4 °C followed by centrifugation at 10 000 *g* for 10 min at 4 °C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total [3 H]E $_2$ solutions using a scintillation counter. The ratio of specifically bound to unbound steroid and the concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, K_D .

Electrophoretic mobility gel-shift assay (EMSA)

In vitro-synthesized murine ERs were used for EMSA. Typically, 1 μ l programmed lysates containing equal amounts of each receptor as determined by polyacrylamide gel electrophoresis of 35 S-methionine labeled protein generated in parallel *in vitro* transcription/translation assays was assayed in EMSA. One microliter of lysate was incubated in a final volume of 20 μ l; the reaction solution was 5 mM Tris chloride, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 5% v/v glycerol and contained 2 μ g polyd(I-C). The binding reaction was initiated by adding 1 μ l (approx. 10 fmol) 5'-[32 P]-end-labeled, double-stranded ERE oligonucleotide (35-mer, 5'-AACTTTGATCAGGTCAGTGTGACCTGACCTTTGGAC-3' containing the vitellogenin A2 ERE sequence), and the mix was incubated at 20 °C for 30 min. DNA-bound complexes were electrophoretically separated on a 4.5% loosely cross-linked acrylamide gel (1:29 bis:acrylamide) at 150 V for 90 min at 20 °C in 0.5 $\times$ TBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1 μ g ER antibody (usually 1 μ l H222 for mER α , or 1 μ l PAI-310 for mER β 1 and mER β 2) were run to determine the presence of super-shifted antibody-bound ER-ERE complexes. Reticulocyte lysates containing *in vitro*-translated ER proteins were incubated with or without saturating concentrations of ligand (estrogen or antiestrogen) at 4 °C overnight to allow receptors to bind ligand, followed by EMSA.

DNA bending assay

The DNA bending vector ERE Bend I (kindly provided by Dr A Nardulli, University of Illinois, Urbana, IL, USA) (Nardulli & Shapiro 1992) was digested with EcoRI and EcoRV to produce a

FIGURE 1. Relative expression of mER β 1 and mER β 2 mRNA in various mouse tissues. Total RNA extracted from normal mouse tissues was reversed transcribed and radioactive PCR amplification was carried out using primers located in exons 5 and 6. The PCR products migrating at 177 bp and 123 bp correspond to a 54 bp inserted mER β 2 variant and the mER β 1 or wild-type cDNA, respectively. The signals were quantified by scanning the autoradiograph using NIH Image 161/ppc software, and the relative ratio of mER β 2 to mER β 1 calculated from these signals. Two independent experiments were performed and quantified. The relative ratios shown represent the mean of the two experiments.

430 bp DNA fragment with a single consensus ERE either at the end (EcoRI fragment) or in the middle (EcoRV fragment). The fragments were then gel purified, labeled by incubation with polynucleotide kinase in the presence of [γ - 32 P]ATP, and purified on a G50 Sephadex column. Gel mobility shift assays were carried out essentially as described above. Aliquots of the binding reactions were run on 8% non-denaturing acrylamide gels, dried and exposed to X-ray film. The degree of DNA-bending was determined using the method of Thompson & Landy (1988).

Cells, cell culture and transient transfection

Murine NIH 3T3 cells were obtained from the ATCC (Manassas, VA, USA). The cells were routinely cultured in DMEM containing 10% v/v fetal calf serum, 1% w/v glucose, glutamine and penicillin-streptomycin (CM). For transient transfection experiments, the culture medium of the stock cells was changed to phenol-red-free DMEM supplemented with 10% charcoal dextran-treated FBS, 1% w/v glucose, glutamine and penicillin-streptomycin (CS). Six days later, cells were seeded into six-well plates at 3.5×10^4 per well. When the cells were 30–40% confluent, culture medium was changed and cells were transfected 2 h later with 1–2 μ g reporter plasmid, 0.1–0.2 μ g receptor expression vector, 0.2 μ g *pCH110* reference gene, using

the calcium phosphate/DNA precipitation method (Graham & van der Eb 1973). After 16 h at 37 °C, cell medium was changed and the cells were washed once with PBS. Fresh medium was added and the cells treated with vehicle, 10 nM E $_2$ or 100 nM antiestrogen, unless otherwise stated, for 24 h. Cells were harvested, cell extracts prepared and used to determine CAT and β -galactosidase activity as described previously (Dotzlaw *et al.* 1992). For experiments in which the ability of the various ER isoforms to regulate the transforming growth factor (TGF)- β 3 promoter-driven *CAT* reporter gene (Yang *et al.* 1996) was determined, NIH 3T3 cells were transfected using the Effectene transfection reagent according to the manufacturer's instruction (Qiagen, Mississauga, Ontario, Canada). Briefly, the day before transfection, cells previously depleted of estrogen were seeded in six-well plates at $3\text{--}5 \times 10^4$ cells per well and left overnight. The plates were 30–40% confluent on the day of transfection. The transfection mixture was prepared according to the manufacturer's procedure, then fresh medium (CS) was added to the transfection mixture and 0.5 ml per well of the above mixture (containing 166 ng TGF- β 3-CAT reporter plasmid DNA, 83 ng ER expression plasmid and 83 ng β -galactoside *pCH110* plasmid DNA) was added drop-wise into the medium (CS) and the plates were gently swirled to ensure uniform distribution of the DNA-Effectene complexes. Cells were incubated with the

DNA–Effectene for 24 h. Fresh medium (CS) $\pm$ estrogen or antiestrogen, or both, was then added and left for a further 24 h.

