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A yeast-based bioassay for the determination of functional and non-functional estrogen receptors

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ABSTRACT

The response to endocrine therapy of breast cancer is not entirely predictable from hormone receptor status alone since some point mutated or splicing variants of the estrogen receptor (ER) show altered biological activities. In order to characterize the activities of all forms of ER in a heterogeneous breast tumor, a functional assay in *Saccharomyces cerevisiae* was developed. Total RNA isolated from breast cancer cells and one breast cancer specimen was reverse transcribed and the ER cDNA was amplified by PCR. The products were then cloned into an expression vector by *in vivo* homologous recombination in yeast. The yeast strain carries a reporter gene (*ADE2*) coupled to an estrogen response element. Activation of the reporter by ER yielded white colonies whereas lack of ER activity produced red colonies. This permitted the testing for functionality of individual ER molecules and subsequent analysis by rescuing of the ER expression plasmids and complete DNA sequencing. This simple visual test allows discrimination between wild-type ER, constitutively active ER and inactive ER.

INTRODUCTION

The content of estrogen receptor α (ER) in breast cancer tissues is commonly used to identify patients who will most likely respond to adjuvant antiestrogen treatment and hence have a lower risk of relapse and a better overall survival than patients with non-responding tumors (1). However, most tumors that originally respond to endocrine therapy will develop resistance during the course of the treatment, although ER is often still present in the relapse tissues (2). Therefore, resistance to antiestrogen therapy may not develop due to ER loss but due to changes in the ER structure and function (3).

As of today, the role of ER variants in the development and progression of breast cancer is poorly understood and their clinical significance for therapy failure has not been studied systematically. Co-expression of several ER mRNA species, including forms with base pair insertions, transitions and alternate splicing, have been identified in breast cancer cell lines and human breast cancer biopsy samples (4). Some of these aberrant ER forms are present in neoplastic as well as in normal breast tissues and thus appear to be naturally occurring polymorphisms (5–8). However, recent data suggest that breast cancer cells expressing elevated amounts of one of these ER forms, namely the constitutive activated exon 5 deletion variant, might escape antiestrogen treatment and continue to proliferate (8).

Presently used diagnostic methods for ER determination do not discriminate between wild-type and variant receptor forms. The assessments of ER-induced proteins such as the progesterone receptor (PR) are commonly used to estimate the biological activity of the ER in a breast tumor. Consequently, the PR status of a tumor yields a more reliable prognosis with respect to response to endocrine therapy than the ER status alone. However, transcription of the PR gene is not exclusively regulated by the ER (9–12). Thus, it seems likely that a method distinguishing between functional and non-functional ER would give more information for the selection of the appropriate adjuvant therapy.

The use of homologous recombination in yeast allows efficient cloning of PCR products directly *in vivo*. An assay published earlier uses a technique, called functional analysis of separated alleles in yeast (FASAY), for the investigation of p53 functionality in tissue samples (13). Additionally, Metzger *et al*. demonstrated that the ER can be expressed in yeast and is indistinguishable from the ER expressed in human cells (14). Activation of transcription in the yeast system is strictly hormone-dependent. We combined the two findings using *ADE2* as a reporter gene in yeast and established a new screening method to test for ER functionality. This assay allows determination of the ratio of functional, non-functional and constitutive activated ERs in tumor biopsies. Individual ERs, wild-type or mutant, can be rescued from the yeast colonies and further investigated by sequence analysis.

MATERIALS AND METHODS

Cell line and tumor biopsy

MCF-7 breast cancer cells (Mason Research Institute, Rockville, MD) were grown in a humidified atmosphere, containing 5%

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CO_2, in IMEM-ZO (improved minimal essential medium–zinc option; Biological Industries, Kibbutz Beth Haemak, Israel) as described (15), supplemented with 5% (v/v) fetal calf serum, 10 mM HEPES (pH 7.3), 5 µg/ml insulin (Eli Lilly, Indianapolis, IN), 10 U/ml penicillin, 10 U/ml streptomycin, 2 mM L-glutamine and 13.3 µM phenol red. The medium was switched to serum-free medium after cells reached ~80% confluency (16). After 2 days, cells were harvested by trypsinization, washed three times with phosphate-buffered saline (PBS), pelleted by centrifugation and stored at –70°C. The primary tumor specimen was obtained from the tissue collection of the Stiftung Tumorbank Basel and originates from a breast cancer patient who had been treated surgically by wide local excision. The biopsy was shock frozen in liquid nitrogen and stored at –70°C. ER and PR were determined quantitatively in cytosol preparations of the tissue sample and MCF-7 cells using enzyme immunoassay monoclonal kits (Abbott, Chicago, IL).

