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Generation of Estrogen Receptor Mutants with Altered Ligand Specificity for Use in Establishing a Regulatable Gene Expression System

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Considerable interest exists in developing an artificial system for the control of gene expression, based on the hormone binding domain (HBD) of steroid receptors. In this study we describe a yeast based approach which allows the identification of mutations within the HBD of steroid receptors, in this case the estrogen receptor, which result in altered specificity of the HBD with respect to its activation by ligands. Using this approach in yeast, we identified an estrogen receptor (HBD) mutant (His[524] to Gln) whose activation by 17β-estradiol (E_2) is significantly reduced while activation by a diphenol indene-ol compound (GR132706X) is increased, compared to the wild type estrogen receptor. When the activity of the mutant receptor was tested in mammalian cells the altered specificity was maintained. A chimeric transcription factor was constructed, in which the mutated estrogen receptor HBD was linked to the DNA binding domain of GAL4 and an 11 amino acid transcriptional activation domain of RelA. Reporter gene activation by this chimera was decreased in response to E_2 and increased in response to GR132706X, as compared to the corresponding chimeric transcription factor containing the wild type estrogen receptor HBD. This approach should allow the development of a steroid receptor HBD based regulator of gene expression, whose activity is controlled specifically by a synthetic ligand, that would not affect the activity of endogenous steroid receptors. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

The recent increase in the number of genes sequenced, as well as the identification of genes linked to disease, has had two major consequences. Genes have been identified for which no function is known and many of the disease linked genes are without assigned function. As a consequence, there is increased interest in the means by which gene function can be addressed. A key factor in allowing one to address the question of gene function is one's ability to regulate gene expression *in vivo* in transgenic animals. One potential approach is to use a regulatable transcription factor to induce gene expression. A further use of such a system would be in the control of therapeutic gene expression in gene therapy.

With this in mind, a number of approaches have been taken in an attempt to develop a system for controlling gene expression *in vivo*. Initially, inducible systems were based on the use of promoters that were inducible by endogenous transcription factors in response to, for example, heat shock [1], or heavy metal ions [2]. The major drawback of such systems is that induction ratios are low and the inducer activates a variety of endogenous genes.

To overcome these problems, recent efforts have focused on development of inducible systems which use chimeric transcription factors that combine elements from mammalian, bacterial, yeast and viral transcription factors (for review see, [3]). One such system is based on the lac repressor which is inducible by isopropyl β-D-thiogalactopyranoside (IPTG) [4]. The

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major limitation of this system concerns the toxicity of IPTG. An alternative system, which combines the DNA binding domain of the tetracycline (tet) repressor from *E. coli* with the activating domain of the herpes simplex virus protein, VP16, has also been developed [5]. In this system, the gene of interest is placed downstream of multiple tet operator sequences. In the absence of the antibiotic, tetracycline, the tet/VP16 activator will bind the operator sequence and activate the downstream gene. In the presence of tetracycline, the binding of the tet/VP16 activator is inhibited and the gene downstream of the tet operator remains silent. The ability of this system to control reporter gene expression *in vivo* has been demonstrated in transgenic mice [6]. The disadvantage of this system is the fact that tetracycline is a repressor and must always be present to keep the downstream gene silent. Recently, a mutant tet repressor has been described whose phenotype is reversed, in that the DNA binding of this mutant tet repressor is induced by certain tetracycline derivatives rather than being inhibited [7]. However, some 10–100-fold higher concentrations of the tetracycline derivative are required for induction in comparison to the concentration of tetracycline required in the repressor system.

Another approach for creating an inducible system has been to have the activity of a protein controlled by fusing it with the hormone binding domain (HBD) of a steroid hormone receptor [8]. A number of proteins, when expressed as a fusion with the HBD of a steroid hormone receptor, are inactive in the absence of hormone. However, in the presence of hormone the normal activity of these proteins is restored. The HBD of the estrogen receptor has been used in a number of these studies [9–12]. The disadvantage of the hormone inducible system is that the hormone will also activate endogenous steroid hormone receptors and thereby alter activity of endogenous genes.

Mutations within the HBDs of the glucocorticoid, progesterone and estrogen receptors have been described which have altered specificity with regard to activation by agonists and antagonists. A glucocorticoid receptor mutant has been identified which fails to bind the agonist dexamethasone but is strongly activated by RU 38486, an antagonist of the wild type glucocorticoid receptor [13]. In the case of the progesterone receptor, a carboxy-terminal deletion of 42 amino acids was identified which no longer bound progesterone. On the other hand, RU 486, an antagonist of the wild type progesterone receptor, acted as an agonist of this mutant progesterone receptor [14]. A regulatory system, based on this mutant progesterone receptor, has been developed which is activated by RU486 [8]. The disadvantage of this system is that RU486 is an antagonist of the endogenous progesterone and glucocorticoid receptors, although it appears that the concentration of RU486 required for activation of the mutant receptor is below that required for antagonism of the endogenous receptors. Most recently, mutations within the HBD of the estrogen receptor have been described which result in a reduced response to E_2 whereas the antagonists Tamoxifen and ICI 164,384 function as agonists of this mutant receptor [15].