Western blot analysis

For western blot analysis, $2\text{--}3 \times 10^5$ cells set up in 10 cm diameter dishes were transiently transfected with 4 μ g plasmid. After 48 h the transfected cells were harvested and cell extracts prepared in boiling lysis buffer (1% SDS, 10 mM Tris–HCl, pH 7.4). Aliquots of cell extracts containing 20 μ g protein were then subjected to 10% SDS-PAGE at 200 V for 90 min at room temperature according to the Laemmli method (Laemmli 1970). The separated proteins were then transferred to a nitrocellulose membrane (Micron Separations Inc., Westborough, MA, USA) using CAPS acid transfer buffer (10 mM CAPS, pH 11, 20% methanol) for 2 h at 250 mA at 4 °C. Blots were blocked with 5% skimmed milk/Tris-buffered saline containing 0.5% Tween-20 (TBST) for 1 h at room temperature, and then incubated with the anti-ER primary antibody AER314 for ER α (1:250 in TBST, NeoMarkers, Fremont, CA, USA) or Y19 for ER β (1:250 in TBST, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 16 h at 4 °C. Blots were then incubated with the appropriate peroxidase-conjugated secondary antibody (1:1000 in TBST, donkey antimouse IgG for ER α from Jackson Immuno Research Labs Inc., West Grove, PA, USA, antigoat IgG from Santa Cruz Biotechnology for ER β) for 1 h at room temperature. Detection was carried out using the enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham/Pharmacia Biotech).

RESULTS

Tissue distribution of mER β 1 and mER β 2 mRNA expression

Both mER β 1 and mER β 2 mRNA were detected in several murine tissues following RT-PCR analysis of total (Fig. 1) and polyA-enriched RNA (data not shown). The use of a single primer set that amplifies two products representing a wild-type mRNA and a splice variant in the same tube, in order to determine the relative expression of each mRNA has been validated previously (Daffada *et al.* 1994, 1995, Leygue *et al.* 1996). The data suggest that ovary and lung express ER β 1 and ER β 2 mRNA at similar levels. However placenta, uterus, breast, heart, brain, skin, and kidney expressed higher

FIGURE 2. Determination of estradiol binding to the mER β 1 and mER β 2. Increasing amounts of [3 H]estradiol-17 β (0.01–100 nM) were incubated with a constant amount of each *in vitro* transcribed/translated mER protein. Three separate experiments were performed and a representative Scatchard analysis is shown for mER β 1 (●) and for mER β 2 (■). The inset is the same mER β 2 analysis shown using differently scaled axes. B/F, bound/free; B, bound.

levels of ER β 2 mRNA relative to ER β 1 mRNA, whereas liver, pancreas, gut and bone expressed predominantly ER β 2 mRNA.

Ligand binding activity of mER β 1 and mER β 2 proteins

ER β 1 (human, rat and mouse) has been shown to bind E $_2$ with high affinity and specificity. However, mER β 2 contains an 18 amino acid insertion within the ligand-binding domain (Brzozowski *et al.* 1997). To determine the ligand-binding capability of mER β 2, both mER β 1 and mER β 2 were synthesized *in vitro* and subjected to saturation ligand-binding analyses using [3 H]E $_2$. As shown in Fig. 2, [3 H]E $_2$ demonstrated specific, saturable binding of high affinity (K_D 0.56 ± 0.19 nM, mean $\pm$ s.e.m., $n=3$ independent experiments) for mER β 1, which

was comparable to that published for both rat and murine ERβ1 (Kuiper *et al.* 1996, Tremblay *et al.* 1997). No ligand-binding activity was detected for mERβ2 in the range of 0.01–5 nM of E₂; however,

specific binding activity was detected when input ligand concentrations were greater than 10 nM. The *K_D* for mERβ2 was calculated to be 17.7 ± 4.7 nM (mean ± s.e.m., *n* = 3 independent experiments).

A.

B.

These data suggest that mER β 2 binds estradiol with around 30-fold less affinity than mER β 1.

DNA binding and bending activity of murine ER β 1 and ER β 2

ER β 1 (human, rat and mouse) was shown to bind to classical ERE either as a homodimer or as a heterodimer with ER β using *in vitro* gel-shift assays (Cowley *et al.* 1997). Consistent with these data, both mER β 1 and mER β 2 proteins synthesized *in vitro* were able to bind specifically to a classical ERE (Fig. 3A). The efficiency of mER β 1 and mER β 2 binding to the ERE oligonucleotide was similar, but was markedly less than the efficiency of mER α binding to the ERE, even though the receptors were present in their respective rabbit reticulocyte lysates at similar levels as determined by parallel reactions in which receptor levels were determined by incorporation of [35 S]methionine into the *in vitro*-translated proteins as described in Materials and Methods. The presence of the appropriate ER within the retarded complexes was demonstrated using the ability of specific antibodies to supershift the retarded complex and the specificity of the binding was assessed by competition with excess unlabeled ERE, whereas no competition was seen with an excess of an unlabeled non-specific 33-mer oligonucleotide. The use of liganded receptor via preincubation with estrogen or antiestrogen had little effect on the DNA-binding characteristics of any receptor under the conditions used (data not shown).

DNA bending assays demonstrated that mER α , mER β 1 and mER β 2 were all able to bend DNA as demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison with the mobility of complexes when the ERE is at the end of the DNA fragment (E) (Fig. 3B). No effect of ligand was observed (data not shown). The calculated bending

angle for the mER α was 66 ± 0.60 (mean $\pm$ S.E.M., $n=3$), for mER β 1 was 63 ± 0.50 and for mER β 2 was 64 ± 0.90 . There were no significant differences of the calculated bending angles amongst the different ER isoforms. A lower overall signal of the retarded complexes was seen with mER β 1 and mER β 2 compared with mER α , which probably reflects the lower efficiency of the mER β isoforms compared with the mER α of binding to an ERE (see Fig. 3A).

