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# Cloning and Characterization of Human Estrogen Receptor $\beta$ Isoforms

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**Multiple transcripts which arise from the human estrogen receptor $\beta$ (ER$\beta$) gene have been characterized. Three full length isoforms of the hER$\beta$ gene, designated hER$\beta$1-3, were identified in a testis cDNA library. An additional two isoforms, designated hER$\beta$4 and hER$\beta$5, were identified by PCR amplification from testis cDNA and from the MDA-MB 435 cell line. hER$\beta$1 corresponds to the previously described hER$\beta$. All five isoforms diverge at a common position within the predicted helix 10 of the ligand binding domain of hER$\beta$, with nucleotide sequences consistent with differential exon usage. The hER$\beta$ isoform mRNAs displayed a differential pattern of expression in human tissues and in tumor cell lines when analyzed by RT-PCR. Further characterization of the three full length isoforms, hER$\beta$1-3, by in vitro band shift studies indicated that the isoforms were able to form DNA-binding homodimers and heterodimers with each other and with the ER$\alpha$ subtype.** 

The genes for ER$\alpha$ and ER$\beta$ have been identified as receptors for the female sex hormone estrogen. Like other members of the nuclear receptor superfamily, ER$\alpha$ and ER$\beta$ contain characteristic sequence motifs (1) including a DNA binding domain (DBD), nuclear localization signals, a ligand-binding domain (LBD), and several transcription activation domains (AFs) (2-4). The receptors activate transcription of target genes following ligand activation and direct binding to palindromic DNA elements located in upstream regulatory sequences of these genes (2-4). Several isoforms of the ER$\alpha$ gene have been isolated. Although their biological function has not been fully elucidated, some studies have implicated a role of specific ER$\alpha$ isoforms in the pathogenesis of estrogen dependent breast cancer (5-7). Recently, isoforms of ER$\beta$ gene have been uncovered. In the rat, an ER$\beta$ isoform has been described which contains a 54 nucleotide insertion in the LBD (8,9) and shows reduced affinity for estrogens. A homologous hER$\beta$ isoform has not been reported. However, an hER$\beta$ splice variant containing a 139 nucleotide deletion in the LBD has been described which is expressed in the estrogen independent breast cancer line MDA-MB-231 and in malignant breast tumor specimens (10). We now describe the cloning and expression characteristics of five isoforms of the human ER$\beta$, designated hER$\beta$1-5, which differ in their C-terminal sequences and tissue expression patterns.

## MATERIALS AND METHODS

*Cloning of human ER$\beta$ isoforms.* A human testis cDNA library was screened using Gene Trapper technology (Life Technologies). The library was constructed in the vector pCMV-SPORT (Life Technologies) and was rendered single-stranded via Gene II and Exo III digestion. The single stranded cDNA was screened by solution hybridization using internal hER$\beta$-specific sense oligonucleotides (DM150 5′-GATTATATTTGTCCAGCTAC and DM151 5′- GCTACAAATCAGTGTACAATCG). For screening, the oligonucleotides were gel-purified on acrylamide/urea gels and coupled with biotin-14-dCTP in the presence of terminal deoxynucleotidyl transferase. Gel analysis revealed >80% of the oligonucleotides were biotinylated with the major product containing 3-4 biotin residues. The biotinylated oligonucleotides were annealed to the single-stranded DNA template, and the hybrids subsequently captured by magnetic streptavidin beads. The captured cDNA was then made double-stranded using a thermostable DNA polymerase (Life Technologies) and electroporated into E. coli DH10B. Six independent hER$\beta$ clones were isolated with inserts greater than 1 Kb. Sequence analysis revealed that these clones represented the known hER$\beta$, which we designate hER$\beta$1, and two novel isoforms hER$\beta$2 and hER$\beta$3 (Fig. 1).

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Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AF061054 and AF061055.



