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

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ABSTRACT

We have isolated a highly expressed splice variant mRNA of murine estrogen receptor-β (ERβ), mERβ2, containing an in-frame 54 nucleotide insertion between exons 5 and 6 of wild-type mERβ1. The predicted ERβ2 protein contains 18 amino acids inserted in the ligand binding domain of mERβ1. Recombinant protein generated by \textit{in vitro} transcription/translation showed that mERβ2 had markedly reduced ligand binding (\(K_D=17.7 \pm 4.7\) nM, mean \pm s.e.m., \(n=3\)) compared with mERβ1-bound \(^3\)H-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) \textit{in vitro} and \textit{in vivo}, and similarly bent DNA. Transcriptional activity was assessed using transient transfection analysis into a homologous murine cell line, NIH 3T3 cells. mERβ1 transactivated \(ERE\)-\textit{tk-CAT} reporter genes similarly to mERα, whereas mERβ2 had little activity except at high ligand concentrations. However, under conditions in which mERβ2 is unlikely to be ligand saturated, co-transfected mERβ2 inhibited activity of mERα and possibly mERβ1 on \(ERE\)-\textit{tk-CAT} genes. Using a ‘novel raloxifene responsive’ gene reporter system (TGF-β3-CAT), we found the ability of estradiol and LY117018 to activate both mERα and mERβ1 on this promoter was identical, and mERβ2 activity in the presence of either estradiol or LY117018 was only slightly less than that observed with either mERβ1 or mERα. Both mERβ1 and mERβ2 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-β3 promoter. These data suggest that responsiveness of gene expression to a relatively highly expressed variant murine ERβ isoform, mERβ2, is both ligand and promoter specific. Determination of the relative level of expression of mERβ1 mRNA and mERβ2 mRNA in mouse tissues indicated predominance of mERβ2 mRNA in some but not all tissues. These data suggest that the mERβ2 may have some tissue-specific and promoter-specific modulatory effects.

