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PRINCIPAL INVESTIGATOR(S): Doctor Larry Vickery

CONTRACTING ORGANIZATION: University of California
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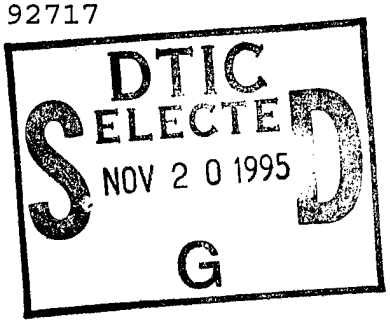

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Irvine, California 92717

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The requisite initial step in estrogen receptor action, ligand binding, is mediated by the ~250 amino acid Hormone Binding Domain (HBD). We have expressed and purified in high yield (~10 mg/liter of culture) two peptides (amino acids 301-551 or 305-551 of the estrogen receptor) in *E. coli*. Both peptides exhibit high affinity estradiol binding (K_d ~0.3 nM) and the ability to discriminate among various ligands comparable to the full-length protein. The isolated HBD (301-551) peptide displayed cooperative estradiol binding suggesting that it dimerizes and undergoes conformational changes required for cooperativity in a manner comparable to the full-length protein. In contrast, the HBD (305-551) peptide exhibited cooperativity only at protein concentrations at least 3-fold higher than those required for the longer peptide, suggesting that the N-terminus of the HBD contributes to dimer interactions. Both of the HBD peptides exhibited binding stoichiometries of 0.5 mol estradiol/mol protein, suggesting that the HBD dimer binds a single molecule of estradiol. These results indicate that the use of fragments of the estrogen receptor expressed in *E. coli* lend insight into the structural features of the protein responsible for its functional properties.

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Table of Contents

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Introduction

The estrogen receptor is a member of a superfamily of proteins that includes the receptors for the steroid hormones, for vitamins A and D, and for thyroid hormone (1,2). The binding of ligands to these receptors is a requisite initial step in a complex series of events culminating in an interaction of the ligand-bound receptor with the transcription machinery and modulation of gene expression. These receptor proteins exhibit four distinct properties required to exert their actions: hormone binding, multimeric complex formation, sequence specific DNA binding, and transcriptional modulation.

The currently proposed schematic structure of these receptor proteins based on sequence identity and deletion analyses (summarized in 2), suggests that these proteins fold into at least three separate structural and functional domains: (i) an N-terminal domain having a highly variable amino acid sequence and believed to mediate much of the transcriptional enhancement activity of the protein, (ii) a highly conserved central domain of ~80 amino acids involved in DNA-binding, and (iii) a somewhat conserved C-terminal domain that is involved in ligand binding and is thought to be responsible for regulating the activity of the remainder of the protein.

The C-terminal hormone binding domain (HBD)[1] of the receptor is of particular interest because, in addition to its ligand binding activity, it appears to contain many of the regulatory functions of the protein. Chimeric constructs containing fusions of the estrogen receptor HBD with unrelated proteins such as the *myc* oncogene product, for example, display hormonal regulation of the activity of the fused gene products (3). This suggests that, even when removed from its normal environment, the HBD is not only capable of specific ligand binding, but must also retain the capacity to undergo the conformational changes that normally regulate the function of the receptor. Furthermore, the finding that

the HBD can affect the activity of unrelated proteins suggests that the ligand-induced alteration in conformation must be a fundamental change in structure.

For the human estrogen receptor mutational and deletion analyses have shown that the HBD resides in amino acids 301-551 (4-6), suggesting that, unique among the receptors for steroids, the estrogen receptor HBD apparently does not extend to the extreme C-terminus of the protein at amino acid 595. In order to obtain preparations of the estrogen receptor HBD for more detailed study, several groups have attempted to express peptides containing the HBD in heterologous systems. Experiments with yeast expression systems have shown that recombinant peptide fragments including the HBD are able to bind physiological and pharmacological ligands with high affinity, thereby establishing that the isolated HBD is capable of exhibiting ligand discrimination similar to that of the full-length receptor protein (7). The amount of active protein produced, however, was too small (generally ~0.1% of the total protein) to be useful as a source for biophysical studies. Expression in a baculovirus-insect cell system has also afforded only low protein yields (8). Somewhat higher yields of receptor protein have been obtained using *Escherichia coli* expression systems: Wooge *et al.* (7) reported expression of a fragment containing the HBD (amino acids 240-595) as a fusion with protein A, and Ahrens *et al.* (9) described expression of a fragment containing the residues 241-595 as a fusion with 18 residues of β-galactosidase. In each case some active protein was produced, but the yields were relatively low, ≤~1 mg/liter of culture; moreover, the fusion proteins were not purified and were not cleaved to release the isolated receptor fragment. Recently, Seielstad *et al.* (10) reported expression of an isolated HBD fragment in high yields in *E. coli*; however, the protein produced was insoluble, necessitating the use of urea during purification and characterization.

We describe herein a system which yields high level expression of soluble

human estrogen receptor HBD in *E. coli*. The HBD peptide (residues 301-551 and 305-551) is produced as a fusion protein with the *E. coli* maltose binding protein at levels of ~10% of the total cell protein; the fusion protein can be chemically cleaved to afford micromole quantities of the HBD peptide. The purified HBD peptide exhibits ligand binding properties generally similar to those of the full-length receptor protein and should prove useful for further biophysical characterization of structural and functional properties of the ligand binding domain of the receptor.

Experimental Procedures

Supplies – Restriction endonucleases and other enzymes used for DNA manipulation were obtained from Boehringer-Mannheim Corp. (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), Stratagene Cloning Systems (La Jolla, CA), or United States Biochemical Corp. (Cleveland, OH). Synthetic oligonucleotides were obtained from Operon Technologies (Alameda, CA). Bacterial growth media components were purchased from Difco (Detroit, MI); other reagents were obtained from Sigma Chemical Company (St. Louis, MO). Tritiated estradiol was obtained from Amersham and New England Nuclear. The estrogen antagonist *trans*-4-hydroxytamoxifen was a gift from Dr. Dominique Salin-Drouin (Laboratories Besins-Iscovesco) and ICI 182,780 was a gift from Dr. Alan Wakeling (ICI Pharmaceuticals).

Vector Construction – Unless otherwise noted, all DNA manipulations were carried out by standard techniques (11). A DNA fragment coding for the human estrogen receptor hormone binding domain (amino acids 301-551) was generated by PCR from the HE0 estrogen receptor cDNA plasmid (5) using the following primers: 5′ primer TCTAAGAAGAACAGCCTGGCCTTG, and 3′ primer atcCgAaTtcaCGCATGTAGGCGGTGGGCGTCCAG; lowercase bases in the 3′ PCR primer are mismatches that convert the codon for Pro-552 to a TGA termination codon and create an *Eco*RI site for sub-cloning. The PCR fragment was digested with *Eco*RI and sub-cloned into the pMAL-c2 vector (New England Biolabs) which had been digested with *Xmn* I and *Eco*RI. Following isolation of the insert-containing plasmid, the entire HBD coding region was sequenced to confirm the absence of errors introduced by PCR amplification. The mutation Gly400Val (12) was verified by DNA sequencing to be present in the cDNA; this mutation was reverted to wild-type using the PCR mutagenesis procedure of

Nelson and Long (13), creating the plasmid pMAL-HBD1.