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Partial clones of additional isoforms were isolated after PCR amplification using primers derived from isoform terminal sequences and cloning of the resulting products. hER$\beta$4 was derived from testis cDNA using oligos DM166 (5′ AGTATGTACCCTCTGGTCACAGCG) and KS5 (5′ GGATTACAATGATCCCAGAGGGAAATTG. hER$\beta$5 was derived from MDA-MB 435 cell line cDNA using oligos DM166 and KS12 (5′ CTTTAGGCCACCGAGTTGATTAGAG). The PCR products were cloned into the pBluescript vector and sequence information was obtained. (GenBank accession numbers for the ER$\beta$ isoform sequences are as follows: hER$\beta$1, AF051427; hER$\beta$2, 175236; hER$\beta$3, AF060555; hER$\beta$4, pending; hER$\beta$5, pending.)

*PCR expression studies.* Tissue specific cDNA samples representing a wide range of human tissues were purchased (Clontech, Palo Alto, CA) or cell lines obtained from ATCC (Rockville, MD) and used in a PCR-based experiment to determine the expression pattern of the different hER$\beta$ isoforms. In each reaction, approximately 0.5 - 2 ng of cDNA served as the template, and the sense oligo was derived from sequence that is shared by all the hER$\beta$ isoforms (5′-AGTATGTACCCTCTGGTCACAGCG). The isoform-specific antisense oligos were as follows: hER$\beta$1, 5′-CCAAATGAGGGACCACACAGCAG; and hER$\beta$2, 5′-GGATTACAATGATCCCAGAGGGAAATTG, hER$\beta$3, 5′-GCAGTCAAGGTGTCGACAAAGGCTGC, hER$\beta$4, GGATTACAATGATCCCAGAGGGAAATTG, hER$\beta$5, CTTTAGGCCACCGAGTTGATTAGAG. The negative control in each set is a reaction where the cDNA template is replaced with an equal volume of water, and the positive control contains 5 ng of the human testis cDNA library from which the hER$\beta$ isoforms were originally isolated. The GAPDH gene was amplified as a control housekeeping gene with the sense oligo 5′-ACCACAGTCCATGCCATCAC and the antisense oligo 5′-TCCACCACCCTGTTGCTGTA. The amplification mix was prepared with the Advantage KlenTaq polymerase mix (Clontech) according to manufacturer's instructions, and the conditions of the PCR were as follows: 94°C for 1 min, then 30 cycles (or 22 cycles for the GAPDH primer set) of 94°C for 30 sec, 64°C for 40 sec, and 72°C for 45 sec. PCR products were resolved on a 2% agarose gel in 1× TAE running buffer.

*Gel-shifts.* For gel-shift studies, open reading frames of hER$\alpha$, hER$\beta$1-3 (full-length constructs) or coding regions of each containing a deletion of the A/B domain coding region replaced by a methionine codon at a position 6 amino acids in front of the respective DNA binding domains (truncated constructs) were cloned into pSG5 (Stratagene, La Jolla, CA). Gel-shift studies were performed essentially as described (11). hER$\alpha$ and hER$\beta$ constructs were used to prime coupled transcription/translation reactions (TNT T7 Quick Coupled Transcription/Translation System, Promega Corp., Madison, WI). A parallel control reaction containing [$^{35}$S]-methionine was run for each condition to monitor synthesis of desired protein product. In gel-shift assays, cold lysate was added to approximately 10 ng ($2 \times 10^7$ cpm) [$^{32}$P]-labeled double stranded ERE (5′GATCGACAAAGTCAGGTCACAGTGACCTGATCAAG). In some cases, excess cold (500 ng) ERE or GRE (5′GATCGACAAAGTCAGAACACA-GTGTTCTGATCAAG) was added to the reactions to test binding specificity.

