Yeast Two-hybrid System Demonstrates That Estrogen Receptor Dimerization Is Ligand-dependent in Vivo*

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Previous studies using in vitro procedures have not clearly established whether the estrogen receptor (ER) acts as a monomer or dimer in the cell. We have used the yeast two-hybrid system as an in vivo approach to investigate the dimerization of the estrogen receptor in the absence and presence of estrogen and anti-estrogens. This system is independent of ER binding to the estrogen response element. Two vectors, expressing GAL4 DNA binding domain-human ER and GAL4 transactivation domain-human ER, were constructed. Control experiments showed that each fusion protein had a high affinity binding site for estradiol-17β and could transactivate an ERE-LacZ reporter gene in yeast similar to the wild type ER. The two fusion proteins, GAL4 DB-hER and GAL 4 TA-hER, were expressed in the yeast strain, PCY2, which carries a GAL1 promoter-lacZ reporter. ER dimerization was measured via reconstitution of GAL4 through interaction of the fusion proteins, which transactivates LacZ through the GAL1 promoter. When both ER fusion proteins were expressed, β-galactosidase activity was estradiol-17β-inducible. Furthermore, we showed that both tamoxifen and ICI 182,780 also induced β-galactosidase activity, albeit lower than that induced by estradiol-17β. These results strongly argue that ER dimerization is ligand-dependent and the dimer can be induced by estradiol-17β, tamoxifen, or ICI 182,780. We also treated the yeast containing the two fusion proteins with estradiol-17β and tamoxifen or ICI 182,780 simultaneously to determine the effects on ER dimerization. β-Galactosidase activity was lower when the yeast was treated with a higher ratio of tamoxifen or ICI 182,780 to estradiol than estradiol-17β alone. Taken together, we conclude that ER dimerization is ligand-dependent, with tamoxifen, or ICI 182,780-dependent, and we suggest that estradiol-17β-induced dimers are destabilized when estradiol-17β is used with tamoxifen or ICI 182,780 simultaneously.

The estrogen receptor (ER)² is an intracellular protein that mediates the actions of estrogens in target cells. The ER is a member of a superfamily of related nuclear proteins which includes receptors for steroid hormones, thyroid hormones, vitamin D, the retinoids, and a number of proteins with high sequence homology but as yet unidentified ligands. These receptors are ligand-inducible transcription factors which bind to their specific DNA targets, termed response elements, to regulate transcription. Based on sequence homology and other approaches, the estrogen receptor protein can be divided into six functionally and physically independent domains (A–F) (1, 2). These domains are required for DNA binding (region C), nuclear localization (region D), and steroid binding (region E). The ER has two well characterized transcriptional activation functions, AF-1, which is located in the N-terminal A/B region, and AF-2, which is located in region E and whose activity is ligand-dependent. The DNA binding domain and the hormone binding domain have both been reported to contribute to ER dimerization.

Estrogen action on target cells involves a distinct pathway where estradiol freely diffuses across the cell membrane and binds to its receptor. This ligand-receptor complex is thought to homodimerize and bind tightly to the estrogen response element (ERE). After binding DNA, ER activates transcription of its target genes by as yet unknown mechanisms. In this system, there has been some controversy as to whether the ER acts as a monomer or a dimer in the cell. It has been traditionally believed that estrogen induces dimerization of ER and hence the DNA binding (1). The ER has been shown to form stable homodimers in solution (3), and several studies have given evidence that the ER and other nuclear receptors bind to response elements as dimers (4–7). However, Gorski et al. (8) have proposed a model where the ER protein binds to an ERE as a monomer or perhaps forms heterodimers with other nuclear proteins, as is the case with thyroid hormone receptors (9). Most of the data for and against dimerization of ER have been shown using gel mobility shift assays or complex assays in vitro, where the assay itself requires ER to bind DNA. These assays have given conflicting evidence in demonstrating whether estrogen is required (4) or not required (10–12) for high affinity binding of the ER to the ERE. It still remains unclear if the ER can form a dimer in vivo, whether estrogen has any effects on dimerization and whether the dimer is formed before or after binding the ERE.

Using similar in vitro assays, the action of anti-estrogen on the ER pathway has been investigated at the levels of dimerization and/or DNA binding of ER. The ER, bound with an anti-estrogen such as tamoxifen or ICI 164,384, was shown to form a ligand-ER complex which can bind the ERE (13, 14). Furthermore, ICI 164,384 and tamoxifen have been shown to induce DNA binding (15) and activate ER transactivation in yeast (16). However, Parker and co-workers (6, 17–19) reported that ICI 164,384 and ICI 182,780 prevent the ER from binding

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1 The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; GAL4 TA, GAL4 transactivation domain; GAL4 DB, GAL4 DNA-binding domain; ERE-17β, estradiol-17β; ICI, ICI compound; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; ONPG, o-nitrophenyl-β-D-galactoside.
ERE. Based on their previous study of mutant mouse ERs and in vitro DNA binding ability of ER, which had assumed that an ER dimer is required for binding to DNA (6, 20), they concluded that ICI 164,384 and ICI 182,780 prevent ER dimerization.

