Pergamon

PII: S0960-0760(97)00154-4

J. Steroid Biochem. Molec. Biol. Vol. 64, No. 3-4, pp. 129–135, 1998

Printed in Great Britain
0960-0760/98 $19.00 + 0.00

Random Mutagenesis of Human Estrogen Receptor Ligand Binding Domain Identifies Mutations that Decrease Sensitivity to Estradiol and Increase Sensitivity to a Diphenol Indene-ol Compound: Basis for a Regulatable Expression System

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We have used low fidelity polymerase chain reaction amplification to generate mutations in the human estrogen receptor ligand binding domain (LBD). Screening of libraries of mutants in yeast revealed a variety of phenotypic changes including decreased responsiveness to estradiol and increased responsiveness to synthetic compounds. Identification of the mutations responsible for these phenotypic changes indicated discrete regions of the LBD that are important for human estrogen receptor function. Cumulative rounds of mutagenesis and screening allowed us to produce a mutant estrogen receptor that was of reversed specificity as compared with the wild type LBD, in that it was more responsive to a diphenol indene-ol than to estradiol. This mutant may form the basis of a useful regulatable expression system in mammalian cells. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

We are interested in creating a regulatable expression system for use in human gene therapy. A detailed description of our approach has been given elsewhere [1]. In brief, our system has three components; a non-toxic synthetic small molecule which acts as an inducer, a synthetic transcription factor which is activated by the inducer and an artificial promoter containing non-mammalian DNA motifs to which the activated transcription factor specifically binds. This design allows precise inducer-dependent regulation of a transgene driven by the artificial promoter, without non-specific cellular effects [2]. There have been several systems of this or similar design produced in recent years [3–9] but none have been ideal for purposes of human therapy; for example, the transactivator proteins used may be toxic [3] or probably immunogenic [7], or the inducers are not always benign [5, 6, 8, 9] or are likely to activate cellular promoters as well as the transgene promoter [4].

Our approach is to use the ligand-binding domain (LBD) of the human estrogen receptor (hER) as a component of our synthetic transcription factor, the aim being to mutate the LBD until it responds to a synthetic inducer and not to estradiol. We have already demonstrated the principle of this approach [1]. Here we describe LBD mutations that change the responsiveness of the hER such that it is more sensitive to a diphenol indene-ol compound than to its natural ligand, estradiol; we propose that this mutated LBD, when incorporated into the synthetic transcription factor that we have previously described [1] will form the basis of a useful regulatable expression system. Furthermore, our data suggest that particular regions of the LBD are critical in hER structure and function and may be of use in the rational design of anti-estrogens for the therapy of breast cancer [10].

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Received 23 May 1997; accepted 14 Oct. 1997.

MATERIALS AND METHODS

Our materials and methods have been previously described in detail [1] and therefore are only briefly summarized here.

Materials

The Glaxo compounds GR132706X [Fig. 1(A)] and CCI5309 [Fig. 1(B)] are small synthetic compounds produced by GlaxoWellcome Research and Development (Stevenage, U.K.). Salmon sperm DNA, 17β-estradiol and *o*-nitrophenyl-β-galactoside (ONPG) were from Sigma Chemical Co. and zymolyase was from Seikagaku Corporation.

The two plasmid constructs used in these experiments were YEpCuphER and YCpERElacZ [1]. YepCuphER contains the human estrogen receptor, while YCpERElacZ contains a β-galactosidase gene under the control of an estrogen responsive promoter. In yeast cells containing both YEpCuphER and YCpERElacZ, β-galactosidase activity is a reflection of the extent of activation of hER. In addition, YEpCuphER contains the LEU gene and YCpERElacZ contains the URA gene; growth of yeast containing these plasmids can therefore be selected for in medium deficient in URA and LEU.