## RESULTS AND DISCUSSION

### *Cloning of hER$\beta$ Isoforms*

A screen for ER$\beta$ clones from a human testis cDNA library was performed by solution hybridization. Based on the nucleotide sequence contained within the LBD of hER$\beta$1 (3), oligo pairs were made to direct isolation of full-length cDNA clones using solution hybridization GeneTrapper Technology. Three full-length hER$\beta$ isoform cDNAs were isolated and designated hER$\beta$1-3 (Fig. 1A). All three isoforms contained the additional

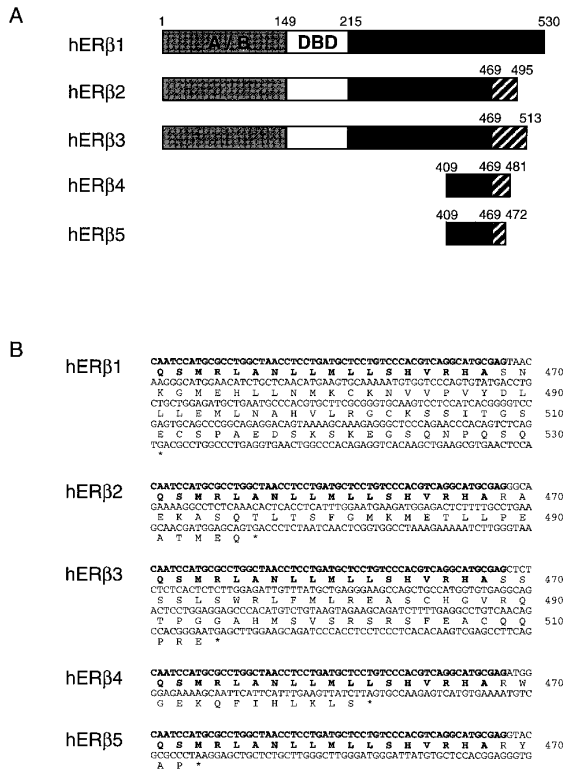


**FIG. 1.** hER$\beta$ isoforms. A. Comparison of hER$\beta$ isoforms. A/B is the N-terminal domain, DBD is the DNA-binding domain, LBD is the ligand binding domain. The solid bar represents conserved LBD sequences and the striped bar shows divergent sequences among the isoforms. B. The C-terminal nucleotide and deduced amino acid sequences of the hER$\beta$ isoforms. Amino acids in boldface represent shared sequences among the isoforms.

N-terminal sequences which were recently reported (12) and were absent in the earlier clones (2-4). PCR amplification using cDNA derived from testis and MDA-MB 435 cell line as template identified partial sequences of two additional C-terminal isoforms of the hER$\beta$ gene, designated hER$\beta$4 and hER$\beta$5 (Fig. 1A). The predicted amino acid sequences of hER$\beta$1-5 diverge at amino acid 469 within the LBD and extend to the C-terminus (Fig. 1B). The nucleotide boundaries are consistent with a 5′ splice junction sequence suggesting that the isoforms are generated by differential splicing of exons (13). Sequence alignment with hER$\alpha$ suggests that amino acid 469 of hER$\beta$ resides within the middle of helix 10 of the LBD (14).

### *Differential RNA Expression of hER$\beta$ Isoforms*

The expression of hER$\beta$1-5 mRNA was examined by PCR analysis (Fig. 2). After 30 cycles of PCR, products of the expected sizes were observed for hER$\beta$1 (408 base pairs), hER$\beta$2 (291 base pairs), hER$\beta$3 (400 base pairs), hER$\beta$4 (608 base pairs), and hER$\beta$5 (372 base