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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-alpha (ERα) and estrogen receptor-beta (ERβ) (Katzenellenbogen & Korach 1997, Giguere \textit{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 \textit{et al}. 1998). However, several splice variants for both ERα and ERβ have been characterized (Chu & Fuller 1997, Murphy \textit{et al}. 1997, Moore \textit{et al}. 1998, Vladusic \textit{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 ERβ (rERβ2) was cloned and characterized (Chu & Fuller 1997, Petersen \textit{et al}. 1998). In addition, we have recently isolated and characterized several splice variants of the murine ERβ, including murine...
ERβ2, 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 ERβ1 (mERβ1) (Lu et al. 1998). The murine ERβ2 (mERβ2) is analogous to the rat ERβ2 (rERβ2), although two amino acids within the inserted sequence differ between the two species (Lu et al. 1998). Exon-deleted splice variants of ERβ were detected in both human and murine tissues, but the human homolog of mERβ2 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 ERβ2 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 ERβ variants could exist. Previous studies in which the putative functions of rERβ2 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 mERβ1 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 mERβ1 and mERβ2 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 17β-estradiol (E2), diethylstilbestrol (DES), 4-hydroxytamoxifen (4-OH-Tam) and 3-cyclohexylamino-1-propanesulfonic acid (CAPS) from Sigma Chemical Co. (St Louis, MO, USA). IC1182,780 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). All other cell culture ingredients were obtained from Gibco Brl (Burlington, Ontario, Canada). All other cell culture reagents were purchased from Flow Laboratories (Mississauga, Ontario, Canada). ERα H222 antibody, which recognizes an epitope in the E-domain of ERα, was a gift from Dr G Greene (University of Chicago, Chicago, IL, USA). ERβ antibody PAI-310 raised to a synthetic peptide corresponding to rERβ C-terminal amino acids 467–485 (as determined from the rERβ sequence quoted by Kuiper et al. (1996)) was from Affinity BioReagents (Golden, CO, USA). The mERα expression plasmid (pcDNA3/wild-type vector) (Couse et al. 1995) was a gift from Dr K Korach (NIHES, Research Triangle, NC, USA) and the mERβ1 expression plasmid (pCMX–mERβ1) (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 ERβ1. The murine ERβ2 expression plasmid (pCMX–mERβ2) was constructed by replacement of the murine ERβ1 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 ERβ2 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 ± 18) amino acid murine ERβ2 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′–AGTCACGTGTGACC–3′), was a gift from Dr W Wahli (Seiler-Tuyns et al. 1986). The ERE<sub>as2</sub>-delta-TCO plasmid containing two vitellogenin A2 (−333/-288, 5′-GGTCACAG TGACC-3′) EREs upstream of a chloramphenicol acetyltransferase (CAT) reporter was a gift from Dr P Webb (Webb et al. 1992). CMV-(ERE<sub>2</sub>)-CAT (Reese & Katzenellenbogen 1992) was a gift from Dr B Katzenellenbogen (University of Illinois, Urbana, IL, USA), respectively. The TGF-β3–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 µg per reaction) was reverse-transcribed as previously described (Lu et al. 1998). One microliter of this reaction was amplified by PCR incorporating [α-32P]dCTP in a final volume of 10 µl. 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 ERβ1 and ERβ2 at the same time are located in exons 5 and 6 respectively, defined by analogy to the human ERβ gene (Enmark et al. 1997). Bands corresponding to ERβ2 and ERβ1 PCR products were quantified by scanning and the relative ratio of ERβ2 to ERβ1 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 ERβ (sense) 5’-GCTGATGGTGGGGCTGATGT-3’ (priming site in exon 5, nucleotides 890–909, as numbered in reference (Tremblay et al. 1997)); murine ERβ (antisense) 5’-ATGCCAAAGATTTCGCAG 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 ERα (pcDNA3/wild-type vector), murine ERβ1 (pCMX–mERβ1), and murine ERβ2 (pCMX–mERβ2) 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 [3H]E2 (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 E2. Unbound steroid was removed by addition of 500 µl 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 [3H]E2 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 µl programmed lysates containing equal amounts of each receptor as determined by polyacrylamide gel electrophoresis of 35S-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 µl; the reaction solution was 5 mM Tris chloride, pH 7·5, 100 mM KCl, 2 mM dithiothreitol, 5% v/v glycerol and contained 2 µg polyd(I-C). The binding reaction was initiated by adding 1 µl (approx. 10 fmol) 5’-[32P]-end-labeled, double-stranded ERE oligonucleotide (35-mer, 5’- AACTTTTGATCAGGTCACTGTGACCTGA TTTTGAC-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 5% loosely cross-linked acrylamide gel (1:29 bis:acrylamide) at 150 V for 90 min at 20 °C in 0·5 × TBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1 µg ER antibody (usually 1 µl H222 for mERα, or 1 µl PAI-310 for mERβ1 and mERβ2) 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
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 \([\gamma^{32}\text{P}]\text{ATP}\), 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). For experiments in which the ability of the various ER isoforms to regulate the transforming growth factor (TGF)-\(\beta3\) promoter-driven \(\text{CAT}\) 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 \(3\times10^4\) 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-\(\beta3\)–\(\text{CAT}\) reporter plasmid DNA, 83 ng ER expression plasmid and 83 ng \(\beta\)-galactosidase 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 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 E2 or 100 nM antiestrogen, unless otherwise stated, for 24 h. Cells were harvested, cell extracts prepared and used to determine CAT and \(\beta\)-galactosidase activity as described previously (Dotzlaw et al. 1992). For experiments in which the relative ratio of mER\(\beta2\) to mER\(\beta1\) 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 \(3\times10^4\) cells per well and left overnight. The plates were 30–40% confluent on the day of transfection. 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DNA–Effectene for 24 h. Fresh medium (CS) ± estrogen or antiestrogen, or both, was then added and left for a further 24 h.