Protein products of pMAL-c2 derived plasmids consist of the maltose binding protein fused to the desired protein with a linker peptide consisting of $(Asn)_{10}$-Leu-Gly-Ile-Glu-Gly-Arg; the terminal four residues of the peptide comprise a Factor X_a cleavage signal. Factor X_a hydrolysis of the expressed fusion protein resulted in heterogeneous, largely inactive peptides; we therefore modified the linker region to generate the sequence Asn-Gly, which can be cleaved by hydroxylamine (14). Bases encoding residues Leu-Gly-Ile-Glu of the Factor X_a recognition sequence were mutated to Asn codons by site-directed mutagenesis using the unique site-elimination procedure (15) with the Clontech kit. The entire coding region of the mutagenesis product was sequenced; the modified DNA was found to encode a linker peptide of $(Asn)_{14}$-Gly-Arg. This plasmid was designated pMAL-HBD2.

A third HBD expression plasmid was also constructed. The Factor X_a recognition sequence of the parent pMAL-c2 vector was mutated (Ile-*Glu*-Gly-Arg to Ile-*Asn*-Gly-Arg) to create a hydroxylamine cleavage site; this mutation does not affect the restriction sites used for sub-cloning fragments into the vector. The modified vector was then digested with *Xmn* I and *Eco*RI and ligated to an *Eco*RI-digested PCR fragment coding for residues 305-551 of the HBD; following sequencing of the construct, this plasmid was designated pMAL-HBD3.

Protein Expression and Purification – Competent TOPP2 cells (Stratagene) were transformed with the expression plasmids pMAL-HBD1, pMAL-HBD2, or pMAL-HBD3. Cells containing the plasmid were grown in TB media to an OD_{600} of ~1.5 at 37°C; protein expression was induced by the addition of IPTG to a final concentration of 0.25 mM and cultures were grown overnight at ambient temperature (usually ~27°C). Cells were lysed using a lysozyme protocol in the

presence of 1 mM AEBSF (Cal Biochem). The protein in the supernatant from a 40,000 x g centrifugation of the lysate was precipitated with 50% ammonium sulfate and then dialyzed against TEAD buffer (20 mM Tris HCl, 1 mM EDTA, 1 mM NaN_3, and 0.2 mM DTT/L, pH 7.3). The protein was then applied to a DEAE-cellulose column (Whatman) and eluted with a 0.05 to 0.3 M NaCl linear gradient in TEAD buffer. The MBP-HBD fusion protein eluted at ~0.15-0.18 M NaCl; the pooled fractions were diluted and applied to an amylose resin column (New England Biolabs) in TEAD + 0.2 M NaCl. (An alternative procedure was also used, in which the lysate supernatant was applied directly to a DEAE-cellulose column; in this case the unbound material was then applied to the amylose column.) After washing with 2-4 column volumes of TEAD + 0.2 M NaCl, the fusion protein was eluted with 10 mM maltose in the same buffer. The fusion protein was then concentrated to ~20 mg/ml using either ultrafiltration or precipitation with 60% ammonium sulfate, and was digested for 60-72 hours at ambient temperature with hydroxylamine (final concentration: 2 M hydroxylamine-HCl, 0.2 M Tris-HCl, pH 9.0). The cleaved HBD peptide was separated from the maltose binding protein by Sephadex G-100 gel filtration chromatography. The final preparation of the purified HBD peptide from all three plasmids was stable and could be stored at 4°C in TEAD + 0.2 M NaCl for several months.

Spectroscopy – Absorbance spectra were obtained using a Cary 17D spectrophotometer at ambient temperature via an On-Line Instrument Systems (Jefferson, GA) interface to a personal computer. The concentration of purified MBP-HBD fusion proteins and of isolated HBD peptides were determined spectrophotometrically assuming ε_{280} = 89,365 $(M \bullet cm)^{-1}$ for the fusion protein, and 23,745 $(M \bullet cm)^{-1}$ for the HBD peptides; these values are based on a composition of 11 tryptophan and 20 tyrosine residues (fusion protein) or 3

tryptophan and 5 tyrosine residues (HBD peptide) predicted from the cDNA sequence and on average extinction coefficients for tryptophan (5615 $(M \bullet cm)^{-1}$) and tyrosine (1380 $(M \bullet cm)^{-1}$) (16-17).

Radioreceptor Assay – The HBD peptide was incubated overnight with various concentrations of [6,7-^{3}H]-estradiol at 4°C in TEAD buffer including 0.2 M NaCl and 1 mg/ml gelatin; unbound steroid was removed by addition of dextran-coated charcoal (0.625% charcoal, 0.125% dextran) in the same buffer without gelatin. In all experiments using purified and partially purified protein, the binding of radioactive estradiol in the presence of a 100-fold excess of unlabeled estradiol was equivalent to the non-specific binding observed in the absence of any added HBD protein. The presence of a carrier protein was found to be necessary to obtain reproducible results with the purified HBD peptide; porcine gelatin (1 mg/ml), bovine γ-globulin (4 mg/ml), or bovine serum albumin (4 mg/ml) gave similar results.

For competitive ligand binding experiments, the HBD peptide was incubated with various concentrations of ligand in the presence of a constant amount of tritiated estradiol. For each ligand the EC_{50} value was determined by approximating the tritiated estradiol binding data as a hyperbolic function of the total competing ligand concentration.

Results

Expression of the HBD peptide – The construction of expression vectors for producing the HBD peptide as a fusion protein with the maltose binding protein is shown schematically in Fig. 1. The region encoding amino acids 301-551 of the human estrogen receptor was amplified by PCR and ligated into the multiple cloning site in the expression vector pMAL-c2. The maltose binding protein is stable and can be purified by amylose affinity chromatography. The linker peptide is designed to be cleavable by the endoproteinase Factor X_a to release the fused protein without additional N-terminal residues.

Induction of protein expression from all of the pMAL-HBD plasmids with IPTG produced significant quantities of fusion protein, estimated to comprise approximately 10% of the cell protein (Fig. 2, Lane 2). Essentially all of this fusion protein appeared to be soluble in the cells. Initial attempts to purify the MBP-HBD fusion from crude cell homogenates by amylose affinity chromatography, however, were unsuccessful; binding of the fusion protein to the affinity column was incomplete, and the eluted protein was not highly purified. For this reason, the cell lysate was first subjected to anion exchange chromatography (either with or without prior ammonium sulfate fractionation). The partially purified fusion protein in the eluent from the anion exchange column bound the amylose column nearly quantitatively, and upon elution from the column exhibited only minor contaminants (Fig. 2, Lane 3).

Attempts to cleave the partially purified MBP-HBD fusion protein with Factor X_a were not successful. The fusion protein proved resistant to cleavage; moreover, prolonged treatment resulted in degradation to heterogeneous products that exhibited markedly reduced ability to bind estradiol (data not shown). Because the HBD region of the full-length receptor has been shown to be somewhat trypsin

resistant (18), we also tested trypsin for cleaving the fusion protein. Analysis of limited tryptic digests showed release of the HBD peptide of the expected size, but significant degradation of the HBD was also observed prior to complete cleavage of the fusion protein (data not shown).