The ability of these receptors to bind to classical EREs *in vivo* was examined by co-transfection of receptor expression plasmids with interference reporter plasmids (Reese & Katzenellenbogen 1992) into NIH 3T3 cells. The interference reporter plasmid containing two EREs between the CMV promoter and start site of transcription of the CAT reporter gene has been described and validated previously (Reese & Katzenellenbogen 1992). The relative binding activity of each ER to the EREs was measured by the decrease of CAT gene expression as previously described (Reese & Katzenellenbogen 1992). The data presented in Fig. 4 suggest that both mER β 1 and mER β 2 could bind to ERE in the presence or absence of ligand. Under these *in vivo* conditions, the ability of both mER β 1 and mER β 2 to bind to DNA is comparable to that of mER α . It is concluded that mER β 1 and mER β 2 have similar abilities to bind to and bend DNA containing ERE, both *in vitro* and *in vivo*.

Transcriptional activity of mER β 1 and mER β 2 from ERE-containing promoters

A dose-response analysis of the ability of estradiol to activate transcription of mER β 1 and mER β 2 on an ERE reporter gene after transient transfection into NIH 3T3 cells is shown in Fig. 5. Maximal transcriptional activation of mER β 1 is observed with approximately 10^{-9} M estradiol, whereas activation of mER β 2 is only observed with 10^{-6} M estradiol. These data are consistent with the

FIGURE 3. Determination of the ability of mER α , mER β 1 and mER β 2 to bind to and bend DNA. (A) Autoradiograph of an electrophoretic mobility gel shift analysis of *in vitro* transcribed/translated mER α , mER β 1 and mER β 2 proteins binding to a 35-mer double-stranded ERE oligonucleotide containing the vitellogenin A2 ERE sequence. Free ERE and the shifted complexes are indicated. The presence of the appropriate mER isoform in the shifted complex was determined by the ability of a specific antibody (H222 for ER α ; PA1 for the ER β proteins) to super-shift the complex. Super-shifted complexes are indicated. Specificity of the complexes was determined by the ability of a 200-fold excess of the unlabelled ERE (specific competitor) to compete for the shifted complex and non-specific interactions were determined using a 200-fold excess of unlabeled non-specific 33-mer oligonucleotide (non-specific competitor). (B) Ability of mER α , mER β 1 and mER β 2 to bend DNA. *In vitro* transcribed/translated mER isoforms were preincubated with 10 nM estradiol-17 β , followed by incubation with radiolabeled ERE Bend fragments as described in the Materials and Methods and subjected to electrophoretic gel mobility shift analysis. DNA bending was demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison with the mobility of complexes when the ERE is at the end of the DNA fragment (E). RRL, rabbit reticulocyte lysate alone.

FIGURE 4. Determination of the ability of mER α , mER β 1 and mER β 2 to bind to DNA *in vivo*. A promoter interference assay as previously described and validated (Reese & Katzenellenbogen 1992) was used to determine the ability of the mER isoforms to bind to an ERE *in vivo*. We co-transfected 300 ng control or mER expression vector plus 300 ng pCH110 with 700 ng pCMV-(ERE)₂-CAT into NIH 3T3 cells in the presence (■, +) or absence (□, -) of 10 nM estradiol-17 β , and CAT expression was determined 48 h later. Each transfection was performed in triplicate and two independent experiments were performed. Data are expressed as a percentage of the control (empty expression vector alone) activity, and represent the mean $\pm$ range of values obtained from two independent experiments.

markedly reduced affinity for estradiol of mER β 2 compared with mER β 1. Both natural and synthetic (DES) estrogens activated transcription of the mER β 1 (data not shown). Neither steroidal (ICI182,780) nor non-steroidal (4-OH-Tam) anti-estrogens activated transcription of either mER β isoform (data not shown). It is unlikely that the differences seen between mER β 1 and mER β 2 are due to different levels of expression of the ER isoforms, as western blot analysis of extracts of transiently transfected NIH 3T3 cells showed similar levels of expression of both mER β 1 and mER β 2 (Fig. 6).

As shown in Table 1, under maximal conditions mER β 1 induced a fivefold and an 11-fold increase in

FIGURE 5. Transcriptional activity of mER β 1 and mER β 2 on ERE-regulated CAT reporter gene. Ligand dose-dependent ability of mER β 1 (■) and mER β 2 (□) to activate transcription of vitellogenin A2 ERE-containing CAT reporter gene transfected into NIH 3T3 cells. Results show the mean fold induction $\pm$ s.d. of two independent experiments. β gal, β -galactosidase.

FIGURE 6. Western blot analysis of NIH 3T3 cell extracts (20 μ g protein) 48 h after transfection of 4 μ g mER α , mER β 1 and mER β 2 expression vectors. Lane 1 is empty expression vector alone; lane 2 is mER α expression vector; lane 3 is mER β 1 expression vector; lane 4 is mER β 2 expression vector. ER- α antibody is a blot visualized with the ER α -specific AER314 antibody; ER- β antibody is a blot visualized with the ER β -specific Y19 antibody. Arrows on the left hand side indicate mobility of molecular mass standards, and arrows on the right hand side indicate the calculated molecular mass of the ER-immunoreactive proteins.