In this study we have been interested in developing a systematic approach which would allow one to identify mutations within steroid receptor HBDs that result in altered specificity of the receptor with respect to activation by ligands. Working with the estrogen receptor, we describe a yeast-based screening approach which has allowed us to identify a mutation within the HBD which results in a reduced response of the receptor to E_2 while activation of this mutant receptor is increased in response to the weak agonist of the wild type receptor, a diphenol-indene-ol, GR132706X.

MATERIALS AND METHODS

Yeast strain and growth conditions

The yeast strain YPH499 (a, ura3-52, lys2-801$_a$, ade2-101$_o$, trp1-Δ63 his3-Δ200 leu2-Δ1) [16] was used in all experiments. Cultures were propagated at 30°C in minimal yeast medium supplemented with appropriate amino acids.

Plasmid constructs

The yeast estrogen receptor expression plasmid (YEpCUPhER) has the estrogen receptor gene under control of yeast copper metalothionein promoter (CUP1). The original human estrogen receptor cDNA had a valine for glycine point mutation at amino acid position 400 [17]. The wild type estrogen receptor was constructed by replacing the mutant fragment to generate the wild type receptor. The reporter plasmid has three copies of the ere consensus sequence from the *Xenopus laevis* vitellogenin promoter upstream of the β-galactosidase reporter gene.

The Gal4-ER(HBD)-VP16 expression vector was prepared by releasing the Gal4-ER(HBD)-VP16 fragment [10] from the plasmid SP64 (Promega), by restriction digestion with *Bam HI*, and subcloning this fragment into the *Bgl II* site downstream of the CMV promoter in the mammalian expression vector CMV4T [18]. To create the Gal4-ER(HBD)-RelA expression vector, the VP16 activation domain was removed from Gal4-ER(HBD)-VP16 expression vector by digestion with *Psh AI* and *Mlu I*. Double-stranded oligonucleotides were synthesized, annealed, and ligated into the *Psh AI/Mlu I* site so as to encode two copies of the 11 amino acid RelA activation domain, in frame with Gal4-ER(HBD). The CAT reporter plasmid has four copies of the GAL4 consensus binding sequence linked to the thymidine kinase promoter and the bacterial chloramphenicol acetyltransferase gene [8].

Transformation of yeast

Yeast cells were transformed using the LiAc procedure [19]. Cells from an overnight culture were inoculated into 50 ml of YEPD medium and grown to 1×10^7 cells/ml (3–4 h). Cells were harvested by centrifugation and washed twice in sterile water. Cells were then transferred to a microfuge tube and washed twice with 1 ml of 1X TE/LiAc (made fresh from 10XTE (100 mM Tris, 10 mM EDTA, pH 7.5) and 10X LiAc (1M LiAc pH 7.5)), then resuspended in 1 ml of 1X TE/LiAc. Fifty microliters of yeast cell suspension were then added to a microfuge tube containing 10 μg of plasmid DNA and 10 μg of single stranded salmon sperm carrier DNA (which had been placed at 100°C for 5 min). Three-hundred microliters of 40% PEG 6000/LiAc solution (40% PEG 6000, 1XTE, 1XLiAc) were then added and the samples were mixed by vortexing. Samples were then incubated at 30°C for 30 min followed by heat shock at 42°C for 20 min. Yeast cells were then spun down in a microfuge and resuspended in 1 ml of sterile water. A proportion of each sample was then plated onto selective medium.

For *in vivo* recombination repair of the estrogen receptor expression plasmid with mutagenized PCR product, an 84 bp fragment was removed from the estrogen receptor HBD by restriction digestion with *Nco I* and *Bgl II* followed by gel purification of the vector backbone. This cut plasmid (200 ng) was then transformed, as outlined, together with mutagenized PCR fragment (400 ng).

Mutagenic polymerase chain reaction

Mutagenic PCR was performed exactly as outlined by Cadwell and Joyce [20]. The mutagenic PCR reaction buffer contains $MgCl_2$ (7 mM), KCl (50 mM), Tris (10 mM) (pH 8.3), 0.01% gelatin and $MnCl_2$ (0.5 mM). Deoxy-nucleotides were present at the following concentrations, dGTP and dATP (0.2 mM), dCTP and dTTP (1 mM). The concentration of Taq polymerase used in the reaction was increased to 5 units. The PCR reaction was incubated for 30 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1 min. The primers used for the PCR correspond to nucleotides 1133–1150 (sense) and 1891–1874 (antisense) within the estrogen receptor HBD (numbering according to [21]).

β-Galactosidase assay

β-Galactosidase assays were performed basically as outlined previously [22]. For β-galactosidase assay of liquid cultures, yeast strain YPH499 containing both the estrogen receptor expression plasmid and reporter plasmid were inoculated in 5 ml of selective medium and grown at 30°C overnight. The overnight culture was then diluted (1:10). From the diluted culture, 2 ml cultures were set up and treated with ligand as desired. Cultures were then grown for an additional 16 h at 30°C. The optical density of the overnight cultures were determined and cells from 1 ml of culture were pelleted by centrifugation in microfuge. The cell pellet was washed by resuspending in LacZ buffer (10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol and 100 mM $NaPO_4$, pH 7.0). Following recentrifugation, the cell pellet was resuspended in 50 μl LacZ buffer. Cells were permeabilized by addition of 50 μl of $CHCl_3$ and 20 μl of 0.1% SDS and vortexing. Cells were equilibrated at 30°C for 5 min prior to addition of 0.5 ml of 2 μg/ml substrate, *o*-nitrophenyl-β-galactosidase (ONPG) in LacZ buffer. The reaction was allowed to proceed for 5 min prior to stopping the reaction by addition of 0.5 ml of 1 M Na_2CO_3. Samples were then spun briefly, to remove $CHCl_3$, supernatant was transferred to cuvettes and the optical density was read at 420 nm. β-Galactosidase units are defined as 10^3 times the change in optical density at 420 nm divided by the product of the assay duration, times the culture volume, times the OD at 600 nm of the culture.