Chemical cleavage methods were then tested as alternatives to proteolytic digestion. Hydroxylamine preferentially hydrolyzes the peptide bond of Asn-Gly sequences, although solvent-exposed Asn-Xaa sequences are hydrolyzed at lower rates (14). The HBD peptide does not contain any Asn-Gly sequences and would therefore be expected to be resistant to hydroxylamine. The linker peptide of the fusion protein, on the other hand, contains the sequence $(Asn)_{10}$-Leu-Gly-Ile-Glu-Gly-Arg and would be expected to the cleaved adjacent to one of the Asn residues. In initial tests with the fusion protein derived from the pMAL-HBD1 plasmid, hydroxylamine treatment was found to cleave the HBD from the MBP without apparent loss of ligand binding activity. Due to the large number of potential Asn-Xaa cleavage sites present in the linker peptide of the pMAL-HBD1 protein product, the coding sequence of the pMAL-HBD1 was altered to $(Asn)_{14}$-Gly-Arg by site-directed mutagenesis, thereby creating an Asn-Gly sequence expected to undergo more rapid cleavage. Protein produced from this pMAL-HBD2 construct was cleaved at the predicted site (following the Asn of the Asn-Gly). N-terminal sequence analysis of the cleaved protein, however, revealed that about 10-20% of the protein was also cleaved between Asn-304 and Ser-305 of the HBD sequence resulting in a peptide six residues shorter than the desired product. An additional plasmid (pMAL-HBD3) was therefore constructed, in which the Asn-Gly of the pMAL immediately preceded Ser-305 of the HBD; N-terminal sequence analysis of the cleaved peptide from pMAL-HBD3 revealed apparently quantitative cleavage at the correct site, yielding a final peptide with Gly-Arg followed by amino acids 305-551 of the HBD (see Fig. 2, Lane 4 for the results of the cleavage reaction). Figure 1

shows the protein sequences surrounding the junction between the MBP and the HBD for the three expression plasmids.

The cleaved HBD peptide was separated from the MBP by gel filtration using a Sephadex G-100 column. Both the HBD (301-551) and HBD (305-551) peptides were found to elute near the void volume of the column, well ahead of the ~40 kDa MBP. This suggests that the HBD (monomer ~29 kDa) exists as a multimeric complex under the conditions used for the gel filtration chromatography (~100 μM peptide concentration).

Analysis of the final preparation of the HBD (305-551) peptide by SDS-PAGE is shown in Fig. 2, Lanes 5-7, with the HBD (301-551) peptide in lane 8. Both peptides have an apparent mass similar to the predicted ~29 kDa. Based on the staining intensities observed the overall purity is estimated to be >90%. The final yield of purified HBD was typically ~10 mg of HBD protein per liter of bacterial culture for several preparations; this corresponds to approximately a 40% yield from the total amount of fusion protein estimated to be present in the cells.

Properties of the purified HBD peptide – The ultraviolet absorption spectrum of the purified HBD (301-551) protein is shown in Fig. 3; the spectrum for the HBD (305-551) peptide is similar. The spectrum exhibits a broad peak with a maximum at 282 nm and a shoulder at 292 nm, characteristic of tryptophan. The extinction coefficient at 280 nm was calculated to be 23,745 $(M \cdot cm)^{-1}$ based on a predicted composition of three tryptophan and five tyrosine residues (see Methods). The slight absorbance at wavelengths longer than 300 nm is presumed due to light scattering from small amounts of aggregated protein although it could not be removed by centrifugation at 100,000 x g for 1 hour or by additional chromatographic procedures; extrapolation of the turbidity-induced 300-320 nm optical density to 280 nm has a small effect (~5%) on the calculated concentrations

of the HBD peptide.

The purified HBD peptides and the MBP-HBD fusion proteins were assayed for their ability to bind estradiol. Figure 4 shows the results of typical Scatchard analyses of ^{3}H-estradiol binding using low concentrations (2.5 nM) of the HBD (301-551) peptide and the corresponding MBP-HBD fusion protein. The average K_d values obtained for the isolated peptides and the MBP-fusion proteins are given in Table I. These values obtained (0.3-0.5 nM) are similar to those reported for the native estrogen receptor protein obtained from human cells (*cf.* 7) indicating that the estrogen binding properties of the HBD are not affected either by isolation from other parts of the receptor or by fusion to the maltose binding protein. The B_{max} values determined in these experiments, however, corresponded to a binding stoichiometry of ~0.5 mol of estradiol bound per mol HBD fragment (Table I). Values near 0.5 were obtained for several different preparations of all three constructs. Although it is possible that the calculations of HBD peptide concentration based on the predicted extinction coefficient are in error, protein determinations by the methods of Lowry (19) and Bradford (20) gave similar values for the protein concentration. The agreement between the stoichiometry obtained with the MBP-HBD fusion proteins and the isolated HBD peptides (with extinction coefficients that differ by ~3.5-fold) also makes it unlikely that errors in concentration determination are large enough to account for the discrepancy. No significant changes in stoichiometry have been observed during purification procedures or following the hydroxylamine cleavage step suggesting that the effect does not arise from denaturation during the isolation procedure.

Ligand discrimination by the HBD peptides – The ability of the HBD peptides to discriminate between different ligands was assessed by competition binding assays using ^{3}H-estradiol. Competition binding curves for the HBD (301-551)

peptide are presented in Fig. 5, and the EC_{50} values for the different ligands (relative to that for unlabeled estradiol) are summarized in Table II for both HBD (301-551) and HBD (305-551). The ligand discrimination profiles exhibited by the two peptides exhibited minor differences with one another but are generally similar those reported for the full-length native receptor (7). The weak agonist estrone exhibits about 10-fold lower affinity than estradiol whereas testosterone (and progesterone, data not shown) does not appear to compete significantly even at concentrations 35,000-fold greater than those of estradiol. The steroidal antagonist ICI 182,780 binds with an affinity intermediate between that of estradiol and estrone. The non-steroidal antagonists *trans*-tamoxifen and *trans*-4-hydroxytamoxifen are both effective competitors, with *trans*-4-hydroxytamoxifen having a similar affinity to that of estradiol (see Table II).

Effect of HBD concentration on estradiol binding – The full-length native estrogen receptor protein has been shown to exhibit positive cooperativity (8,21) and has been proposed to require dimerization in order to function (2,22). During gel filtration chromatography we observed that both the various MBP-HBD fusion proteins and the HBD peptides migrated as dimers or larger multimers suggesting that the residues in the HBD region might be sufficient to mediate cooperative interactions between receptor molecules. In order to investigate this, Scatchard analyses were carried out using several different concentrations of HBD peptides. Fig. 6A shows the results of a typical experiment at two concentrations of the HBD (301-551) peptide. At the lower concentration (2.5 nM HBD), the best fit to the data is a straight line similar to that presented in Fig. 3. In contrast, at the higher concentration (20 nM HBD), the data exhibit convex curvature indicative of positive cooperativity. The inset in Fig. 6A shows a Hill plot of the data from the higher concentration of the HBD (301-551) peptide; the line determined from this set of data has a slope (Hill coefficient) of 1.57, indicating

positive cooperativity. At both concentrations, the B_{max} corresponds to ~0.5 mol estradiol/mol peptide.

In similar experiments for the HBD (305-551) peptide (Fig 6B), positive cooperativity was only observed at significantly higher concentrations of the HBD (305-551) protein than those required for HBD (301-551). Linear Scatchard plots were observed for concentrations from 2.5 to 20 nM peptide, with significant cooperativity observed only at 40 nM. The inset in Fig. 6B shows a Hill plot for the data from the highest HBD (305-551) peptide concentration; the line determined from this set of data has a slope of 1.23.

The estradiol binding data for the HBD peptides were further analyzed to determine the degree of cooperativity. For both HBD peptides, the Hill coefficient (n) and concentration of estradiol required for half-maximal binding ($F_{0.5}$) increased with increasing protein concentration (Fig. 7). However, the concentrations required for cooperativity were lower for HBD (301-551) than for HBD (305-551). Over the apparent B_{max} range of 2-20 nM, the Hill coefficient for HBD (301-551) increased from 1.0 (*i.e.* non-cooperative) at the lowest concentration to ~1.5 at the highest concentration assayed (Fig. 7A), and the $F_{0.5}$ value increased approximately 10-fold (0.2 to 2.1 nM, Fig. 7B). In contrast, evidence for cooperativity by HBD (305-551) required an apparent B_{max} greater than ~12 nM, and the $F_{0.5}$ values for HBD (305-551) increased more slowly over the same concentration range.