CAT expression through the vitellogenin B1 ERE- and A2 ERE-containing promoters respectively (Table 1). Similarly, 10 nM E₂ was able to increase the transcriptional activity of mER α on both vitellogenin B1- and A2 ERE-driven promoters. It was noted, however, that mER α demonstrated a greater apparently ligand-independent basal transcriptional activity compared with either mER β 1 or

TABLE 1. Determinations of the ability of mERα, mERβ1 and mERβ2 to activate transcription from classical ERE-regulated reporter genes. Empty expression vector (Control, 166 ng) or the expression vectors for mERα, mERβ1 and mERβ2 were co-transfected into NIH 3T3 cells together with 166 ng pCH110 and 1.3 µg CAT reporter vectors containing the vitellogenin B1-ERE (A), or the vitellogenin A2-ERE (B) in the promoter, in the presence (+) or absence (–) of 10 nM estradiol-17β (E₂). Each assay was done at least in duplicate and often in triplicate. The mean ± s.d. of three independent experiments (*n*=3) is shown in panel A. The results shown in panel B represent the mean ± range of two independent experiments (*n*=2)

	+E ₂	CAT activity/βgal (fold induction)	
		A	B
Empty Vector	–	1 ± 0.3	1 ± 0.1
	+	1 ± 0.3	1 ± 0.2
mERα	–	6.4 ± 1.9††	9.6 ± 2.7
	+	13.4 ± 3.9*	58 ± 21
mERβ1	–	2.1 ± 0.9	3.1 ± 0.2
	+	9.9 ± 1.6**	34 ± 1.2
mERβ2	–	1.4 ± 0.3	1.9 ± 0.2
	+	1.3 ± 0.1	4.2 ± 0.8

Statistically significant differences (unpaired two-tailed *t*-test) from the appropriately non-estrogen-treated cells: **P*<0.05, ***P*<0.01.

††Statistically significant difference (unpaired two-tailed *t*-test) between untreated mERα-transfected and untreated empty vector (control) transfected cells.

mERβ2 (Table 1, data in the absence of added E₂). In addition, mERβ2 showed little, if any, ligand-independent transcriptional activity under these conditions.

Our data so far suggest that mERβ2 has markedly reduced ligand-binding activity compared with mERβ1 and, under conditions in which ligand maximally activated mERβ1- and mERα-induced transcription (i.e. 10 nM E₂), mERβ2 alone had no transactivating activity. However, our data also suggest that mERβ2 bound to DNA both *in vitro* and *in vivo* (see Figs 3 and 4) with an efficiency similar to that of mERβ1 and could bend DNA to a similar extent *in vitro*. As co-expression of both mERβ1 and mERβ2 mRNA was found in most murine tissues so far examined, the data suggest the possibility that, if the mRNA levels reflect the protein concentrations of these ERβ isoforms, under some physiological conditions mERβ1 could be maximally activated by ligand, whereas mERβ2 would remain inactive, although it might act as a modulator of ligand-activated mERβ1 function. To test this hypothesis, transient co-transfection studies were conducted using a constant level of mERβ1 expression vector together with increasing amounts of mERβ2 expression vector. A dose-

TABLE 2. Effect of increasing amounts of co-expressed mERβ2 on the ability of mERα (A) and mERβ1 (B) to activate transcription from an ERE (vitellogenin A2)-regulated CAT receptor gene in the presence (+) and absence (–) of 10 nM estradiol-17β (E₂) after co-transfection into NIH 3T3 cells. Each assay was performed in triplicate and the results show the mean ± range of two independent experiments (*n*=2). ANOVA (one-way) showed that the effect of mERβ2 on mERα (A) was significantly significant (*P*<0.0001), whereas the effect of mERβ2 on mERβ1 (B) did not reach statistical significance

mERα (83 ng)	mERβ1 (83 ng)	mERβ2 (x ng)	+E ₂	CAT activity/βgal (fold induction)	
				A	B
+	+	–	–	1 ± 0.2	1 ± 0.5
+	+	–	+	8.8 ± 1	28.5 ± 15.8
+	+	16	+	8.1 ± 0.01	21.6 ± 16.8
–	–	16	+	1.2 ± 0.02	0.9 ± 1.0
+	+	83	+	4.7 ± 0.2	11.6 ± 7.3
–	–	83	+	1 ± 0.1	1.1 ± 0.2
+	+	416	+	2.2 ± 0.2	2.3 ± 0.5
–	–	416	+	1 ± 0.2	1.7 ± 0.9
+	+	833	+	1.8 ± 0.5	7.8 ± 3.9
–	–	833	+	1.1 ± 0.1	3.6 ± 0.5

dependent inhibition of estrogen-induced mERβ1 transcriptional activity by mERβ2 was found (Table 2). This occurred under conditions in which mERβ2 alone had no effect on transcription. In addition, under similar conditions, a dose-dependent inhibition of estrogen-induced mERα transcriptional activity by mERβ2 was found (Table 2). The data are consistent with the ability of mERβ2 to act as a negative regulator of mERβ1 and mERα transcriptional activity on promoters containing classical EREs, in NIH 3T3 cells.

Transcriptional activity of mERβ1 and mERβ2 from a raloxifene-responsive region containing promoter (TGF-β3)

The transcriptional activity of mERβ1 and mERβ2 was examined using promoters regulated by so-called 'non-classical' estrogen-responsive promoters – the raloxifene-responsive region in the TGF-β3 promoter (Yang *et al.* 1996). As we were unable to obtain measurable activity of the TGF-β3–CAT reporter using calcium phosphate-mediated transfection, we used the methodology based on Effectene reagent transfection (see Materials and Methods) for the ensuing studies. To ensure that this change in experimental procedure did not substantially alter the previously observed differences between mERβ1 and mERβ2 at an ERE-tk-CAT reporter gene in NIH 3T3 cells, we repeated

the dose-response experiments using Effectene reagent transfection methodology. The results are shown in Fig. 7A. As expected, a dose-dependent increase in transcriptional activity of mER β 1 was observed, with maximum activity with approximately 10^{-9} M estradiol, which was similar to that seen previously (see Fig. 5). Furthermore, also as seen previously, estradiol at 10^{-6} M or greater concentrations transcriptionally activated mER β 2 at classical ERE-regulated reporter genes. This activity appears to be specific to estradiol, as no effect of 10^{-5} M cortisone was observed under these conditions. Therefore, the differences observed between mER β 1 and mER β 2 at ERE-regulated promoters were not substantially altered by the use of methodology based on Effectene reagent transfection, and we continued with the analysis of mER β 1 and mER β 2 activity at the raloxifene responsive-

region in the TGF- β 3 promoter using the TGF- β 3-CAT reporter.

