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Transcriptional Regulation of the Estrogen-inducible pS2 Breast Cancer Marker Gene by the ERR Family of Orphan Nuclear Receptors[1]

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ABSTRACT

The estrogen-receptor-related receptors (ERRs) α, β, and γ are orphan nuclear hormone receptors that share significant homology with the estrogen receptors (ERs) but are not activated by natural estrogens. In contrast, the ERRs display constitutive transcriptional activity in the absence of exogenously added ligand. However, the ERRs bind to the estrogen response element and to the extended half-sites of which a subset can also be recognized by ERα, suggesting that ERRs and ERs may control overlapping regulatory pathways. To test this hypothesis, we explored the possibility that ERRs could regulate the expression of the estrogen-inducible *pS2* gene, a human breast cancer prognostic marker. Transfection studies show that all of the ERR isoforms can activate the pS2 promoter in a variety of cell types, including breast cancer cell lines. Surprisingly, sequence analysis combined with mutational studies revealed that, in addition to the well-characterized estrogen response element, the presence of a functional extended half-site within the pS2 promoter is also required for complete response to both ER and ERR pathways. We show that ERR transcriptional activity on the pS2 promoter is considerably enhanced in the presence of all three members of the steroid receptor coactivator family but is completely abolished on treatment with the synthetic estrogen diethylstilbestrol, a recently described inhibitor of ERR function. Finally, we demonstrate that ERRα is the major isoform expressed in human breast cancer cell lines and that diethylstilbestrol can inhibit the growth of both ER-positive and -negative cell lines. Taken together, these results demonstrate that estrogen-inducible genes such as *pS2* can be ERR targets and suggest that pharmacological modulation of ERRα activity may have therapeutic value in the treatment of breast cancer.

INTRODUCTION

Estrogens have long been recognized to play a predominant role in controlling the growth and development of malignant human breast epithelium (1). Consequently, substantial efforts have been devoted to study the molecular mechanisms underlying estrogen-regulated pathways and exploring ways to block their activity in breast cancer cells. The biological activity of estrogens are now known to be mediated by two receptors (ER[3]α and β) that are members of the superfamily of nuclear receptors (reviewed in Ref. 2). ERα and β, either as homodimers or heterodimers, regulate gene transcription through binding to short sequences within the promoter of target genes, referred to as EREs, that are composed of inverted repeats of the core half-site motif AGGTCA (3–6). On estrogen binding, ERα and β acquire the ability to interact with members of the family of SRC proteins through conformational changes of the receptors (7, 8). Coactivator/receptor interactions can be abrogated by antiestrogens, a mechanism that contributes to their therapeutic effect in the treatment of breast cancer. However, breast cancer often progresses from hormone dependence to hormone independence, which limits the use of tamoxifen and toremifene, the only antiestrogens currently approved for the treatment of breast cancer (9). This biological phenomenon is complex and involves both genetic and epigenetic changes in breast cancer cells and remains, for the most part, unresolved.

The ERRs α, β, and γ are orphan members of the superfamily of nuclear receptors (10–13). Despite being most closely related to ERα and β, the ERRs are not activated by known natural estrogens but rather display constitutive transcriptional activity that appears to be isoform-, cell context-, and promoter-dependent (10, 12, 14–17). However, like the ERs, the ERRs recognize the ERE, suggesting that these receptors may control overlapping regulatory pathways (18). In addition, ERRs bind to extended half-sites with the consensus sequence TCAAGGTCA referred to as an ERRE (16, 17, 19–21). ERα but not ERβ has recently been shown to recognize a subset of ERRE and stimulate the transcriptional activity of promoters containing such sites, reinforcing the concept that the two classes of related receptors share common target genes (22). Moreover, our laboratory has recently discovered that the synthetic estrogen DES can act as an inverse agonist or antagonist on all three of the ERR isoforms, leading to the dissociation of coactivator proteins and loss of transcriptional activation function (23). Taken together, these findings indicate a closer functional kinship between the two receptor subclasses than originally anticipated. Consequently, we have begun to investigate whether the ERRs may participate in classical ER-mediated pathways involved in the initiation and progression of breast cancer. Here, we demonstrate that the orphan nuclear receptors ERRα, β, and γ can regulate the transcriptional activity of the human breast cancer marker gene *pS2* and, unexpectedly, that the full transcriptional activity of the ERs and the ERRs on the *pS2* gene is dependent on the presence of an ERRE unidentified previously within its promoter. We also show that the transcriptional activity of ERRs on the *pS2* gene can be abrogated by DES, suggesting that ERRα, the major isoform expressed in breast cancer cell lines, may constitute a therapeutic target in the treatment of breast cancer.