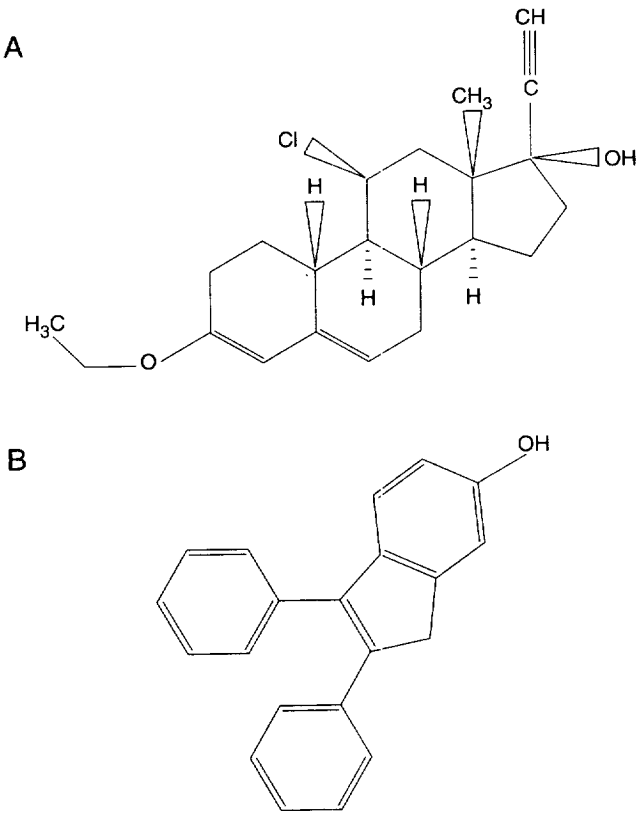

Fig. 1. Structures of the Glaxo compounds used in this study. (A) Structure of GR132706X; formula = $C_{21}H_{16}O$, mol. wt. = 284.36; (B) Structure of CCI5309; formula = $C_{22}H_{29}ClO_2$, mol. wt. = 360.93.

Methods

Mutagenic PCR. Our protocol was based on a published method [11]. Briefly, the PCR buffer contains 0.5 mM $MnCl_2$ and 7 mM $MgCl_2$ to decrease polymerase template specificity and stabilize mismatches, respectively; *Taq* polymerase in excess (5 units in 100 μl) to encourage chain extension beyond mismatches and a biased nucleotide mixture (dCTP and dTTP at 1 mM each, dATP and dGTP at 0.2 mM each) to encourage misincorporation. The amplification reaction consisted of 30 cycles of 94°C/1 min, 45°C/1 min, and 72°C/1 min. The template DNA used in the low-fidelity PCR reactions was the 1.6 kb fragment produced by *Asc*I/*Sac*I digestion of YEpCuphER. The primers used were as follows: (5') TCTAAGAAGAACAGCCTG and (3') AGTGGGCGCATGTAGGCG.

Yeast strain, growth conditions and transfections. In all cases the *Saccharomyces cerevisiae* yeast strain YPH499 [12] was used. Yeast cells were grown at 30°C in minimal yeast medium (0.67% [w/v] yeast nitrogen base without amino acids and 2% [w/v] glucose) supplemented with the required amino acids. Yeast were transformed by a lithium acetate procedure based on a published method [13]. Briefly, a logarithmically growing culture of optical density 0.5–0.6 (600 nm) was washed in 0.1 M LiAc in TE (10 mM Tris, 1 mM EDTA, pH 7.5) and incubated in 500 μl of 0.1 LiAc/TE at 30°C for 30–90 min. 100 μl of these cells was mixed with up to 10 μg of plasmid DNA, 10 μg of salmon sperm DNA and 500 μl of 50% (w/v) polyethylene glycol 6000 in 0.1 M LiAc/TE (filter sterilized) and incubated with agitation for 30 min at 30°C, followed by a 20 min heat shock at 42°C. Cells were pelleted gently and PEG removed prior to resuspension in sterile water and plating out on selective medium.

In these transformations, plasmid DNA consisted of a 2:1 mixture of mutagenic PCR product and YEpCuphER plasmid, the latter having been gapped with *Nco*I and *Bgl*II; this enzyme treatment removes an 84 bp sequence from the ER LBD in YEpCuphER. The purpose of gapping YEpCuphER prior to transformation is to favour yeast-mediated homologous recombination between the PCR product and the plasmid backbone.