**Western blot analysis**

For western blot analysis, 2–3 × 10^5 cells set up in 10 cm diameter dishes were transiently transfected with 4 µg 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 µg 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 ERα (1:250 in TBST, NeoMarkers, Fremont, CA, USA) or Y19 for ERβ (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 ERα from Jackson Immuno Research Labs Inc., West Grove, PA, USA, antigoat IgG from Santa Cruz Biotechnology for ERβ) 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 mERβ1 and mERβ2 mRNA expression**

Both mERβ1 and mERβ2 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 ERβ1 and ERβ2 mRNA at similar levels. However placenta, uterus, breast, heart, brain, skin, and kidney expressed higher levels of ERβ2 mRNA relative to ERβ1 mRNA, whereas liver, pancreas, gut and bone expressed predominantly ERβ2 mRNA.

**Ligand binding activity of mERβ1 and mERβ2 proteins**

ERβ1 (human, rat and mouse) has been shown to bind E2 with high affinity and specificity. However, mERβ2 contains an 18 amino acid insertion within the ligand-binding domain (Brzozowski et al. 1997). To determine the ligand-binding capability of mERβ2, both mERβ1 and mERβ2 were synthesized in vitro and subjected to saturation ligand-binding analyses using [3H]E2. As shown in Fig. 2, [3H]E2 demonstrated specific, saturable binding of high affinity (K_D 0.56 ± 0.19 nM, mean ± s.e.m., n=3 independent experiments) for mERβ1, 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 $17·7 \pm 4·7$ nM (mean ± s.e.m., $n=3$ independent experiments).
These data suggest that mERβ2 binds estradiol with around 30-fold less affinity than mERβ1.

**DNA binding and bending activity of murine ERβ1 and ERβ2**

ERβ1 (human, rat and mouse) was shown to bind to classical ERE either as a homodimer or as a heterodimer with ERβ using *in vitro* gel-shift assays (Cowley *et al.* 1997). Consistent with these data, both mERβ1 and mERβ2 proteins synthesized *in vitro* were able to bind specifically to a classical ERE (Fig. 3A). The efficiency of mERβ1 and mERβ2 binding to the ERE oligonucleotide was similar, but was markedly less than the efficiency of mERα 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 [35S]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 c 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 binding assays demonstrated that mERα, mERβ1 and mERβ2 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 mERα was 66 ± 0.60 (mean ± s.e.m., n=3), for mERβ1 was 63 ± 0.50 and for mERβ2 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 mERβ1 and mERβ2 compared with mERα, which probably reflects the lower efficiency of the mERβ isoforms compared with the mERα 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 mERβ1 and mERβ2 could bind to ERβ in the presence or absence of ligand. Under these *in vivo* conditions, the ability of both mERβ1 and mERβ2 to bind to DNA is comparable to that of mERα. It is concluded that mERβ1 and mERβ2 have similar abilities to bind to and bend DNA containing ERE, both *in vitro* and *in vivo*.

**Transcriptional activity of mERβ1 and mERβ2 from ERE-containing promoters**

A dose–response analysis of the ability of estradiol to activate transcription of mERβ1 and mERβ2 on an ERE reporter gene after transient transfection into NIH 3T3 cells is shown in Fig. 5. Maximal transcriptional activation of mERβ1 is observed with approximately $10^{-9}$ M estradiol, whereas activation of mERβ2 is only observed with $10^{-6}$ M estradiol. These data are consistent with the
markedly reduced affinity for estradiol of mERβ2 compared with mERβ1. Both natural and synthetic (DES) estrogens activated transcription of the mERβ1 (data not shown). Neither steroidal (ICI182,780) nor non-steroidal (4-OH-Tam) anti-estrogens activated transcription of either mERβ isoform (data not shown). It is unlikely that the differences seen between mERβ1 and mERβ2 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 mERβ1 and mERβ2 (Fig. 6).