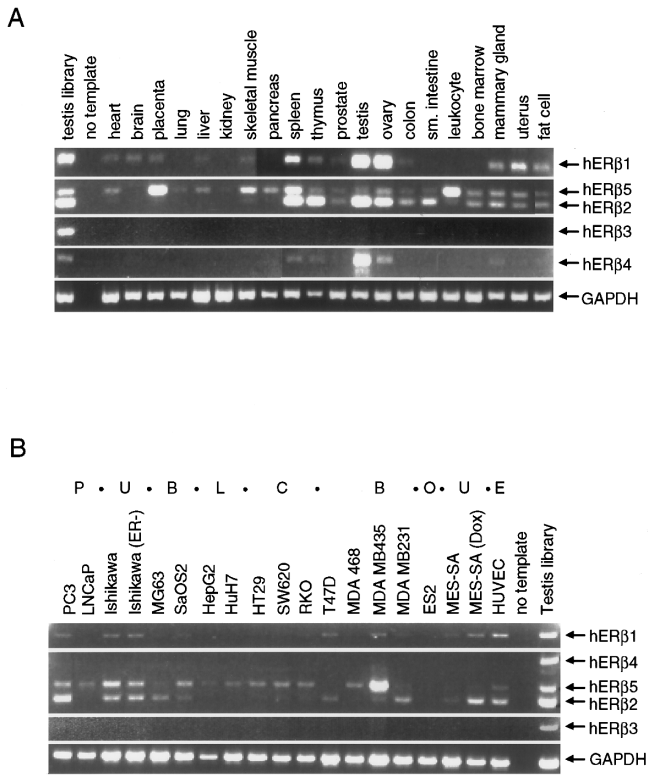


**FIG. 2.** hER$\beta$ expression analysis. RT-PCR analysis of hER$\beta$ expression in normal tissues (A) and in cell lines (B). PCR amplification was carried out for 30 cycles using primers designed to identify hER$\beta$1-5. The tissues from which cDNA template was derived are indicated below the lanes. The negative control consisted of substituting water for cDNA template and the positive control was primed with template from a testis cDNA library from which all of the hER$\beta$ isoforms were originally cloned. The cell lines are grouped by tissue origin: P, prostate; U, uterus; B, bone; L, liver; C, colon; Br, breast; E, endothelial.

pairs). The hER$\beta$ isoforms displayed striking differences in their patterns of expression. In normal tissues, the highest hER$\beta$1 expression was detected in testis and ovary, with lower levels of expression detected in heart, brain, placenta, liver, skeletal muscle, spleen, thymus, prostate, colon, bone marrow, mammary gland, uterus, and fat. hER$\beta$2 was detected at highest levels in spleen, thymus, testis and ovary, with lower levels of expression in skeletal muscle, prostate, colon, small intestine, leukocytes, bone marrow, mammary gland, uterus, and fat. By contrast, expression of hER$\beta$3 was restricted to the original testis cDNA library from which it was isolated. hER$\beta$4 was detected at highest levels in testis, and at lower levels in spleen, thymus, ovary, mammary gland, and uterus. hER$\beta$5 was detected in all tissues probed except for brain, with highest levels in placenta, spleen and leukocytes. In addition to normal tissues, transformed cell lines representing prostate, uterine, bone, liver, colon, breast, and ovarian tumors were analyzed for expression of hER$\beta$ isoforms. hER$\beta$1 was detected in prostate, uterine, bone, breast, and ovarian tumor cell lines but not in colon and liver tumor cell lines. hER$\beta$2 and hER$\beta$5 were detected in a wide spectrum of cell lines. Interestingly in the MDA MB435 breast cancer cell line hER$\beta$5 appeared to be expressed at relatively high levels. hER$\beta$3 and hER$\beta$4 were not detected in any of the cell lines screened. We conclude that the hER$\beta$ isoforms are differentially expressed at the RNA level, and that multiple hER$\beta$ isoforms may contribute to estrogen