The *in situ* plate β-galactosidase assay is performed by transferring yeast colonies from the original transformation plate on to replica plates, containing the desired ligand, using the standard velvet transfer procedure. Colonies were then allowed to grow on replica plates overnight at 30°C. The replica plates were then overlaid with a molten solution of 0.5% agarose containing 0.5 M $NaPO_4$ (pH 7.0), 0.1% SDS, 2% dimethylformamide and 0.05% X-Gal. Plates were then incubated at 30°C to allow color to develop (1–2 h). Following identification of a mutant of interest, the corresponding yeast colony was picked from the original plate, plasmid DNA isolated, amplified in *E. coli*, and the mutations within estrogen receptor (HBD) were determined by sequencing.

Preparation of yeast extracts for receptor binding assay

Yeast extracts were prepared from 50 ml culture at 0.5–0.9 OD_{600}, as described previously, with some minor modifications [23]. Cells were pelleted by centrifugation and washed in water (ice cold). Cells were then resuspended in 300 ml receptor buffer (50 nM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DDT, 15 mM $MgCl_2$, 20 mM sodium molybdate, 20% glycerol, 0.25 mM of the protease inhibitor, 4-(2-aminoethyl)-denzenesulfonyl fluoride hydrochloride (AEBSF)(Pentapharm)). Cells were transferred to a 15 ml round bottom tube and a 1.5 ml volume of dry (acid washed) glass beads were added. Cells were then vortexed vigorously for 1 min and then placed on ice for 1 min. This was repeated another three times to effect cell lysis. A hole was punched through the bottom of the 15 ml tube with a 20-gauge needle and the cell lysate was harvested by brief centrifugation of the 15 ml tube inside a 50 ml tube with adapter. The lysate was then transferred to a microfuge tube and centrifuged at $12{,}000 \times g$ for 10 min at 4°C. The supernatant was then

transferred to a fresh tube and the protein concentration determined.

Receptor binding assay

Cell extract (300 μg) was incubated with radiolabeled E_2 (0.5 nM) in the absence or presence of increasing concentrations of unlabeled ligand, in a final volume of 100 μl of receptor buffer. After incubation on ice for approximately 18 h, the extract was mixed with an equal volume of 10 mg/ml acid washed charcoal suspended in receptor buffer, incubated on ice for 10 min, and centrifuged at $12{,}000 \times g$ for 2 min. The supernatant was then spotted onto Whatman 2.4 cm GF/C filters. Specific binding was quantitated by scintillation counting of filters.

DNA transfection and CAT analysis

Approximately 12 h prior to transfection HeLa cells were plated at a density of 0.5×10^5/100 mm diameter plate. Transfection of plasmid DNA into HeLa cells was carried out by the calcium phosphate coprecipitation method as described previously [24]. CAT assays were carried out 48 h post transfection essentially as described [25]. CAT enzymatic activity was quantitated by measuring conversion of chloramphenicol to its acetylated forms by using an Ambis (CA) radioanalytic imaging system according to the manufacturer's instructions.

RESULTS

GR132706X is a weak estrogen receptor agonist

To establish a system for measurement of estrogen receptor activation, yeast (*Saccharomyces cerevisiae*) were transformed with a multicopy estrogen receptor expression plasmid and a reporter plasmid having three tandem copies of the consensus estrogen response element (ere), upstream of the β-galactosidase reporter gene (*lac-Z*) (Fig. 1A). The estrogen receptor expression and reporter plasmids encode LEU2 and URA3 respectively to complement the *leu2* and *ura3* defect in the parent yeast strain. These plasmids were maintained in transformed yeast by growth on minimal medium lacking both leucine and uracil. Yeast were grown in liquid cultures, in the presence of either E_2 or GR132706X at indicated concentrations, for approximately 16 h prior to extract preparation and measurement of β-galactosidase activity. Half-maximal activation in response to E_2 and GR132706X was obtained at approximate concentrations of 100 pM and 100 nM respectively (Fig. 1B). Therefore, half maximal activation of reporter gene activity required about a 1000-fold higher concentration of GR132706X compared to E_2. We tested whether the weaker response to GR132706X reflected a lower affinity of this compound for the receptor, rather than poor permeability of yeast for the compound, by measuring the receptor binding affinity of GR132706X. The weak response to GR132706X in the yeast strain was reflected by a similar weak affinity of GR132706X ($K_d = 0.44$ mM) in comparison to E_2 ($K_d = 0.78$ nM) for the receptor (Fig. 1C).

An important criterion in compound selection for an inducible system based on a steroid hormone receptor HBD is that the compound does not act as an antagonist of the wild type receptor. To address this question with respect to GR132706X, we tested GR132706X for antagonistic activity on E_2 activation of the estrogen receptor in this yeast strain. When this yeast strain was treated with GR132706X, at a concentration which alone does not activate the estrogen receptor, it did not antagonize receptor activation in response to E_2 present at half-maximal activation concentration (Fig. 1D).

