

# Cloning, Chromosomal Localization, and Functional Analysis of the Murine Estrogen Receptor $\beta$

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**Estrogen receptor  $\beta$  (ER $\beta$ ) is a novel steroid receptor that is expressed in rat prostate and ovary. We have cloned the mouse homolog of ER $\beta$  and mapped the gene, designated *Estrb*, to the central region of chromosome 12. The cDNA encodes a protein of 485 amino acids that shares, respectively, 97% and 60% identity with the DNA- and ligand-binding domains of mouse (m) ER $\alpha$ . Mouse ER $\beta$  binds to an inverted repeat spaced by three nucleotides in a gel mobility shift assay and transactivates promoters containing synthetic or natural estrogen response elements in an estradiol (E<sub>2</sub>)-dependent manner. Scatchard analysis indicates that mER $\beta$  has slightly lower affinity for E<sub>2</sub> [dissociation constant (K<sub>d</sub>) = 0.5 nM] when compared with mER $\alpha$  (K<sub>d</sub> = 0.2 nM). Antiestrogens, including 4-hydroxytamoxifen (OHT), ICI 182,780, and a novel compound, EM-800, inhibit E<sub>2</sub>-dependent transactivation efficiently. However, while OHT displays partial agonistic activity with ER $\alpha$  on a basal promoter linked to estrogen response elements in Cos-1 cells, this effect is not observed with mER $\beta$ . Cotransfection of mER $\beta$  and H-Ras<sup>V12</sup> causes enhanced activation in the presence of E<sub>2</sub>. Mutagenesis of a serine residue (position 60), located within a mitogen-activated protein kinase consensus**

**phosphorylation site abolishes the stimulatory effect of Ras, suggesting that the activity of mER $\beta$  is also regulated by the mitogen-activated protein kinase pathway. Surprisingly, the coactivator SRC-1 up-regulates mER $\beta$  transactivation both in the absence and presence of E<sub>2</sub>, and *in vitro* interaction between SRC-1 and the ER $\beta$  ligand-binding domain is enhanced by E<sub>2</sub>. Moreover, the ligand-independent stimulatory effect of SRC-1 on ER $\beta$  transcriptional activity is abolished by ICI 182,780, but not by OHT. Our results demonstrate that while ER $\beta$  shares many of the functional characteristics of ER $\alpha$ , the molecular mechanisms regulating the transcriptional activity of mER $\beta$  may be distinct from those of ER $\alpha$ . (Molecular Endocrinology 11: 353–365, 1997)**

## INTRODUCTION

Estrogens regulate female reproductive functions through a nuclear receptor that belongs to a superfamily of ligand-activated transcription factors that includes receptors for steroids, thyroid hormone, retinoids, prostanoids, and vitamin D (1, 2). Like other members of the superfamily, the estrogen receptor (ER) has a modular structure consisting of distinct functional domains (3). The DNA-binding domain (DBD) enables the receptor to bind to its cognate

target site consisting of an inverted repeat of two half-sites with the consensus motif AGGTCA (or closely related sequences) spaced by 3 bp and referred to as an estrogen response element (ERE). The ligand-binding domain (LBD) also harbors a nuclear localization signal as well as sequences necessary for dimerization and transcriptional activation (AF-2). A second activation function, AF-1, is present in the amino-terminal domain of the receptor. Although transcriptional activation is mediated through both AF-1 and AF-2, only activation by AF-2 requires hormone binding (4, 5). Studies using the estrogen antagonists 4-hydroxytamoxifen (OHT) and ICI 164,384 indicated that although both compounds blocked estrogen effects, their mode of action differed: the mixed agonist/antagonist OHT inhibited only AF-2 function while the pure antiestrogen ICI 164,384 inhibited activation by both AF-1 and AF-2 (6).

Additional signals that modulate nuclear receptor function have been described and commonly involve phosphorylation. Steroid receptors, including the progesterone and glucocorticoid receptors, have an increased state of phosphorylation upon ligand binding (7). Similar results have been described for ER where treatment of cells with peptide growth factors or agents that alter cellular cAMP levels result in up-regulation of the receptor coupled with an increase in its phosphorylation state (8, 9). These reports, as well as subsequent studies (10), demonstrated that antiestrogens also caused an increase in receptor phosphorylation albeit to a lesser extent than estradiol ( $E_2$ ), even though they inhibited transactivation. Deletion mapping and mutagenesis of human ER revealed that phosphorylation at Ser<sup>118</sup> was required for full activity of AF-1 (10). Furthermore, this residue was shown to be a direct substrate for mitogen-activated protein (MAP) kinase, providing an *in vivo* link between estrogen action and the Ras-MAP kinase signaling cascade (11, 12). These results have begun to shed light on the molecular events responsible for regulation and proliferation of different cell types by ER in response to estrogens and growth factors.

Transactivation by nuclear receptors has recently been shown to be modulated by a growing family of coregulators (13). These include corepressors such as N-CoR and silencing mediator for retinoid and thyroid hormone receptors (SMRT) (14–16), which participate in the ligand-independent silencing functions of TR and retinoic acid receptor, and several coactivators: TRIP1/SUG-1 (17, 18), ERAP-140 (19), RIP-140 and RIP-160 (20, 21), TIF1 (22), and the related TIF2 (23), GRIP1 (24), SRC-1, and ERAP-160 (19, 25, 26). The LBD of ER was demonstrated to specifically interact with many of these coactivators, including steroid receptor coactivator-1 (SRC-1), ERAP-160, and RIP-140, and the strength of interaction was increased by  $E_2$  but not by the antiestrogens OHT and ICI 164,384 (20, 25, 27). The significance of these coactivators in nuclear receptor function is underscored by recent

results demonstrating that SRC-related proteins interact with the nuclear integrators, CBP and p300, to augment nuclear receptor transactivation in mammalian cells (26, 28, 29). The activation domain of CBP can interact with TFIIB (30), thus providing evidence for a link between nuclear receptors and the basal transcription machinery. This growing network of interacting factors has increased our understanding of how steroids such as  $E_2$  are able to alter the expression of specific genes at the molecular level.

Although human and mouse ER cDNAs were cloned several years ago (31, 32), RT-PCR of rat prostate mRNA has revealed the presence of a second ER, referred to as ER $\beta$  (33). This novel receptor was found to bind  $E_2$  with relatively high affinity and to be capable of activating transcription of a reporter gene in the presence of this ligand. *In situ* hybridization of rat tissues indicated that ER $\beta$  is expressed in female animals in primary, secondary, and mature follicles as well as granulosa cells in the ovary and in the prostate of male rat. As an initial step toward elucidating the physiological role of this second ER and understanding its functional relationship with ER $\alpha$ , we report the cloning of the murine homolog of ER $\beta$ . We show that mER $\beta$  is able to bind to an ERE in electrophoretic mobility shift assays as well as to transactivate, in an  $E_2$ -dependent manner, promoters containing either synthetic or natural EREs in Cos-1 and HeLa cells. In addition,  $E_2$ -induced mER $\beta$  activity is inhibited by several antiestrogens that have been previously shown to be selective for ER $\alpha$ . We also demonstrate that SRC-1 interacts *in vitro* with the mER $\beta$  LBD in a ligand-dependent manner. However, SRC-1 modulates mER $\beta$  transcriptional activity in intact cells both in the presence and absence of ligand. Finally, we show that the  $E_2$ -induced transcriptional activity of mER $\beta$  is enhanced by cotransfection with Ras. The functional differences observed between the two ER isoforms are discussed.

## RESULTS

### The Mouse ER $\beta$ cDNA

The mER $\beta$  cDNA contains an open reading frame of 1455 nucleotides encoding a protein of 485 amino acids (Fig. 1). Alignment of mER $\beta$  with mER $\alpha$  indicates that the receptors share 97% and 60% amino acid similarity in the DBD and the LBD, respectively (Fig. 2). The AF-2 core sequences in each receptor are virtually identical; however, no significant regions of homology could be detected between the two amino-terminal domains, a region of the protein that contains the AF-1 in ER $\alpha$ . Two potential MAP kinase phosphorylation sites (Ser<sup>60</sup> and Ser<sup>94</sup>) are present in the amino-terminal region of the mER $\beta$ .