Total RNA isolation and reverse transcription

Total RNA was extracted from 10^6 cells or 30 mg of pulverized tissue with a RNeasy kit (Qiagen, Hilden, Germany). RNA quantification was performed by mixing 2 µl RNA with 200 µl DEPC-treated water and SYBR Green II (Molecular Probes Inc., Eugene, OR) in a 96-well plate. Yeast total RNA (Sigma, St Louis, MO) ranging from 5 ng to 12.5 µg was used as a standard. After 30 min incubation at room temperature fluorescence was measured with a Fluorostar plate reader (Perkin Elmer, Foster City, CA). For cDNA synthesis, 1 µg RNA was diluted in DEPC-treated water to a final volume of 12.5 µl, denatured at 70°C for 2 min and subsequently chilled on ice. Reverse transcription was performed in the presence of 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP, 0.5 mM dGTP, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 U/µl ribonuclease inhibitor (RNAsin; Promega, Madison, WI), 20 pmol random hexamer primers and 200 U M-MLV reverse transcriptase (Promega, Madison, WI) for 1 h at 42°C. The reaction was terminated by heating for 5 min at 94°C. A quantitative and qualitative control of the obtained cDNA was performed as described earlier (8).

Primers and PCR conditions

cDNA abundance was calculated according to the amount of the control PCR product (glyceraldehyde-3-phosphate dehydrogenase). Equal amounts of cDNA were used for PCR amplification of the ER coding sequences with primer p1 (5′-ATG ACC ATG ACC CTC CAC ACC AAA G-3′, nt 233–257, codons 1–9) and primer p2 (5′-CTC AGA CTG TGG CAG GGA AAC CCT-3′, nt 1998–2021, codons 589–596). PCR amplifications were performed with 0.625 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany) in a total volume of 25 µl, in PCR buffer supplemented with Q-Solution (Qiagen, Hilden, Germany) and 1 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP and 0.3 µM each primer. Each PCR consisted of 36 cycles (12 cycles with 1 min at 94°C, 4 min at 68°C, followed by 24 cycles with 1 min at 94°C, 4 min, plus a time increase of 15 s per additional cycle at 68°C). Conditions for PCR amplifications with 1.625 U of cloned *Pfu* DNA polymerase (Stratagene Cloning Systems, La Jolla, CA) were similar except that each PCR consisted of 40 cycles (45 s at 95°C, 1 min at 60°C, 4 min at 72°C). PCR products were stained with SYBR Green I nucleic acid stain (Molecular Probes Inc., Eugene, OR) and then separated on 1% agarose gels by electrophoresis. The concentrations of the amplification products (1789 bp) were evaluated by densitometric measurements. Quantification of the signals was performed using the Molecular Analyst software (BioRad, Hercules, CA).

Plasmids

Plasmid pU/ERE-Ade2 was constructed by substituting a single estrogen response element (ERE) for the p53 binding sites in plasmid pLS210 (17). The key features of plasmid pU/ERE-Ade2 are: an ERE upstream of the minimal *CYC1* promoter driving the expression of *ADE2* and the *URA3* marker. The β-galactosidase reporter plasmid pUCΔSS-ERE has been described earlier (18). The gap repair vector pG/hERΔ was derived from the expression vector pG/ER(G) (19) by deleting the *Not*I–*Pfl*MI fragment comprising codons 68–503 of ER; the gap repair vector retains a unique *Not*I site at the junction of the first 200 bp and the last 280 bp of the ER coding sequence at the 5′- and 3′-ends, respectively. Both pG/ER(G) and pG/hERΔ are high copy number yeast episomes with the 2µ replicon and the *TRP1* marker. The vector pG/hERΔ was prepared for transformation by digestion with *Not*I, filling the cohesive ends with Klenow fragment of DNA polymerase I and dephosphorylation of the resulting blunt ends with shrimp alkaline phosphatase (Boehringer Mannheim, Germany). The plasmid pG/ERΔ5 harbors the human ER exon 5 deletion variant.