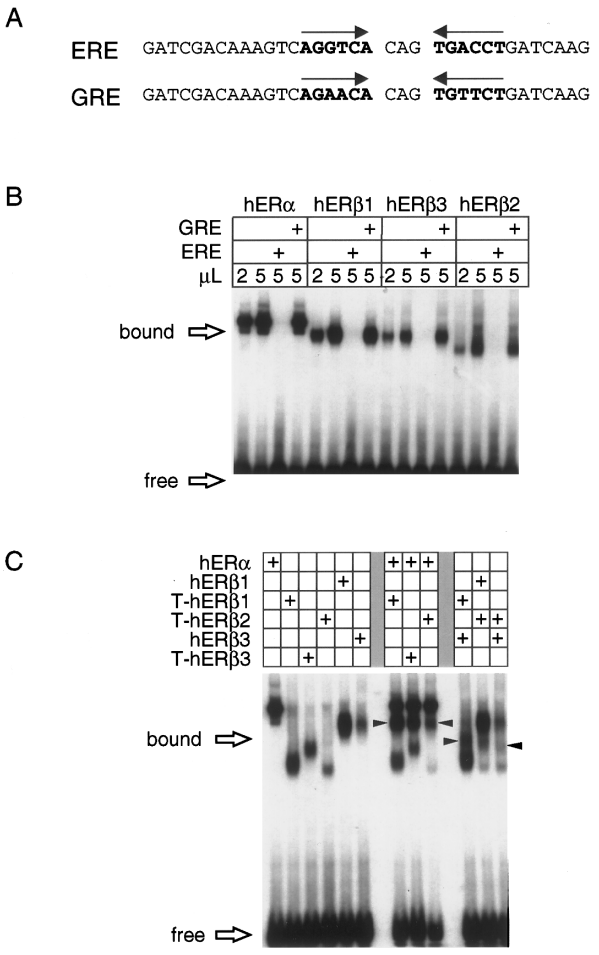


**FIG. 3.** Gel-shift analysis. A. Nucleotide sequences of the synthetic ERE and GRE. Boldfaced nucleotides and arrows indicate the inverted repeat motifs. B. Gel-shift studies of full length hER subtypes and isoforms. Gel-shifts were performed using [$^{32}$P]-labeled ERE oligonucleotides and rabbit reticulocyte lysate containing in vitro transcribed and translated product from hER$\alpha$, hER$\beta$1, hER$\beta$2, or hER$\beta$3. Quantities refer to the amount of lysate added to the gel. The presence of excess cold ERE or GRE is indicated by a plus sign. Bound and free probe is indicated. C. Gel-shift studies of truncated hER$\beta$ isoforms. Gel-shifts were performed using [$^{32}$P]-labeled ERE oligonucleotides. The presence of the relevant hER protein is indicated by a plus sign. Proteins with the A/B domains deleted are noted by a T. Arrows indicate bands corresponding to hER$\alpha$/hER$\beta$ heterodimers or hER$\beta$ isoform heterodimers.

pharmacology in those tissues and cells where the corresponding proteins are expressed.

### *hERβ Isoforms Bind to an ERE as Homodimers and Heterodimers*

Helix 10 of the LBD has been demonstrated to form part of the dimerization interface involved in ERα homodimerization and ERα/ERβ heterodimerization (15). Since the hERβ isoforms diverge in sequence in the middle of helix 10, we examined whether the dimerization function was affected. Dimerization is known to affect DNA binding, therefore gel mobility shift assays were employed to study the DNA affinity of the hERβ isoforms for which full length cDNAs had been isolated. Using a canonical ERE sequence hERβ1, hERβ2 and hERβ3 were each able to bind to DNA and the size of the complexes, relative to the hERα controls, was consistent with the hERβ isoforms binding as homodimers (Fig. 3B). The binding could be abolished by addition of unlabeled ERE competitor oligonucleotide, but not by a GRE oligonucleotide indicating that DNA binding was specific for the ERE motif. Heterodimerization was studied using a standard approach where the N-terminal A/B domain was deleted from the hERβ2 and hERβ2 isoforms to allow size discrimination and identification of complexes formed with full-length hERα and hERβ1. Control experiments demonstrated that truncation of the A/B domain caused no obvious diminution in the ability of hERβ2 and hERβ2 to bind to an ERE (Fig. 3C). Gel-shift analysis indicated that heterodimeric complexes were formed between hERα and each of the hERβ1-3 isoforms, and between any combination of the hERβ1-3 isoforms (Fig. 3C). We conclude that all heterodimeric combinations among hERα and the hERβ isoforms can occur in vitro and that sequence variation in helix 10 does not impair homo- or heterodimerization of the receptors on DNA.

In conclusion, additional C-terminal isoforms of the hERβ gene have been identified. The hERβ isoforms show differential expression in tissues and cell lines and are predicted to form DNA-binding heterodimers when coexpressed. These observations suggest that there exists additional complexity in ER signaling pathways. Further characterization of the protein expression, ligand binding and transactivation profiles of these isoforms and their heterodimeric products is currently in progress.

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