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1   tcgaggtcgacATGACATTCTACAGTCTGCTGTGATGAACTACAGTGTCCAGCAGCACCGGTAACCTGGAAGGTGGGCCTGTTCGCCAGACTGCAAG
    M T F Y S P A V M N Y S V P S S T G N L E G G P V R Q T A S 30
101  CCCAAATGTGCTATGGCCAACCTTCTGGACACCTCTCTCCTTTAGCCACCCACTGCCAATCATCGCTTCTCTATGCAGAACCTCAAAGAGTCCCTGGTGT
    P N V L W P T S G H L S P L A T H C Q S S L L Y A E P Q K S P W C 63
201  GAAGCAAGATCACTAGAACACACCTTGCCTGTAAACAGAGAGACCCCTGAAGAGGAAGCTTGGCGGGAGCGGTTGTGCCAGCCCTGTACTAGTCCAAGCG
    E A R S L E H T L P V N R E T L K R K L G G S G C A S P V T S P S 96
301  CCAAGAGGGATGCTCATTCTGCGCCGTCTGCAGTGATTATGCATCTGGGTATCATTACGGTGTCTGGTCTGTGAAGGATGTAAGGCCCTTTTTTAAAG
    A K R D A H F C A V C S D Y A S G Y H Y G V W S C E G C K A F F K R 130
401  AAGCATTCAAGAGCATAATGACTATATCTGTCCAGCCACGAATCAGTGTACCATAGACAAGAACCAGCGTAAAAGCTGCCAGCCCTGCCACTTCCGCAAG
    S I Q G H N D Y I C P A T N Q C T I D K N R R K S C Q A C R L R K 163
501  TGTTCAGAAAGTAGGAATGGTCAAGTGTGGATCCAGGAGAGAAAGGTGTGGGTACCGAAATAGTACGAAGACAGAGAAGTGCAGCGAGCAGGTGCATGGCC
    C Y E V G M V K C G S R R E R C G Y R I V R R Q R S A S E Q V H C 196
601  TGAACAAAAGCCAAAGAGAACCAGTGGGCACACCCCGGTGAAGGAGCTACTGCTGAACTCTCTGAGTCCCGAGCAGCTGGTCTCACCTGCTGGAAGC
    L N K A K R T S G H T P R V K E L L L N S L S P E Q L V L T L L E A 230
701  TGAGCCACCAATGTGCTAGTGTAGCCGTCACAGCATGCCCTTCACCGAGGCCCTCCATGATGATGTCCCTCACGAAGCTGGCTGACAAGGAAGTGGTGCAC
    E P P N V L V S R P S M P F T E A S M M M S L T K L A D K E L V H 263
801  ATGATTGGTGGCCAAAGAAAATCCCTGGCTTTGTGGAGCTCAGCCTGTGGACCAAGTCCGCCTCTTGGAAAGCTGCTGGATGGAGGTGCTGATGGTGG
    M I G W A K K I P G F V E L S L L D Q V R L L E S C W M E V L M V 296
901  GGCTGATGTGGCGCTCCATCGACCACCCCGCAAGCTCATCTTTGCTCCAGACCTCGTTCGGACAGGATGAGGGGAAGTGCCTGGAAGGGATTCTGGA
    G L M W R S I D H P G K L I F A P D L V L D R D E G K C V E G I L E 330
1001 AATCTTTGGCATGCTCCTGGCGACGACGGCAGGTTCCGTGAGTTAAAAGTGCAGCACAAAGAATATCTGTGTGTAAGGCCATGATTTCTCTCAACTCC
    I F G M L L A T T A R F R E L K L Q H K E Y L C V K A M I L L N S 363
1101 AGTATGTACCCTTTGGCTACCAGCAAGCCAGGAAGCAGAGAGTAGCCGGAAGCTGACACACCTATTGAACGCAGTGACAGATGCCCTGGTCTGGGTGATTT
    S M Y P L A T A S Q E A E S S R K L T H L L N A V T D A L V W V I 396
1201 CGAAGAGTGAATCTCTCCAGCAGCAGTCAGTCCGTCTGGCCAACTCCTGATGCTTCTTCTCATGTGAGGCACATCAGTAACAAGGGCATGGAACA
    S K S G I S S Q Q Q S V R L A N L L M L L S H V R H I S N K G M E H 430
1301 TCTGCTCAGCATGAAGTGCAAAAATGTGGTCCCGGTGTACGACCTGTGTGGAGATGCTGAATGCTCACACGCTTCGAGGGTACAAGTCTCAATCTCG
    L L S M K C K N V V P V Y D L L L E M L N A H T L R G Y K S S I S 463
1401 GGGTCTGAGTGTGCTGCAGCAGAGGACAGTAAGAGCAAGAGGGCTCCAGAACCTCCAGTACAGTACAGTGCAGGCCAGGCTGGAGCGGATGATTACAATGA
    G S E C C S T E D S K S K E G S Q N L Q S Q * 485
1501 TGGTCAGAAGTGGGACATGTACCCTAGCATCGTGGGCCTCACCTTAGGGCTGACTTGGTCACGTACCCCTTACCCAGCTTGCCTTCCAGGAGTCAGGG
1601 TGGTTGGGTTGGTGTCCCCTGTACCAGGACTTACTGCTGAATGCTGAGTTCTAAGTGTATAACCTTAGAAGGCTCTCGTTGTACTTTCCATCTCTTT
1701 GCCCACTTGGAAAACATCTTAAGAGTTCTGGAATGAAAAGGTCAAAGTCTGACTTGGAAAGGATTGGCCTTGGACAGGAAAAGGAAAGTGACCGCATACAC
1801 CGTCTATAAGAAATGGACTGTAGAACGGTGTGGTTCATCAAATCGACCTTTGAATGGTGTCTTCTAGACCACTTGATTGTAGGACTGAAAACCACATTGAC
1901 AATCAACTCGTTTCGCATTCTACCTCACAGGCTGTGAGGACTCATGACGTGTAATGGGTTATTGTATCAAAGACCAAGAAGATAATGGTCAAGCTTGG
2001 ATGTGACCTTGTCTCTCTCTAGATCCTCTTCTTCTGCTTGGCTTACATAACTAGATTATTCGGTCTGTCTGAATGTGGTCACTGTCTACAGATTAAAG
2101 ATTTAAATATCTTTTAAATGTTGAAAAAAAAAAAAAAAAAAAAAagcgccgcg
    
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**Fig. 1.** Nucleotide Sequence of Murine ER $\beta$   
 Amino acid numbers are indicated on the left. The potential MAP kinase phosphorylation sites are *underlined*. GenBank accession number of the mER $\beta$  sequence is U81451.

**Chromosomal Localization**

The mouse chromosomal location of ER $\beta$  (*Estrb*) was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J  $\times$  *Mus spretus*)F1  $\times$  C57BL/6J] mice. This interspecific backcross mapping panel has been typed for more than 2200 loci that are well distributed among all the autosomes as well as the X chromosome (34). C57BL/6J  $\times$  *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for infor-

mative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA ER $\beta$  probe (positions 816-1361). The 8.4- and 5.1-kb *EcoRI* *M. spretus* RFLPs (see *Materials and Methods*) were used to follow the segregation of the *Estrb* locus in backcross mice. The mapping results indicated that *Estrb* is located in the central region of mouse chromosome 12 linked to *Sos2*, *Spnb1*, and *Fos*. Although 165 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 3), up to 184 mice were typed for some pairs of mark-

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β 104 CAVCSQDYASGYHYGVVWSCGEGCKAFFKRSTQGHNDYICPATNQCTIDKNRR
α 191 CAVCNDYASGYHYGVVWSCGEGCKAFFKRSTQGHNDYMCPATNQCTIDKNRR