The maximal Hill coefficient observed for the HBD (301-551) peptide is similar to that reported for the full-length receptor, and the concentration range over which the behavior changes from noncooperative to cooperative is also similar to that over which the full-length human receptor exhibits cooperativity (8). These results suggest that most or all of the structural features required for cooperative

ligand binding interactions are retained in the HBD (301-551) peptide fragment; the differences in the behavior of the HBD (305-551) peptide suggest that residues in the N-terminus of the HBD are involved in the interactions that result in cooperativity.

Discussion

We have expressed, purified in high yield, and begun to characterize two hormone binding domain peptides from the human estrogen receptor. The yield of soluble HBD obtained (~10 mg/liter of culture) is significantly greater than previously reported, and represents amounts of protein sufficient for a variety of biophysical studies. Previous attempts at expression of the HBD in *E. coli* (7,9) used constructs coding for estrogen receptor peptides comprising amino acids residues 240-595, and therefore sequences of more than 350 amino acids. Moreover, these expressed peptides were fused to either a peptide from β-galactosidase (9) or to Protein A (7), and the HBD products were not reported to be either purified or released from the fusion proteins. Recently, expression of the HBD (amino acids 282-595) in high yield in insoluble form from a T7 RNA polymerase plasmid was reported (10); however, this protein required 1 M urea for solubilization, and 5 M urea for purification. The purified protein was somewhat heterogeneous; the results indicated that the majority of the expressed estrogen receptor proteins were cleaved at positions 569 or 571.[2]

The expression system we describe herein produces active fragments of the estrogen receptor comprising only 253 amino acids (positions 301-551, with an additional Gly-Arg at the amino terminus; note that amino acid 300 of the human estrogen receptor is Arg) or 249 amino acids (positions 305-551 with an additional Gly-Arg at the amino terminus) following purification and cleavage. The reduced size of this construct should simplify interpretation of biophysical data regarding the expressed protein. In addition, this peptide is expressed at high levels and does not require the use of urea in the purification procedure. It is not clear whether the high level of expression we observe is due to the composition of the estrogen receptor-derived peptide, to the efficiently expressed maltose binding protein used as a leader, or to a combination of the two.

A surprising observation is that the ligand binding of these peptides corresponds to only ~0.5 mol estradiol/mol HBD. The reason for this deviation from 1:1 is not understood. It is unlikely to be due to competing ligand originating in *E. coli*, since the binding stoichiometry does not change during the purification procedure. Gel electrophoresis suggests that only relatively small amounts of impurities remain, and therefore the discrepancy is unlikely to be due to the presence of contaminating non-receptor proteins. It is also unlikely to be due to inaccurate determination of protein concentration as three different methods (Lowry and Bradford protein assays, and absorbance at 280 nm based on the calculated extinction coefficient for both the fusion and isolated HBD proteins) all gave similar results. It is also unlikely to be due to inaccurate calculation of the estradiol concentration used, since similar results were observed using ^{3}H-estradiol obtained from different vendors, using ^{3}H-estradiol of nominal specific activities varying by about 3-fold, and using known amounts of unlabeled estradiol in addition to the ^{3}H-estradiol. Another possible explanation is that it could reflect the presence of unfolded protein. Denatured HBD protein, however, might be expected to aggregate and precipitate and therefore be readily separable from the native folded protein. Our experience has been that centrifugation has only limited effects in increasing the observed stoichiometry. It is possible that some of the protein is folded, but binding-incompetent, either due to an incorrect fold, or to a covalent modification. If a subset of the protein is misfolded, it would in principle be possible to refold it into an active conformation. In preliminary experiments using 6 M urea to unfold the HBD, followed by dialysis to remove the urea and allow the protein to refold (unpublished data), however, we have been unable to increase the binding stoichiometry. Covalent modifications are possible, but it seems unlikely that *E. coli* would consistently modify ~50% of the expressed HBD. Moreover, the similarity of the value obtained for both ligand affinities and

the maximal Hill coefficient for the HBD (301-551) to that observed for the native protein suggests that any misfolded or covalently modified protein present does not alter the binding kinetics or cooperativity of the correctly folded protein.

Finally, it is possible that the stoichiometry of 0.5 ligand/HBD molecule is correct, with binding of ligand requiring two protein monomers. When Furlow *et al.* (23) examined estrogen receptor binding to a consensus palindromic estrogen response element, they observed a 1:1 ratio of estradiol/DNA. Their interpretation was that the estrogen receptor was binding the DNA as a monomer, and that the monomer was binding a single estradiol molecule. An alternative interpretation of their data would be that the estrogen receptor was binding as a dimer (as predicted by most studies of estrogen receptor action and expected for the palindromic response element used), and that the dimer only bound a single estradiol molecule. If this single site hypothesis is correct, the active complex must be capable of forming at least a tetramer to account for the cooperative binding we observe at higher protein concentrations. Our data cannot yet distinguish between 1) a preparation consisting of a mixture of inactive protein (*i.e.* protein incapable of binding ligand) and active protein (*i.e.* dimeric protein with two ligand binding sites) and 2) a preparation of fully active tetramer containing two ligand binding sites per tetramer.

Currently available structural data for other members of the steroid receptor superfamily do not assist in differentiating among these competing hypotheses. In a recent report the crystal structure of the HBD of the retinoid-X-receptor-α homodimer was described; however, the protein was crystallized in the absence of ligand (24). In the crystalline state, monomeric thyroid hormone receptor HBD was found to have ligand bound[3]. The full length thyroid hormone receptor is thought to function as a dimer; the stoichiometry of ligand binding to the dimer has not been established.

Although the asymmetrical nature of the high affinity ligands might suggest that this hypothesis is somewhat unlikely, it is not without precedent. Two cases in which a homodimeric protein binds a single ligand have been reported. In one case, the ligand binding site is at the interface between the two monomers, with each contributing residues to the binding site. Examples of this are the Growth Hormone receptor, in which two similarly folded receptor monomers bind a single Growth Hormone molecule (25), and the retroviral aspartyl protease (26), which forms its single active site from residues derived from both subunits of the homodimer. An alternative mechanism requires that the two subunits of the dimer have different three dimensional structures, with only one subunit containing the binding site. This mechanism is used by the HIV reverse transcriptase; the crystal structure of this protein dimer revealed that the two subunits have different folded structures from identical protein sequences (although one monomer has additional sequences not present in the other), such that the dimer contains single binding sites for nucleic acids, nucleotides, and non-nucleoside inhibitors (27).

The isolated estrogen receptor HBD is clearly capable of forming multimeric complexes in solution. At the high concentrations (~10-100 μM) required for gel filtration chromatography, the HBD forms a multimeric complex large enough to elute near the void volume on Sephadex G-100. In addition, the HBD (301-551) peptide exhibited positively cooperative estradiol binding at a concentration range of 1 to 10 nM, with a maximal Hill coefficient of ~1.5 at a B_{max} of 10 nM. Positive cooperativity exhibited by estrogen receptor from calf uterine cytosol has been extensively studied (21,s); the maximal Hill coefficient cited in these reports is ~1.6 at a concentration of about 5 nM receptor. Using recombinant full-length human estrogen receptor expressed in Sf9 insect cells, Obourn et *al.* (8) also found a maximal Hill coefficient of 1.6; their data suggest that at receptor

concentrations below ~1 nM, the receptor does not exhibit cooperativity, while maximal cooperativity is observed at 10 to 20 nM, the same concentration range that we observe for the isolated HBD (301-551).