MATERIALS AND METHODS

Reagents. E_2 and DES were obtained from Sigma Chemical Co. (St. Louis, MO). Klenow and T4 DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany). PFU polymerase was from Strategene (La Jolla, CA). All of the oligonucleotides used in this study were synthesized at the Sheldon Biotechnology Center (McGill University, Montréal, Canada).

Plasmids. Reporter plasmids pS2Luc and pS2ΔERELuc have been described previously (24). pS2ΔERRELuc was generated by PCR mutagenesis using PFU polymerase. A *Pst*I/*Xho*I fragment containing mutated ERRE was sequenced and subcloned back into the template plasmid to eliminate the presence of undesired mutations during the amplification procedure. The oligonucleotide used was: pΔERRE, 5′-GAGTAGGACCTGGATTAATTC-CAGGTTGGAGGAGACTCCC-3′ (changes are underlined). For the construction of the pS2ΔEREΔ/ERRELuc double mutant, the plasmid pS2ΔERRE was digested with *Pst*I and *Xho*I, and the fragment containing the ERRE mutation was subcloned into the corresponding sites of pS2ΔERELuc. The

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[3] The abbreviations used are: ER, estrogen receptor; ERR, estrogen-receptor-related receptor; ERE, estrogen response element; ERRE, estrogen related response element; SRC, steroid receptor coactivator; DES, diethylstilbestrol; E_2, estradiol; RLU, relative luciferase unit; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-PCR.

pCMXhSRC-1 expression vector, pCMXhERα, pCMXmERRα, and pCMXβgal have been described (16, 24). To construct pCMXrERRβ, a 1.7-kb *Eco*RI/*Bam*HI fragment of the HH3 cDNA (10) containing the entire coding sequence of rat ERRβ was subcloned into pCMX (25). To construct pCMXmERRγ, a 2.5-kb murine cDNA encoding the entire coding region of ERRγ isolated by screening a kidney cDNA library with a human expressed sequence tag clone ERRγ was digested with *Xba*I and *Sal*I, end-filled with Klenow, and subcloned into the *Eco*RV site of pCMX. The VP16-mERRα, VP16-rERRβ, and VP16-mERRγ expression vectors were constructed by amplifying the entire coding region of each cDNA and subcloning the resulting blunt-ended fragment into pCMX-VP16, which had been digested previously with *Bam*HI and subsequently blunt-ended with Klenow (gift from G. B. Tremblay, Signalgene, Montréal, Canada). All of the constructs were sequenced to confirm their integrity.

Cell Culture and Transfection. HeLa, Cos-1, and human breast cancer cell lines ZR75.1, MDA-MB-231, HS587T, BT549, SKBr3, T47D, MCF-7, MDA-MB-448, and BT474 were maintained in DMEM supplemented with 10% FBS and 100 μg/ml penicillin and 100 μg/ml streptomycin. MCF-10a and MCF-12 cells were grown in DMEM:F12HAM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. BT20 cells were grown in α-MEM (Life Technologies, Inc.) supplemented with 5% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. MDA-MB-435S and MDA-MB-436 cells were grown in Leibovitz L15 (Life Technologies, Inc.) supplemented with 5% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. For transfection, cells were grown in phenol red-free DMEM (Life Technologies, Inc.) with 10% charcoal dextran-treated FBS for $\geq$24 h before transfection. Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. Transient transfections were performed in 12-well plates. At ~50% confluence, cells were transfected using the FuGene transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instruction, typically with 0.5 μg of reporter plasmid, 0.1–0.2 μg of control plasmid pCMXβgal, 50–400 ng ER or ERR expression plasmids, and carrier DNA pBluescriptKSII for a total of 1 μg/well. After 16 h, the cells were washed and given fresh medium that contained 10% charcoal dextran-treated FBS with 10 nM E_2 or 10 μM DES for 24 h. For luciferase assays, cells were lysed in 0.1 M potassium phosphate buffer pH 7.8 containing 1% Triton X-100, and light emission was detected in the presence of luciferin using a microtiter plate luminometer (Dynex Technologies, Chantilly, VA). Luciferase values were normalized for variations in transfection efficiency using the β-galactosidase internal control and expressed as RLUs. The values for luciferase activity presented in this study as RLUs or fold induction over control represent means of a minimum of three independent transfections performed in duplicate.