Yeast strains

The *Saccharomyces cerevisiae* strain YPH250 (MAT**a** *ade2-101° his3-Δ200 leu2-Δ1 lys2-801^a trp1-Δ1 ura3-52*) (20) was used as parent strain. The ERE–*ADE2* construct pU/ERE-Ade2 was linearized with *Bst*BI within the *URA3* gene and integrated into the *ura3* locus of YPH250 to yield strain DP160 (MAT**a** *ade2-101° his3-Δ200 leu2-Δ1 lys2-801^a trp1-Δ1 ura3-52 URA3*::*ERE-ADE2* [*pU/ERE-Ade2*]). Strain DP161 (MAT**a** *ade2-101° his3-Δ200 leu2-Δ1 lys2-801^a trp1-Δ1 ura3-52 URA3*::*ERE-ade2* [*pU/ERE-Ade2*]/*hER-2µ-TRP1* [*pG/ER(G)*]) was derived from DP160 by the introduction of plasmid pG/ER(G).

Growth media

Handling of strains has been described by others (17). YEPD (Gibco-BRL Life Technologies, Paisley, UK) was used for the culturing of yeast. DP160 was routinely cultured on medium supplemented with adenine (200 µg/ml) to avoid selection of spontaneous suppressors of the endogenous mutant ade2 locus. Solid media for red/white screening consisted of 2% D-glucose, 0.67% yeast nitrogen base without amino acids (DIFCO Laboratories, Detroit, MI), 2% agar, all amino acids except uracil and tryptophan, plus limiting amounts of adenine (5 µg/ml) and optionally 100 nM 17-β-estradiol (Sigma, St Louis, MO).

Transformation of yeast

Plasmids were introduced into yeast by the LiAc/PEG method (21). Aliquots of 50 µl of yeast cells were mixed with 100 µg heat-denatured carrier DNA (sonicated single-strand salmon sperm DNA; Stratagene), 100 ng of linearized gap repair vector pG/hERΔ and 100 ng of unpurified PCR products. After

transformation 50 µl were spread on appropriate minimal medium for selection of plasmids (synthetic complete –ura –trp, 5 µg/ml adenine). After incubation for 72 h at 30°C and 12 h at 4°C colonies were scored for color. Subsequently, colonies were replica plated (Accutran replica plater; Schleicher & Schuell, Switzerland) on selective medium containing 100 nM estradiol (Sigma, St Louis, MO), incubated for 36 h at 30°C and 12 h at 4°C and colonies were scored again for color. With each series of transformation a positive control [300 ng of plasmid pG/ER(G)] and a negative control (100 ng linearized gap repair vector) were included.

Western blot analysis

One milliliter of an overnight yeast culture (DP161) was centrifuged and washed with PBS. Cells were lysed by vortexing for 3 min with acid-washed glass beads in 150 µl TEGMo-buffer (10 mM Tris–HCl pH 7.4, 1.5 mM EDTA, 5 mM disodium molybdate, 10% glycerol, 1 mM monothioglycerol). Lysates of 30×10^6 MCF-7 cells or 30 mg tissue were prepared in TEGMo buffer by mechanical disruption. ER was immunoprecipitated with a polyclonal antibody G-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) directed against the hinge region of the ERα and protein G–Sepharose 4 Fast Flow beads (Pharmacia, Uppsala, Sweden). After an incubation of 2 h at room temperature, the immunoprecipitates were washed and ER was eluted. Eluates were used for analysis by 10% SDS–PAGE and transferred to nitrocellulose membranes by electroblotting. Blots on nitrocellulose membranes (Immobilon; Millipore, Bedford, MA) were probed with a mouse monoclonal antibody against human ER (DAKO-ER 1D5; Dako A/S, Glostrup, Denmark) and alkaline phosphatase-conjugated secondary antibodies (goat anti-mouse IgG; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Detection was performed with the chemiluminescence western blotting kit (BioRad Laboratories, Hercules, CA).

DNA rescue

Yeast colonies were picked from the plate and individually grown overnight in microtiter plates containing 200 µl YEPD medium. Subsequently, cells were pelleted and lysed with 10 U of Zymolase (ICN Biomedicals Inc., Aurora, OH) in the presence of 50 mM β-mercaptoethanol, 10 mM EDTA and 50 mM Tris–HCl (pH 7.5) in a final volume of 240 µl for 30 min at 37°C. Plasmid DNA was isolated using the QIAprep Spin Plasmid kit (Qiagen, Hilden, Germany). Plasmids were transfected into XL-1 Blue bacteria by electroporation. Minipreparation of plasmids was carried out using the QIAprep Spin Plasmid kit (Qiagen, Hilden, Germany). The isolated plasmids were cut with *Bam*HI (New England Biolabs, Beverly, MA), restriction products were stained with SYBR Green I nucleic acid stain (Molecular Probes Inc., Eugene, OR) and separated on a 1% agarose gel by electrophoresis.