Since the effect of ligand on the ER-ERE dimerization is still unclear and the actions of estrogen and anti-estrogens can be hypothesized to involve differences in ER dimerization, which may affect ER/DNA binding, we have used the yeast two-hybrid system, which is independent of the interaction of ER with ERE, to study ER protein dimerization in vivo. The yeast two-hybrid system has been described by Fields and co-workers (21, 22). It involves the expression of a LacZ reporter gene under the control of a GAL4-activated promoter (GAL1 promoter) that depends on the reconstitution of GAL4 activity via protein-protein interactions. This is accomplished by apposition of the GAL4 DNA binding (GAL4 DB) and transcription activation (GAL4 TA) domains via interaction of polypeptides fused to each domain. Colonies containing interacting polypeptides are detected with a chromogenic substrate for β-galactosidase activity via protein-protein interactions. This system has also been used successfully in screening a cDNA library (23–25) and has turned out to be a useful approach to study protein-protein interactions in vivo.

In our experiments, the human ER cDNA was cloned into the GAL4 fusion vectors, pPC62 (GAL4 DB) and pPC86 (GAL4 TA) (24), to study the dimerization of ER and the effects of ligands on dimerization. Using this yeast two-hybrid system, we show that the ER-ER interaction is estrogen-dependent in vivo. We also show that the anti-estrogens, tamoxifen and ICI 182,780, can induce ER dimerization. Dimerization, however, is perturbed when estrogen is used with anti-estrogens simultaneously. The implications of these data on the ER-ER dimer are discussed.

MATERIALS AND METHODS
Yeast Strains and Methods
Yeast strain PCY2 (MATα 3αgal 1gal80 URA3;GAL1-lacZ; lys2-801;咏咏 his3-a2 tcr1-p1-163[au2ade2-101[stry]v]) was obtained from Dr. Nathans and Dr. Chervaj, who are the product of a cross between GGY1::171 (26) and YPH499 (27). Yeast strain RS188N (MATα ade2-1 his3-a1 iura2-112 tcr1-1 ura3-1) is a "leaky" yeast strain, which is a gift from Dr. T. Butt (16). Yeast strains were grown in yeast extract/peptone/dextrose (YPD) or supplemented synthetic dextrose medium (28). Transformation of yeast was carried out using the lithium acetate method with plasmid DNA (28). Yeast colonies transformed with the fusion vectors were selected by culture on synthetic medium lacking uracil, tryptophan, and/or leucine.

cDNA and Constructs
Construction of GAL4-ERE-hER fusion vectors involved cloning human ER cDNA into the pPC62 (GAL4 DB) and pPC86 (GAL4 TA) fusion vectors (kindly provided by Drs. Chernay and Nathans). Human ER cDNA (hER) was kindly provided by Dr. Katzenellenbogen, who was placed into the Sac site of pCMV-5 (Dr. David Russell, University of Texas Southern Medical Center) (29). We subcloned the hER cDNA digested with Sall into pBluescript II SK+ at the Sall site such that its transcription is dependent on T7 polymerase (T7-HER). T7-HER was amplified with T3 primer and a oligonucleotide ('5'-GGGGATCGTC-AGACTCGGTCTGCA-3'). This was designed by inserting a Sac site and an extra T base before the ER start codon to keep the correct reading frame intact in the GAL4-ERE vector. The PCR product was directly cloned into GAL4 DB and GAL4 TA at the Sac site. The GAL4 DB-ERE vector and GAL4 TA-ERE fusion cDNAs were then sequenced to confirm correct reading frame before transforming yeast.

The yeast two-hybrid system control vectors, GAL4 TA-bz-c-jun, GAL4 DB-bz-c-jun, GAL4 TA-bz-cfos, and GAL4 DB-bz-cfos, were kindly provided by Dr. Chernay (24). The yeast ERE-lacZ reporter (YReE2) was used in the leaky yeast experiments were kindly provided by Dr. T. Butt (16).