β 154 KSCQACRLRKCCEYVGMVKCGSRRRERCYRIV--RRQRSASEQVHCLNKAK
α 241 KSCQACRLRKCCEYVGMVKCGSRRRERCYRIV--RRQRSASEQVHCLNKAK

β 202 RTSGHTPRVKELLN-----SLSPFQLVLTLEAEFPNVLVLS
α 288 A-SGDMRAANLWPSPLVIKHTKKNPALSLTADQMVSAALLDAEPPMIYSE

β 239 R-PSMPFTEASMMMSLTKLADKELVHMIGWAKKIPGFVLSLLDQVRLE
α 337 YDPSRPFSEASMMGLTNLADRELVHMINKAKRVPFGDLNLHDQVHLLLE

β 288 SCWMEVLMVGLMWRSDHDPKLIIFAPDLVLDREDEGKCEGILEIFGMLLA
α 387 CAWLELLMIGLVWRSMEHFGKLLFAPNLLDRNQKCEGMEVIFDMLLA

β 338 TTAARFRELKLOHKEYLCKVAMILLNSSMY-PLA-TASQEAESSRKLTHLL
α 437 TSSRFMMNLQGEFVCLKSIILLNSGVYTFSS'TLKSLEEKDH-IHRVL

β 386 NAVTDALVWVVISKSGISSQQQSVRLANLMLLSHVRIHISNKGMEHLISMK
α 486 DKITDTLTHIMAKAGLTLQQOHRRLAQLLLLSHIRHMSNKGMEHLYNMK

β 436 KKNVVPVYDLEMLNAHTLRGYKSSISGSECCSTEDSKSKEGSQNLQSQ*
α 536 KKNVVPVYDLEMLDAHRLHAPASRMGVPPEEPSQTLATTSSTSAHSL
    
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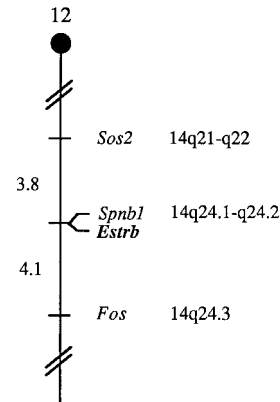
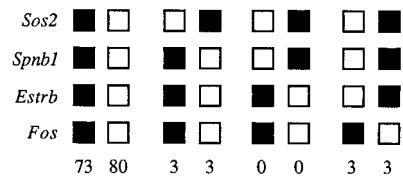
**Fig. 2.** Amino Acid Sequence Comparison between Murine ERβ with ERα

The alignment begins with the first Cys residue of the respective DBDs. Identities are indicated with vertical bars, biochemically similar residues by vertical dots, and gaps by dashed lines. The stop codon in ERβ is indicated by an asterisk. The AF-2 domain is underlined.

Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere - *Sos2* - 7/184 - *Spnb1* - 0/181 - *Estrb* 7/173 - *Fos*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the SE] are - *Sos2* - 3.8 ± 1.4 - [*Spnb1*, *Estrb*] - 4.1 ± 1.5 - *Fos*. No recombinants were detected between *Spnb1* and *Estrb* in 181 animals typed in common, suggesting that the two loci are within 1.7 cM of each other (upper 95% confidence limit).

**The mERβ Gene Expresses Several Transcripts**

A Northern analysis was performed to determine the size of the message encoding mERβ using the 550-bp PCR fragment that spans the LBD of mERβ as a probe. As shown in Fig. 4, mouse ovary expressed at least four predominant transcripts of approximately 3.5, 4.6, 7.2, and 9.5 kb and a weaker message of about 4.9 kb. In contrast, only one transcript could be detected in ovary mRNA when probed with the mERα cDNA. Analysis of total RNA from several other mouse tissues including liver, heart, kidney, skeletal muscle, thymus, spleen, and brain were all negative for presence of the ERβ transcripts indicating that expression of ERβ was below the level of detection by Northern blotting in these tissues (data not shown).

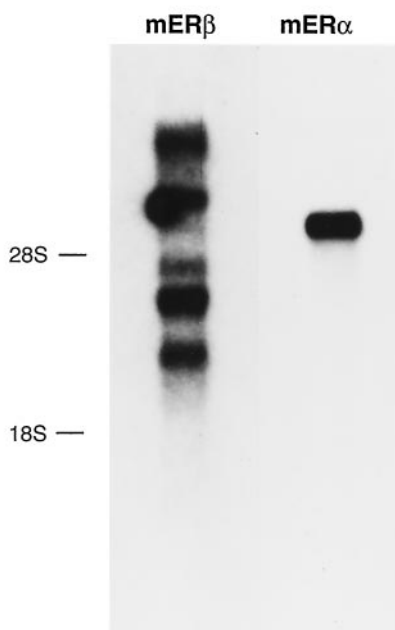


**Fig. 3.** Chromosomal Localization of the ERβ Gene

The ERβ gene, *Estrb*, maps to the central region of mouse chromosome 12. *Estrb* was placed on mouse chromosome 12 by interspecific backcross analysis. The segregation patterns of *Estrb* and flanking genes in 165 backcross animals that were typed for all the loci are shown at the top of the figure. For individual pairs of loci, more than 165 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F<sub>1</sub> parent. The shaded boxes represent the presence of C57BL/6J allele, and open boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 12 linkage map showing the location of *Estrb* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

**Mouse ERβ Binds to an ERE**

The homology between the DBDs of mERα and β indicated that the receptors most likely bind to the same type of response element, namely an inverted repeat spaced by three nucleotides (IR-3). We tested this by conducting electrophoretic mobility shift assays (EMSA) with both mouse receptors. Figure 5 demonstrates that both ER isoforms produced *in vitro* by rabbit reticulocyte lysates were able to bind specifically to a vitellogenin A2 (vitA2)-ERE (35) probe. It was noted that the mERβ did not bind as strongly to the element as mERα (compare lanes 2–5 with lanes 7–10) even though both receptors were present at



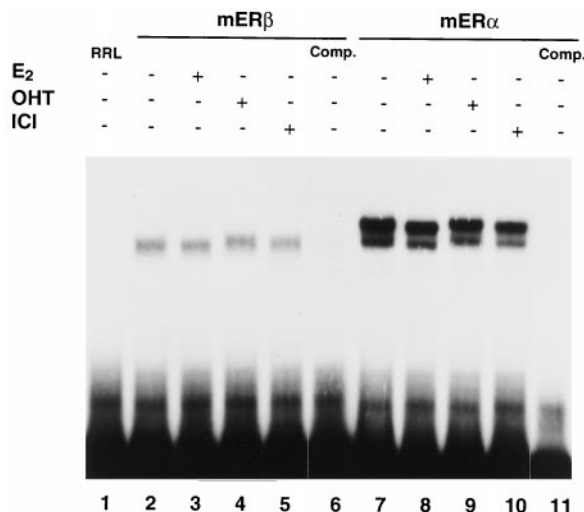
**Fig. 4.** Northern Blot Analysis of ER $\beta$  mRNA  
 Five micrograms of mouse ovary poly-A<sup>+</sup> RNA were probed with a 550-bp cDNA fragment encoding the mER $\beta$  LBD and a full-length mER $\alpha$  cDNA. The blot was hybridized with the mER $\beta$  cDNA fragment, stripped, and subsequently probed with the mER $\alpha$  cDNA. Exposure was carried out for the same length of time in each case. The position of 18S and 28S rRNAs are indicated on the left.

roughly the same level in the crude lysates as shown by [<sup>35</sup>S]methionine-labeled proteins (data not shown). In addition, both receptors were able to bind to the pS2-ERE (36) but less efficiently than to the vitA2-ERE (data not shown), which can most likely be accounted for by the fact that the pS2-ERE contains an imperfect consensus sequence in the second half-site.

Addition of E<sub>2</sub> to the binding reactions did not have an effect on binding under the conditions used (Fig. 5, lanes 3 and 8) nor was there any effect on binding upon addition of specific antagonists such as OHT (lanes 4 and 9) and ICI 182,780 (lanes 5 and 10), although the migration rate of the protein/DNA complexes were differentially altered by each ligand. However, as previously observed with ER $\alpha$  (37), a severe decrease in binding by ER $\beta$  was observed when the preincubation step was conducted at room temperature, 37 C, or for shorter periods of time. The formation of the ER $\beta$ /DNA complexes could be partially restored in the presence of E<sub>2</sub> at these temperatures (data not shown).