In situ β-galactosidase assay. In order to screen the yeast libraries for mutant LBDs that possessed potentially useful ligand specificities, the libraries were replicated onto solid media containing the appropriate amino acid mix plus either estradiol, Glaxo compound or their diluents (ethanol and DMSO, respectively) as controls. Concentrations of Glaxo compound and estradiol used in the first round of *in situ* screens were, respectively, 250 and 0.5 nM; in subsequent screens of libraries containing mutated LBDs of improved phenotype, concentrations were

changed to 1 nM estradiol and 25 nM Glaxo compound. After growth for 10–16 h, colonies on these replica plates were assayed *in situ* for β-galactosidase activity according to a published method [14]. In brief, the agar plates supporting the colonies were covered with a warm solution of 0.5% agarose, 0.1% SDS, 2% dimethylformamide, 0.5 M phosphate pH 7 and 0.05% X-gal and incubated for 2–4 h. This mixture provokes lysis of the cells and thus allows β-galactosidase to come into contact with X-gal substrate, resulting in a blue colouration the intensity of which is proportional to the quantity of β-galactosidase in the yeast, which is itself proportional to the activation of the ER. In this way colonies were picked from the original plates for further analysis on the basis of, for example, a strong coloration in the presence of Glaxo compound and a weak coloration in the presence of estradiol.

Quantitative β-galactosidase assays. These were performed according to a published method [14]. Briefly, yeast clones identified using the *in situ* screen were grown overnight in selective medium and then diluted to an optical density of 0.1. Subcultures of 2 ml volume were set up and allowed to grow for up to 16 h in the presence of various concentrations of estradiol, Glaxo compound or controls. After recording the optical density at 600 nm of each of the subcultures, yeast cells were pelleted, washed in lacZ buffer (10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol and 100 mM phosphate, pH 7) and resuspended in 50 μl of lacZ buffer. After addition of 50 μl of chloroform and 20 μl of 0.1% SDS, cells were vortexed for 1 min to ensure lysis. Cell lysates were then warmed to 30°C and mixed with 0.5 ml of 2 mg/ml ONPG (substrate). After 10 min at 30°C, the reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3, the chloroform removed by centrifugation and the yellow colouration quantified by reading the optical density at 420 nm. Units of β-galactosidase are then defined by 1000× change in OD at 420 nm divided by (assay duration in min × culture volume in ml × OD at 600 nM).

Plasmid rescue from yeast. Yeast clones were grown to saturation (48 h at 30°C) in a volume of 15 ml, then pelleted, washed in water and cell walls dissolved by incubation for 1 h in 1 ml of zymolyase solution (2 mg/ml zymolyase, 100 mM KH_2PO_4, 1.2 M sorbitol, pH 7.5) at 37°C. Lysis was then effected by the addition of 200 μl of lysis solution (ProMega "Magic" Minipreps) and incubation of the cells for 5 min at 65°C. Subsequent procedure was according to ProMega "Magic Miniprep" instructions. Rescued plasmid was transformed into commercially available DHSa bacteria (Gibco BRL) according to the manufacturers instructions and plasmid DNA prepared by normal methods.

Table 1. Incidence of mutation types using low-fidelity PCR

Mutation	%
G–A	13.5
G–T	4.8
G–C	1.9
A–G	10.6
A–T	16.5
A–C	3.8
T–G	3.8
T–A	17.4
T–C	13.5
C–A	2.9
C–T	12.6
C–G	0

RESULTS

Mutagenic PCR

We determined the types and frequencies of mutations obtained with low fidelity PCR mutagenesis by sequencing hER-containing plasmids from a number of yeast clones that showed loss of responsiveness to estradiol in the *in situ* β-galactosidase screen. The results are summarised in Table 1 and Figs 2 and 3. Table 1 shows that, with the exception of C–G, all