Ligand Treatment and β-Galactosidase Activity Assays
In all cases, β-galactosidase activity, which was the product of LacZ driven by the GAL1 promoter, was used to reconstitute the transcriptional activity of the two fusion proteins. β-Galactosidase activity was quantified and qualified in various tests. For qualifying activity, yeast colonies transformed with the fusion vectors were selected by culture on synthetic medium lacking uracil, tryptophan, and/or leucine and transferred to nitrocellulose filters. The yeast colonies on the filters were cultured by placing the filters on Whatman No. 3MM filter paper soaked with medium with or without the addition of steroid hormones for 3–12 h. Estradiol-17β, tamoxifen, progesterone, dexamethasone, testosterone, and medroxyprogesterone acetate were obtained from Sigma, and ICI 182,780 was obtained from ICI Pharmaceuticals. The yeast treated with 0.1–0.3% ethanol (the final concentration) was used as the vehicle control. Yeast colonies on the nitrocellulose filter were then frozen with liquid nitrogen for a few seconds followed by the X-gal induction reaction. β-Galactosidase activity of liquid yeast cultures was quantified as described (28).

Transformed yeast were selected and then cultured in synthetic medium (in the case of β-galactosidase activity assays, glucose was replaced with galactose). The agonist and/or antagonists were added to the yeast liquid culture after the cells had to late log phase. After 60 min of incubation, the cells were assayed by the β-galactosidase activity assay. The results were collected with low speed centrifugation. The yeast cells were resuspended in an equal volume of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol, pH 7.0) and placed on ice. The 1 ml of reaction mixture was made up of 0.1 or 0.05 ml of cells in Z-buffer (the value of β-galactosidase activity is nitrogen for a few seconds followed by the X-gal induction reaction. β-Galactosidase activity of liquid yeast cultures was quantified as described (28).

Quantitative Aspects of GAL4-HER Fusion Proteins
Ligand Binding Assays—The estradiol-17β binding capability of GAL4 DB-HER and GAL4 TA-HER were characterized from yeast extract (30, 31). The yeast extract were obtained by "glass bead vortexing" the transformed yeast cells in 10 ml Tris-HCl, pH 7.4, 400 mM KCl, and 1 mM PMSF. After centrifugation (14,000 × g, at 4°C for 30 min, the supernatant was incubated overnight at 4°C with [1H]estradiol-17β (Amersham Corp., 93 Ci/mmol) in presence (nonspecific binding) or absence (specific binding) of 100-fold molar excess of unlabeled estradiol-17β. The free and bound ligands were separated using dextran-coated charcoal. Radioactivity of each fraction was determined, and the specific binding was obtained by subtraction of nonspecific from total binding. The Kd of the GAL4-HER fusion proteins was determined by Scatchard analysis.

GAL4-HER transactivation activity in vivo—The transcriptional activity of GAL4-HER fusion proteins upon treatment of various concentrations of estradiol-17β was tested using the yeast strain RS188N (16). In this yeast strain, response to estradiol-17β was measured at the physiological concentration of the hormone (16). The GAL4 TA-HER and/or GAL4 DB-HER vectors were transformed into yeast strain RS188N with ERE-lacZ. The transfectants were selected using synthetic medium lacking tryptophan, leucine, and uracil. The yeast carrying HER fusion protein(s) and ERE-lacZ reporter were cultured in liquid medium and treated with various concentrations of estradiol-17β for 6 h. The transcriptional activity in response to estrogen was analyzed using the ONPG reaction.

Quantitative Aspects of GAL4-HER Fusion Proteins
Immunoblotting was used to quantify the amount of intact GAL4-HER fusion proteins. Total protein was extracted from the yeast as follows. The yeast liquid culture stimulated with estrogen agonist or antagonists for various times (described above) were collected by low speed centrifugation. The yeast pellets were washed with the same volume of cold acetone, allowed to air dry, and resuspended in 0.2 ml SDS-polyacrylamide gel electrophoresis sample buffer. Equal amounts of total protein were analyzed on 8% SDS-PAGE gels, electrophoresed and transferred to polyvinylidene difluoride membrane (Immobilon-P Millipore). After transfer, the blots were stained with...
The percentage of β-galactosidase-positive colonies after various treatments on the filter for X-gal reaction.

| Treatment | GAL4 TA-hER | GAL4 DB-hER* | GAL4 TA-hER and GAL4 DB-hER |
|-----------|-------------|--------------|---------------------------|
| No treatment | 0% | 0% | 1.3% |
| 0.2% ethanol | 0% | 0% | 2.3% |
| 0.1 µM E_2-17β | 0% | 0% | 100% |
| 100 µM E_2-17β | 0% | 0% | 100% |
| 5 µM TAM | 0% | 0% | 100% |
| 5 µM ICI | 0% | 0% | 100% |
| 100 µM TAM + 1 µM E_2-17β | 0% | 0% | 100% |
| 100 µM ICI + 1 µM E_2-17β | 0% | 0% | 100% |
| 5 µM TAM + 5 µM ICI + 1 µM E_2-17β | 0% | 0% | 100% |

* Some yeast colonies (<3%) carrying only GAL4 DB-hER showed pale blue when the X-gal reaction was taken for a longer time (>72 h). The quantitative value of β-galactosidase activity were determined using the ONPG reaction (see Figs. 1, 5, and 6).