Sequence analysis

Inserts of the rescued plasmids were amplified by PCR (see Primers and PCR conditions) and purified PCR products were sequenced. Cycle sequencing was performed with 30–50 ng purified PCR product using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Division, Perkin Elmer, Foster City, CA). Sequences were analyzed on an ABI 310 automated sequencer and assembled with the Auto Assembler software (Perkin Elmer, Foster City, CA).

RESULTS

Control of *ADE2* gene expression in the yeast *Saccharomyces cerevisiae*

In the *S.cerevisiae* strain YPH250 (20) the endogenous *ADE2* gene is mutated and an ERE-controlled *ADE2* gene (reporter) was stably introduced by chromosomal integration at the *URA3* locus. Thus, the activation of the reporter gene is dependent on the activity of ER expressed in yeast. The *ADE2* gene encodes an enzyme of the adenine biosynthesis pathway. Lack of the enzyme yields an accumulation of a red colored intermediate metabolite of adenine metabolism (22). Therefore, colonies grown on an appropriate medium containing limiting amounts of adenine turn red (23). The wild-type ER expression vector was introduced by transformation, yielding the yeast strain DP161. The estradiol-activated wild-type ER forms homodimers which bind to ERE and stimulate transcription of the reporter gene resulting in white colonies, whereas lack of ER activity yielded red colonies. Without addition of estradiol all yeast colonies were red whereas upon addition of 1 nM estradiol to the medium all yeast colonies turned white (Fig. 1). This sensitivity for estradiol was confirmed by a β-galactosidase assay performed with the same yeast strain containing a β-galactosidase reporter plasmid (pUCΔSS-ERE; 18; results not shown). Furthermore, co-transformation of gap repair vector and PCR products obtained from a plasmid harboring the ER exon 5 deletion variant (pG/ERΔ5) produced exclusively white colonies in the presence and absence of estradiol, indicating that constitutive active forms of the receptor show the expected phenotype in the assay (data not shown). These findings indicate that the *ADE2* reporter in yeast was able to visualize the functionality of the wild-type ER in a strictly hormone-dependent manner.

Expression of ER in yeast

The yeast strain DP161 expressed 195 fmol ER/mg cytosolic protein, as determined by enzyme immunoassay. Figure 2 shows a western blot of cytosol extracts of the yeast strain DP161, of the breast cancer cell line MCF-7 and of a breast cancer tissue. Figure 2 confirms that the human ER is expressed in yeast at amounts similar to cell lines and that the 65 kDa molecular weight of the ER protein in yeast corresponds to the ER expressed in mammalian cells (24–26).

Gap repair assay for the functional screening of ER

A schematic diagram of the method is depicted in Figure 3. Total RNA was isolated from frozen samples and reverse transcribed. With the obtained cDNA, PCR amplification of the ER coding sequence was performed. The unpurified PCR products were transformed into yeast cells together with a linearized gap repair expression vector. ER coding sequences were integrated into the expression vector by homologous recombination in yeast generating circular plasmids. Yeast colonies growing on selective medium (ura^-, trp^-, limiting adenine) repaired their ER expression plasmid resulting in expression of the ER proteins in yeast. By transferring the transformed colonies from medium lacking estrogen to estrogen-containing medium, three different phenotypes can be expected (Table 1). Colonies that are red on medium

Figure 1. The *ADE2* reporter is estrogen-dependent. In the recipient yeast strain, the endogenous *ADE2* has been inactivated and, subsequently, an ERE-controlled *ADE2* gene (reporter) has been introduced at the *URA-3* locus. The wild-type ER was introduced and cells were grown on indicator medium containing various amounts of the ligand estradiol. Absence of *ADE2* expression led to the formation of red colonies on medium with limiting exogenous adenine. Addition of estradiol to the culture medium activated the ER and produced white colonies.