For cell proliferation assays in the presence of DES and E_2, MCF-7 and MDA-MB-231 cells were seeded in 96-well plates at a density of 10,000 cells/100 μl/well in phenol red-free DMEM containing 2% charcoal dextran-treated FBS. One day later and at days 3 and 6, cells were treated with ethanol or estrogens as indicated. At 3, 6, and 9 days after initiation of the treatment, a colorimetric proliferation assay was performed using the CellTiter 96 AQueous nonradioactive cell proliferation assay as directed by the manufacturer (Promega, Madison, WI).

EMSA. *In vitro* translated ERRα, ERRβ, and ERRγ were generated from plasmids pCMXmERRα, pCMXrERRβ, and pCMXmERRγ using the TNT reticulocyte lysate kit (Promega). For preparation of probes, sense and antisense strands of oligonucleotides were annealed and radiolabeled by end filing with Klenow. Aliquots (5 μl) of *in vitro* translated proteins were preincubated in binding buffer [10 mM Tris-HCl (pH 8.0), 40 mM KCl, 10% glycerol, 1 mM DTT, and 0.05% NP40] containing 2 μg of poly (dI-dC)$_2$, 0.1 μg of denatured salmon sperm DNA, and 10 μg of BSA for 20 min on ice. Probe (~40,000 cpm) was added and allowed to bind for 20 min at room temperature. Complexes were resolved on a 5% polyacrylamide gel in 0.5 $\times$ Tris borate-EDTA and electrophoresed at 150 V at room temperature. Competition experiments were performed by adding the indicated amounts of unlabeled oligonucleotides to the reaction before adding the probe. The following oligonucleotides and their complements were used as probes: pS2-ERE, 5′-TCGACCCTGCAAGGTCACGGTGGCCACCCCGTG-3′; and pS2-ERRE, 5′-TCGACACCTGGATTAAGGTCAGGTTGGAG-3′.

Semiquantitative RT-PCR of pS2 Expression. MDA-MB-231 cells were grown in phenol red-free DMEM (Life Technologies, Inc.) with 10% charcoal dextran-treated FBS for $\geq$24 h before transfection. Cells were transiently transfected with 5 μg of expression plasmids using FuGene transfection reagent as described above in 50 $\times$ 10-mm plates. After 16 h, the cells were washed and given fresh medium that contained 10% charcoal dextran-treated FBS with either 100 nM E_2 or an ethanol vehicle for 24 h. Total RNA was extracted from transfected cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. The cDNA was synthesized from 2 μg of total RNA using Superscript reverse transcriptase (Life Technologies, Inc.) and oligo(dT) according to manufacturer's instructions. The 252-bp pS2 fragment was amplified with the following primers: pS2 sense, 5′-ATGGCCACCATGGAGAACAAGG-3′; and pS2 antisense, 5′-CTAAAATTCACACTCCTCTTCTGG-3′. The amplification of a 534-bp human acidic ribosomal phosphoprotein 36B4 cDNA fragment was used as an internal PCR control. The primer pair used was: 36B4 sense, 5′-TGTTTCATTGTGGGAGCAGAC-3′; and 36B4 antisense, 5′-AAGCACTTCAGGGTTGTAGAT-3′. The reaction mixtures (50 μl) were subjected to 25, 30, and 35 amplification cycles of 45 s at 94°C, 60 s at 58°C, and 90 s at 72°C to determine the linearity of the PCR reaction. Aliquots (7 μl) of the PCR reactions taken at 30 amplification cycles were then analyzed by electrophoresis in a 1.2% agarose gel. Quantification of the electrophoresis data were performed using the ImageQuant software from images obtained with a Typhoon phosphorimager apparatus (Pharmacia Biotech, Piscataway, NJ). Two distinct transfection experiments were performed, and pS2 expression was analyzed each time by two separate RT-PCR analyses to ensure reproducibility.