**Transcriptional Activity of mER $\beta$  on Synthetic and Natural Promoters**

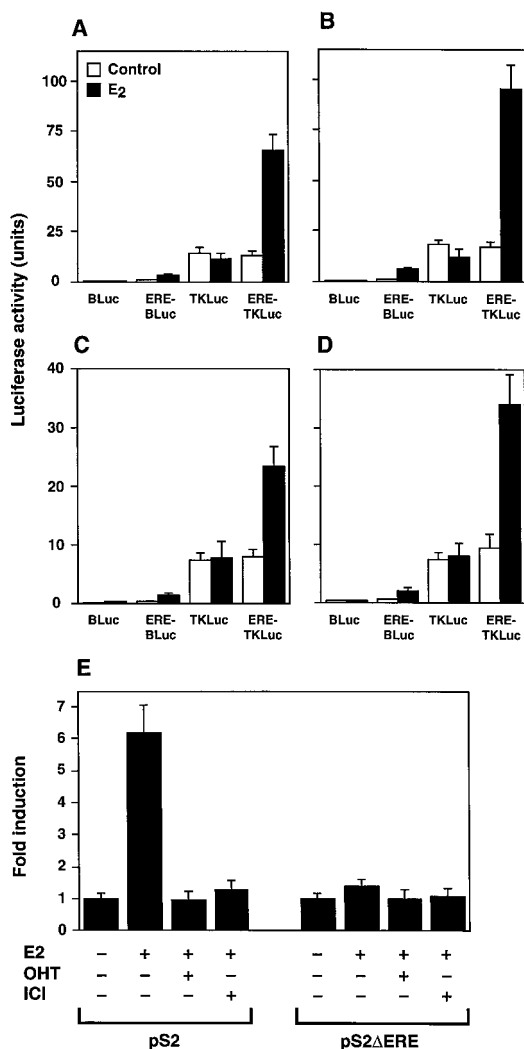
To test whether the vitA2-ERE could mediate mER $\beta$  transcriptional activity, we linked one copy of the ERE



**Fig. 5.** Binding of mER $\beta$  and mER $\alpha$  to an ERE  
 Each receptor produced by rabbit reticulocyte lysates (RRL) was incubated at room temperature with approximately 0.1 ng of labeled vitA2-ERE in the absence (lanes 2 and 7) and presence of 100 nM E<sub>2</sub> (lanes 3 and 8), 100 nM OHT (lanes 4 and 9), and 100 nM ICI 182,780 (lanes 5 and 10). Unprogrammed RRL was used as a negative control (lane 1) and specificity was determined in the presence of 100 $\times$  unlabeled vitA2-ERE (compare lanes 6 and 11).

to either a basal promoter containing a TATA box (BLuc) or to the more complex viral thymidine kinase promoter (TKLuc) driving the expression of the luciferase reporter gene for transactivation studies. In Cos-1 cells, mER $\beta$  induced a 5- to 10-fold response on all the ERE-containing reporters tested when 10 nM E<sub>2</sub> was added to the medium (Fig. 6A). mER $\alpha$  produced a slightly larger fold induction of the ERE-containing reporters when studied under the same transfection conditions (Fig. 6B). In addition, E<sub>2</sub>-dependent transcriptional activity of mER $\beta$  was also observed in HeLa cells (Fig. 6C), suggesting a cell type-independent effect. It is noteworthy that, as already observed in Cos-1 cells, the levels of E<sub>2</sub>-induced activation in HeLa cells were somewhat higher when mER $\alpha$  was used as compared with mER $\beta$  (Fig. 6D), although it is not known whether similar levels of ER proteins were present in these transient transfections.

We next investigated whether mER $\beta$  could modulate the transcriptional activity of an ERE-containing promoter in its natural context. The pS2 promoter has been shown to respond to hER $\alpha$  (36). As shown in Fig. 6E, mER $\beta$  was able to transactivate the pS2 promoter in HeLa cells with a 6-fold induction when E<sub>2</sub> was added. In addition, OHT and ICI 182,780 efficiently blocked the E<sub>2</sub>-induced effect of mER $\beta$  on pS2. This interaction is mediated through the ERE since its deletion abolished the response to E<sub>2</sub> (Fig. 6E).



**Fig. 6.** E<sub>2</sub>-Dependent Transcriptional Activities of mERβ vs. mERα

A, Cos-1 cells were transfected with 2 μg of the various reporter plasmids carrying either one copy of the vitA2-ERE (ERE-BLuc and ERE-TKLuc) or vector only (BLuc and TKLuc), along with 1 μg pCMX-mERβ. Cells were treated with or without 10 nM E<sub>2</sub> for 12 h before being assayed for luciferase activity. B, Transfection conditions were as in panel A, except that a pCMX-mERα expression vector was used. C and D, Transfection conditions were as in panels A and B, respectively, except that HeLa cells were used. E, mERβ transactivates the pS2 promoter. HeLa cells were transfected with 500 ng pCMX-mERβ and 1 μg pS2Luc (pS2) or pS2ΔERELuc (pS2ΔERE) reporter plasmids and incubated with or without 10 nM E<sub>2</sub> in the presence or absence of 100 nM of the indicated antagonists, 4-hydroxytamoxifen (OHT) and ICI 182,780 (ICI).

### Ligand Binding

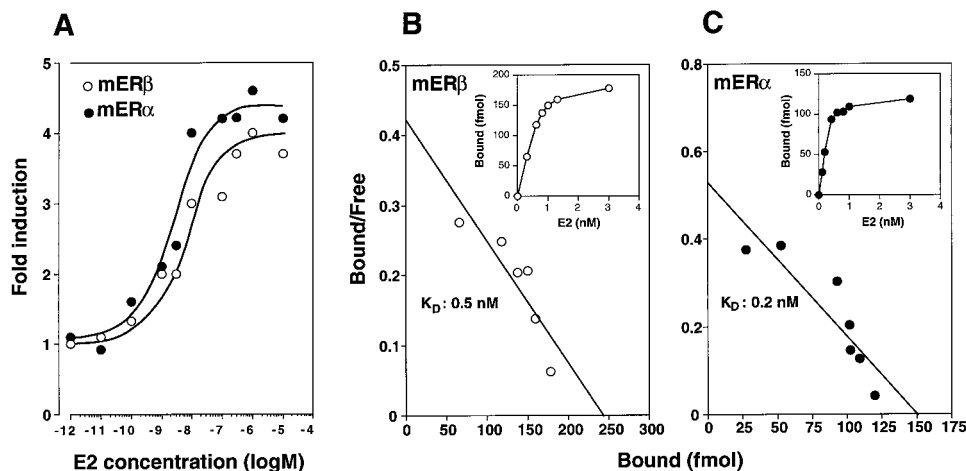
To ascertain whether there was a dose dependency of E<sub>2</sub> for mERβ and mERα, we tested the activity of both receptors in the presence of increasing E<sub>2</sub> concentrations using the vitA2-ERE-TKLuc reporter in Cos-1

cells. Comparison of the dose-response curves of Fig. 7A shows a shift of approximately 4-fold of E<sub>2</sub> concentration required to achieve half of the maximal level of induction between the two receptors. These results suggested that mERβ may have lower affinity for E<sub>2</sub> than measured for mERα. To verify if the difference in E<sub>2</sub> responsiveness was due to a difference in ligand binding, we performed a binding analysis on both mERβ and mERα. [<sup>3</sup>H]E<sub>2</sub> was used to conduct binding studies with mERβ, and results were plotted by the method of Scatchard. As shown in Fig. 7B, this analysis yielded an average dissociation constant (K<sub>d</sub>) of 0.5 nM for E<sub>2</sub> when performed on receptor prepared from rabbit reticulocyte lysates. This value is comparable to that obtained for the rat ERβ, which was reported to be 0.6 nM (33). We obtained an average K<sub>d</sub> of 0.2 nM for mERα (Fig. 7C), which is well within the range of previously published determinations for the cloned human receptor (38). Therefore, this slightly reduced affinity of mERβ for E<sub>2</sub> may provide an explanation for the shift in E<sub>2</sub> responsiveness indicated by the dose-response curves (Fig. 7A).

### Estrogen Antagonists Block the E<sub>2</sub>-Dependent Activity of mERβ

Estrogen antagonists such as OHT and ICI 164,384 are known to interact with hERα by blocking its transcriptional activity. More precisely, OHT is a mixed agonist-antagonist and blocks the activity of AF-2 but not of AF-1, whereas ICI 164,384 and 182,780 are pure antagonists that block both AF-1 and AF-2 activities (Refs. 6 and 39 and see below). We tested the effects of OHT and ICI 182,780 along with other antagonist compounds on mERβ transactivation, and the results are shown in Fig. 8. All antagonists tested, including OHT, ICI 182,780, hydroxy-toremifen, raloxifene, and EM-652 (the active derivative of the novel nonsteroidal antiestrogen EM-800), effectively inhibited E<sub>2</sub> activity (Fig. 8A). In contrast to its stimulatory effect on mERα, OHT did not display an agonistic activity on mERβ when tested with a vitA2-ERE-BLuc reporter in either Cos-1 (Fig. 8B) and HeLa cells (data not shown).