0.5% Ponceau S Red to monitor transfer efficiencies and subsequently probed with the anti-ER antibody.

**RESULTS**

Neither GAL4 DB-hER nor GAL4 TA-hER Alone Activates the Transcription of GAL1 Promoter Driving LacZ—Since ER has two independent transcriptional activation domains (AF-1 and AF-2), as well as a DNA binding domain, it was unknown whether ER itself could activate transcription of the GAL1-lacZ reporter gene. Therefore, we first examined β-galactosidase activity in yeast carrying only one of the fusion proteins. Human ER cDNA was cloned into the GAL4 fusion vectors (GAL4 DB and GAL4 TA) and introduced into PCY2 yeast to express GAL4 DB-hER or GAL4 TA-hER, respectively. β-Galactosidase activity in the yeast colonies selected with Trp or Leu dropout medium was determined using X-gal indicator (Table I). No blue colony was found on filters where yeast containing only GAL4 TA-hER were growing. After the yeast was treated with estrogen, the same result was observed. GAL4 TA-hER yeast in absence or presence of estrogen showed no significant β-galactosidase activity (less than 0.01 unit). Because this fusion protein does not have the GAL4 DNA binding domain, it fails to localize to the specific upstream DNA sequences necessary for GAL1-lacZ transcription. In yeast carrying only the GAL4 DB-hER, no blue colony was found during our assay period (Table I). Only during prolonged treatment times (72 h) were a few pale blue colonies found (usually <1%) in the absence or presence of E_2-17β. This fusion protein can bind to upstream sequences in the GAL1-lacZ promoter, but does not significantly activate the reporter gene. This result is supported by the finding that GAL4 transactivation is believed to be mediated by its acidic stretches of amino acids in the activation domain, whereas the two independent transcription domains of human ER are non-acidic (32). In control yeast cotransformed with the fusion vectors alone (GAL4 DB and GAL4 TA), there was no detectable β-galactosidase activity. We conclude that GAL4 DB-hER or GAL4 TA-hER fusion proteins alone do not contain significant GAL4 transactivation properties.

The Interaction of GAL4 DB-hER and GAL4 TA-hER Fusion Proteins in Yeast Is Estradiol-17β-dependent—Next, we sought to examine the interaction of GAL4-hER fusion proteins in yeast in the absence and presence of estradiol-17β. For this purpose, PCY2 yeast was cotransformed with GAL4 DB-hER and GAL4 TA-hER and selected in Trp and Leu dropout medium. β-Galactosidase activity was then measured before and after the treatment of estradiol-17β (1 µM). The estradiol-17β treatment led to a dramatic change in the percentage of blue colonies: 100% of the yeast carrying both GAL4 DB-hER and GAL4 TA-hER were blue (Table I). In comparison, the yeast carrying only GAL4 DB-hER showed 3% blue colonies even after longer exposure (>72 h) to X-gal, and the color remained very light. The result was confirmed by the β-galactosidase activity assay of liquid culture of 1 µM estrogen-treated yeast with or without GAL4 TA-hER and/or GAL4 DB-hER (Fig. 1). We also tested the role of varying concentrations of estradiol-17β on induction of β-galactosidase activity. In this experiment, β-galactosidase activity was measured at different times after adding estradiol-17β (0.1% ethanol)-treated yeast (4.0 units). These results indicate that β-Galactosidase activity was determined using ONPG reaction. The yeast carrying only GAL4 DB-hER was also used as a control (● treated with 10^{-6} M of estradiol-17β). β-Galactosidase activity was measured as a function of time after the yeast was stimulated with estradiol-17β. The error bar stands for the S.D. of population (n = 8).

![FIG. 1. The induction of β-galactosidase by estradiol-17β in the yeast (PCY2) carrying both GAL4 DB-hER and GAL4 TA-hER fusion vectors. The yeast liquid culture was treated with different concentrations of estradiol-17β (●, 10^{-6} M; ■, 10^{-7} M; ◆, 10^{-8} M; ○, 10^{-5} M), and β-galactosidase was determined using ONPG reaction. The percentage of β-galactosidase-positive colonies after various treatments on the filter for X-gal reaction was taken for a longer time (>72 h). The quantitative value of β-galactosidase activity were determined using the ONPG reaction (see Figs. 1, 5, and 6).](image)
β-β-Galactosidase activity induced by the ER dimerization compared with that induced by the β-J un/β-Fos (100%) and the β-J un/β-Fos dimers in the same yeast strain (PCY2). The yeast, PCY2, was cotransfected with GAL4 DB-βz-c-Fos and GAL4 TA-βz-c-Fos and GAL4 TA-βz-c-Fos showed no significant value of β-galactosidase. The β-J un/β-Fos heterodimer induced activity was about 20% of wild type GAL4. These data were similar to Chevray and Nathans (24). A asterisk, the yeast was treated with 1 μM of estradiol-17β (final concentration) for 12 h. The error bar represents the S.D. (n = 4).

tosidase activity of that by the J un/Fos dimer and was 25 times stronger than that by the J un/Jun dimer (Fig. 2). We conclude that the ability of ER to dimerize is significantly enhanced by estradiol-17β. In the absence of estrogen, ER seems to exist as a monomer (or forms a heterodimer/oligomer with other proteins), but there seems to be little or no direct ER-ER interaction.