As shown in Table 1, under maximal conditions mERβ1 induced a fivefold and an 11-fold increase in 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 mERα on both vitellogenin B1- and A2 ERE-driven promoters. It was noted, however, that mERα demonstrated a greater apparently ligand-independent basal transcriptional activity compared with either mERβ1 or mERβ2 (Fig. 6).

**FIGURE 4.** Determination of the ability of mERα, mERβ1 and mERβ2 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-17β, 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 ± range of values obtained from two independent experiments.

**FIGURE 5.** Transcriptional activity of mERβ1 and mERβ2 on ERE-regulated CAT reporter gene. Ligand dose-dependent ability of mERβ1 (■) and mERβ2 (□) to activate transcription of vitellogenin A2 ERE-containing CAT reporter gene transfected into NIH 3T3 cells. Results show the mean fold induction ± s.d. of two independent experiments. βgal, β-galactosidase.

**FIGURE 6.** Western blot analysis of NIH 3T3 cell extracts (20 µg protein) 48 h after transfection of 4 µg mERα, mERβ1 and mERβ2 expression vectors. Lane 1 is empty expression vector alone; lane 2 is mERα expression vector; lane 3 is mERβ1 expression vector; lane 4 is mERβ2 expression vector. ER-α antibody is a blot visualized with the ERα-specific AER314 antibody; ER-β antibody is a blot visualized with the ERβ-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.
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-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 ERs, 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

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**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).

<table>
<thead>
<tr>
<th>mERα (83 ng)</th>
<th>mERβ1 (83 ng)</th>
<th>mERβ2 (sx ng)</th>
<th>CAT activity/βgal (fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+E₂</td>
<td>A</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>Empty Vector</td>
<td>−</td>
<td>1 ± 0·3</td>
<td>1 ± 0·1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1 ± 0·3</td>
<td>1 ± 0·2</td>
</tr>
<tr>
<td>mERα</td>
<td>−</td>
<td>6·4 ± 1·9††</td>
<td>9·6 ± 2·7</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13·4 ± 3·9*</td>
<td>58 ± 21</td>
</tr>
<tr>
<td>mERβ1</td>
<td>−</td>
<td>2·1 ± 0·9</td>
<td>3·1 ± 0·2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9·9 ± 1·6**</td>
<td>34 ± 1·2</td>
</tr>
<tr>
<td>mERβ2</td>
<td>−</td>
<td>1·4 ± 0·3</td>
<td>1·9 ± 0·2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1·3 ± 0·1</td>
<td>4·2 ± 0·8</td>
</tr>
</tbody>
</table>

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.

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**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.

<table>
<thead>
<tr>
<th>mERα (83 ng)</th>
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<th>mERβ2 (sx ng)</th>
<th>CAT activity/βgal (fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+E₂</td>
<td>A</td>
<td>B</td>
<td>+</td>
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<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>1 ± 0·2</td>
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<tr>
<td></td>
<td>+</td>
<td>8·8 ± 1</td>
<td>28·5 ± 15·8</td>
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<tr>
<td></td>
<td>+</td>
<td>8·1 ± 0·01</td>
<td>21·6 ± 16·8</td>
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<tr>
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<td>−</td>
<td>1·2 ± 0·02</td>
<td>0·9 ± 1·0</td>
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<td></td>
<td>+</td>
<td>83</td>
<td>4·7 ± 0·2</td>
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<tr>
<td></td>
<td>−</td>
<td>83</td>
<td>1·0 ± 0·1</td>
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<td></td>
<td>+</td>
<td>416</td>
<td>2·2 ± 0·2</td>
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<td>1·0 ± 0·2</td>
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<td>−</td>
<td>833</td>
<td>1·0 ± 0·1</td>
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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 mERβ1 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 mERβ2 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 mERβ1 and mERβ2 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 mERβ1 and mERβ2 activity at the raloxifene responsive-

region in the TGF-β3 promoter using the TGF-β3–CAT reporter.