RESULTS

ERRs Activate the pS2 Promoter. The *pS2* gene was originally identified as an estrogen-inducible transcript in the human breast cancer cell line MCF-7 (26). Molecular characterization of the estrogen response showed that the *pS2* gene is a direct target of the ERs, because its promoter encodes a functional ERE located 407 bp upstream of the transcriptional start site (Fig. 1*A;* Ref. 27). Thus, the *pS2*

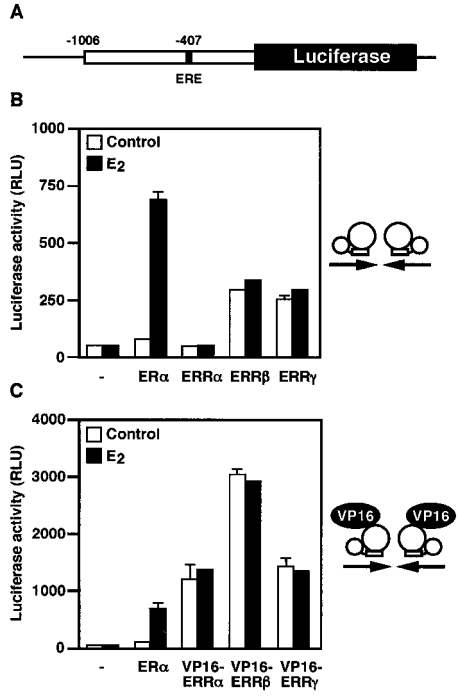

Fig. 1. ERRs and VP16-ERR chimeras mediate activation of the pS2 promoter. *A*, schematic representation of the pS2 promoter used in this study. The relative position of ERE is indicated. *B* and *C*, HeLa cells were transfected with 0.5 μg of pS2Luc reporter plasmid and 0.1 μg of expression plasmid pCMXhERα, pCMXmERRα, pCMXrERRβ, pCMXmERRγ, pCMXVP16-mERRα, pCMXVP16-rERRβ, and pCMXVP16-mERRγ. After transfection, cells were treated with either 10 nM E_2 or ethanol vehicle. Cells were harvested 24 h after treatment, and cell extracts were assayed for luciferase and β-galactosidase activities.

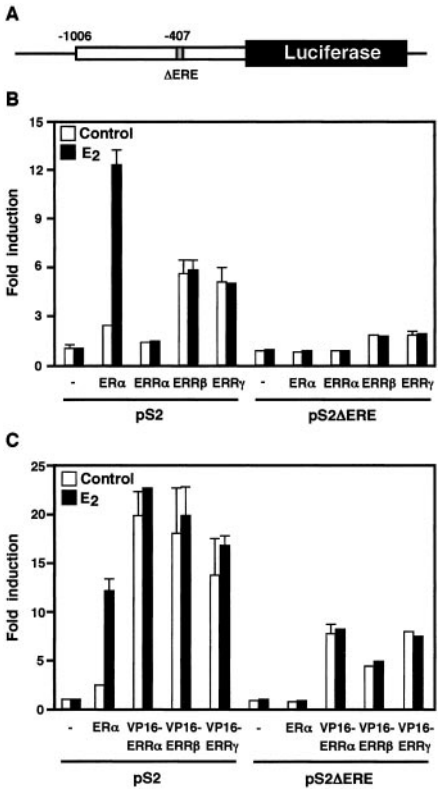

Fig. 2. Effects of pS2-ERE mutation on the transcriptional activities of ERRs and VP16-ERR chimeras. *A*, schematic diagram of the pS2ΔERE reporter. *B*, the reporter plasmid pS2Luc and pS2ΔERE were transfected into HeLa cells along with pCMX-based expression plasmids for ERα, ERRα, ERRβ, and ERRγ. Transfected cells were treated with 10 nM E_2 or the carrier (ethanol) for 24 h after transfection. *C*, the transfection conditions were identical to *panel B* except that pCMX-based expression plasmids for VP16-ERRα, β, and γ chimeras were used.

gene also represents a potential target for members of the ERR family. We first tested whether the three ERR isoforms could activate the pS2 promoter when transfected in HeLa cells, an ER-negative cell line originally used for the characterization of the pS2 promoter (28). As expected, ERα induces a ~12-fold stimulation of the activity of the pS2 luciferase reporter in a E_2-dependent manner (Fig. 1*B*). In contrast, two of the ERR isoforms, ERRβ and γ, constitutively stimulate by ~4-fold the activity of the pS2 promoter (Fig. 1*B*), and the presence of E_2 had no effect on their activity. The lack of constitutive ERRα transcriptional activity is in agreement with previous studies from our laboratory (16). However, VP16-mERRα as well as VP16-rERRβ and VP16-mERRγ chimeras, constructs that allow evaluation of receptor-promoter interactions in the absence of potential ligands or cell-specific cofactors, increase transactivation of the pS2 reporter above the level achieved with ERα in the presence of E_2 (Fig. 1*C*). Taken together, these results demonstrate that the pS2 promoter can be considered a valid target of all three members of the ERR family.