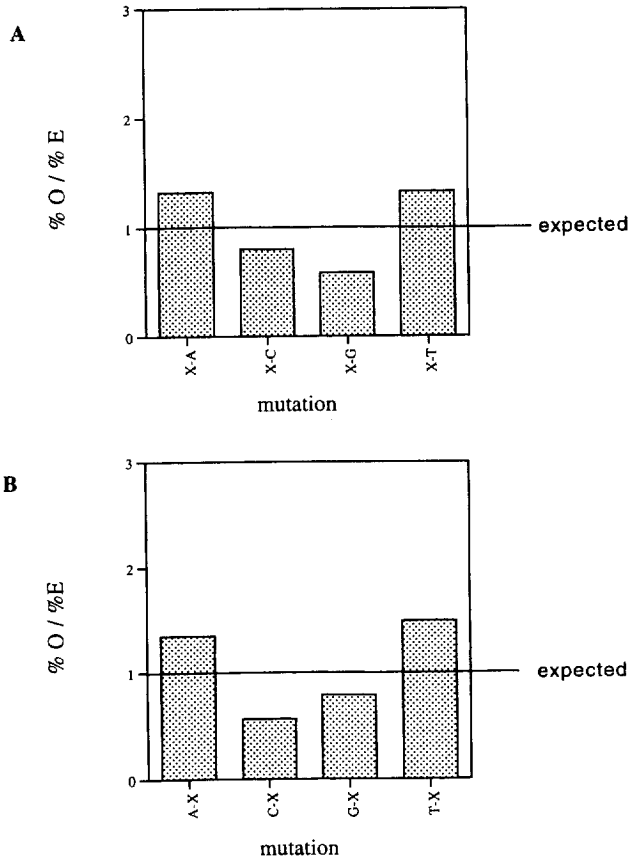

Fig. 2. Comparison of observed and expected mutation types obtained with low fidelity PCR. (A) Mutations of the form X to N; (B) mutations of the form N to X (N = G, A, C, T; X = any nucleotide except N). %O/%E = observed percentage incidence divided by expected percentage incidence.

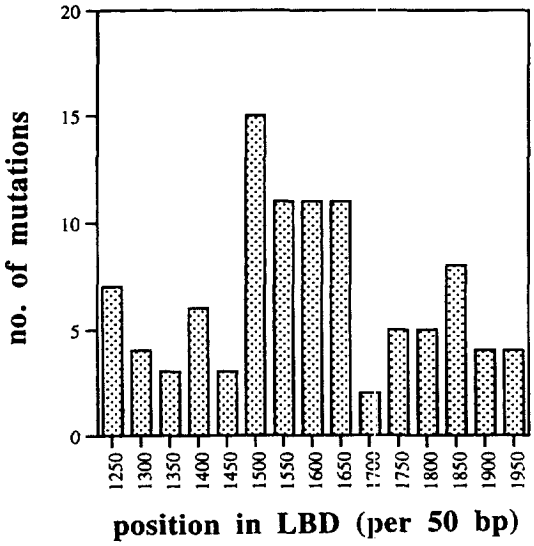

Fig. 3. Distribution of observed mutations per 50 bp of the hER LBD.

types of mutation were generated. There were however distinct variations in the relative proportions of mutation types, with T–A for example being about 8× as frequent as G–C. The overall result is a slight bias towards mutations to or from A and T, and a corresponding bias against mutations to or from C and G (Fig. 2). There was however no strong bias towards either transitions or transversions; using data from Table 1, the transitions/transversions ratio is 0.93. These results are in broad agreement with those of Cadwell and Joyce [15]. We also checked the distribution of mutations within the ER LBD. It can be seen that there is a slight bias towards finding mutations towards the centre of the target sequence (Fig. 3). However the bias is not so strong as to constitute a significant problem as, on the 50 bp scale shown in Fig. 3, mutations occur in all regions of the LBD.

Screening of yeast libraries

From the *in situ* assays, colonies were picked that showed either a decrease in sensitivity to estradiol without a decrease in sensitivity to compound, or that showed an increase in sensitivity to compound. After this initial cursory screen, the mutants were grown up in liquid culture to confirm the ER phenotype by the more sensitive quantitative β-galactosidase assay.

Treatment with synthetic compound CCI5309. A library screened on plates containing 250 nM CCI5309 or 0.5 nM estradiol revealed two colonies, named D1 and D2, that were blue in the presence of CCI5309 but not in the presence of estradiol. Quantitative β-galactosidase assays of liquid cultures of these clones confirmed a significant decrease in responsiveness to estradiol (in both cases half-maximal activation occurred at approximately 5 nM, as compared with approximately 0.1 nM for the wt) with an unchanged responsiveness to CCI5309 (half-maximal activation at approximately 100 nM) (data not shown). This change in phenotype was due in D1 to one or both of the mutations V368E and L387Q and in D2 to the mutation M421T (numbering according to Green [16]). We used the D1 and D2 sequences as templates for further mutagenesis, with the aim of increasing their responsiveness to CCI5309 while retaining their decreased responsiveness to estradiol. However, substantial efforts including the use of DNA shuffling [17] failed to produce any such improved phenotypes and these mutants were therefore discarded.