In contrast to activity at an ERE-containing promoter, no differences in the ligand-independent or basal transcriptional activity amongst mERα, mERβ1 and mERβ2 were found (data not shown). All receptor isoforms in the presence of estradiol were able to increase transcription from the TGF-β3–CAT reporter in a dose-dependent fashion (Fig. 7B), although the dose–response curve for mERβ2 compared with mERα and mERβ1 was moved to the right, consistent with the reduced affinity of mERβ2 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-β3–CAT reporter in the presence of an empty expression vector (X in Fig. 7B and C). The half-maximal activation for mERα and mERβ1 was achieved with similar concentrations of estradiol and the maximum fold

**Figure 7.** Ability of mERα, mERβ1 and mERβ2 to regulate transcription from a novel raloxifene-responsive region-regulated reporter gene. (A) mERβ1 (■) and mERβ2 (○) 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-β3–CAT experiments shown below. Results represent the mean ± 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 mERα (▲), mERβ1 (■) and mERβ2 (○) (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-β3 promoter. The cells were treated with increasing concentrations of estradiol-17β. The results represent the mean ± 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 mERα (▲), mERβ1 (■) and mERβ2 (○) (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-β3 promoter. The cells were treated with increasing concentrations of the raloxifene analog LY117018. The results represent the mean ± 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 mERβ2 half-maximally, and the maximum fold induction was less than that achieved with mERα and mERβ1. In contrast, in the presence of the raloxifene analog, LY117018, all three isoforms showed similar maximum activities (Fig. 7C) and potencies, with mERβ2 being less active only at the lowest concentration of LY117018 used. The ability of estradiol and LY117018 to activate both mERα and mERβ1 on the TGF-β3 promoter appeared to be identical, and mERβ2 activity in the presence of ligand – either estradiol or LY117018 – was only slightly less than that observed with either mERβ1 or mERα. This was in contrast to the markedly reduced potency of estradiol to induce transcription via the mERβ2 at an ERE-regulated reporter gene. The activity of the estradiol-mERβ2 complex at the TGF-β3 promoter appeared greater than that observed at the ERE-regulated promoter (compare Fig. 7A with Fig. 7B). Furthermore, both mERβ1 and mERβ2, 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-β3 promoter. These data suggest that responsiveness of gene expression to a relatively highly expressed variant murine ERβ isoform, mERβ2, 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 ERβ. This murine ERβ splice variant, now referred to as murine ERβ2, contains an in-frame 54 nucleotide insertion between exon 5 and exon 6 sequences of the wild-type murine ERβ, now referred to as ERβ1. Interestingly, we have not detected the equivalent of rodent ERβ2 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 ERβ2 mRNA would contain an 18 amino acid insertion in the ligand-binding domain of wild-type ERβ1. The mERβ2 is the mouse homolog of a recently described rERβ2 (Chu & Fuller 1997, Petersen et al. 1998). The 18 amino acid insertion in the mERβ2 has 89% amino acid identity with the rERβ2 (Lu et al. 1998). By analogy to ERα, 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 mERβ2 has markedly reduced ligand-binding affinity compared with mERβ1, as classically measured using saturation analysis with radiolabelled ligand in vitro. These data are similar to the results obtained for the rERβ2 (Petersen et al. 1998), with which reduced but measurable binding affinity for radiolabeled estradiol was demonstrated. Both murine and rat ERβ1 and ERβ2 appear to bind to ERE oligonucleotides with similar affinity in the presence and absence of ligand in EMSA, although they are less efficient than ERα in doing so. Furthermore, the mERβs were also able to bind to DNA in vitro 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 mERβ2 is an ERβ-like protein that can bind to and bend ERE-containing DNA sequences both in vitro and in vivo, in a fashion similar to mERβ1, despite its affinity for estradiol being significantly less than that of mERβ1. These data suggested that ERβ2 might have either constitutive transcriptional activity or may interfere with the transcriptional activity of ERβ1.