Identification of an estrogen receptor (HBD) mutant with altered specificity for activation by ligands

We wished to develop a rapid and efficient method to identify estrogen receptor mutants which had altered specificity for activation by ligands. In particular, we wished to isolate estrogen receptor mutants having increased response to GR132706X, while at the same time having a reduced response to E_2. To identify mutants with altered ligand specificity we coupled random mutagenesis with a genetic screen in yeast. Random mutagenesis allows the generation of a large number of mutations within defined regions of DNA. One can then use the genetic capabilities of yeast for rapid and efficient screening of large numbers of mutations, as a step towards identifying the individual amino acids which give rise to altered ligand specificity of the steroid receptor.

We have chosen to use the mutagenic polymerase chain reaction (PCR) approach to generate random mutations. This method exploits the inherent infidelity of *Taq* DNA polymerase during the reaction. Initially, the error rate of *Taq polymerase* under standard conditions was insufficient to generate a diverse library of variant sequences. Furthermore, under standard PCR conditions, errors made by *Taq polymerase* are heavily biased toward A/T to G/C transitions. By altering the PCR reaction conditions, these drawbacks can be largely overcome [26]. Another problem previously associated with the mutagenic PCR approach has been the need to reclone the mutated PCR fragment into a vector after the PCR reaction. To overcome this we have taken advantage of the high efficiency of homologous recombination in yeast [27]. Co-transformation of the PCR product with the gapped estrogen receptor expression plasmid, containing regions homologous to both ends of mutated PCR product, allows *in vivo* recombination to repair the gap with the mutagenized DNA (Fig. 2A). Therefore, this procedure is efficient, allows targeting of mutations to

the HBD of the estrogen receptor and requires no sub-cloning steps in *E. coli*.

Following co-transformation of the mutagenic PCR fragment and the gapped estrogen receptor expression plasmid into yeast, the resultant colonies were replica plated on to plates containing either E_2 or GR132706X (Fig. 2B). The activity of the estrogen receptor (HBD) mutants, in response to either E_2 or GR132706X, was then determined by measuring β-galactosidase activity directly on the plates (see Materials and Methods). In response to a concentration of E_2 that results in maximal activation of the wild type receptor, many estrogen receptor mutants should fail to respond to E_2. On the other hand, in response to a concentration of GR132706X that results in weak activation of the wild type receptor, estrogen receptor mutants that have an enhanced response to GR132706X should be found. The mutants of most interest are those that have an increased response to GR132706X while also showing a reduced response to E_2.

Using this approach a mutant estrogen receptor was identified that had a reduced response to E_2 and an increased response to GR132706X. To more accurately asses this alteration in specificity, the activity of this