Treatment with the synthetic compound GR132706X. We have already described an H524Q mutation (from a clone termed 15A) that has the effect of simultaneously increasing responsiveness to GR132706X by 2–5× and decreasing responsiveness to estradiol by 9–10× [1]. We have now used this mutant LBD as template in further rounds of PCR mutagenesis with the object of further decreasing the responsiveness to estradiol and increasing the responsiveness to GR132706X. Quantitative β-galactosidase assays identified four clones which exhibited a profound decrease in responsiveness to estradiol while retaining the increased responsiveness to GR132706X conferred by the 15A mutation (Fig. 4, A–D); these clones were named CA7, CB7, BA7 and BI4. Plasmids were rescued from these clones and sequenced; mutations were identified as M343L (BA7), V533E (CA7), V533M (CB7) and L536F/L345S (BI4). We took the most promising three mutant LBDs (CA7, CB7 and BA7) and used these as template in further rounds of mutagenic PCR; our aim was to identify mutants that showed increased sensitivity to GR132706X while retaining the reduced responsiveness to estradiol exhibited by the above three mutants. In spite of screening a large number of libraries of mutated CA7, CB7 and BA7 LBDs, we identified only one such mutation, H513Q. This was rescued from a clone named F12, and has a halfmaximal response to GR132706X at 2–5 nM (as compared with about 50 nM for the wt) and a half-maximal response to estradiol at about 5 nM (as compared with <0.1 nM for the wt). F12 was created by mutagenesis of the CA7 LBD; the F12 LBD therefore contains three mutations, H524Q (from 15A), V533E (from CA7) and H513Q. Clearly F12 is now more responsive to synthetic activator than to estradiol (Fig. 5). We have therefore succeeded in our intention of reversing the human ER LBD specificity by successive rounds of mutagenesis and screening.

DISCUSSION

Although our protocol does not produce exactly equal numbers of all forms of mutation, equally dis-