We further evaluated the specificity and potency of OHT and ICI 182,780 on both ER isoforms. Increasing concentrations of ICI 182,780 and OHT led to a complete inhibition of the E<sub>2</sub>-induced mERβ activity in Cos-1 cells (Fig. 8C). Furthermore, when added at 10 nM and above, ICI 182,780 and OHT lowered mERβ activity even below its basal level (compared with untreated in Fig. 8C). We observed a similar dose response for OHT and ICI 182,780 using either receptor (Fig. 8, D and E). However, for OHT, there was an apparent shift in the dose response toward the lower concentrations for mERβ as compared with mERα (Fig. 8D).



**Fig. 7. Dose-Response and Binding Properties of mER $\beta$**

A, Cos-1 cells were transfected with 500 ng mER $\beta$  (open circles) or mER $\alpha$  (closed circles) expression vectors and 1  $\mu$ g vitA2-ERE-TKLuc and then incubated for 12 h with increasing concentrations of E<sub>2</sub> as indicated. B, Specific binding of [2,4,6,7-<sup>3</sup>H]-17 $\beta$ -estradiol ([<sup>3</sup>H]E<sub>2</sub>) to mER $\beta$  was determined using receptors generated from rabbit reticulocyte lysates as described in *Materials and Methods*. Binding was determined over a concentration range of 0.01–3 nM [<sup>3</sup>H]E<sub>2</sub> in the absence or presence of a 200-fold excess of unlabeled E<sub>2</sub>. The saturation plot is shown in the *inset*, and results were plotted by the method of Scatchard. Each point was determined in triplicate in each experiment, and the above results are representative of at least two separate experiments. C, Specific binding to mER $\alpha$  using the conditions described in panel B.

**Ras Enhances E<sub>2</sub>-Induced Transcriptional Activity of mER $\beta$**

Phosphorylation of serine residues, in particular Ser<sup>118</sup>, has been shown to be necessary for maximal activity of AF-1 in the hER $\alpha$  and to mediate the effect of the Ras-Raf-1-mitogen-activated protein kinase (MAPK) kinase and MAPK pathway on the transcriptional activity of the ER (10–12, 40, 41). In an attempt to investigate the effect of the activation of this pathway on mER $\beta$  activity, we used H-Ras<sup>V12</sup>, a dominant active form of H-Ras, in transactivation studies. As shown in Fig. 9A, H-Ras<sup>V12</sup> acted to further increase by a factor of 3 the E<sub>2</sub>-induced activation of mER $\beta$  using the vitA2-ERE-TKLuc reporter in Cos-1 cells. ICI 182,780, but not OHT, completely abolished the E<sub>2</sub>-dependent induction of mER $\beta$  (Fig. 9A) and mER $\alpha$  (Fig. 9B) by Ras, suggesting that the effect of Ras on mER $\beta$  activity is mediated by a putative AF-1 present in the amino-terminal domain. These results also show that ICI 182,780 suppresses transactivation mediated by both AF-1 and AF-2.

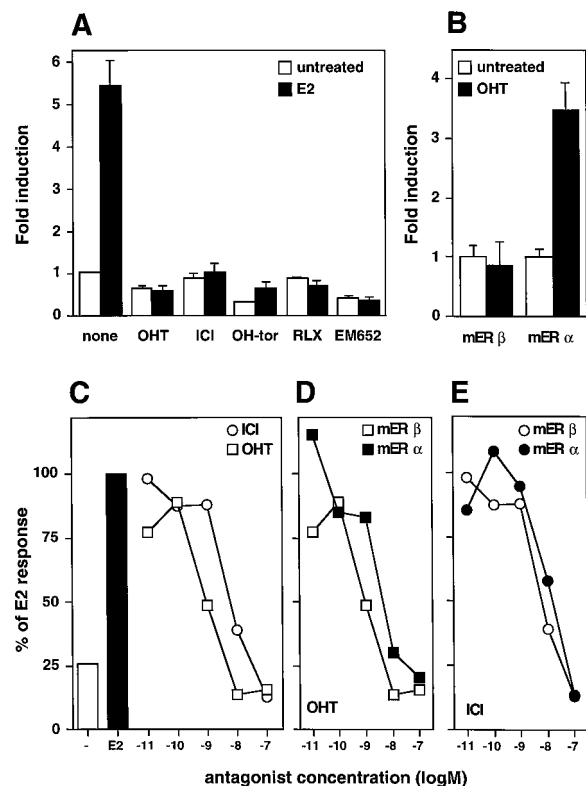
Two potential serines at positions 60 and 94 in the mER $\beta$  amino-terminal domain matched the consensus MAPK phosphorylation site (42) (Fig. 9C). To determine whether one or both serine residues were involved in the Ras-mediated induction, we mutated Ser<sup>60</sup> to Ala (S60A) and Ser<sup>94</sup> to Ala (S94A) in mER $\beta$  and used both mutants in transactivation studies. Figure 9D shows that H-ras had no inducing effect on the S60A mutant in the presence of E<sub>2</sub> while the S94A mutant retained its responsiveness to H-ras as compared with the wild type mER $\beta$  (Fig. 9A). This suggests that Ser<sup>60</sup> in mER $\beta$  is a potential target for

phosphorylation by the Ras-Raf-1-MAPK kinase-MAPK pathway.

**SRC-1 Interacts with and Augments Transcriptional Activity of mER $\beta$**

The contribution of the AF-2 domain to mER $\beta$  activity was investigated by examining the effect of the coactivator SRC-1, which has been shown to interact with a number of nuclear receptors including hER $\alpha$  (25, 43). Glutathione-S-transferase (GST) fusion proteins were generated with both the mER $\beta$  and mER $\alpha$  LBDs (Fig. 10A) and tested in a GST-pull down experiment (Fig. 10B). GST-mER $\beta$ EF and GST-mER $\alpha$ DEF were expressed in *Escherichia coli*, purified with GST-Sepharose, and incubated with [<sup>35</sup>S]methionine-labeled SRC-1. As shown in Fig. 10B, the LBD of mER $\beta$  interacted weakly with SRC-1 in the absence of E<sub>2</sub> (lane 3), whereas addition of E<sub>2</sub> caused an increase in interaction between the two proteins (lane 4). As expected, estrogen antagonists that affect the AF-2 of ER, namely OHT (lane 5) and ICI 182,780 (lane 6), do not promote a ligand-dependent SRC-1 interaction. The E<sub>2</sub>-dependent interaction with SRC-1 was also efficiently blocked in the presence of 10-fold higher concentration of the various antagonists (data not shown). Protein-protein interactions between SRC-1 and the mER $\alpha$  LBD paralleled those observed with mER $\beta$  (see Fig. 10B, lanes 8–12).

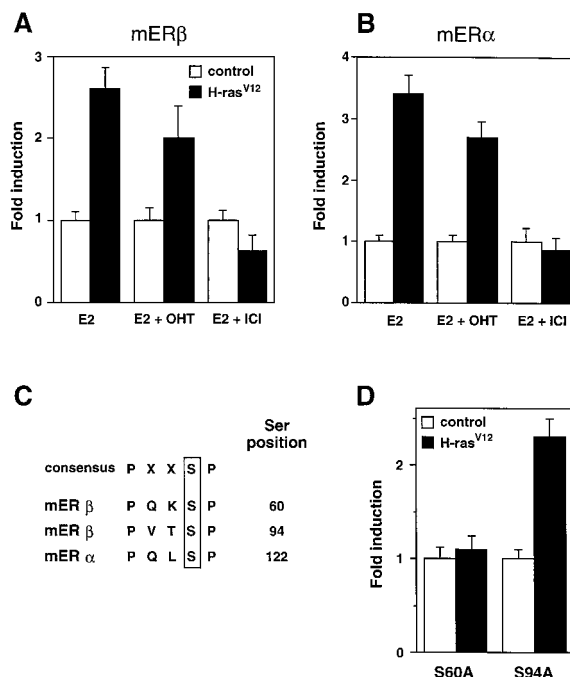
The contribution of SRC-1 on mER $\beta$  activity was analyzed *in vivo* by transfection studies. When SRC-1 was cotransfected with mER $\beta$  and vitA2-ERE-TKLuc in Cos-1 cells, a 2- to 3-fold induction over that of E<sub>2</sub> alone was observed (Fig. 10C). A similar response was