Identification of a Functional ERRE within the pS2 Promoter. We next tested whether, as anticipated from our knowledge of the DNA-binding properties of the ERR family, the ERE was sufficient to transduce ERR transcriptional activity to the pS2 promoter. We used a mutant of the pS2 promoter in which the ERE was rendered nonfunctional (pS2ΔERE; Fig. 2*A*). As expected, the pS2ΔERE reporter failed to respond to ERα in the presence of E_2 (Fig. 2*B*). Surprisingly, the pS2ΔERE reporter was still significantly responsive to the presence of ERRβ and γ, albeit at a lower level than that observed with the intact reporter. This observation was even more striking when transcriptional activity was assessed using the VP16-ERR chimeras (Fig. 2*C*). All three of the VP16-ERR chimeras generated between 5- and 8-fold induction of the luciferase activity from the mutant pS2 promoter. These results indicate that whereas the pS2 promoter is a direct target of the ERR isoforms and that the ERE participates in that response, the presence of additional response element(s) appears to be necessary to generate a full response from the ERR isoforms.

The observation that the ERE is not absolutely required to generate a transcriptional response from the ERR isoforms led us to reexamine the sequence of the pS2 promoter for the presence of related elements. Scanning of the pS2 promoter sequence revealed the presence at position −269/−260 of a TTAAGGTCA motif that corresponds to the consensus ERRα binding site TNAAGGTCA as defined previously by selected and amplified binding analysis (Fig. 3*B*; Ref. 16). To test whether the ERR isoforms can interact with the pS2 ERE and this newly identified element, we preformed EMSA using *in vitro*-synthesized ERRα, β, and γ and synthetic ^{32}P-labeled oligonucleotides representing the pS2 ERE, as well as the putative ERRE. As shown in Fig. 3*C*, all three of the ERR isoforms bind to the pS2 ERE, and the binding is specifically competed by addition of increasing amounts of unlabeled synthetic ERE oligonucleotides. Similarly, the ERR isoforms bind to the pS2 ERRE, and in each case binding is competed by unlabeled ERRE oligonucleotides.

To examine whether the ERRE is functional *in vivo*, HeLa cells were transfected with pS2 reporter constructs in which the ERRE or both the ERE and ERRE were mutated (Fig. 4*A*). Mutation of the ERRE resulted in more than a 60% decrease in ERRβ- and ERRγ-dependent transcriptional activity, whereas mutation of both elements