GAL4-ER Fusion Proteins Bind Estradiol-17β with High Affinity and Are Transcriptionally Active in Yeast—Since the concentration of estrogen necessary for significant β-galactosidase activity was higher than the typical mammalian physiological concentration (1 nM), and induction time was at least 3 h, we decided to measure the affinity of the fusion proteins for estradiol and to determine if these proteins were transcriptionally active. Saturation and Scatchard plots were used to determine the binding capability of ER to estradiol-17β, showing a high affinity binding site having a KD of 0.84 nM for GAL4 TA-HER and 1.4 nM for GAL4 DB-HER (Fig. 3). These values show a slightly reduced affinity for estradiol when compared with previous data regarding hER from MCF7 cells (30, 31); KD of 0.6 nM (30, 31); KD of 0.6 nM (30, 31); KD of 0.6 nM (30, 31); KD of 0.6 nM (30, 31); KD of 0.6 nM (30, 31).

To determine if GAL4 DB-HER and GAL4 TA-HER were functional as compared with wild type hER, we measured the ability of these proteins to transcriptionally activate an ERE-lacZ reporter in hyperpermeable (leaky) yeast. Using leaky yeast allowed us to eliminate any questions regarding the ability of the yeast to take up estradiol-17β. Unfortunately, a leaky yeast strain is not available currently for use in the two-hybrid system. ERE-lacZ was cotransformed into RS188N yeast either with GAL4 DB-HER or with GAL4 TA-HER. The ability of each fusion protein to activate transcription of the reporter gene was highly regulated by estradiol-17β, showing similar activity in response to the various concentrations of estradiol-17β, from 1 nM to 1 μM (Fig. 4). These data are in good agreement with the transcriptional activity reported with the wild type hER used in the same yeast strain (16). Therefore, we can conclude that GAL4 DB-HER and GAL4 TA-HER fusion proteins expressed in yeast are functional; they bind estradiol-17β with high affinity and show similar estrogen-dependent gene regulation as compared with the wild type hER.

The GAL4-hER expression vectors together with ERE-lacZ reporter were introduced into the leaky yeast strain RS188N. For estradiol-17β concentrations of 10−6 to 10−4 M for 6 h as described (16). The transcriptional activity of GAL4-HER fusion proteins, via β-galactosidase activity under the control of ERE, was determined using the ONPG reaction described under “Material and Methods.” The error bar represents the S.D. (n = 4). The longer time treatments of ligand (9, 12, and 24 h) showed similar results (data not shown).

The Dimerization of ER Can Be Induced by Tamoxifen or ICI 182,780—Since anti-estrogens may mimic or inhibit estrogen induction at various levels in the estrogen-dependent transactivation pathway, we next examined whether the anti-estrogens tamoxifen and ICI 182,780 induce β-galactosidase activity via the ER dimerization. Because tamoxifen is a partial anti-estrogen (36), and ICI 182,780 is a pure anti-estrogen (37, 38), it was likely that β-galactosidase activity could be positively affected in the tamoxifen induction and negatively affected in the ICI induction. In this experiment, we treated yeast (PCY2) harboring GAL4 DB-HER and GAL4 TA-HER with three different concentrations of anti-estrogens, 10−5 M, 10−6 M, and 10−7 M. Compared with the activity induced by estradiol-17β after 2 h, we observed that there was about 20% β-galactosidase ac-
tivity in ICI 182,780 induction and 15% activity in tamoxifen induction (10^{-6} M ligand treatment) (Fig. 5). These relative anti-estrogen-induced β-galactosidase activities indicated that the ER-ER interaction could be induced by anti-estrogens. These results were also corroborated by counting the blue yeast colonies on the filter using X-gal induction (Table I). There was no difference in the percentage of blue colonies on the filter treated with estradiol-17β, tamoxifen, or ICI 182,780; all were 100%. We did notice, however, that the color of the colonies treated with anti-estrogens were lighter than those treated with estradiol-17β during the same exposure time to X-gal. As for the control, all three compounds showed no significant induction of β-galactosidase activity in the yeast carrying only one of the fusion proteins. Moreover, these compounds did not affect the growth property of yeast.