In contrast to activity at an ERE-containing promoter, no differences in the ligand-independent or basal transcriptional activity amongst mER α , mER β 1 and mER β 2 were found (data not shown). All receptor isoforms in the presence of estradiol were able to increase transcription from the TGF- β 3-CAT reporter in a dose-dependent fashion (Fig. 7B), although the dose-response curve for mER β 2 compared with mER α and mER β 1 was moved to the right, consistent with the reduced affinity of mER β 2 for estradiol. The dependence of this activity on the expression of an ER isoform was demonstrated by the inability of estradiol and LY117018 to activate the TGF- β 3-CAT reporter in the presence of an empty expression vector (X in Fig. 7B and C). The half-maximal activation for mER α and mER β 1 was achieved with similar concentrations of estradiol and the maximum fold

FIGURE 7. Ability of mER α , mER β 1 and mER β 2 to regulate transcription from a novel raloxifene-responsive region-regulated reporter gene. (A) mER β 1 (■) and mER β 2 (●) were co-transfected into NIH 3T3 cells together with 83 ng pCH110 and 333 ng ERE (vitellogenin A2)-CAT reporter, using the same transfection conditions as for the TGF- β 3-CAT experiments shown below. Results represent the mean $\pm$ s.d. fold induced CAT activity of three independent experiments each performed in duplicate. The differences between the effects of estrogen on the two receptors was significant at the $P < 0.0001$ level (ANOVA, two-way). (B) Expression vectors for mER α (★), mER β 1 (■) and mER β 2 (●) (83 ng) were co-transfected into NIH 3T3 cells together with 83 ng pCH110 and 333 ng CAT reporter vectors containing a novel raloxifene-responsive region present in the TGF- β 3 promoter. The cells were treated with increasing concentrations of estradiol-17 β . The results represent the mean $\pm$ s.d. fold induced CAT activity of three independent experiments each performed in triplicate. X represents CAT activity after transfection of the reporter with empty expression vector alone followed by treatment with the indicated concentration of estradiol. (C) Expression vectors for mER α (★), mER β 1 (■) and mER β 2 (●) (83 ng) were co-transfected into NIH 3T3 cells together with 83 ng pCH110 and 333 ng CAT reporter vectors containing a novel raloxifene-responsive region present in the TGF- β 3 promoter. The cells were treated with increasing concentrations of the raloxifene analog LY117018. The results represent the mean $\pm$ s.d. fold induced CAT activity of three independent experiments each performed in triplicate. X represents CAT activity after transfection of the reporter with empty expression vector alone, followed by treatment with the indicated concentration of LY117018.

induction was also similar. However, a greater concentration of estradiol was required to activate the mER β 2 half-maximally, and the maximum fold induction was less than that achieved with mER α and mER β 1. In contrast, in the presence of the raloxifene analog, LY117018, all three isoforms showed similar maximum activities (Fig. 7C) and potencies, with mER β 2 being less active only at the lowest concentration of LY117018 used. The ability of estradiol and LY117018 to activate both mER α and mER β 1 on the TGF- β 3 promoter appeared to be identical, and mER β 2 activity in the presence of ligand – either estradiol or LY117018 – was only slightly less than that observed with either mER β 1 or mER α . This was in contrast to the markedly reduced potency of estradiol to induce transcription via the mER β 2 at an ERE-regulated reporter gene. The activity of the estradiol–mER β 2 complex at the TGF- β 3 promoter appeared greater than that observed at the ERE-regulated promoter (compare Fig. 7A with Fig. 7B). Furthermore, both mER β 1 and mER β 2, when liganded with LY117018 inhibited transcription at an ERE-regulated promoter (data not shown) under these transfection conditions, which is in marked contrast to their stimulatory effect at the TGF- β 3 promoter. These data suggest that responsiveness of gene expression to a relatively highly expressed variant murine ER β isoform, mER β 2, is both ligand and promoter specific.

DISCUSSION

In this study, we have investigated some of the potential functional characteristics of the protein encoded by a naturally occurring, abundantly expressed mRNA splice variant of the mouse ER β . This murine ER β splice variant, now referred to as murine ER β 2, contains an in-frame 54 nucleotide insertion between exon 5 and exon 6 sequences of the wild-type murine ER β , now referred to as ER β 1. Interestingly, we have not detected the equivalent of rodent ER β 2 transcripts in any human tissue so far analyzed, suggesting a species-specific alternative splicing mechanism and, possibly, function (Lu *et al.* 1998). The protein predicted from ER β 2 mRNA would contain an 18 amino acid insertion in the ligand-binding domain of wild-type ER β 1. The mER β 2 is the mouse homolog of a recently described rER β 2 (Chu & Fuller 1997, Petersen *et al.* 1998). The 18 amino acid insertion in the mER β 2 has 89% amino acid identity with the rER β 2 (Lu *et al.* 1998). By analogy to ER α , the 18 amino acid insertion is within the E domain of the receptor, which contains the ligand-binding

domain, dimerization function and a ligand-inducible transactivating function (Brzozowski *et al.* 1997, Parker *et al.* 1993). Therefore, the insertion of 18 amino acids might be predicted to alter some of the functional characteristics of the E domain. Our data suggest that the mER β 2 has markedly reduced ligand-binding affinity compared with mER β 1, as classically measured using saturation analysis with radiolabelled ligand *in vitro*. These data are similar to the results obtained for the rER β 2 (Petersen *et al.* 1998), with which reduced but measurable binding affinity for radiolabeled estradiol was demonstrated. Both murine and rat ER β 1 and ER β 2 appear to bind to ERE oligonucleotides with similar affinity in the presence and absence of ligand in EMSA, although they are less efficient than ER α in doing so. Furthermore, the mER β s were also able to bind to DNA *in vivo* as assessed in whole cells using a previously described promoter interference assay (Reese & Katzenellenbogen 1992) and this activity was again unaffected by treatment with estradiol under these experimental conditions. The three ER isoforms were also found to bend DNA in a similar fashion. From these data, we conclude that the mER β 2 is an ER β -like protein that can bind to and bend ERE-containing DNA sequences both *in vitro* and *in vivo*, in a fashion similar to mER β 1, despite its affinity for estradiol being significantly less than that of mER β 1. These data suggested that ER β 2 might have either constitutive transcriptional activity or may interfere with the transcriptional activity of ER β 1.