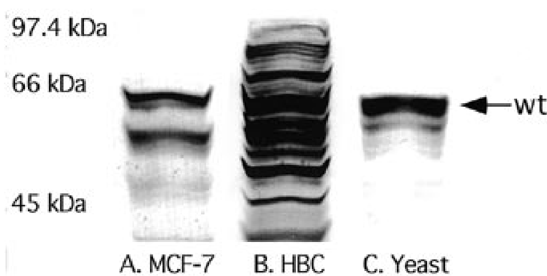

Figure 2. Western blot of ER from different sources. Aliquots of 50 µg total protein each from cytosolic extracts of MCF-7, one human breast cancer tissue sample and from yeast strain DP161 were separated by 10% SDS–PAGE. Western blot analysis was performed using a monoclonal antibody directed against the hinge region of the ER (wild-type ER, M_r 65 000).

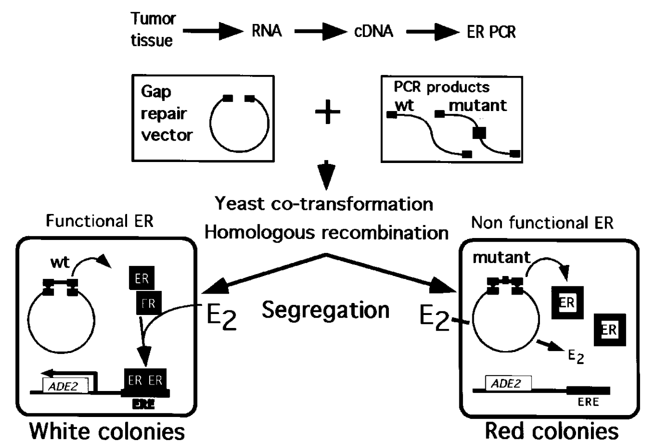

Figure 3. Functional display of ER molecules in yeast. For explanations see text.

lacking estrogen and turn white in the presence of estrogen represent wild-type ER. Colonies that are white without estrogen and stay white in the presence of estrogen contain constitutively active ER. Colonies that are red in the presence and absence of estrogen contain either non-functional ER or self-ligated empty vectors. A fourth putative phenotype, an ER that is active in the absence of hormone but becomes inactive in the presence of hormone, is not expected to be found.

Table 1. Predicted phenotypes of yeast colonies

Without estradiol	With estradiol White phenotype	Red phenotype
Red phenotype	Wild-type	Non-functional ER Gap repair vector (background)
White phenotype	Constitutively active ER	?

Controls for the assay

Co-transformation of 25 ng gap repair vector with 25 ng of unpurified PCR product gave rise to 200–600 colonies on selective medium. Controls consisting of linearized gap repair vector only (negative control) or wild-type ER plasmid (positive control) were transformed with each series. The vector alone produced little background (1–2%) whereas the wild-type ER produced exclusively red and, after replica plating on medium containing estradiol, exclusively white colonies. To test for false red or false white colonies due to PCR-induced mutations, wild-type ER from plasmid pHEGO (27) was amplified with *Taq* DNA polymerase or with a polymerase with proofreading ability (*Pfu* DNA polymerase). Different amounts (0.13, 0.013 and 0.0013 pg) of template DNA were used for the PCR reaction (Table 2). From these experiments the amount of ER template cDNA used in the assays was estimated and yielded a value of ~0.1 pg, since we obtained amounts of PCR products (2.5 µg with *Taq* DNA polymerase) similar to the reaction with 0.13 pg wild-type ER. By using 10–100 times less template in the PCR reaction, the number of errors introduced by the polymerases could in theory increase. Therefore, yeast was transformed with 100 ng of unpurified products from the PCRs with different amounts of template and as a control 100 ng of wild-type ER cut from pHEGO. In all cases, on medium without estradiol all colonies were red whereas after replica plating on medium containing estradiol, 95% or more of the colonies turned white (Table 2). The colonies with religated gap repair vector are included in this 5% background. The experiment was repeated three times and standard errors below 1% were observed. This indicates that the error rate resulting from the PCR reaction

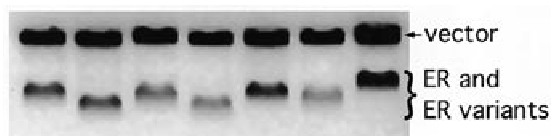

Figure 4. Restriction products of rescued ER clones originating from MCF-7 cells. A selection of plasmids derived from individual yeast colonies were cut with *Bam*HI and separated on 1% agarose gels. Restriction products represent vector and their respective ER inserts.