**Fig. 8.** Effect of Antagonists on mER $\beta$ -Mediated Transactivation

A, Cos-1 cells cotransfected with pCMX-mER $\beta$  and vitA2-ERE-TKLuc plasmids were incubated for 12 h in the presence or absence of 10 nM E<sub>2</sub> or 100 nM of the indicated antagonists: 4-hydroxytamoxifen (OHT), ICI 182,780 (ICI), hydroxy-toremifen (OH-tor), raloxifen (RLX), and EM652. Results are expressed as fold response over the mER $\beta$  basal level in the absence of E<sub>2</sub>; this value was set arbitrarily to 1. B, Cos-1 cells cotransfected with pCMX-mER $\beta$  or pCMX-mER $\alpha$  and ERE-BLuc plasmids were incubated for 12 h in the presence or absence of 100 nM OHT. C, Dose response of OHT and ICI in the presence of 10 nM E<sub>2</sub> on mER $\beta$  activity with the ERE-TKLuc in Cos-1 cells. The maximal induction by E<sub>2</sub> alone was arbitrarily set at 100%. The untreated mER $\beta$  basal level is also shown. Compounds within a panel are differentiated by open squares (OHT) and circles (ICI). D, Comparative panel of the dose responses to OHT in the presence of 10 nM E<sub>2</sub> between mER $\beta$  (open squares) and mER $\alpha$  (filled squares). Results are expressed as in panel B. E, Comparative panel of the dose responses to ICI in the presence of 10 nM E<sub>2</sub> between mER $\beta$  (open circles) and mER $\alpha$  (filled circles).

also seen with mER $\alpha$  in the presence of E<sub>2</sub> (Fig. 10C). However, SRC-1 alone efficiently increased the basal level of transcriptional activity by mER $\beta$ , suggesting a ligand-independent effect of SRC-1 on mER $\beta$  (Fig. 10C). The basal transcriptional level of mER $\alpha$  was not affected by the addition of SRC-1. We also studied the effect of SRC-1 on the pS2 promoter in HeLa cells and found a similar ligand-independent activation of mER $\beta$  by SRC-1 as well as a potentiation of the E<sub>2</sub>-induced activity (Fig. 10D). These results suggest that SRC-1 does not act on mER $\beta$  in a promoter- and cell-specific



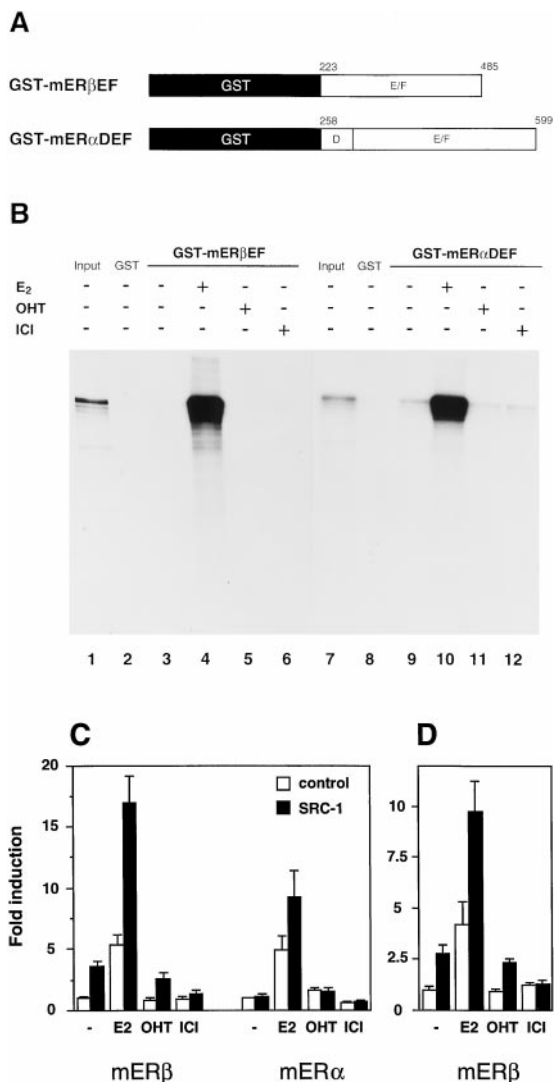
**Fig. 9.** Effects of H-Ras on E<sub>2</sub>-Induced mER $\beta$  Transcriptional Activity

A, Cos-1 cells were cotransfected with 1  $\mu$ g ERE-TKLuc, 500 ng pCMX-mER $\beta$ , and with or without H-Ras<sup>V12</sup> expression plasmid. The cells were then grown in the presence or absence of 10 nM E<sub>2</sub> or 100 nM of the indicated antagonists, 4-hydroxytamoxifen (OHT), and ICI 182,780 (ICI). B, Similar to panel A except that mER $\alpha$  was used. C, Potential MAP kinase phosphorylation sites in the mouse ER $\alpha$  and  $\beta$ . D, Mutation of Ser<sup>60</sup> abolishes the stimulatory effect of H-Ras<sup>V12</sup> on E<sub>2</sub>-induced mER $\beta$  transcriptional activity. Transfection conditions were as in panel A, except that mER $\beta$  mutants were used as indicated.

manner. Furthermore, ICI 182,780, but not OHT, was able to abrogate the ligand-independent effect of SRC-1 on mER $\beta$  transcriptional activity (Fig. 10, C and D).

## DISCUSSION

The physiological actions of E<sub>2</sub> are mediated through a member of the steroid hormone receptor family, ER $\alpha$ , which, for many years, has remained the only nuclear receptor known to have E<sub>2</sub> as a ligand. Recently, it has been shown that a second receptor, ER $\beta$ , is also able to respond to this hormone. ER $\beta$  was cloned by degenerative PCR from rat prostate (33) and more recently from human testes (44), but its physiological role remains to be elucidated. As a first step toward investigating the role of ER $\beta$  in development and homeostasis, we report the cloning of the murine homolog. The availability of the murine cDNA provides us with the means to characterize this receptor utilizing both biochemical and genetic methodologies and



**Fig. 10.** SRC-1 Interacts with mER $\beta$  and Induces Its Transcriptional Activity

A, Structure of the GST fusion proteins used in the analysis. B, GST pull-down experiments. The purified fusion proteins were incubated with labeled SRC-1 in the absence (lanes 3 and 9) or presence of 10 nM E<sub>2</sub> (lanes 4 and 10), 10 nM each of OHT (lanes 5 and 11) and ICI 182,780 (ICI) (lanes 6 and 12). The input lane represents 10% of the total amount of labeled SRC-1 used in each binding reaction. An equivalent amount of protein extract was used in the samples containing only GST (lanes 2 and 9). C, Cos-1 cells were cotransfected with mER $\beta$  and vitA2-ERE-TKLuc in the presence or absence of 1  $\mu$ g CMX-SRC-1. Cells were incubated with or without 10 nM E<sub>2</sub> or 100 nM antagonists as indicated. Separate experiments were also done with mER $\alpha$  as shown. D, Similar to panel A except that pS2Luc was used as the reporter and HeLa cells were transfected.

will allow us to study its relationship, if any, with ER $\alpha$  in normal reproductive physiology.

We first used a fragment of the LBD of the mouse ER $\beta$  cDNA to establish the localization of the gene to chromosome 12. We have compared our interspecific

map of chromosome 12 with a composite mouse linkage map location of many uncloned mouse mutations (provided from Mouse Genome Database, computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). *Estrb* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown). The central region of mouse chromosome 12 shares a region of homology with human chromosome 14q, suggesting that *Estrb* will reside on 14q as well.

The mER $\beta$  cDNA was used in a Northern analysis on murine ovary mRNA that revealed that the mER $\beta$  gene generates several transcripts as compared with a single message for mER $\alpha$  in this tissue. Partial cDNAs obtained from an ovary library all contained a 3'-untranslated region (UTR) of 657 bp followed by a polyA<sup>+</sup> tail, suggesting that the different transcripts seen in the Northern analysis may differ at their 5'-ends or in the coding region. Although we were unable to detect any messages in the total RNA from other mouse tissues, the hER $\beta$  homolog appears to be expressed in mRNA isolated from testes, spleen, and thymus in addition to ovary (44).