The Effects of Tamoxifen and ICI 182,780 on Estradiol-17β-induced ER Dimerization in Yeast—Since it is widely believed that anti-estrogens can affect the estrogen-induced pathway through interaction with ER, we decided to test the hypothesis that among other possible mechanisms, anti-estrogens destabilize the ER-ER dimer formation. To test our hypothesis, we treated the yeast with a mixture of estrogen and anti-estrogen, with ratios of 1:0, 1:0.5, 1:1, 1:5, and 1:10 respectively, where the concentration of estradiol-17β was used as 1 μM. Using the ONPG assay, we measured the β-galactosidase activity in yeast cells containing fixed concentrations of estradiol-17β with increasing concentrations of either tamoxifen or ICI 182,780 (Fig. 6). Both tamoxifen and ICI compound caused a decrease in β-galactosidase activity, reflecting either de-dimerization or prevention of dimerization. Whereas tamoxifen caused a 70% decrease, ICI 182,780 caused a 50% decrease in β-galactosidase activity. These results would suggest that tamoxifen is significantly more effective in destabilizing estradiol-17β-induced ER-ER dimer formation. To determine whether the lower β-galactosidase activity induced by the mixture of higher ratio of anti-estrogen to estrogen was related to the anti-estrogen-induced rapid degradation of ER, immunoblotting was used to show that there was no ligand induced rapid turnover of the receptor and that the GAL4-ER fusion protein levels were not effected by estrogen or anti-estrogen (Fig. 7).

### Table I

| Hormone | Control | Estradiol-17β | ICI 182780 | Tamoxifen |
|---------|---------|--------------|------------|-----------|
| Concentration (M) | 0.2% Ethanol | 10^{-6} | 10^{-6} | 10^{-6} | 10^{-6} | 10^{-6} |

FIG. 5. Induction of β-galactosidase activity in the yeast carrying both GAL4 DB-hER and GAL4 TA-hER fusion vectors by estrogen agonist and antiestrogens. The yeast liquid culture was treated with different concentration (nM) of estradiol-17β, ICI 182,780, or tamoxifen as indicated. The activity was determined at different times after the yeast was stimulated with hormone. The error bar stands for the S.D. of population (n = 8).

FIG. 6. Induction of β-galactosidase activity in the yeast carrying both GAL4 DB-hER and GAL4 TA-hER fusion vectors treated with a mixture of estrogen agonist and antiestrogens. Yeast co-transformed with GAL4 DB-hER and GAL4 TA-hER was exposed simultaneously to a combination of varying concentrations of estrogen and anti-estrogens. After 6, 9, and 12 h, the activity of β-galactosidase was determined in each treatment group. Relative β-galactosidase activity is presented. Asterisk, the blank control (without any treatment); Double asterisks, 0.3% ethanol treatment was used as a vehicle control. The error bar stands for the S.D. of population (n = 8).

FIG. 7. Levels of GAL4 fusion proteins in the yeast treated with estradiol-17β, ICI 182,780, or tamoxifen. The GAL4 DB-hER (756 amino acids) and GAL4 TA-hER (747 amino acids) fusion proteins were detected using anti-hER antibody H222 using Western blot analysis. The protein samples were extracted from the yeast carrying only GAL4 TA-hER (lane 1), GAL4 DB-hER (lane 2), or both GAL4 TA-hER and GAL4 DB-hER (lanes 3–12). The yeast carrying both fusion proteins had been treated with 10^{-6} M of estradiol-17β (lanes 4–6), ICI 182,780 (lanes 7–9), or tamoxifen (lanes 10–12) for different time as indicated. The yeast carrying the GAL4 DB fusion vector (pPC 62) was used as a negative control (lane 0).

DISCUSSION

Previous studies using in vitro procedures have not clearly established whether the ER acts as a monomer or dimer in the cell. We have used the yeast two-hybrid system as an in vivo approach to examine the dimerization of the ER in response to estrogen and anti-estrogens. Because the yeast two-hybrid system is performed in vivo, the proteins involved are more likely to be in their native conformations than in an in vitro assay. In this system, the dimerization of the ER is ligand-dependent, as measured by the reconstitution of GAL4 activity from GAL4-ER fusion protein interaction. Estradiol-17β is quite effective in inducing dimerization of the ER, whereas tamoxifen and ICI 182,780 also induce ER dimerization, but less effectively.

Understanding the action of transcription factors such as the estrogen receptor in vivo will greatly enhance our ability to design therapeutic modalities for breast and uterine cancers. Analysis of steroid receptor interactions in a simple eukaryote,
like Saccharomyces cervisiae offers the ease with which multiple analyses can be performed and the molecular genetics of the system exploited. The basic transcription machinery is remarkably conserved between mammals and yeast. The large subunit of RNA polymerase II from human cells and yeast show considerable homology (39, 40). Besides the high degree of structural homology in the functional region, the TATA box-binding protein, TFIIID, is functionally interchangeable between yeast and human TFIID (41). As for the yeast transcriptional factors, GAL4 regulation functions in animal system (42). Conversely, the human estrogen receptor has been shown to activate transcription in a hormone-dependent manner in yeast (30, 31, 43, 44). Yeast have also been used as a system to overexpress functional ER (45) and to study the effects of estrogen agonist and antagonists on ER-dependent transactivation (16).