In contrast to the results obtained with the HBD (301-551), the HBD (305-551) peptide exhibited cooperativity only at higher concentrations of protein. These proteins differ only at the N-terminus, with the (305-551) protein being four residues shorter and having the sequence Gly-Arg in place of Lys-Asn immediately upstream of the Ser-305. The data suggest that steric and/or charge alterations introduced by the removal of the four residues affect the interaction between receptor monomers, and therefore that some of the residues required for cooperativity and/or dimerization are present at the N-terminal region of the HBD. The HBD residues previously suggested to be involved in the dimerization of the estrogen receptor are located in the C-terminal region (near positions 500-520) (29,30). However, the retinoic acid receptor HBD structure (26) suggests that the N-terminal residues of each monomer are in fairly close proximity to one another in the dimer, and that the N-terminal region of the HBD may indeed mediate some of the dimer interface interactions.

Taken together, our results suggest that the isolated HBD contains the amino acid sequences both necessary and sufficient for dimerization, that it undergoes the conformational changes required for cooperativity in a manner comparable to the full-length protein, and that the extreme N-terminus of the HBD plays a role in interprotein interactions. We consistently observe a ratio of 0.5 mol estradiol bound/mol HBD; future experiments will attempt to elucidate the physical basis of this phenomenon. The production of purified isolated HBD will allow further biophysical and biochemical characterization of the protein aimed at understanding the mechanism by which ligands bind and alter the activity of the rest of the receptor protein.

Footnotes

[1] The abbreviations used are: AEBSF, [4-(2-aminoethyl)-benzenesulfonylfluoride]; DTT, dithiothreitol; HBD, hormone binding domain; PCR, polymerase chain reaction.

[2]We have also found that whole cells expressing constructs intended to produce protein extending to the C-terminus of the estrogen receptor at position 595 contain variously sized estrogen receptor protein products, with the major product ending near position 567 based on SDS-PAGE analysis. Constructs ending at amino acid 567 or 551 appear to yield significantly higher levels of MBP-HBD fusion protein expression than do constructs intended to extend to position 595.

[3]Reported by J.D. Baxter, R.C.J.Ribeiro, J.W Apriletti, B.L. West, F. Schaufele, R.L. Wagner, and R.J. Fletterick in a symposium at the 77th meeting of the Endocrine Society.

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Figure Legends

Figure 1. Construction of the expression vectors. A fragment of the cDNA corresponding to the HBD was amplified by PCR and sub-cloned into pMAL-c2. The protein sequences near the cleavage site for the fusion protein are shown for each expression vector; the HBD protein sequence is underlined, and the sites cleaved by hydroxylamine are shown in bold.

Figure 2. SDS-PAGE analysis of HBD peptides during purification. A 12% polyacrylamide gel stained with Coomassie blue is shown: Lane 1: whole cells from an overnight culture of TOPP2 *E. coli* harboring the pMAL-HBD3 expression plasmid; Lane 2: whole cells from an overnight culture following induction with 0.25 mM IPTG; Lane 3: partially purified fusion protein; Lane 4: the product of the hydroxylamine cleavage reaction; Lanes 5-7: purified HBD (305-551) peptide, and Lane 8: purified HBD (301-551) peptide. Note: lanes 1-7 on this gel represent protein produced from pMAL-HBD3; lanes 1-3 appeared identical using all three pMAL-HBD constructs.

Figure 3. Absorption spectrum of the purified HBD. The protein concentration calculated from the A_{280} is 29 μM, corresponding to a HBD peptide concentration of 1 mg/ml. The spectrum shown is that of the 301-551 peptide; the spectrum of the 305-551 peptide is similar.

Figure 4. Scatchard analysis for estradiol binding to the MBP-HBD (301-551) fusion protein and the purified HBD (301-551) peptide. A radioreceptor assay was performed using 2.5 nM MBP-HBD protein or 2.5 nM purified HBD peptide. Linear regression of the data yields apparent K_d values of 0.28 nM (B_{max} = 1.4 nM) and 0.25 nM (B_{max} = 1.2 nM) for the fusion protein and peptide, respectively.

Figure 5. Competition ligand binding curves for the purified HBD (301-551) peptide. Assays were performed using 2.5 nM purified peptide (apparent B_{max} = ~1.2 nM) and 1.4 nM ^{3}H-estradiol. The curves were generated from the fit of the corresponding data to a rectangular hyperbolic function of total competitor concentration (see methods). The data shown represent the average of two experiments.

Figure 6. Scatchard analyses for estradiol binding to the HBD peptides. Panel A: Two concentrations of the HBD (301-551) peptide showing apparent positively cooperative estradiol binding. The lines represent curves generated by fits of the data to the Hill equation. The open symbols correspond to an apparent B_{max} of 1.1 nM, and a Hill coefficient of 1.0; the closed symbols correspond to an apparent B_{max} of 10.5 nM and a Hill coefficient of 1.57. The inset shows a Hill plot of the data for the higher protein concentration.

Panel B: Scatchard analysis for three concentrations of the HBD (305-551). The lines represent curves generated by fits of the data to the Hill equation. The open squares correspond to an apparent B_{max} of 1.3 nM and a Hill coefficient of 1.0, the open diamonds to an apparent B_{max} of 8.3 and a Hill coefficient of 1.0, and the closed squares to an apparent B_{max} of 16.6 and a Hill coefficient of 1.23. The inset shows a Hill plot of the data for the highest protein concentration.

Figure 7. Summary of the Hill analyses for the HBD peptides. Panel A. Variation of Hill coefficient with concentration of HBD (301-551) (closed symbols), and HBD (305-551) (open symbols). Panel B. Variation of the concentration of estradiol required for half-maximal binding with concentration of HBD (301-551) (closed symbols), and HBD (305-551) (open symbols).

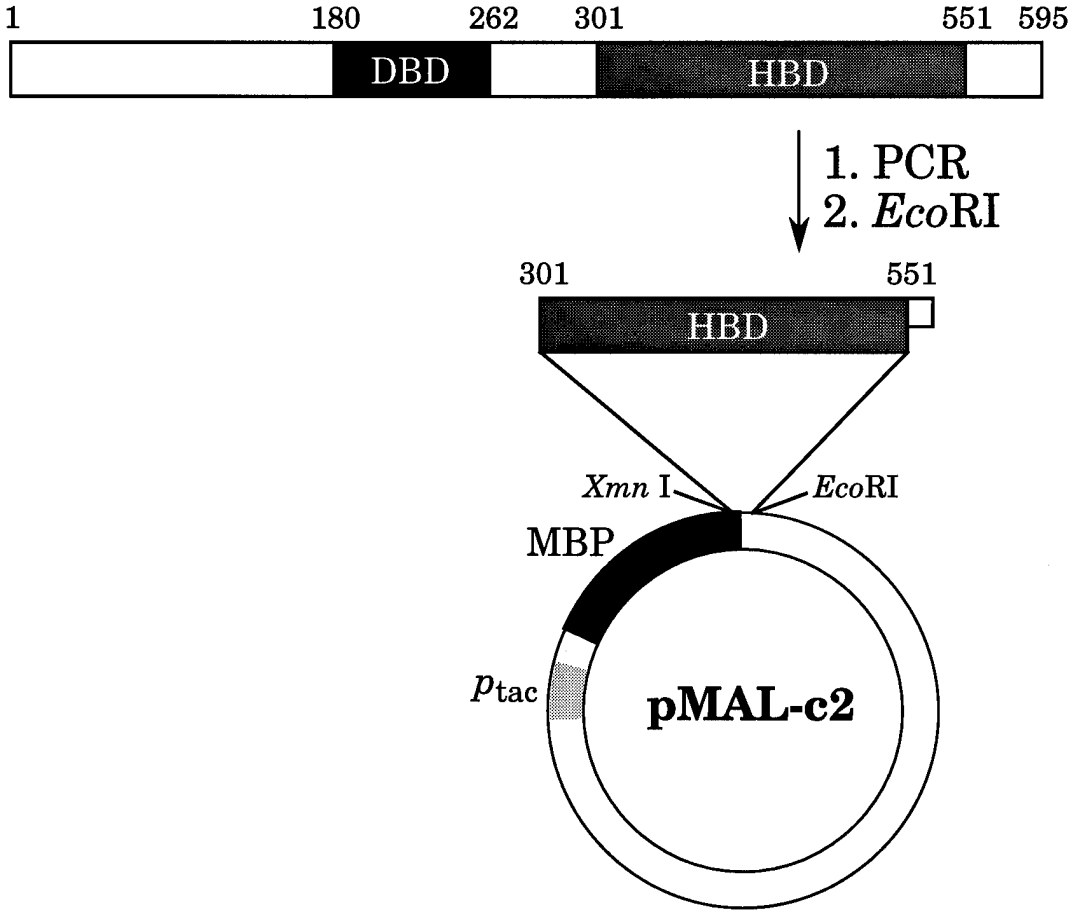
Table I.
Summary of Estradiol Binding Affinity and Stoichiometry