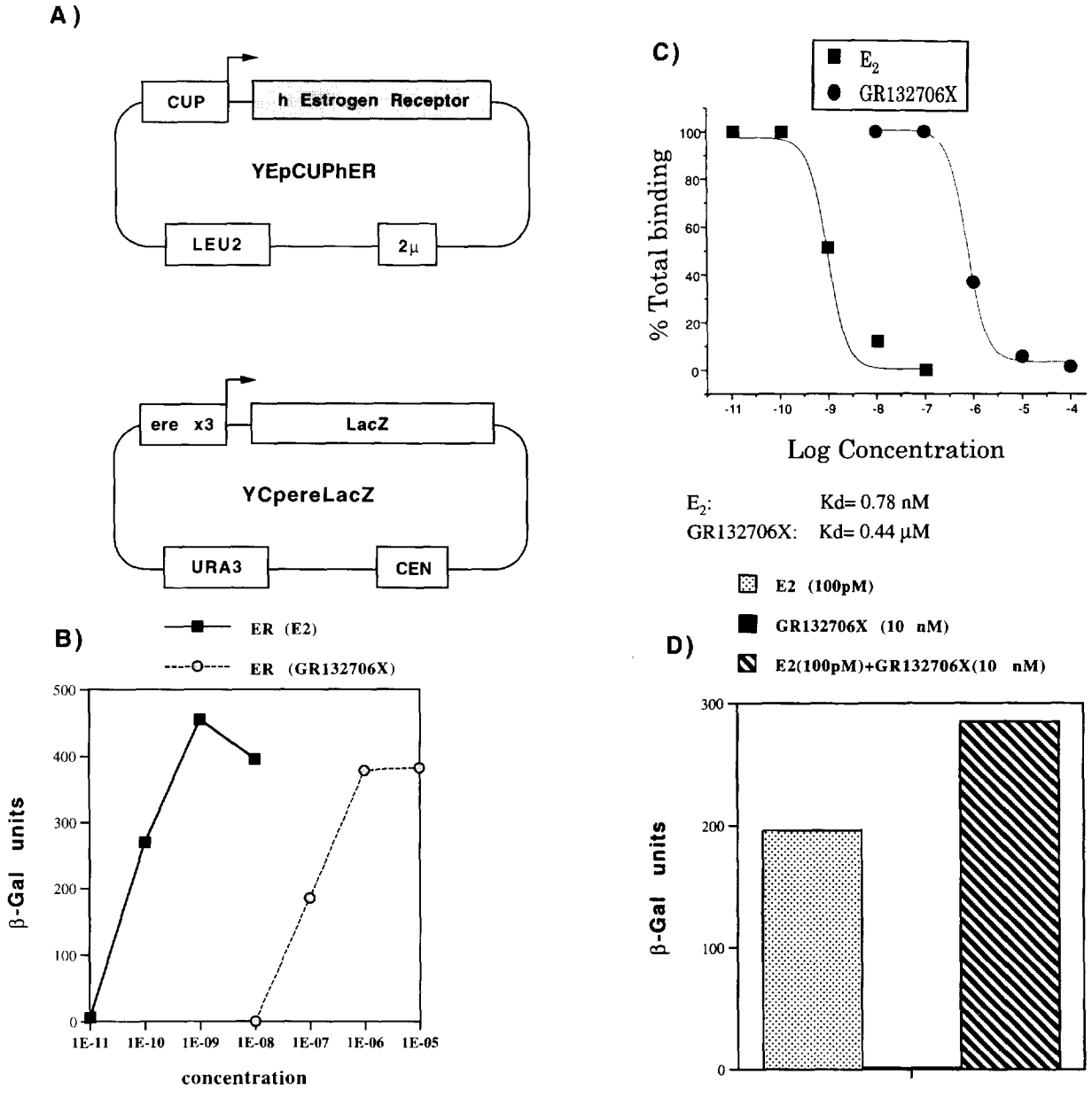


Fig. 1. Characterizing activity of GR132706X on estrogen receptor. (A) A schematic representation of the estrogen receptor expression plasmid and LacZ reporter plasmid. (B) β-Galactosidase assay measurement of estrogen receptor activation in response to E_2 and GR132706X. (C) Receptor binding analysis to measure affinity of E_2 and GR132706X for estrogen receptor. K_d of E_2 and GR132706X is the mean of three independent experiments and was determined using the equilibrium binding data analysis (EBDA) program (Biosoft, Cambridge). (D) β-Galactosidase assay to determine if GR132706X can antagonize estrogen receptor activation by E_2.

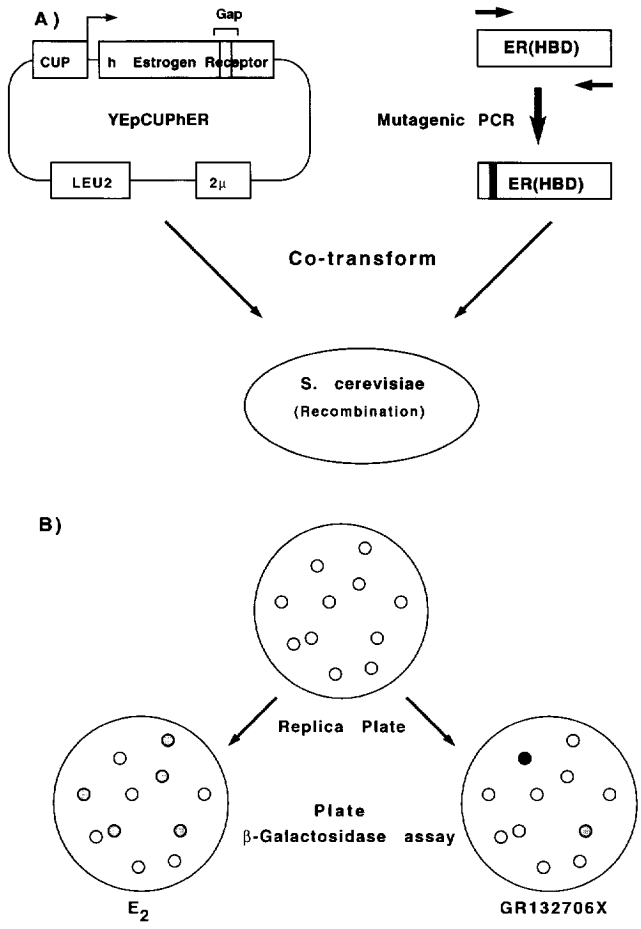
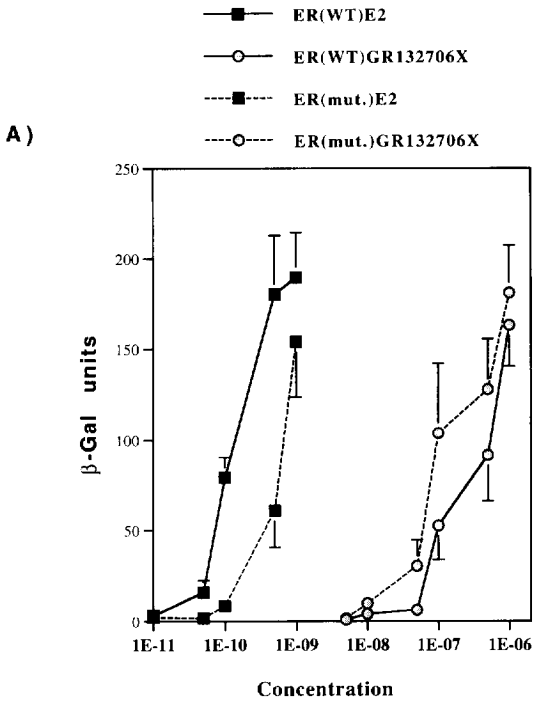


Fig. 2. Outline of mutagenesis and yeast screening approach. (A) A schematic outline of the procedure to create a library of estrogen receptor HBD mutants in yeast. (B) A schematic outline of the plate β-galactosidase assay procedure to detect estrogen receptor mutants with altered specificity for activation by ligands. Gray/black indicates the intensity of blue color of the colonies in the plate β-galactosidase assay.