Consistent with its low binding affinity for estradiol, the mER β 2 has no significant transcriptional activity of its own on promoters containing EREs, except at high (>1 μ M) concentrations of added estradiol (see Figs 5 and 7A). Interestingly, our data suggest that mER β 2, under conditions in which little, if any, of the protein is likely to be bound to estradiol, inhibits the ligand-induced transcriptional activity of mER β 1 and mER α at this type of promoter. Our data suggest that mER β 2 inhibited the estrogen-induced mER β 1 transcriptional activity by 50% when expressed at a 1:1 ratio, and achieved complete inhibition when present at five times the amount of mER β 1. If the relative RNA levels reflect the relative protein concentrations, our data suggest that, in some tissues, mER β 2 can be expressed in at least three times greater levels than mER β 1. Therefore, in some tissues, mER β 2 could have a significant modulatory effect over the ability of mER β 1 to respond to estrogenic compounds, under conditions in which mER β 2 is unlikely to be bound to ligand. In addition, although our data do not concern this issue, it may be speculated that, under conditions in

which high concentrations of ligand occur, both receptor isoforms would be liganded and could possibly be additive in their regulation of gene expression. In contrast to our data, rER β 2 was not shown to have significant inhibitory effects on rER β 1 in the study reported by Peterson *et al.* (1998). However, their study used a heterologous system for the analyses – rER β proteins were analyzed in human cell lines – whereas our analyses were carried out in a homologous system – mER β proteins expressed in a murine cell line. Such experimental variations may underlie some of the observed differences between the studies. Not only have species-specific differences between ER α proteins been shown to occur (Curtis *et al.* 1997), but it is also well known that differential activities of estrogen agonists and antagonists via the ER α occur, which are promoter and often cell type specific (Martin & Middleton 1978, Tzukerman *et al.* 1994, Curtis *et al.* 1997). While our studies were being carried out, a report was published demonstrating that rER β 2 had a dominant negative activity on rER β 1 and rER α (Maruyama *et al.* 1998), which is consistent with the findings from our mouse studies. However, the significance of the ability of mER β 2 to exert a dominant negative effect on mER α will depend on their co-expression in the same cell, in addition to the relative expression of the two proteins.

In our hands, the apparent ligand-independent activity (basal) of mER β 1 and mER β 2 on ERE-containing promoters was also significantly less than that observed for mER α , which is in contrast to results previously obtained with mER β 1 (Tremblay *et al.* 1997). Again, the differences may reside in the use of a heterologous system – that is, mouse proteins expressed in human cell lines (Tremblay *et al.* 1997) rather than mouse proteins expressed in murine cell lines (present study). It is interesting that studies reported in abstract form (Galluzzo & McDonnell 1998) have shown that, in human cell lines, hER α has significantly greater ligand-independent activity than hER β 1, which would be consistent with our murine studies. The differences in the ligand-independent activities of mER α and mER β isoforms could also contribute to tissue-specific estrogenic action and the ability of the estrogen signaling mechanism to cross-talk with other signal transduction systems (El-Tanani & Green 1997, Tremblay *et al.* 1997).

We have also investigated the transcriptional activity of the three mER isoforms on the TGF- β 3 promoter, which has been reported to be regulated by hER α , using a novel mechanism (Yang *et al.* 1996). In contrast to previous studies using the hER α in which the estradiol liganded receptor was

much less efficient in activating transcription than the raloxifene-liganded receptor, the activity of the mER α receptor liganded with either estradiol or the raloxifene analog, LY117018, on the TGF- β 3 promoter was similar, despite the previously observed lower relative binding affinity of LY117018, compared with that of estradiol, for the rat and human ER α determined *in vitro* at 4 °C (Wakeling *et al.* 1984). However, raloxifene has a greater affinity for rER α than has LY117018 (Jones *et al.* 1984), and the affinity of both these compounds for ER α markedly increases and exceeds that of estradiol at 30 °C (Black *et al.* 1981, 1983) mainly because of reduced dissociation rates compared with estradiol at higher temperatures. Therefore, the greater stability of the LY117018/ER α complex may also occur when LY117018 (or its analogs) is bound to the mER β isoforms.

The action of the liganded mER isoforms at the TGF- β 3 promoter is apparently distinct from that of so-called ‘classical’ ERE and other ‘non-classical’ estrogen-regulated promoters. First, LY117018 bound to all ER isoforms inhibits transcription of ERE-regulated promoters, but stimulates transcription of the TGF- β 3 promoter. Secondly, although antiestrogens stimulate transcription when liganded to all ER isoforms at AP-1 regulated promoters (Paech *et al.* 1997), in contrast to the TGF- β 3 promoter, estradiol-liganded ER β isoforms inhibit transcription at AP-1 regulated promoters (Paech *et al.* 1997). Thirdly, the relative potency of estradiol to activate transcription of mER β 2 at the TGF- β 3 promoter is significantly greater than its ability to activate transcription of mER β 2 at an ERE-regulated promoter. This suggests that conformational changes induced by estrogen binding to mER β 2 are sufficient *in vivo* to drive significant transcriptional activity at the TGF- β 3 promoter but unable to affect an ERE-driven promoter, except at very high, and under most circumstances non-physiological, concentrations of estradiol. The data also suggest that, in a promoter-specific fashion, mER β 2 could, at low ligand levels, have an inhibitory action on both mER β 1 and mER α , whereas at higher ligand concentrations it could be additive to, or possibly synergistic with, the action of mER β 1 and possibly mER α . The data are also consistent with the idea that the mechanisms by which ER isoforms regulate the TGF- β 3 promoter are quite distinct from those by which ER isoforms affect either AP-1-regulated promoters or ERE-regulated promoters.