accounted for only a few colonies per transformation plate and produced only inactive phenotypes. The use of *Taq* polymerase resulted in a slightly higher apparent error rate than with *Pfu* (Table 2). The differences in error rate between the two polymerases were, however, small and indicated that *Taq* polymerase with its higher efficiency can be used for the assay. Transformation of 500 ng of a PCR product that was not ER related (erbB4 fragment) yielded the same rate of background colonies indicating that foreign DNA was not introduced into the gap repair vector by homologous recombination (not shown). The assay was then tested with ER-positive MCF-7 cells. After transformation, a selection of colonies was grown and their plasmids were rescued. Each individual yeast colony contained only one type of ER insert as tested by restriction digestion (Fig. 4). Thus, the individual yeast colonies contained ER plasmids derived from a single recombination event indicating an efficient segregation of the different PCR products. Reamplifications of these individual ER inserts were performed and their phenotypes were confirmed by retesting these ER variants with the yeast bioassay. Additionally, size variations of the inserts might originate from different ER variants (Fig. 4).

Table 2. Rate of false phenotypes

DNA polymerase	Input DNA[a] (pg)	Without estradiol (red) (%)	With estradiol (white)[b] (%)
Taq	0.13	100	96.3 ± 0.9
	0.013	100	95.0 ± 0.8
	0.0013	100	95.0 ± 0.3
Pfu	1.3	100	98.8 ± 0.2
	0.13	100	98.9 ± 0.6
	0.013	100	96.3 ± 0.7
Control[c]		100	0

Different amounts of wild-type ER as templates were amplified with *Taq* or with *Pfu* DNA polymerases. Yeast was transformed with 100 ng of the respective, unpurified PCR products and 100 ng of wild-type ER cut from pHEGO (control), respectively. On medium without estradiol all colonies were red whereas after replica plating on medium containing estradiol, 95% or more of the colonies turned white.
[a]Template, wild-type coding sequence of ER.
[b]Mean percentage of white colonies ± SD ($n = 3$).
[c]100 pg of ER fragment, isolated by restriction digestion from plasmid pHEGO.

ER heterogeneity in MCF-7 breast cancer cells and in one human breast cancer specimen

Transformations of ER PCR products from both the MCF-7 cells and one human breast cancer tissue resulted in a similar mixture of different phenotypes (Figs 5A and 6A). The percentages of functional, non-functional and constitutive active ERs in these samples were calculated and are shown in Table 3. Using a cut-off value of 20 fmol ER/mg cytosolic protein, both the MCF-7 cells and the primary breast cancer sample were defined as ER-positive (156 and 181 fmol ER/mg cytosolic protein, respectively). However, our yeast assay revealed that only 33% of ER molecules in MCF-7 cells and only 40% in the tissue sample were responding to estrogen with transcriptional activity (Table 3).

Table 3. ER phenotypes in the MCF-7 breast cancer cell line and one breast cancer specimen

Sample	Constitutively active (%)	Inactive (%)	Wild-type (%)
MCF-7	10	57	33
Breast cancer specimen	6	54	40

ER profile in MCF-7 cells and a human breast cancer tissue

Plasmids from several randomly picked yeast colonies were isolated and analyzed by PCR and DNA sequencing. A series of PCR products of a yeast transformation assay with MCF-7 cDNA is shown in Figure 5B. Functional ER (from white colonies in the presence of estradiol; lanes 1–12) mainly showed the size of the wild-type PCR product. The plasmid inserts with a size that deviated from the wild-type size were then analyzed by sequencing. One PCR product (lane 10) was identified as the exon 5 deletion variant, appearing as a constitutively active phenotype in the functional assay. Analysis of a PCR product slightly larger in size than the wild-type (lane 12) revealed an incomplete duplication of exon 4 resulting in the translation of a truncated protein due to a frameshift. In the yeast functional bioassay this truncated protein was also constitutively active. Rescued non-functional ERs (from red colonies in the presence of estradiol; Fig. 5B, lanes 13–26) were either of wild-type size, corresponding most likely to inactive ER mutants, or of sizes different from the wild-type, indicating the presence of inactive ER splicing variants. Lane 24 corresponds to an empty self-religated gap repair vector. Sequence analysis of PCR products smaller than the wild-type size revealed that they were all deletion variants (lane 17, exon 4 deletion; lane 20, exon 3 deletion; lane 21, exon 6 deletion) containing frameshifts which lead to non-functional truncated proteins. Two non-functional ER with wild-type size were sequenced and contained an ER with an insertion of an additional cytosine (Fig. 5B, lane 16) at position 1202 (codon 324) or an ER with a deletion of one adenine (Fig. 5B, lane 22) at position 699 (codon 156). These point mutations changed the reading frame and resulted in truncated non-functional proteins. These findings demonstrate that the assay is able to detect inactivating point mutations.