mutant estrogen receptor was quantified by measuring β-galactosidase activity in liquid cultures exposed to a range of E_2 and GR132706X concentrations (Fig. 3A). Upon quantification of β-galactosidase activity, it was observed that the mutant receptor had a 9–10-fold decrease in response to E_2, at half-maximal concentration (100 pM). On the other hand, this mutant had a 2-5-fold increase in response to GR132706X, at half maximal concentration (50–100 nM). Sequencing of the HBD of this mutant estrogen receptor revealed a single nucleotide mutation (T to G) at base position 1804 (numbering according to [21]). This mutation gives rise to a histidine to glutamine mutation at amino acid position 524 within the estrogen receptor (HBD) (Fig. 3B). The altered amino acid falls within the region (between amino acids 507 and 538) that has previously been defined as being critical for hormone binding [28]. Although the trans-activating activity of this mutant estrogen receptor is altered significantly, in comparison to the wild type receptor, the binding affinity of both E_2 and GR132706X for this mutant receptor was not altered (data not shown). We conclude that this mutation (His^{524} to Gln) alters the trans-activating function of the receptor, but not the binding affinity of ligand for the receptor (see discussion).

Generating a regulator of gene expression based on the estrogen receptor HBD

Systems for control of gene expression based on the HBD of steroid receptors have been described (see introduction). A chimeric receptor which consists of the DNA-binding domain of the yeast transcription factor Gal4, linked to the HBD of the estrogen receptor, has been shown to stimulate transcription from Gal4 responsive reporter genes in a hormone-dependent manner [29]. Although the HBD of the estrogen receptor contains a trans-activation domain (TAF-2), between amino acids 534 and 548, this is a relatively weak trans-activating domain, and activity appears to be



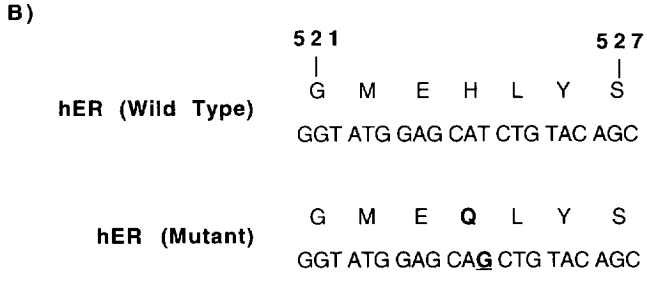


Fig. 3. Estrogen receptor HBD mutant with altered response to ligands in yeast. (A) Activity of estrogen receptor mutant (ER(mut.)) in response to E_2 and GR132706X in comparison to the response of the wild type estrogen receptor (ER(WT)) as determined by β-galactosidase assay. (B) Amino acid and DNA sequence of wild type and mutant estrogen receptors.

cell type-dependent [29]. In order to improve the trans-activating potential of the Gal4-ER(HBD) chimeric receptor, and make it cell type-independent, Braselmann *et al.* incorporated the strong 71 amino acid trans-activation domain of the herpes simplex viral protein VP16 onto the C-terminus [10]. The Gal4-ER(HBD)-VP16 chimeric receptor was capable of inducing expression from a Gal4 responsive promoter up to 100-fold in transiently transfected cells. However, expression of even moderate levels of proteins containing the strong trans-activating domain of VP16 is toxic to at least some cell types [30]. Transcriptional 'squelching' by the trans-activating domain of VP16 is believed to be the cause of this toxicity. Therefore, use of the VP16 trans-activating domain is likely to be of limited use in the development of a regulator of gene expression *in vivo*.

To address this question we have examined the potential of using alternative trans-activating domains. Several recent studies of mammalian transcription factors have defined small regions (less than 20 amino acids) that independently function as strong trans-activators [31, 32]. One of these is an 11 amino acid region of the RelA subunit of the NFκB transcription factor. Furthermore, three mutations within this domain, making it more acidic, enhanced its trans-activating potential [31]. Based on this information, we constructed a vector encoding a chimeric estrogen receptor (HBD) based transcription factor where two copies of the mutated trans-activating domain from RelA were substituted for the VP16 trans-activating domain (Fig. 4A). We then compared the ability of these chimeras to induce expression of a Gal4 responsive reporter gene, in response to E_2, in transiently transfected HeLa cells (Fig. 4B). The Gal4-ER(HBD), containing only the estrogen receptor TAF-2 trans-activating domain, was capable of mediating only a weak 2.5-fold induction of the reporter gene in response to E_2. The chimera containing the VP16 trans-activating domain was capable of inducing reporter gene expression 12.5-fold in response to E_2. The chimera containing the RelA trans-activating domain was capable of inducing reporter gene expression 7.5-fold in response to E_2. Therefore, the small RelA domain is capable of mediating significant transcriptional activation in response to E_2 and represents an alternative to the potentially toxic VP16 trans-activating domain. Furthermore, a chimeric regulator of gene expression containing the RelA trans-activating domain, as opposed to that of VP16, is likely to be less immunogenic when expressed *in vivo*.