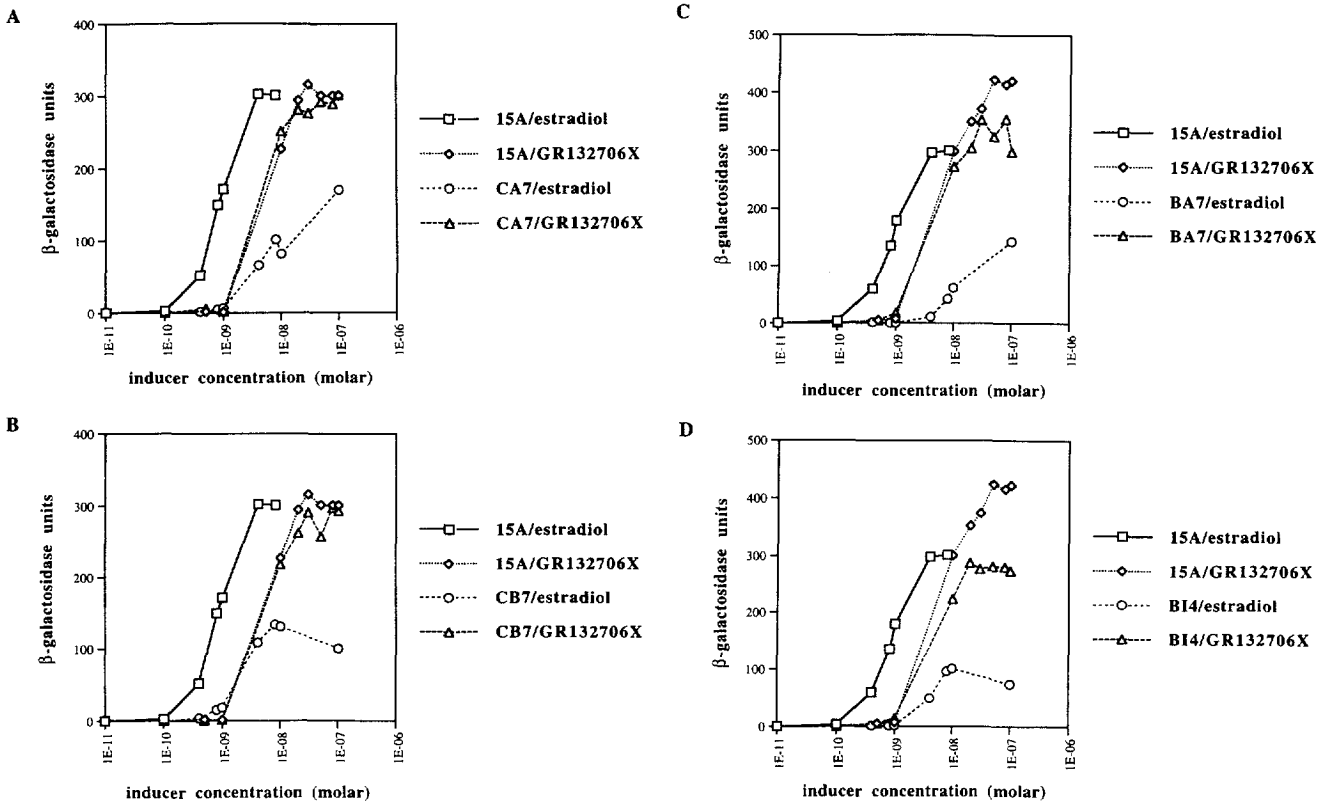

Fig. 4. Phenotypes, as determined by quantitative β-galactosidase assay, of four mutant hERs that exhibit profoundly decreased responsiveness to estradiol, but unchanged (A, B, C) or only slightly decreased (D) responsiveness to GR132706X. The phenotype of the parental mutant, 15A, is shown for comparison. A = mutant CA7, B = mutant CB7, C = mutant BA7, D = mutant BI4.

tributed throughout the LBD, nevertheless we find a wide variety of mutations (Table 1, Fig. 2) produced in a broad range of locations (Fig. 3). We conclude therefore that this method constitutes a useful mechanism for generating mutations in a relatively unbiased fashion. This, together with the rapid yeast screening system we use, makes our method eminently applicable to the *in vitro* evolution of the ER LBD.

We found several mutants exhibiting decreased responsiveness to estradiol, without any concomitant decreases in responsiveness to GR132706X. Sequencing of those mutants with the most promising phenotypes (i.e. most decreased response to estradiol and least decreased response to GR132706X) indicated the following. Mutations that decrease responsiveness to estradiol but not to GR132706X lie in two discrete regions of the LBD, namely amino acids 533–536 and 343–345 (Figs 4 and 6). In the case of CA7 and CB7, different base changes altered the same amino acid (V533E or V533M), emphasising the importance of this residue in estradiol recognition. In all these cases the loss of response to estradiol was such that the mutants were more responsive to synthetic compounds than to their natural ligand. However, an ideal gene regulation system would need to respond strongly to the synthetic activator at concentrations lower than those required to activate these mutants. We therefore took the most promising three mutant LBDs (CA7, CB7 and BA7) and used these as template in further rounds of mutagenic PCR, in order to produce mutants of further increased responsiveness to GR132706X. Only one such mutant was found, namely F12; this is clearly more responsive to synthetic activator than to estradiol (Fig. 5). We have therefore succeeded in our intention of reversing the human ER LBD specificity by successive rounds of mutagenesis and screening.

Previously, we have used the 15A mutant LBD as a component of a synthetic transcription factor in a regulatable expression system and have shown that reporter gene expression in mammalian cells can be controlled by administration of GR132706X [1]. We therefore now suggest that replacement of the 15A LBD in this synthetic transcription factor by the F12 LBD would give a protein that would be even more responsive to GR132706X and less responsive to estradiol and which would result in an improved regulatable expression system.