The relevance of the data presented in this study and of other data in the literature concerning the putative function of the ER β 2 resides in the detection of a stably expressed protein predicted

from the ER β 2 mRNA. Although there are no unequivocal data that directly prove the detection of the ER β 2 protein, our data suggest that the ER β 2 protein can be transiently expressed and detected in mouse NIH 3T3 cells using an expression vector construct (see Fig. 6). This is consistent with other published transient transfection data (Maruyama *et al.* 1998, Petersen *et al.* 1998). Furthermore, some published western blot analyses of rodent tissues or cell extracts, which used ER β -specific antibodies, sometimes identified a doublet around 66 kDa that could be consistent with ER β -like proteins representing the wild-type ER β 1 and a slightly larger ER β 2 variant (Grohe *et al.* 1998, Fitzpatrick *et al.* 1999, O'Brien *et al.* 1999). However, other possibilities such as post-translational modifications cannot be ruled out at this stage.

In conclusion, our data suggest that the variant mER β 2 can exert both ligand-independent and ligand-dependent effects at promoters known to contain both ERE and non-ERE estrogen-responsive elements, and that the effect is promoter specific. In addition, this novel mER β variant isoform can exert dominant negative transcriptional effects on both mER β 1 and mER α . The physiological and pathophysiological importance of these activities will depend, not only on the relative expression of all the mER isoforms, but also on the co-expression of these ERs in the same cells. Data presented in this study, together with those in the literature, would suggest that differential tissue expression of the mER isoforms probably occurs, which leads to the speculation that this may in part contribute to the mechanisms associated with tissue-specific estrogenic and, possibly, antiestrogenic action.

ACKNOWLEDGEMENTS

This work was supported by the US Army Medical Research and Materiel Command (USAMRMC) and the Canadian Breast Cancer Research Initiative (CBCRI). B L is a recipient of a Medical Research Council of Canada (MRC) Studentship, L C M is an MRC Scientist, E L is a recipient of a USAMRMC Postdoctoral Fellowship.

REFERENCES

- Black L, Jones C & Goode R 1981 Differential interaction of antiestrogens with cytosol estrogen receptors. *Molecular and Cellular Endocrinology* **22** 95–103.
- Black L, Jones C & Falcone J 1983 Antagonism of estrogen action with a new benzothioophene derived antiestrogen. *Life Sciences* **32** 1031–1036.
- Brzozowski A, Pike A, Dauter Z, Hubbard R, Bonn T, Engstrom O, Ohman L, Green G, Gustafsson J-A & Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389** 753–758.
- Chu S & Fuller P 1997 Identification of a splice variant of the rat estrogen receptor β gene. *Molecular and Cellular Endocrinology* **132** 195–199.
- Couse J, Curtis S, Washburn T, Lindzey J, Golding T, Lubahn D, Smithies O & Korach K 1995 Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene. *Molecular Endocrinology* **9** 1441–1454.
- Cowley S, Hoare S, Mosselman S & Parker M 1997 Estrogen receptors alpha and beta form heterodimers on DNA. *Journal of Biological Chemistry* **272** 19858–19862.
- Curtis S, Shi H, Teng C & Korach K 1997 Promoter and species specific differential estrogen mediated gene transcription in the uterus and cultured cells using structurally altered agonists. *Journal of Molecular Endocrinology* **18** 203–211.
- Daffada AA, Johnston SR, Nicholls J & Dowsett M 1994 Detection of wild type and exon 5-deleted splice variant oestrogen receptor (ER) mRNA in ER-positive and -negative breast cancer cell lines by reverse transcription/polymerase chain reaction. *Journal of Molecular Endocrinology* **13** 265–273.
- Daffada AA, Johnston SR, Smith IE, Detre S, King N & Dowsett M 1995 Exon 5 deletion variant estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. *Cancer Research* **55** 288–293.
- Dotzlaw H, Miller T, Karvelas J & Murphy LC 1990 Epidermal growth factor gene expression in human breast cancer biopsy samples: relationship to estrogen and progesterone receptor gene expression. *Cancer Research* **50** 4204–4208.
- Dotzlaw H, Alkhalaf M & Murphy LC 1992 Characterization of estrogen receptor variant mRNAs from human breast cancers. *Molecular Endocrinology* **6** 73–785.
- Dotzlaw H, Leygue E, Watson P & Murphy L 1997 Expression of estrogen receptor-beta in human breast tumors. *Journal of Clinical Endocrinology and Metabolism* **82** 2371–2374.
- El-Tanani M & Green C 1997 Two separate mechanisms for ligand independent activation of the estrogen receptor. *Molecular Endocrinology* **11** 928–937.
- 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.
- Fitzpatrick S, Funkhouser J, Sindoni D, Stevis P, Deecher D, Bapat A, Merchenthaler I & Frail D 1999 Expression of estrogen receptor β protein in rodent ovary. *Endocrinology* **140** 2581–2591.
- Galluzzo J & McDonnell D 1998 Estrogen receptors alpha and beta are mechanistically distinct in their responses to agonists and antagonists. *Xth International Congress on Hormonal Steroids*, Quebec City. Abstract 8.
- Giguere V, Tremblay A & Tremblay G 1998 Estrogen receptor β : re-evaluation of estrogen and antiestrogen signalling. *Steroids* **63** 335–339.
- Graham F & van der Eb A 1973 A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52** 456–457.
- Grohe C, Kahlert S, Lobbert K & Vetter H 1998 Expression of oestrogen receptor α and β in rat heart: role of local oestrogen synthesis. *Journal of Endocrinology* **156** R1–R7.