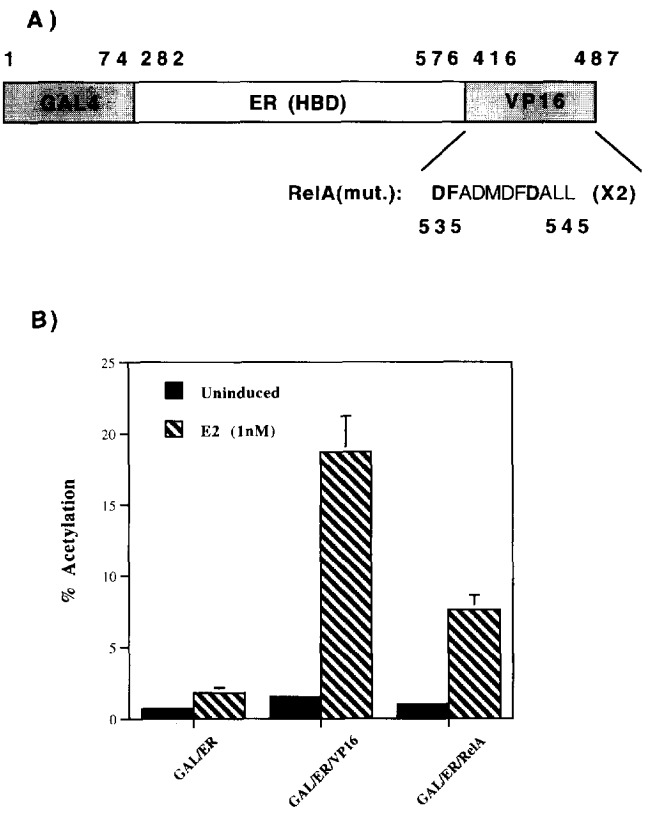


Fig. 4. Activation of gene expression by estrogen receptor HBD based chimera. (A) Diagram of chimeric transcription factors with numbers referring to the amino acid positions within the different proteins. (B) CAT analysis of activation of chimeric transcription factors by E_2. HeLa cells were transfected with CAT reporter plasmid (5 μg) and chimeric transcription factor expression plasmid (2 μg). Transfected DNA was made up to total of 10 mg with empty expression vector (CMV4T). Results are the means, ± standard errors of the mean, for three independent experiments.

Incorporation of the mutant estrogen receptor (HBD) into a chimeric regulator

Having identified a mutation within the estrogen receptor HBD which altered the specificity of activation in response to ligands in yeast, we wished to verify that this altered specificity was maintained in mammalian cells. Therefore, we created a chimeric Gal4-ER (HBD)-RelA expression vector having a histidine to glutamine mutation at amino acid 524 within the estrogen receptor HBD. The ability of this mutant chimera to mediate reporter gene activation, in response to both E_2 and GR132706X, was compared with the response of the chimera containing the wild type estrogen receptor HBD. As was observed in yeast, the mutant estrogen receptor HBD mediated a reduced response to E_2 and an enhanced response to GR132706X (Fig. 5). The response of the mutant chimera was approximately 7-fold lower than the wild type chimera in response to E_2, at half maximal concentration. This is similar to what was observed in yeast (Fig. 3A). The response of the mutant chimera was approximately 8-fold higher than the wild type chimera in response to GR132706X, at half maximal concentration. Thus, the enhanced response of the mutant to GR132706X in mammalian cells is greater than that observed in yeast (2–5-fold versus 8-fold). From this result, we can conclude that the altered response of the mutant estrogen receptors identified in

the yeast screen is maintained in mammalian cells. Moreover, this approach should prove extremely useful in the development of specific regulators of gene expression.

DISCUSSION

We have described a rapid and efficient method for the identification of mutant steroid receptor HBDs with altered specificity for activation by ligands. We used mutagenic PCR conditions that generate a large number of unbiased mutations throughout a defined region of the receptor (the HBD), plus the high efficiency of homologous recombination in yeast, to create a large library of estrogen receptor mutants. A plate β-galactosidase assay was used to rapidly examine the response of these mutants to selected ligands. Using an estrogen receptor HBD mutant isolated by this approach (His^{524} to Gln), we have shown that this HBD can be incorporated into a modified regulator of gene expression (Gal4-ER(HBD)-RelA) and mediate reporter gene activation in response to ligand. This approach should prove very useful in attempting to develop regulators of gene expression which are responsive specifically to selected compounds.

The estrogen receptor mutant examined in this study had a single amino acid mutation (His^{524} to Gln) within the HBD. This demonstrates that subtle changes in the HBD are sufficient to significantly alter the response of the receptor to ligand. Previous studies have shown that subtle changes can significantly alter the binding of both agonists and antagonists to the estrogen receptor HBD (for review see [28]) Interestingly, the mutation described in this study, although demonstrating a