We feel that we have described a promising technique for the *in vitro* evolution of proteins to desired ends. However, it should be noted that attempts to

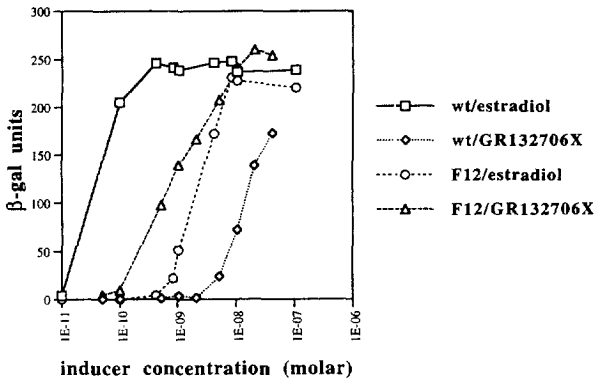

Fig. 5. Phenotype of mutant hER F12, as determined by quantitative β-galactosidase assay. The phenotype of the wt hER is shown for comparison. Note that concentrations of estradiol that give maximal activation of the wt hER (about 0.5 nM) do not activate the F12 mutant hER, while concentrations of GR132706X that give half-maximal activation of the F12 mutant hER (2–5 nM) give only minimal activation of the wt hER.

produce ER mutants highly sensitive to CCI5309, from the estrogen-insensitive mutants D1 and D2, were unproductive. Possible further improvement in sensitivity to CCI5309 is impossible in the presence of D1 and D2 mutations; i.e. these mutants may represent evolutionary dead-ends in this context. Alternatively, the structure of CCI5309 may be inherently unsuitable as a ligand for ER-based receptor mutants. In any event, it would seem that multiple rounds of mutagenesis are not guaranteed to improve the phenotype of the estrogen receptor.

Our data may also be relevant to the structure–function relationships of the human ER. The results we describe suggest that there are at least four discrete regions of the LBD that are important in hER function. These are (a) a region around amino acids 343–345 (in which mutations M343L (BA7) and possibly L345S (one of the two B14 mutations) result

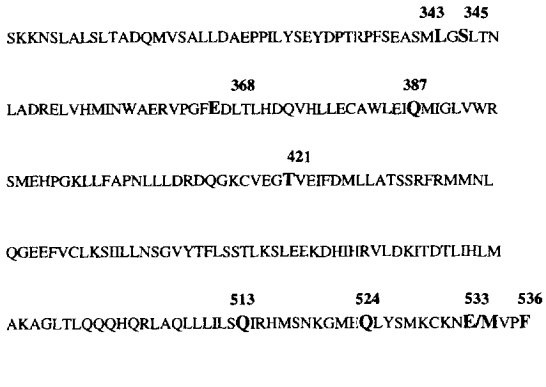

Fig. 6. Positions of mutations identified in this study. Mutated amino acids are shown in large font and bold type and their positions are defined by number above each mutation. Two mutations, V–E and V–M, were both found at 533. See text for details.

in decreased responsiveness to estradiol but do not decrease responsiveness to GR132706X), (b) a region between 368 and 387 (in which one or both of the D1 mutations decrease responsiveness to estradiol), (c) a region around 421 (in which the D2 mutation decreases response to estradiol) and (d) a region around 513–533 (in which mutations V533E (CA7) and V533M (CB7) result in decreased response to estradiol, but not to GR132706X; mutation H524Q (ISA) results in both a decreased response to estradiol and an increased response to GR132706X and mutation H513Q (F12) results in an increased response to GR132706X and a smaller increase in response to estradiol). The region from 507–538 has previously been proposed to be critical for hormone binding, as receptors containing 121–538 display estradiol binding of appropriate affinity, whereas receptors containing 121–507 show no estradiol binding at all [18]. Also, other studies have reported point mutations in this region which alter estradiol affinity, sometimes without affecting anti-estrogen affinity [19–21]. Our data therefore confirm the importance of this region in ligand recognition and define previously unreported critical residues within this sequence. Mutations at 364 and 383 have also been shown to be of importance in ligand binding [20] and this supports our data suggesting the involvement of residues 368 and 387 in hER function. Similarly, a previous study [20] has indicated that mutations at 351 and 426 decrease affinity for estradiol and this is not incompatible with our data suggesting that residues 343/345 or 421 are important in hER function.

In conclusion, we have used repeated rounds of PCR mutagenesis and yeast library screening to create a modified hER that is more responsive to a synthetic compound than to its natural ligand, estradiol. The LBD from this receptor is likely to form the basis of a synthetic transcription factor for a regulatable expression system. Furthermore, our data, in combination with data from other studies, indicates four regions of the LBD which are critical to hER function and which may be involved in hormone binding; within these regions, we have defined particular residues which are important in hER function. These residues have not to our knowledge previously been reported to be directly involved in hER function. Such data may be of use in the rational design of anti-estrogens for the sequestration of ER in breast cancer therapy.