Consistent with its low binding affinity for estradiol, the mERβ2 has no significant transcriptional activity of its own on promoters containing EREs, except at high (>1 μM) concentrations of added estradiol (see Figs 5 and 7A). Interestingly, our data suggest that mERβ2, under conditions in which little, if any, of the protein is likely to be bound to estradiol, inhibits the ligand-induced transcriptional activity of mERβ1 and mERα at this type of promoter. Our data suggest that mERβ2 inhibited the estrogen-induced mERβ1 transcriptional activity by 50% when expressed at a 1:1 ratio, and achieved complete inhibition when present at five times the amount of mERβ1. If the relative RNA levels reflect the relative protein concentrations, our data suggest that, in some tissues, mERβ2 can be expressed in at least three times greater levels than mERβ1. Therefore, in some tissues, mERβ2 could have a significant modulatory effect over the ability of mERβ1 to respond to estrogenic compounds, under conditions in which mERβ2 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, rERβ was not shown to have significant inhibitory effects on rERα in the study reported by Peterson et al. (1998). However, their study used a heterologous system for the analyses – rERβ proteins were analyzed in human cell lines – whereas our analyses were carried out in a homologous system – mERβ 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 ERα 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 ERα 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 rERβ2 had a dominant negative activity on rERβ1 and rERα (Maruyama et al. 1998), which is consistent with the findings from our mouse studies. However, the significance of the ability of mERβ2 to exert a dominant negative effect on mERα 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 mERβ1 and mERβ2 on ERE-containing promoters was also significantly less than that observed for mERα, which is in contrast to results previously obtained with mERβ1 (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, hERα has significantly greater ligand-independent activity than hERβ1, which would be consistent with our murine studies. The differences in the ligand-independent activities of mERα and mERβ 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-β3 promoter, which has been reported to be regulated by hERα, using a novel mechanism (Yang et al. 1996). In contrast to previous studies using the hERα in which the estradiol liganded receptor was much less efficient in activating transcription than the raloxifene-ligated receptor, the activity of the mERα receptor liganded with either estradiol or the raloxifene analog, LY117018, on the TGF-β3 promoter was similar, despite the previously observed lower relative binding affinity of LY117018, compared with that of estradiol, for the rat and human ERα determined in vitro at 4°C (Wakeling et al. 1984). However, raloxifene has a greater affinity for rERα than has LY117018 (Jones et al. 1984), and the affinity of both these compounds for ERα 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/ERα complex may also occur when LY117018 (or its analogs) is bound to the mERβ isoforms.

The action of the liganded mER isoforms at the TGF-β3 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-β3 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-β3 promoter, estradiol-ligated ERβ isoforms inhibit transcription at AP-1 regulated promoters (Paech et al. 1997). Thirdly, the relative potency of estradiol to activate transcription of mERβ2 at the TGF-β3 promoter is significantly greater than its ability to activate transcription of mERβ2 at an ERE-regulated promoter. This suggests that conformational changes induced by estrogen binding to mERβ2 are sufficient in vivo to drive significant transcriptional activity at the TGF-β3 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, mERβ2 could, at low ligand levels, have an inhibitory action on both mERβ1 and mERα, whereas at higher ligand concentrations it could be additive to, or possibly synergistic with, the action of mERβ1 and possibly mERα. The data are also consistent with the idea that the mechanisms by which ER isoforms regulate the TGF-β3 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 ERβ2 resides in the detection of a stably expressed protein predicted
from the ER\(\beta\)2 mRNA. Although there are no unequivocal data that directly prove the detection of the ER\(\beta\)2 protein, our data suggest that the ER\(\beta\)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\(\beta\)-specific antibodies, sometimes identified a doublet around 66 kDa that could be consistent with ER\(\beta\)-like proteins representing the wild-type ER\(\beta\)1 and a slightly larger ER\(\beta\)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\(\beta\)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\(\beta\) variant isoform can exert dominant negative transcriptional effects on both mER\(\beta\)1 and mER\(\beta\)\(\alpha\). 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.

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