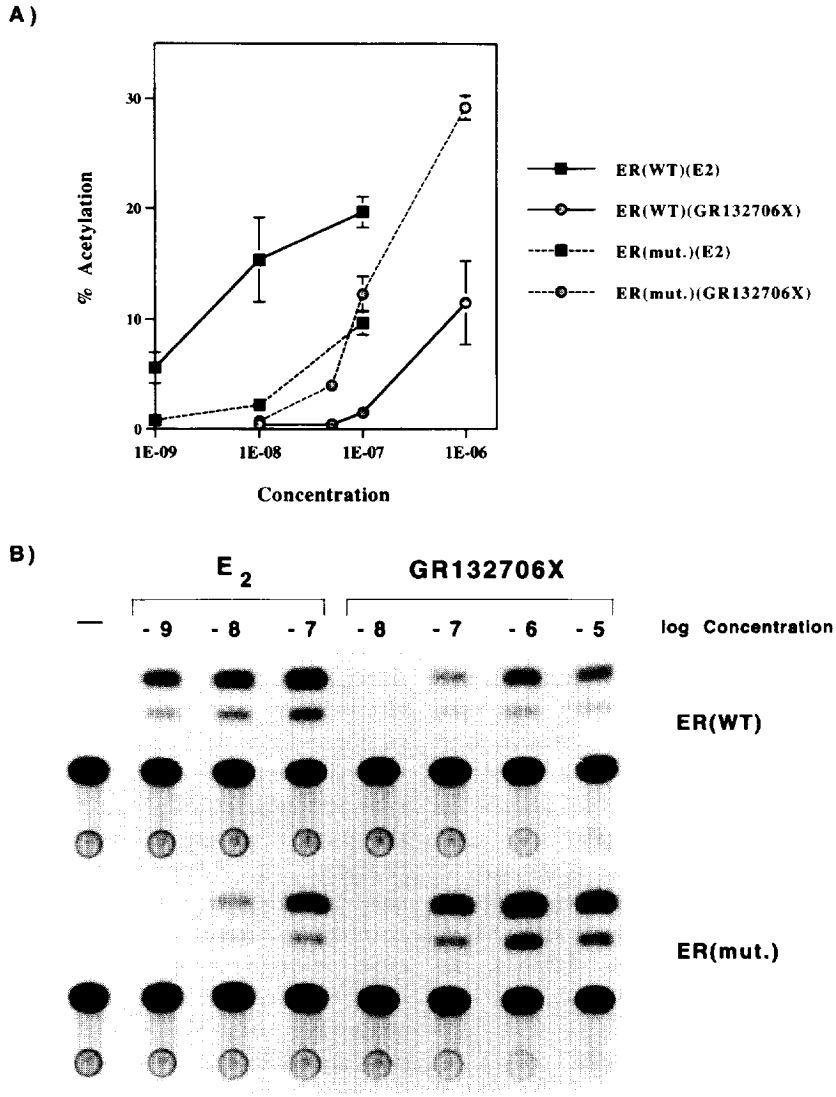


Fig. 5. Estrogen receptor HBD mutant demonstrates altered specificity in mammalian cells. (A) CAT analysis to measure activation of chimeric transcription factor containing either the wild type (ER(WT)) or mutated (ER(mut.)) HBD by E_2 and GR132706X. HeLa cells were transfected with CAT reporter plasmid (5 μg) and chimeric transcription factor expression plasmid (2 μg). Cells were treated with ligand approximately 18 h prior to extract preparation. Results are the means, ± standard errors of the means, for three independent experiments. (B) Results of a representative experiment are shown.

significant change in response to ligands, does not demonstrate any significant alteration in the binding affinity for either E_2 or GR132706X. Previously, estrogen receptor mutants have been described which have reduced response to E_2 without a reduction in the binding affinity for the ligand. However, the mutations responsible for this reduction in the trans-activating ability of the receptor, without altering E_2 binding affinity, all lie within the TAF-2 trans-activating domain (amino acids 534–548) [33]. Recently, a number of estrogen receptor associated proteins have been identified which appear to be important in mediating transcriptional activation [34, 35]. The mutation described in this study (His^{524} to Gln) may alter the ligand induced interaction with one or more of these receptor associated proteins and thereby alter the trans-activating function of this mutant without affecting ligand binding affinity.

The mutation at position 524 lies close to the cysteine at position 530 which is the site of covalent binding by aziridine derivatives of estrogen to the receptor [36]. Furthermore, changes in amino acids close to Cys^{530} reduce the binding affinity of the receptor for estrogen without altering the affinity for anti-estrogen's. For this reason, amino acids near Cys^{530} are believed to be involved in receptor discrimination between estrogen and anti-estrogens [37]. The mutation discussed here (His^{524} to Gln), having altered specificity in response to ligands, lies close to Cys^{530} and thus agrees with this proposal.

The objective of the strategy was to develop an approach that allows the identification of mutations within the HBD of a steroid receptor which give large shifts in specificity with regards to activation of the receptor by ligands. The mutation described in this study has demonstrated the feasibility of such an approach. However, to develop a very specific regulator of gene expression, with the long term objective of being able to use such a regulator for control of gene expression in gene therapy, mutants with significantly larger shifts in response than that shown here are required. One of the important advantages of the mutagenic PCR approach is that it allows repeated rounds of mutagenesis without isolating clones and obtaining sequence information. Once the library of receptor mutants has been generated and the phenotypic yeast screen carried out to identify mutants with the appropriate shift in specificity, these mutants can be used directly as an imput for a second round of mutagenic PCR. Repeating the cycle of selection and mutagenesis allows for *in vitro* evolution of the receptor. Another approach would be to apply the recently described technique of DNA shuffling into the *in vitro* evolution of the receptor. DNA shuffling has been shown to be an extremely powerful approach for the *in vitro* molecular evolution of proteins [38, 39]. By combining multiple rounds of both mutagenic PCR and DNA shuffling the large shifts in steroid receptor specificity that are required for the development of specific regulators of gene expression should be realized.

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