The human breast cancer sample showed a similar variety in rescued ER products (Fig. 6). In the series of ER variants derived from the human breast cancer specimen, a non-functional splicing variant, namely the exon 7 deletion variant, was identified twice (Fig. 6B, lane 1 and 3).

DISCUSSION

FASAY (13) was recently used for the investigation of ER in breast cancer biopsies (28). However, this approach was based on a growth selection for yeast colonies with functional ER. Yeast colonies containing non-functional ER molecules were lost on

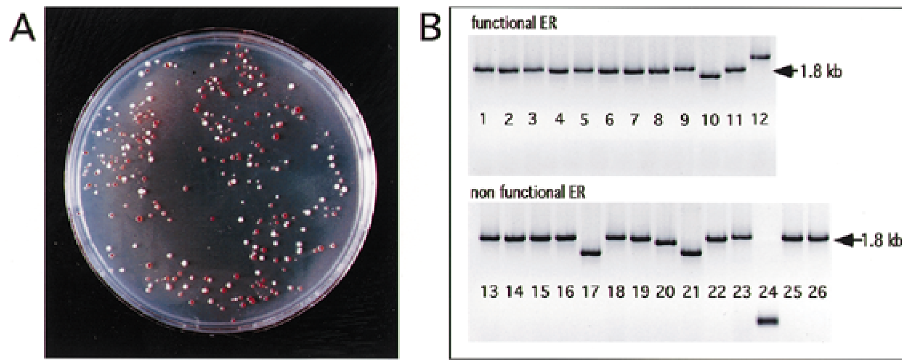

Figure 5. ER profile of MCF-7 cells. (**A**) Colonies transformed with gap repair vector and MCF-7 ER PCR products. Colonies were grown on indicator medium containing 100 nM estradiol (phenotype score, Table 3). (**B**) ER inserts obtained from yeast colonies transformed with MCF-7 ER PCR products. Lanes 1–12, a selection of rescued functionally intact ER; lanes 13–26, a selection of non-functional ER. Lane 24 shows the band corresponding to an empty gap repair vector (background). Indicated by the arrow is the wild-type ER size. An insertion of a C (lane 16) and a deletion of an A (lane 22) changed the reading frame and resulted in truncated, non-functional proteins.

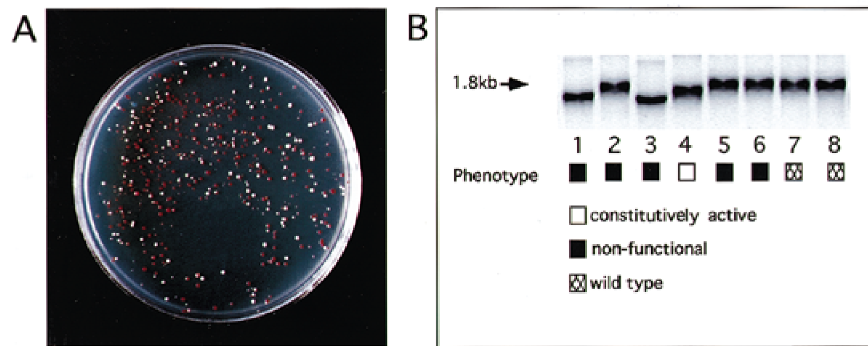

Figure 6. ER profile of a primary human breast tumor. (**A**) Colonies transformed with gap repair vector and ER PCR products from a human primary breast cancer sample. Colonies were grown on indicator medium containing 100 nM estradiol (phenotype score, Table 3). (**B**) ER inserts obtained from yeast colonies transformed with ER PCR products from the human breast cancer specimen showing a selection of functional, non-functional and constitutively active ERs.