The two mouse ER isoforms are closely related to each other in their LBD and DBD, indicating that these receptors may regulate common gene networks and respond to similar ligands. Using EMSA, a ligand-binding assay, and transient transfection experiments, we showed that mER $\beta$  has slightly lower affinities than mER $\alpha$  for ERE and E<sub>2</sub> binding *in vitro*, but that mER $\beta$  can transactivate reporter genes driven by synthetic and natural E<sub>2</sub>-responsive promoters *in vivo* as efficiently as mER $\alpha$  in both Cos-1 and HeLa cells. However, the two ERs display no sequence homology in their amino-terminal domains, suggesting that each receptor may possess distinct transactivation functions. We made use of the differential mode of action of the estrogen antagonists OHT and ICI 182,780 and activation of receptor activity by the Ras-Raf-1-MAPK pathway to demonstrate functional similarities and differences between mER $\beta$  and mER $\alpha$ . We first showed that, when assayed in Cos-1 cells with an E<sub>2</sub>-responsive reporter gene driven by a basal promoter, OHT does not display an agonistic activity with ER $\beta$ . It has recently been shown that a specific region of the mER $\alpha$  AF-1 is required for OHT agonism (45): the absence of a corresponding domain in mER $\beta$  may explain the present observation. We demonstrated that mER $\beta$ , as previously shown for ER $\alpha$  (11, 12), can be activated by the Ras-Raf-1-MAPK pathway. We have identified a serine residue in mER $\beta$  that could be the target of phosphorylation upon cotransfection with Ras. This was demonstrated by the fact that a mutation at Ser<sup>60</sup> in the amino terminus of mER $\beta$  eliminates the effect seen with Ras. Although another serine residue at position 94 also matches the MAP kinase consensus, mutation at this position has no effect on Ras-mediated activation. Moreover, alignment of the mouse, rat, and human amino acid se-

quences in this region indicates that Ser<sup>60</sup> is conserved in all species whereas Ser<sup>94</sup> is replaced by a glycine in the human ER $\beta$ . Finally, we showed that the induction of the E<sub>2</sub>-dependent activation of mER $\beta$  by Ras can be abolished by ICI 182,780, but not OHT, an observation that provides further evidence of the involvement of an AF-1-like domain in regulating mER $\beta$  functions.

Antiestrogens play an important role in the treatment of breast cancer. We therefore monitored the efficacy of a series of antiestrogens previously shown to be selective for ER $\alpha$  on mER $\beta$  and found that all compounds tested, including OHT, ICI 182,780, hydroxy-toremifen, raloxifen, and the novel nonsteroidal antiestrogen EM-800, inhibited E<sub>2</sub>-dependent activation by mER $\beta$ . We showed that OHT is also AF-2 selective on mER $\beta$ , and that ICI 182,780 inhibits both activation functions, displays no estrogenic activity, and thus can be considered as a pure antagonist of mER $\beta$  activity. However, in dose-response studies, OHT proved to be a more potent inhibitor of mER $\beta$  than of mER $\alpha$ . In addition, we show that the mode of action of ICI 182,780, as previously observed for ICI 164,384, involves the inhibition of both AF-1 and AF-2 activity of ER $\alpha$ .

A growing network of coactivators that interact with ER $\alpha$  has now been cloned. These include RIP140 (20), TIF2 (23), and SRC-1 (25). However, only SRC-1 has been shown to up-regulate ER-stimulated transcription (25, 43). mER $\beta$  transcriptional activity could also be stimulated by SRC-1 in a ligand-dependent manner in cotransfection assays as well as *in vitro* where we observed a very strong ligand-dependent interaction with a GST-mER $\beta$  LBD fusion protein. Surprisingly, we also observed ligand-independent SRC-1 enhancement of mER $\beta$  transcriptional activity. These results suggest that SRC-1 may interact with a region, other than the AF-2, of the mER $\beta$  protein. McInerney *et al.* (43) have shown that constructs expressing the ABCD (no LBD) and EF (LBD only) domains of hER $\alpha$  separately are able to transactivate in the presence of E<sub>2</sub>, and that SRC-1 can enhance the level of activation under these conditions. These results led them to suggest that SRC-1 may act as an adapter to promote AF-1 and AF-2 receptor activities and are in agreement with our suggestion that SRC-1 may interact with several regions of nuclear receptor proteins.

The identification of a second ER in mammals is very exciting, and these and previous studies have only begun to define the putative role that it plays in the mediation of estrogen action. Clearly, further studies into the mechanism of action of ER $\beta$  are required if we are to understand how it functions *in vivo*. The results we present in this paper indicate that ER $\beta$  and  $\alpha$  may have both redundant and distinct functions, as exemplified by the manner with which they respond to OHT, Ras, and SRC-1, and suggest that ER $\beta$  plays an unique role in the physiological actions of natural estrogens.

## MATERIALS AND METHODS

### Cloning of the Mouse ER $\beta$ cDNA

A combination of PCR and cDNA library screening was used to obtain the full-length cDNA encoding the murine ER $\beta$ . All oligonucleotides used in this study were synthesized at the Sheldon Biotechnology Center, McGill University. Total RNA was prepared from several mouse ovaries and used to isolate poly-A<sup>+</sup> RNA over two oligo-dT columns (Pharmacia, Piscataway, NJ). An initial 550-bp fragment was amplified from 100 ng poly-A<sup>+</sup> RNA using degenerate primers specific for the rat ER $\beta$  LBD (33). The 5'- and 3'-sequence of these primers spanned amino acids 269–277 (KKIPGVE) and 443–450 (YDLLLEML, noncoding strand), respectively. The reaction was carried out for 40 cycles using Pfu polymerase (Stratagene, La Jolla, CA) at an annealing temperature of 54 C and for an extension time of 1 min. The PCR product was separated on low melt agarose (Life Technologies, Gaithersburg, MD) and subcloned into pBluescriptKSII (Stratagene). The sequence was determined on both strands using the T3 and T7 primers and found to encode an open reading frame that was highly homologous to the rat ER $\beta$ . This 550-bp fragment served as a probe to screen a mouse ovary cDNA library constructed using the Superscript cDNA Synthesis System (Life Technologies). Three strong positives were plaque purified, subcloned into pBluescriptKSII, and sequenced on both strands. Each of these clones started in the LBD and ended with a poly-A tail. A PCR primer was designed based on the sequence of the 3'-UTR of the partial mouse ER $\beta$  cDNA and used to synthesize first-strand cDNA from mouse ovary poly-A<sup>+</sup> RNA. The cDNA served as a template in a PCR reaction using a primer that spans the first 21 bases of the 5'-UTR of rat ER $\beta$  ending with the putative initiator codon and a second specific mouse ER $\beta$  3'-UTR primer. This reaction yielded a single product of 2.1 kb that was subcloned into pBluescriptKSII directionally using *Sal*I and *Eco*RI sites that were designed in the 5'- and 3'-PCR primers, respectively, and sequenced on both strands. Sequencing was conducted at the sequencing facility at the Sheldon Biotechnology Center, McGill University.

### Chromosomal Localization of the ER $\beta$ Gene

Interspecific backcross progeny were generated by mating (C57BL/6J  $\times$  *M. spretus*) F1 females and C57BL/6J males as described (34). A total of 205 N<sub>2</sub> mice were used to map the *Estrb* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (46). All blots were prepared with Hybond N<sup>+</sup> nylon membrane (Amersham). The probe, a 550-bp *Hind*III/*Eco*RI fragment of the mouse cDNA, was labeled with [<sup>32</sup>P]- $\alpha$ -dCTP using a random-primed labeling kit (Stratagene); washing was done to a final stringency of 0.8 $\times$  SSCP, 0.1% SDS, at 65 C. Fragments of 13.5 and 5.5 kb were detected in *Eco*RI-digested C57BL/6J DNA, and fragments of 8.4 and 5.1 kb were detected in *Eco*RI-digested *M. spretus* DNA. The presence or absence of the 8.4- and 5.1-kb *Eco*RI *M. spretus*-specific fragments, which cosegregated, was followed in the backcross mice.

A description of the probes and RFLPs for the loci linked to *Estrb* including *Sos2*, *Spcb1*, and *Fos* has been reported previously (47). Recombination distances were calculated as described (48) using the computer program SPRETUS MAD-NESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

### Northern Analysis

Five micrograms of mouse ovary poly-A<sup>+</sup> RNA were electrophoresed on a 1% formaldehyde-agarose gel and blotted onto Hybond-N<sup>+</sup>. The entire mouse ER $\alpha$  cDNA (49) and the 550-bp PCR product encoding mER $\beta$  were radiolabeled with [<sup>32</sup>P]- $\alpha$ -dCTP (Amersham, Arlington Heights, IL) by random priming (Pharmacia). Prehybridization was carried out for 4 h in 50% formamide, 5 $\times$ SSPE, 5 $\times$  Denhardt's solution, 1% glycine, and 100  $\mu$ g/ml denatured salmon sperm DNA at 42 C. Hybridization was conducted overnight at the same temperature in 50% formamide, 5 $\times$  SSPE, 1 $\times$  Denhardt's solution, 10% dextran sulfate, 0.3% SDS, 100  $\mu$ g/ml denatured salmon sperm DNA, and 2  $\times$  10<sup>6</sup> cpm/ml denatured probe. The membrane was washed to high stringency (0.1 $\times$  SSC, 0.1% SDS at 65 C for 30 min) and exposed to X-OMAT AR film (Kodak) overnight at -85 C. The positions of the 18S and 28S rRNAs were determined by the electrophoresis of total mouse ovary RNA on the same gel.