Peptide	**K_d** (nM)	**Binding Stoichiometry** (mol Estradiol/mol peptide)
MBP-HBD (301-551)[a]	0.40 ± 0.16	0.53 ± 0.15
HBD (301-551)[b]	0.33 ± 0.19	0.55 ± 0.19
MBP-HBD (305-551)[c]	0.46 ± 0.13	0.51 ± 0.16
HBD (305-551)[d]	0.28 ± 0.12	0.42 ± 0.06

[a] 7 determinations; 5 separate preparations.
[b] 12 determinations; 3 separate preparations.
[c] 6 determinations; 2 separate preparations.
[d] 20 determinations; 2 separate preparations.

Table II.
Summary of Competitive Binding Experiments

Ligand	**Relative EC_{50}** (301-551)	**Relative EC_{50}** (305-551)
17β-Estradiol	1.0 ± 0.18	1.0 ± 0.07
trans-4-hydroxytamoxifen	0.81 ± 0.24	1.60 ± 0.12
ICI 182780	3.69 ± 1.32	1.23 ± 0.18
Estrone	11.18 ± 1.38	13.20 ± 1.75
trans-tamoxifen	100.82 ± 40.30	67.39 ± 24.89



	301	305
pMAL-HBD1	AsnLeuGlyIleGluGlyArgSerLysLysAsnSerLeu	
pMAL-HBD2	AsnAsnAsnAsn**AsnGly**ArgSerLysLys**AsnSer**Leu	
pMAL-HBD3	AsnAsnAsnAsnAsnLeuGlyIle**AsnGly**ArgSerLeu	

Figure 1

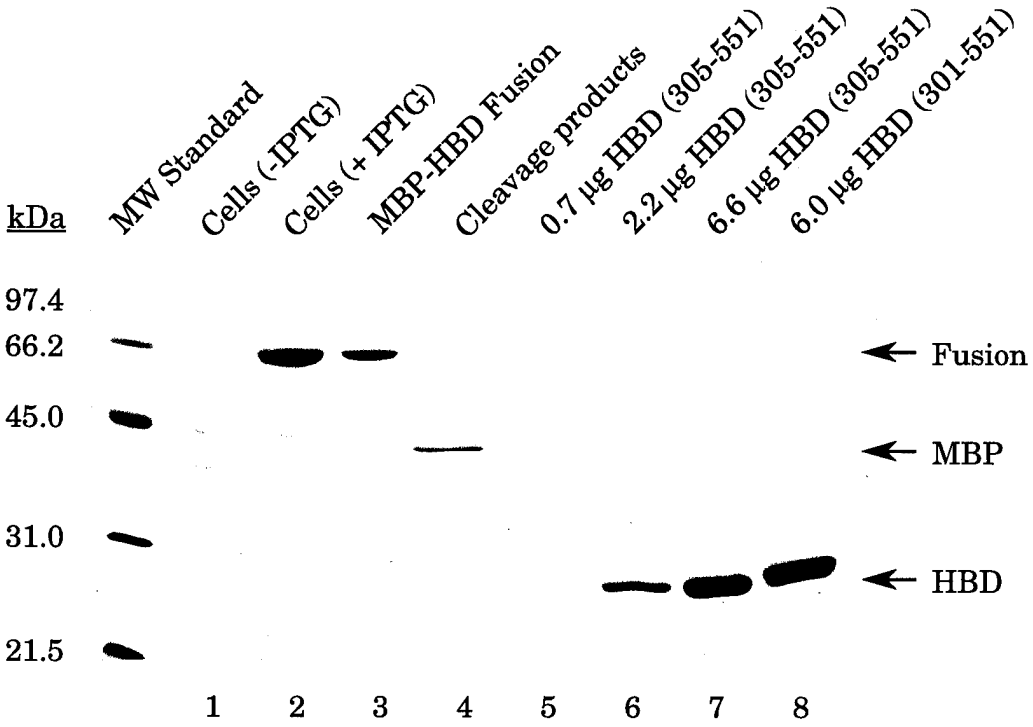

kDa
MW Standard
Cells (-IPTG)
Cells (+ IPTG)
MBP-HBD Fusion
Cleavage products
0.7 µg HBD (305-551)
2.2 µg HBD (305-551)
6.6 µg HBD (305-551)
6.0 µg HBD (301-551)
97.4
66.2
45.0
31.0
21.5
Fusion
MBP
HBD
1 2 3 4 5 6 7 8

Figure 2

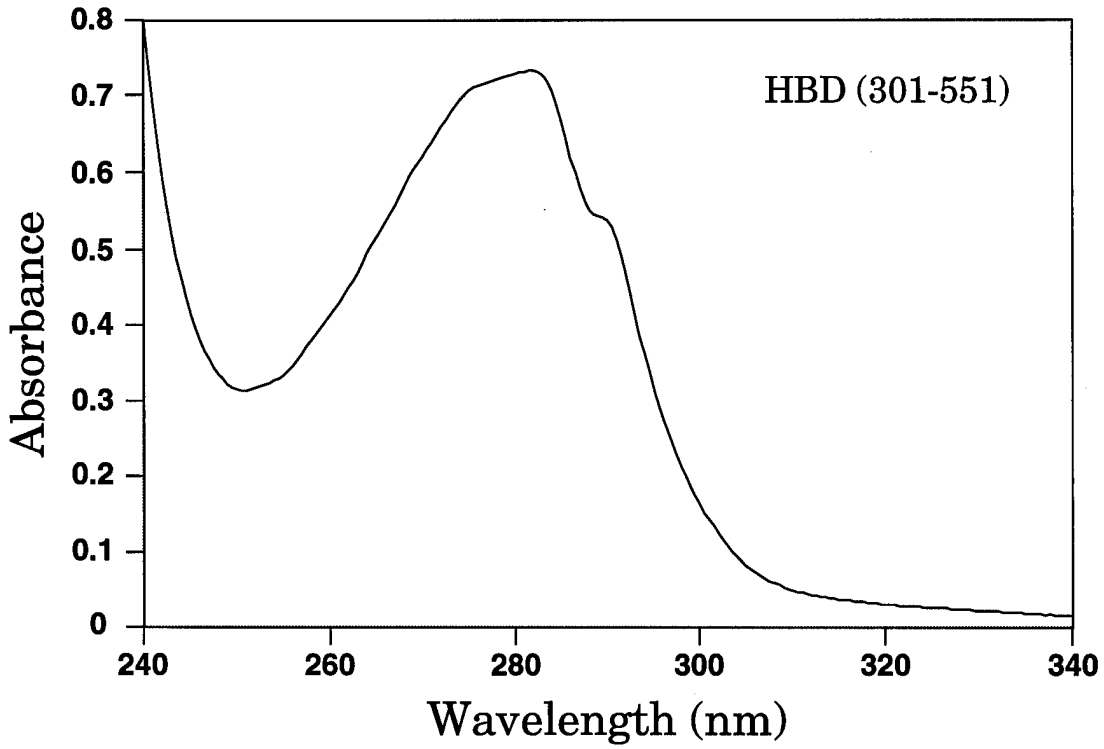

HBD (301-551)
Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
240
260
280
300
320
340
Wavelength (nm)