In this report, we constructed GAL4-hER fusion proteins to investigate ER dimerization in the yeast two-hybrid system. Since dimerization of the ER has been highly correlated to the DNA binding domain (46), it has been difficult to study the dimerization and the DNA binding independently. Previous studies have relied upon the gel retardation assay to investigate dimerization of the ER in vitro. Evidence for the existence of the ER dimer has depended on its DNA binding ability (4, 6, 20). In addition, the hormone binding domain has also been thought to contribute to ER dimerization (47). Recently, using in vitro experiments, it has been reported that the human ER hormone binding domain dimersize independently of ligand activation (35). In contrast, other DNA binding assays have shown that ER is capable of binding as a monomer to a thyroid hormone response element consisting of an inverted palindrome without spacing (48), and that ERE binding requires neither the ER-ER homodimer nor estrogen (49). Furthermore, the ER also binds as a monomer to half-palindromic ERES (50), and a half-site of ERE in the c-jun gene turns out to be a strong regulatory element in response to estrogen induction (51). Therefore, it seems that the dimerization of ER is not directly correlated to its DNA binding capability and transactivation, and a DNA band shift or ER transactivation cannot be used reliably as an indicator for the existence of an ER dimer in vivo. We have exploited the yeast two-hybrid system, which is independent of this ERE binding requirement, as an in vivo system to investigate the dimerization of the ER.

We have clearly shown that \( \beta \)-galactosidase activity was under estrogen control through ER interaction. Other steroid hormones such as progesterone, medroxyprogesterone acetate, dexamethasone, and testosterone do not induce ER interaction as measured in these yeast. Furthermore, we examined the effects of tamoxifen and ICI 182,780 on ER dimerization, and the results strongly argued that ER dimerization is ligand-dependent in each case. However, the effective concentrations (\( 10^{-5}, 10^{-6}, 10^{-7} \)m) used in our experiments were much higher than the mammalian physiological concentration (\( 10^{-9} \)m). It is possible that the estrogen agonist and antagonists cannot sufficiently penetrate the yeast, especially through the cell wall, and thus the low concentration was ineffective. Indeed, most work using estradiol-17\( \beta \) in yeast to investigate ER has used concentrations at \( 10^{-6} \)m due to the low permeability of yeast to various compounds (30, 31, 52). Recent work has been done using hyperpermeable (leaky) yeast, in which the physiological concentration (\( 10^{-9} \)m) of estradiol-17\( \beta \) is enough to induce significant values of ER-dependent transcription (16).

Using the same leaky yeast strain (RS188N), we showed that the GAL4-hER fusion proteins were functional, being able to transactivate an ERE \( \beta \)-galactosidase reporter at the physiological concentration of estradiol-17\( \beta \) (Fig. 4). It is evident that these ER fusion proteins respond to estrogen normally as compared with wild type human ER expressed in yeast (16). Ligand binding assays show that the GAL4 DB-hER (KD = \( 0.84 \times 10^{-9} \)m) and GAL4 TA-hER (KD = \( 1.4 \times 10^{-9} \)m) fusion proteins each have a high affinity binding site for estradiol, slightly lower than that reported for the wild type hER expressed in MCF-7 cells (KD = \( 6.0 \times 10^{-9} \)m) or in yeast (KD = \( 0.5 \times 10^{-9} \)m) (30, 31). It is reasonable to suggest that the higher KD may have necessitated higher concentrations of ligand needed to contribute to dimerization in our assay, which would not be available if the in vivo concentration of ligand was lower than that in the medium due to low permeability to these compounds in the PCY2 yeast strain. In addition, the requirement of high concentration of ligand could also have resulted from the limitation of the sensitivity of the assay, since we can only detect about 50\% of the potential dimers formed. GAL4 DB-hER/GAL4 TA-hER, GAL4 TA-hER/GAL4 TA-hER, and GAL4 DB-hER/GAL4 TA-hER can each dimerize, but only the last two can be detected using this system. As for the low anti-estrogen-induced \( \beta \)-galactosidase activity, it might be argued that intake of anti-estrogens into yeast is lower than that of estradiol-17\( \beta \) (52). However, the decreased activities in the mixture of estradiol-17\( \beta \) and anti-estrogen strongly support our contention that there is no significant difference between estradiol-17\( \beta \) and tamoxifen or ICI in entering yeast.