REFERENCES

1. Whelan J. and Miller N., Generation of estrogen receptor mutants with altered ligand specificity for use in establishing a regulatable gene expression system. *Journal of Steroid Biochemistry and Molecular Biology* **58** (1996) 3–12.
2. Miller N. and Whelan J., Progress in transcriptionally targeted and regulatable vectors for gene therapy. *Human Gene Therapy* **8** (7) (1997) 803–815.

3. Gossen M. and Bujard H., Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the U.S.A.* **89** (1992) 5547–5551.
4. Braselmann S., Graninger P. and Busslinger M., A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. *Proceedings of the National Academy of Sciences of the U.S.A.* **90** (1993) 1657–1661.
5. Wang Y., O'Malley B. W., Tsai S. Y. and O'Malley B. W., A regulatory system for use in gene transfer. *Proceedings of the National Academy of Sciences of the U.S.A.* **91** (1994) 8180–8184.
6. Delort J. P. and Capecchi M. R., TAXI/WAS: A molecular switch to control expression of genes *in vivo*. *Human Gene Therapy* **7** (1996) 809–820.
7. No D., Yao T.-P. and Evans R. M., Ecdysone-inducible expression in mammalian cells and transgenic mice. *Proceedings of the National Academy of Sciences of U.S.A.* **93** (1996) 3346–3351.
8. Rivera V. M., Clackson T., Natesan S., Pollock R., Amara J. F., Keenan T., Magari S. R., Phillips T., Courage N. L., Cerasoli F., Holt D. A. and Gilmen M., A humanised system for pharmacologic control of gene expression. *Nature Medicine* **2** (1996) 1028–1032.
9. Wang Y., DeMayo F. J., Tsai S. Y. and O'Malley B. W., Ligand-inducible and liver-specific target gene expression in transgenic mice. *Nature Biotechnology* **15** (1997) 239–243.
10. Parczyk K. and Schneider M. R., The future of antihormone therapy: Innovations based on an established principle. *Journal of Cancer Research and Clinical Oncology* **122** (1996) 383–396.
11. Cadwell R. C. and Joyce G. F., Mutagenic PCR. *PCR Methods and Applications* **3** (1994) S136–S140.
12. Sikorski R. S. and Hieter P., A system of shuttle vectors and yeast strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122** (1989) 19–27.
13. Geitz D., St. Jean A., Woods R. A. and Schiestl R. H., Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research* **20** (1992) 1425.
14. Garabedian M. J., Genetic approaches to mammalian nuclear receptor function in yeast. *Methods* **5** (1993) 138–146.
15. Cadwell R. C. and Joyce G. F., Randomization of genes by PCR mutagenesis. *PCR Methods and Applications* **2** (1992) 28–33.
16. Green S., Walter P., Kumar V., Krist A., Bornert J. M., Argos P. and Chambon P., Human estrogen receptor cDNA: Sequence, expression and homology to verb-A. *Nature* **320** (1986) 134–139.
17. Stemmer W. P. C., Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370** (1994) 389–391.
18. Fawell S. E., Lees J. A. and Parker M. G., A proposed consensus steroid binding sequence: A reply. *Molecular Endocrinology* **3** (1989) 1002–1005.
19. Pakdel F. and Katzenellenbogen B. S., Human estrogen receptor mutants with altered estrogen and anti-estrogen ligand discrimination. *Journal of Biological Chemistry* **267** (1992) 3429–3437.
20. Wrenn C. K. and Katzenellenbogen B. S., Structure–function analysis of the hormone binding domain of the human estrogen receptor by region-specific mutagenesis and phenotypic screening in yeast. *Journal of Biological Chemistry* **268** (1993) 24089–24098.
21. Danielan P. S., White R., Hoare S. A., Fawell S. E. and Parker M. G., Identification of residues in the estrogen receptor that confer differential sensitivity to estrogen and hydroxytamoxifen. *Molecular Endocrinology* **7** (1993) 232–240.