Figure 3

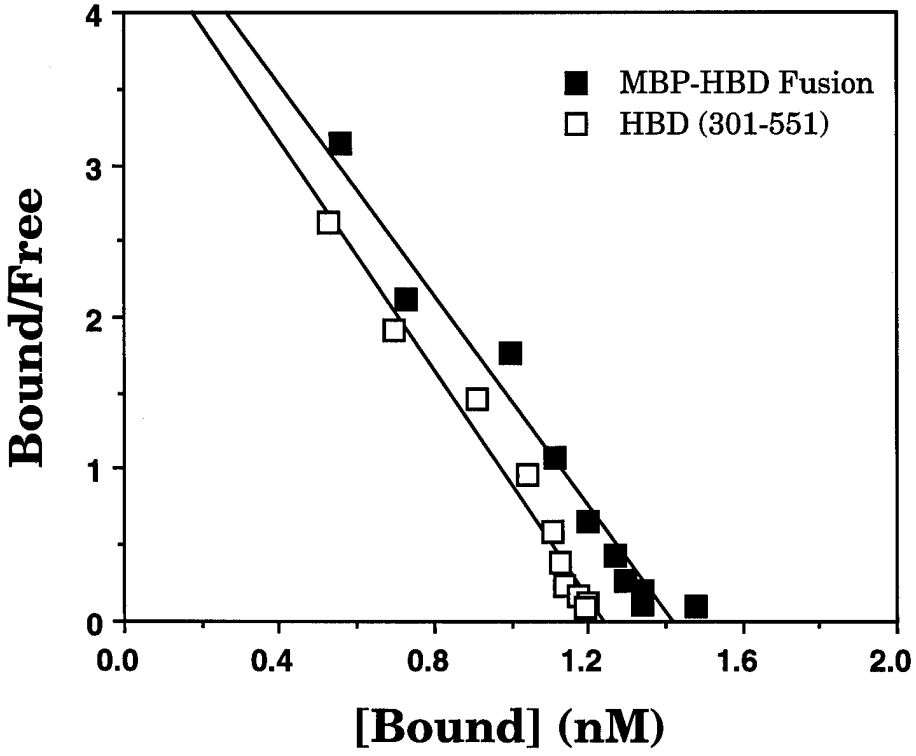

MBP-HBD Fusion
HBD (301-551)
Bound/Free
[Bound] (nM)
0
1
2
3
4
0.0
0.4
0.8
1.2
1.6
2.0

Figure 4

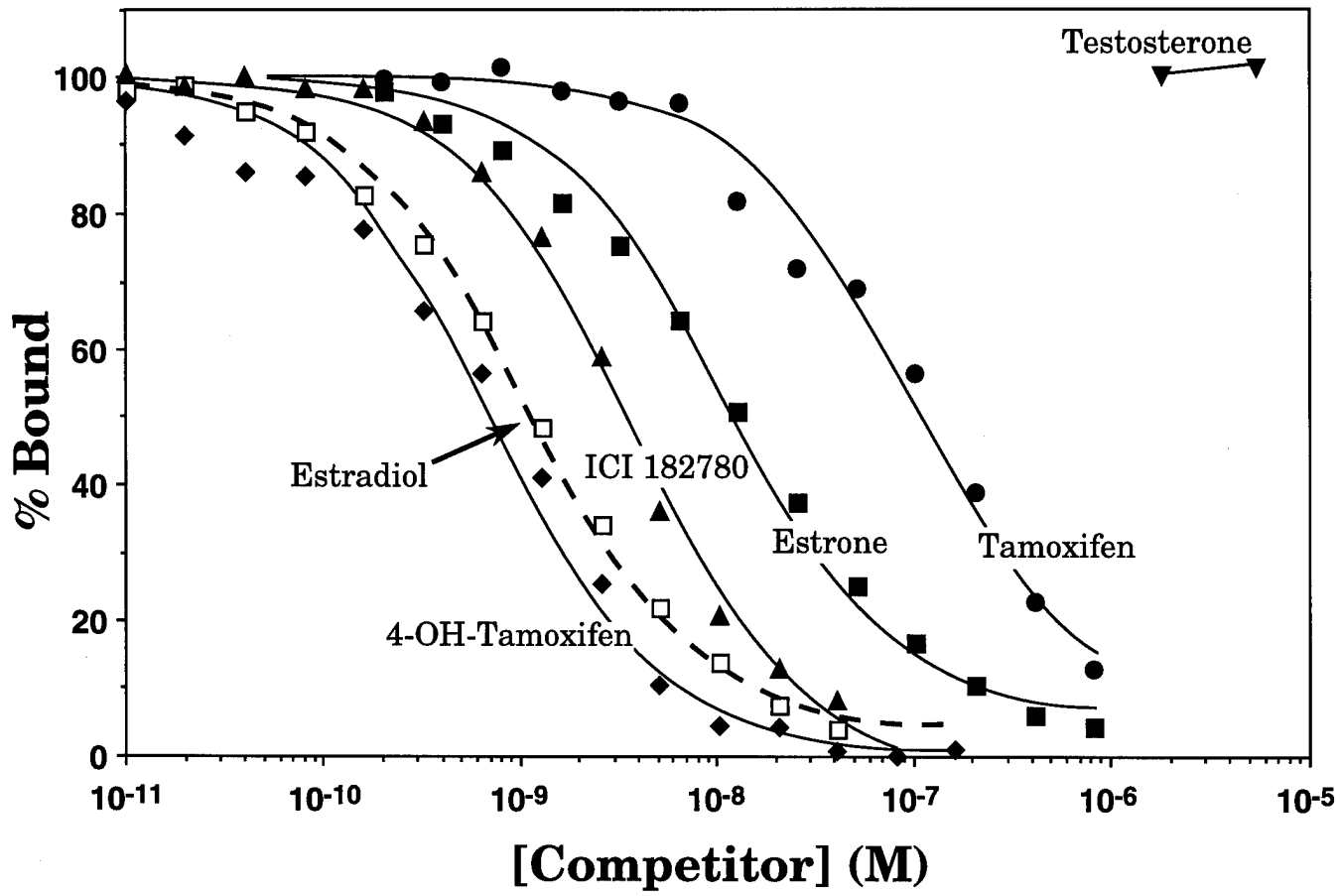

Testosterone
100
80
60
40
20
0
% Bound
Estradiol
ICI 182780
Estrone
Tamoxifen
4-OH-Tamoxifen
10-11
10-10
10-9
10-8
10-7
10-6
10-5
[Competitor] (M)