### Plasmids

The expression vector pCMX-mER $\alpha$  was constructed by ligating a 1.9-kb *NaeI*-*EcoRI* fragment of mER $\alpha$  (49) into the appropriate sites of the eukaryotic expression vector, pCMX (50). pCMX-mER $\beta$  was constructed in a similar fashion by subcloning the 2.1-kb *Sall*-*EcoRI* fragment described above into pCMX. Fusion proteins were generated between the mER $\beta$  and mER $\alpha$  LBDs and GST by subcloning fragments of the mouse cDNAs into the pGEX-2T expression vector (Pharmacia). Briefly, a unique *PvuII* site located at position 678 and an *EcoRI* site in the vector were used to excise the LBD (domains E and F) from the full-length mER $\beta$  cDNA. The fragment was then subcloned into the *SmaI* site of pGEX to produce pGST-mER $\beta$ EF. For mER $\alpha$ , the hinge region and the LBD (domains D, E, and F) were amplified from the full length mER $\alpha$  cDNA using PCR and subcloned directionally into the *Bam*HI and the blunted *EcoRI* sites of the pGEX vector (pGST-mER $\alpha$ DEF) (see Fig. 9A). Both constructs were verified to be in-frame with GST by sequencing. vitA2-ERE-BLuc and vitA2-ERE-TKLuc were constructed by ligating the vitA2-ERE oligonucleotide (see below for sequence) into *Sall*-*Bam*HI-digested TKLuc vector. pS2-ERELuc contains the ~1050-bp pS2 promoter (36) preceding the luciferase reporter of pGL3 (Promega, Madison, WI). The pS2 $\Delta$ ERELuc, in which the ERE was replaced by sequences encoding *EcoRI*-*EcoRV* sites, was generated by PCR mutagenesis using the ExSite kit from Stratagene as described by the manufacturer. The serine to alanine mutants at positions 60 and 94 of mER $\beta$  were also generated by PCR mutagenesis. The oligonucleotides used were: S60A, 5'-CTCTATGCAGAACCCTCAAAG-GCTCCTTGGTGTGAAGC-3'; S94A, 5'-GGTTGTGCCAGC-CCTGTTACTGCTCCAAGCGCCAAGAGG-3'. The H-Ras<sup>V12</sup> expression plasmid was a generous gift from Dr. Morag Park, McGill University.

### Chemicals

E<sub>2</sub> was obtained from Sigma Chemical Co. (St. Louis, MO). EM-800, EM-652, ICI-182780, and OH-toremifene were synthesized in the medicinal chemistry division of the Laboratory of Molecular Endocrinology, CHUL Research Center, Québec, Canada. OHT was kindly provided by Dr. D. Salin-Drouin, Besins-Iscovesco, Paris, France.

### EMSA

mER $\beta$  and mER $\alpha$  proteins were synthesized by *in vitro* transcription-translation using rabbit reticulocyte lysates (Promega) and pCMX-mER $\beta$  and pCMX-mER $\alpha$ , respectively, as templates. Typically 5  $\mu$ l of programmed lysate were used in

each binding reaction. DNA-binding reactions were carried out in binding buffer (5 mM Tris, pH 8.0, 40 mM KCl, 6% glycerol, 1 mM dithiothreitol, 0.05% Nonidet P-40), 2  $\mu$ g of poly(deoxyinosinic-deoxycytidylic)acid, 0.1  $\mu$ g of denatured salmon sperm DNA, and 10  $\mu$ g of BSA with 0.1 ng probe that was labeled by end-filling with Klenow in the presence of [<sup>32</sup>P]- $\alpha$ -dCTP. Preincubations containing ligand and/or cold competitor as indicated were conducted on ice for 30 min, after which the probe was added and allowed to bind for 30 min at room temperature. The entire reaction (20  $\mu$ l) was loaded onto a 4% polyacrylamide gel and electrophoresed at 150 V at room temperature. Gels were dried and exposed overnight at -85 C. The following oligonucleotides and their compliments were used as probes and competitors: vitA2-ERE, 5'-TCGACAAAGTCAGGTACACAG-TGACCTGAT-CAAG-3' (51); pS2-ERE, 5'TCGACCCCTGCAAGGTACCGGT-GGCCA-CCCCGTG-3' (36); IR3, 5'-TCGACGTGTAGGTCA-CAGTGACCTCTTCA-3'.

### Scatchard Analysis

Ligand binding studies were conducted essentially as previously described (52) with the following modifications. mER $\beta$  and mER $\alpha$  were produced using rabbit reticulocyte lysates, diluted 12-fold in TEG buffer (10 mM Tris, pH 7.5, 1.5 mM EDTA, 10% glycerol) and kept on ice until use. One hundred microliters of this dilution were used in each binding reaction at 4 C overnight containing [2,4,6,7-<sup>3</sup>H]17 $\beta$ -estradiol concentrations ranging from 0.01–3 nM. Nonspecific binding was assessed by including 200-fold excess E<sub>2</sub> in a parallel set of samples. Unbound steroids were removed with dextran-coated charcoal and counts per min were determined by liquid scintillation counting.

### Cell Culture, DNA Transfection, and Luciferase Assay

For transfection, Cos-1 and HeLa cells were seeded in six-well plates in phenol red-free DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% charcoal dextran-treated FBS, and 100  $\mu$ g/ml penicillin and 100  $\mu$ g/ml streptomycin. At 50–60% confluency, cells were transfected with 1–2  $\mu$ g of reporter plasmid, 0.5–1  $\mu$ g receptor expression vector, 1  $\mu$ g CMX- $\beta$ gal or RSV- $\beta$ gal, and 6–7.5  $\mu$ g pBluescriptKSI, using the calcium phosphate/DNA precipitation method (53). After 8–16 h, cells were washed and typically 10 nM E<sub>2</sub> or 100 nM antiestrogens, unless otherwise stated, were added to the growth medium for 16 h. For luciferase assay, cells were lysed in potassium phosphate buffer containing 1% Triton X-100, and light emission was detected using a luminometer after addition of luciferin. Values are expressed as arbitrary light units normalized to the  $\beta$ -galactosidase activity of each sample.

### In Vitro Protein-Protein Interactions

Fusion proteins were expressed in *E. coli* DH5 $\alpha$  as follows. An overnight culture was diluted 10-fold in 500 ml prewarmed LB containing 100  $\mu$ g/ml ampicillin and incubated for 1 h at 37 C. Isopropyl- $\beta$ -D-thiogalactopyranoside was added to a final concentration of 1 mM to induce expression, and the culture was allowed to grow for a further 3 h. Cells were cooled on ice for 10 min and centrifuged at 2500  $\times$  g for 20 min. The pellet was resuspended in 12 ml ice-cold PBS and sonicated. After the addition of one tenth volume of 10% triton, the extract was centrifuged at 12,000  $\times$  g for 20 min at 4 C. Clarified extracts were aliquoted in 1-ml samples and frozen at -85 C.

The 3.3-kb SRC-1 cDNA encoding the predicted 1061 amino acid open reading frame as originally cloned (25) was digested with *XbaI* and *Sall*, blunt ended with Klenow, ligated into pCMX, and labeled with [<sup>35</sup>S]methionine (Amersham) *in vitro* using rabbit reticulocyte lysates as described above.

Approximately 300  $\mu\text{g}$  of total protein from extracts containing the GST fusion proteins were loaded onto glutathione-Sepharose 4B (Pharmacia) for 30 min at 4 C with gentle agitation. After a short centrifugation, the beads were resuspended in 150  $\mu\text{l}$  IPAB buffer (20) containing 10 nM ligand or antagonists and 4  $\mu\text{l}$  of labeled SRC-1 crude lysate and allowed to bind for 90 min at 4 C. Beads were washed twice in the presence of IPAB and twice with IPAB without BSA, dried briefly, and resuspended in 30  $\mu\text{l}$  loading buffer. Bound proteins were analyzed by SDS-PAGE. The gel was treated with Amplify (Amersham), dried, and exposed at  $-85\text{ C}$ .

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