- Hanstein B, Liu H, Yancisin M & Brown M 1999 Functional analysis of a novel estrogen receptor- β isoform. *Molecular Endocrinology* **13** 129–137.
- Jones C, Jevnikar M, Pike A, Peters M, Black L, Thompson A, Falcone J & Clemens J 1984 Antiestrogens. 2. Structure–activity studies in a series of 3-aroyle-2-arylbenzo[β]thiophene derivatives leading to [6-hydroxy-2-(4-hydroxyphenyl)benzo[β]thien-3-yl] [4-[2-(1-piperidinyl)ethoxy]-phenyl] methanone hydrochloride (LY156758), a remarkably effective estrogen antagonist with only minimal intrinsic estrogenicity. *Journal of Medicinal Chemistry* **27** 1057–1066.
- Katzenellenbogen BS & Korach KS 1997 A new actor in the estrogen receptor drama – enter ER- β . *Endocrinology* **138** 861–862 (editorial).
- Kuiper G, Enmark E, Peltö-Huikko M, Nilsson S & Gustafsson J-A 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *PNAS* **93** 5925–5930.
- Laemmli U 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.
- Leygue ER, Watson PH & Murphy LC 1996 Estrogen receptor variants in normal human mammary tissue. *Journal of the National Cancer Institute* **88** 284–290.
- Lu B, Leygue E, Dotzlaw H, Murphy L, Murphy L & Watson P 1998 Estrogen receptor- β mRNA variants in human and murine tissues. *Molecular and Cellular Endocrinology* **138** 199–203.
- Martin L & Middleton E 1978 Prolonged oestrogenic and mitogenic activity of tamoxifen in the ovariectomized mouse. *Journal of Endocrinology* **78** 125–129.
- Maruyama K, Endoh H, Sasaki-Iwaoka H, Kanou H, Shimaya E, Hashimoto S, Kato S & Kawashima H 1998 A novel isoform of rat estrogen receptor β with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regulator of estrogen action. *Biochemical and Biophysical Research Communications* **246** 142–147.
- Moore J, McKee D, Slentz-Kesler K, Moore L, Jones S, Horne E, Su J-L, Kliewer S, Lehmann J & Willson T 1998 Cloning and characterization of human estrogen receptor β isoforms. *Biochemical and Biophysical Research Communications* **247** 75–78.
- Murphy L, Dotzlaw H, Leygue E, Douglas D, Coutts A & Watson P 1997 Estrogen receptor variants and mutations: a review. *Journal of Steroid Biochemistry and Molecular Biology* **62** 363–372.
- Nardulli A & Shapiro D 1992 Binding of the estrogen receptor DNA-binding domain to the estrogen response element induces DNA bending. *Molecular and Cellular Biology* **12** 2037–2042.
- O'Brien M, Park K, In Y & Park-Sarge O 1999 Characterization of estrogen receptor- β (ER β) messenger ribonucleic acid and protein expression in rat granulosa cells. *Endocrinology* **140** 4530–4541.
- Paech K, Webb P, Kuiper G, Nilsson S, Gustafsson J, Kushner PJ & Scanlan TS 1997 Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science* **277** 1508–1510.
- Parker MG, Arbuckle N, Dauvois S, Danielian P & White R 1993 Structure and function of the estrogen receptor. *Annals of the New York Academy of Sciences* **684** 119–126.
- Petersen D, Tkalecic G, Koza-Taylor P, Turi T & Brown T 1998 Identification of estrogen receptor β 2, a functional variant of estrogen receptor β expressed in normal rat tissues. *Endocrinology* **139** 1082–1092.
- Reese JC & Katzenellenbogen BS 1992 Examination of the DNA-binding ability of estrogen receptor in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens. *Molecular and Cellular Biology* **12** 4531–4538.
- Seiler-Tuyns A, Walker P, Martinez E, Merillat A, Givel F & Wahli W 1986 Identification of estrogen-responsive DNA sequences by transient expression experiments in a human breast cancer cell line. *Nucleic Acids Research* **14** 8755–8770.
- Thompson J & Landy A 1988 Empirical estimation of protein induced DNA bending angles: applications to 1 site specific recombination complexes. *Nucleic Acids Research* **16** 9687–9705.
- Tremblay G, Tremblay A, Copeland N, Gilbert D, Jenkins N, Labrie F & Giguere V 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β . *Molecular Endocrinology* **11** 353–365.
- Tzukerman M, Esty A, Santiso-Mere D, Danielian P, Parker M, Stein R, Pike J & McDonnell D 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Molecular Endocrinology* **8** 21–30.
- Vladusic E, Hornby A, Guerra-Vladusic F & Lupu R 1998 Expression of estrogen receptor β messenger RNA variant in breast cancer. *Cancer Research* **58** 210–214.
- Wakeling A, Valcaccia B, Newbould E & Green L 1984 Non steroidal antiestrogens – receptor binding and biological response in rat uterus, rat mammary carcinoma and human breast cancer cells. *Journal of Steroid Biochemistry* **20** 111–120.
- Webb P, Lopez G, Greene G, Baxter J & Kushner P 1992 The limits of the cellular capacity to mediate an estrogen response. *Molecular Endocrinology* **6** 157–167.
- Yang NN, Venugopalan M, Hardikar S & Glasbrook A 1996 Identification of an estrogen response element activated by metabolites of 17- β estradiol and raloxifene. *Science* **273** 1222–1225.

REVISED MANUSCRIPT RECEIVED 13 April 2000