Figure 5

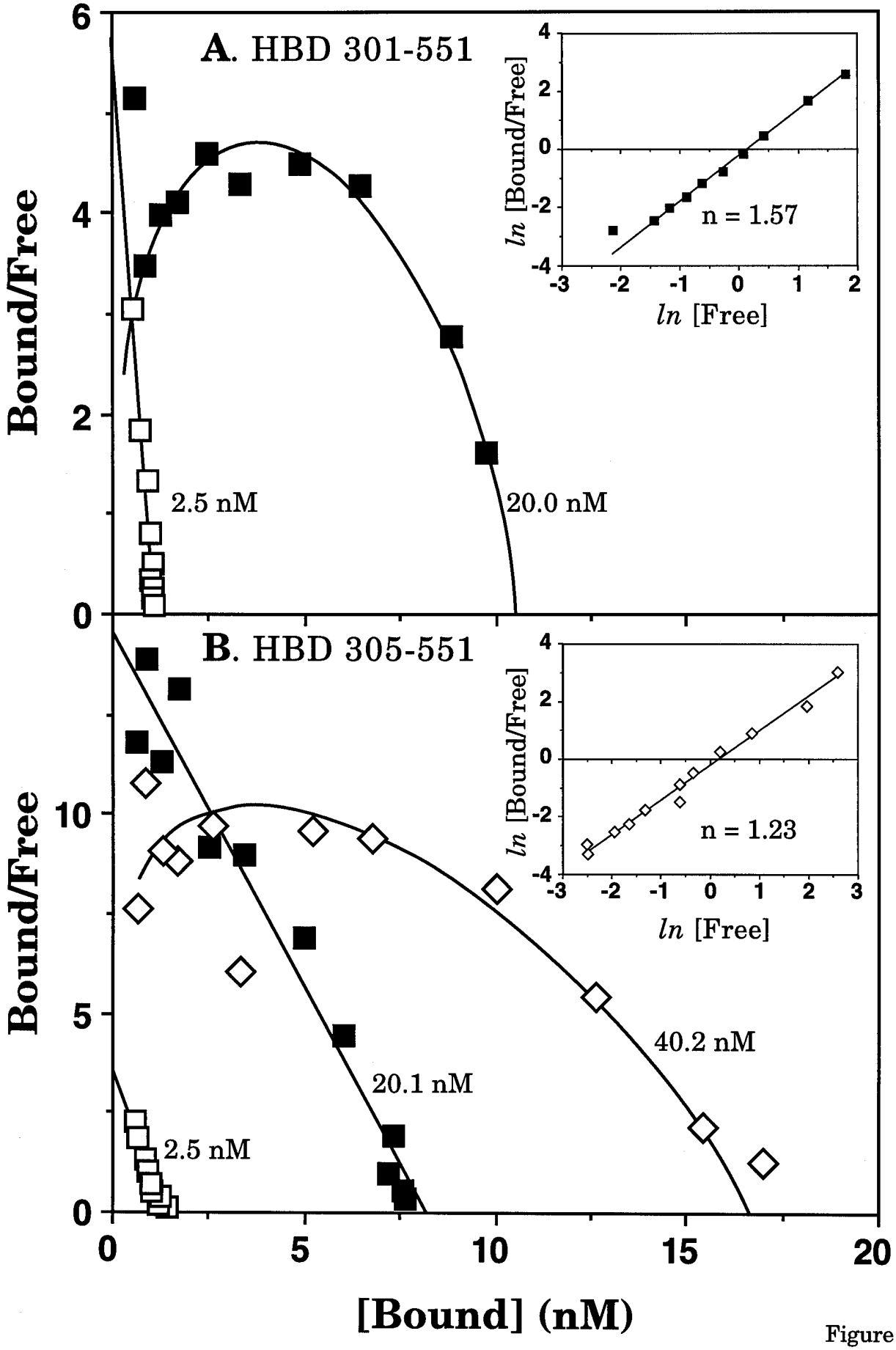

A. HBD 301-551
Bound/Free
2.5 nM
20.0 nM
ln [Bound/Free]
ln [Free]
n = 1.57
B. HBD 305-551
Bound/Free
2.5 nM
20.1 nM
40.2 nM
ln [Bound/Free]
ln [Free]
n = 1.23
[Bound] (nM)

Figure 6

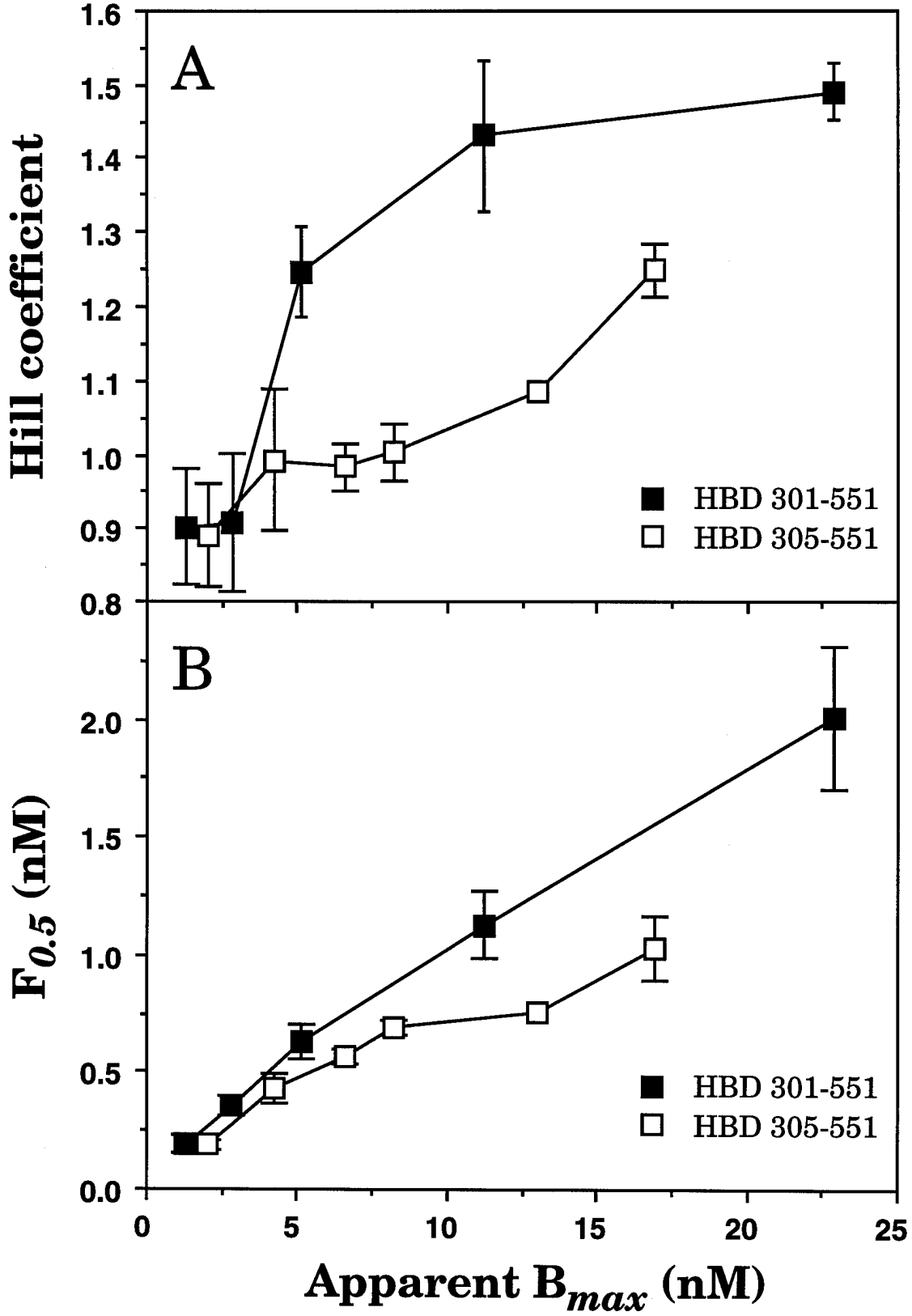

A
B
Hill coefficient
$F_{0.5}$ (nM)
Apparent B_{max} (nM)
HBD 301-551
HBD 305-551
HBD 301-551
HBD 305-551
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25

Figure 7