selective plates. In contrast, the assay described in this report takes advantage of the adenine biosynthesis system in *S.cerevisiae* and the *ADE2* gene as a reporter (17) coupled to an ERE. Activation of gene transcription led to the formation of white colonies whereas lack of activation produced red yeast colonies. Combination of this system with gap repair and RT–PCR allowed a simple visual distinction between hormone-dependent (wild-type), non-functional and constitutively active ER. Thus, this assay allows the investigation of ER and its variant forms in human breast tumors since the PCR amplification products reflected quantity and type of ER receptor in the tumor. Consequently, the segregation of the amplification products into individual yeast colonies resulted in a population of yeast colonies representing the ER mRNA of the original cells or tumors. However, ER variants deleted in the primer binding sites will not be amplified and therefore not detected by the yeast assay. Furthermore, point mutations in the recombination regions of 200 bp at the 5′-end and 280 bp at the 3′-end of the coding region will not be detected in all cases and if detected their abundance might be under-represented in the yeast colonies.

Testing the antiestrogens tamoxifen and ICI 182780 has not been possible in any *in vivo* yeast assay described so far, including the one presented here (29–32). Additions of various concentrations of these antiestrogens to yeast medium were also tested in a yeast β-galactosidase assay (not shown). Neither the partial antagonists tamoxifen and hydroxytamoxifen nor the full antagonist ICI 182780 inhibited the action of estradiol. On the contrary, the antiestrogens tamoxifen and hydroxytamoxifen acted as partial agonists for the ER as also demonstrated by others (29,31,33). Explanations for the failure to inhibit estrogen activity with antiestrogens might be insufficient uptake of these compounds into the yeast cells, metabolic changes of the compounds in yeast cells resulting in the formation of agonists, lack of co-repressor recruitment or other yeast-specific mechanisms (34). Further investigations are needed to enable the use of antiestrogens in yeast assays.

The findings with MCF-7 breast cancer cells demonstrate that a large heterogeneity with respect to ER is present in tumor cells. MCF-7 cells are in principle clonal and therefore should show a homogeneous phenotype. However, whether the polymorphism found is due to the prevalence of ER variants in single cells or derives from ER variants from different cells remains to be investigated. The yeast assay allowed detection of novel mutant ER forms as well as previously described mutations or splice

variants (Fig. 6C). Additionally, the biological function of these forms could be easily determined. For example, a duplication of exon 4 resulting in a constitutively active ER was found. Duplications of whole exons of the ER sequence have been described earlier (35) although their functional effects were not addressed. The phenotype of some splicing variants could be confirmed, such as the exon 7 deletion variant that lacks the dimerization ability of the receptor and therefore is not capable of binding to DNA. We also detected two novel point mutations (codon 324, 1202insC; codon 156, 699delA). The fact that the resulting truncated ER proteins have no intact DNA-binding domain and have lost their C-terminal transactivation domain makes them possible candidates for dominant negative ERs (36). The possibility that these point mutated ERs might be artifacts due to PCR amplification is unlikely for several reasons: (i) the *Taq* DNA polymerase-produced mutations are rather misincorporations than deletions or insertions; (ii) *Taq* has an error rate of 1.8–2 × 10^{-5}/nucleotide/cycle (37,38) and under the conditions we used, this leads to only a few percent inactive phenotypes (Table 2); (iii) under the conditions used, false non-functional ERs represent only a small proportion and are mostly due to background (religated empty gap repair vector). Potentially important inactivating mutations are visualized in the assay (red colonies) whereas functionally silent mutations appear as wild-type phenotypes. Furthermore, the assay gives information about the frequency of non-functional ER forms in tumors and therefore determines the predominant phenotype of ER in a specimen.

To this date ER variants have been investigated mainly at the DNA and RNA/cDNA levels. Therefore, it is still not clear whether the identified mutations or splice variants are actually translated into proteins and to what extent. The yeast assay allows the investigation of ER variants at the protein level since the function of the protein is measured. Approximately 200 fmol/mg cytosolic protein are expressed in yeast (Fig. 2) which corresponds to the average ER concentration in breast cancer tissues (39,40). The biological activity of the ER is routinely assayed by the determination of ER-induced proteins. The tumor sample tested with the functional assay was positive with respect to PR and ER as determined by enzyme immunoassay, indicating a population of biologically active ER. However, the yeast bioassay revealed that the tumor sample contained 54% non-functional ER. These findings suggest that variant mRNAs encoding truncated ER proteins may contribute to misleading determinations if the antibodies used for detection target only the N- or C-terminus (41).

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