A major limitation of our work is that we cannot quantitate the ER dimer in the yeast. We used the homodimerization of c-jun or c-Fos as positive or negative controls, respectively, and compared these levels with those of ER-ER dimerization. Using the yeast two-hybrid system, Chevray and Nathans (24) have shown that c-jun, but not c-Fos, can form stable homodimers. We can see through the yeast system that ER-ER dimerization is 11 times less efficient than c-jun/c-jun interaction, a transcriptional factor that has been shown to act as a homodimer (Fig. 2). Our results show that there is no significant dimerization between c-Fos/c-Fos or ER-ER untreated with steroid hormones. Since approximately 40\% of the hER expressed in yeast has been reported to be able to bind ligand (16), it is possible that the dimerization of ER in estrogen-responsive cells may be somewhat stronger than that measured in our assay.

The molecular mechanism of the different efficiency of dimer formation by the different ligands, however, is still unclear. Since the ability of ER to discriminate between estradiol-17\( \beta \), tamoxifen, and ICI has been shown previously by hormone binding, DNA binding, and transactivation assays, it is likely that estrogen and anti-estrogens bind ER and induce different conformational changes, resulting in different effects upon dimerization. Considering that the data from GAL4-hER fusion proteins are unrelated to ER binding to ERE, our results suggest that the dimerization and the DNA binding (ERE) are separate events. However, whether this is true in an estrogen-responsive mammalian cell is still unclear. It is also unclear if these dimers can bind DNA or are required for DNA binding for the same reason. The dimerization could be affected by DNA binding (ERE), because the hormone binding domain can be allosterically modulated by DNA (ERE) (53). In fact, the evidence that the DNA binding domain is important for dimerization (46, 54) may suggest that the ERE may influence the efficiency of ER dimer formation. The relationship among estrogen- or anti-estrogen-induced dimerization of ER with DNA binding and transactivation deserves further investigation.

In our experiments, we showed that the dimerization is less
effective when estrogen and anti-estrogen are used simultaneously than when estrogen is used alone. It is difficult to explain the ability of anti-estrogen alone to induce ER dimerization. However, the assay used in our experiments argues persuasively that anti-estrogens do induce ER dimerization, albeit to a lower extent. Among several possible explanations to our observation, we offer a model in which the yeast two-hybrid system is detecting protein-protein interaction(s) of one or more ER-associated proteins, which would not require direct ER dimerization, but where ER is involved. Only upon addition of ligand would the proper conformational change of ER take place to allow ER to then bind the protein in question. The protein-binding interaction would then cause subsequent conformational changes in the binding protein, which would then allow, and be required for, the binding protein to dimerize, thus forming an oligomeric complex of GAL4 DB-HER, ER-binding protein:ER-binding protein, GAL4 TA-HER. Dimerization of the binding protein, but not direct dimerization of ER, would thus be detected. Recently, three estrogen receptor-associated proteins of 160, 140, and 80 kDa have been identified (55, 56), each satisfying the requirements of this model. These estrogen receptor-associated proteins only bind to ER in the presence of estrogen, whereas tamoxifen and the pure anti-estrogens block ER-estrogen receptor-associated protein complex formation. This model reflects that ER-ER interaction takes place in a large complex with the aid of adapter proteins, such as the estrogen receptor-associated proteins.

The antagonistic activity of tamoxifen and the ICI compound may occur at several levels of the estrogen transactivation pathway (57). It is obvious that the effects of tamoxifen and ICI compounds on ER dimerization and DNA binding alone are not enough to explain the antagonistic function of anti-estrogens. Instead, anti-estrogens may actually mimic estrogenic action somewhat at the two steps of dimerization and DNA binding. At other levels of the estrogen pathway, the differential structure of ER-anti-estrogen complexes bound to the ERE (14), their different DNA binding abilities (13, 14, 58), as well as the ligand dependence of ER induced changes in chromatin structure (59, 60) have all been proposed to be responsible for the antagonistic activity of anti-estrogens. Furthermore, if ICI-induced fast turnover of ER (61, 62) and the disruption of ER nucleocytoplasmic shuttling (63) have also been suggested to contribute to IC1 antagonism. Therefore, it appears that anti-estrogenic action on the estrogen pathway may be more complex than previously believed.

In conclusion, our observations emphasize that ER dimerization is an estrogen-inducible event in vivo. Tamoxifen or ICI 182,780 also induces ER dimerization at a lower level, and tamoxifen or ICI 182,780 makes the dimerization ineffective when it is used with estrogen simultaneously. The establishment of this inducible ER-ER interaction within the yeast two-hybrid system sets up a good system to further investigate ER and its associated proteins, as well as a possible system to test effectiveness of drugs in disrupting ER dimerization. Further investigation using this system for ligand induced ER-ER dimerization will offer more detail in the understanding of the function of the ER dimer.

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