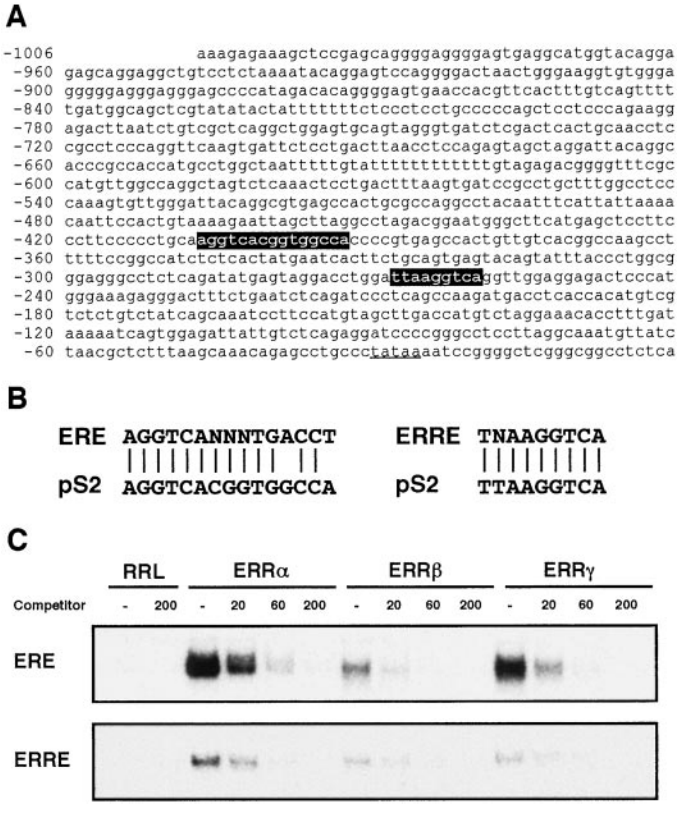

Fig. 3. A functional ERRE within the human pS2 promoter. *A*, the nucleotide sequence of the human pS2 promoter. The ERE and ERRE at position −407 and −269, respectively, are shown in ■. The TATA box sequence is underlined. *B*, comparison between consensus ERE and ERRE and the pS2 ERE and ERRE. *C*, EMSA performed with the pS2 ERE and ERRE probes. The probes were radiolabeled and incubated with *in vitro*-translated ERRα, ERRβ, and ERRγ. Unprogrammed rabbit reticulocyte lysate (*RRL*) was used as a negative control. The unlabeled pS2 ERE or ERRE were used as competitors in 20-, 60- and 200-fold molar excess.

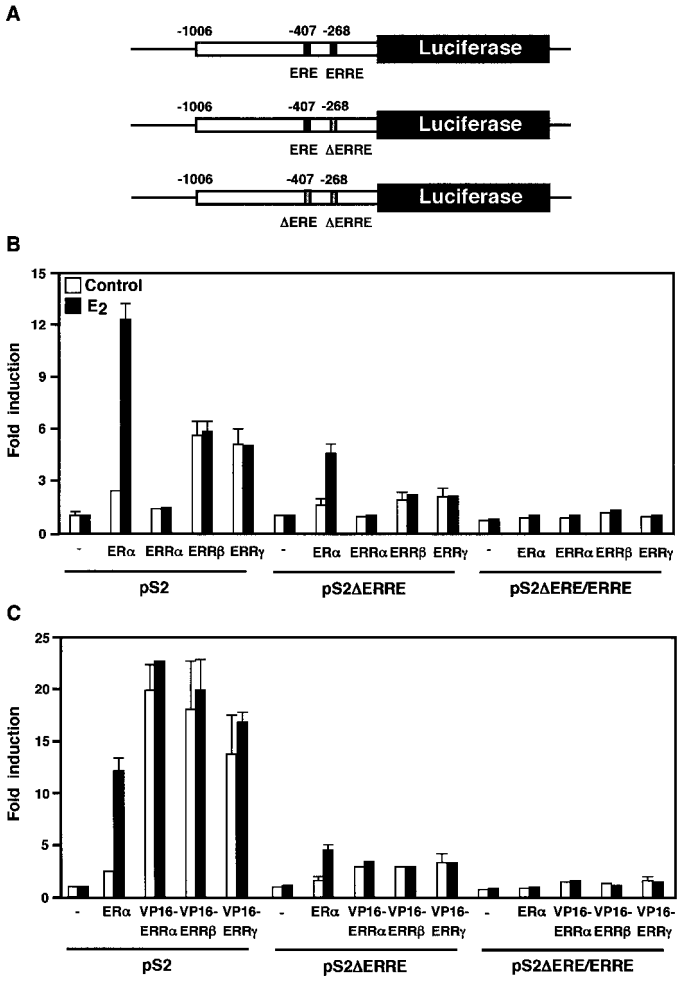

Fig. 4. Functional analysis of the pS2 ERRE. *A*, schematic diagram of pS2Luc, pS2ΔERRE, and pS2ΔERE/ERRE reporters. The position of the ERE and/or ERRE mutations is indicated by ▨. *B*, wild-type and mutated pS2Luc reporter constructs were cotransfected into HeLa cells with expression vectors carrying ERα, ERRα, ERRβ, and ERRγ. Cells were incubated with E_2 (10 nM) or ethanol for 24 h before harvest. *C*, similar to *panel B* except that expression plasmids for VP16-mERRα, VP16-rERRβ, and VP16-mERRγ were used.

completely abolished the ERR-mediated response (Fig. 4*B*). The requirement of the ERRE for the ERR-mediated response is even more striking in the presence of the VP16-ERR chimeras (Fig. 4*C*). In this assay, the ERR responses were diminished by up to 80% in the absence of a functional ERRE and completely lost when both ERRE and ERE were mutated. We also observed that mutation of the ERRE resulted in a 50% diminution of the E_2 response mediated by transfected ERα, suggesting that the pS2 ERRE is also a functional ERα response element.

ERRs Are Transcriptionally Active in Human Breast Cancer Cell Lines. Having shown that ERR isoforms are able to regulate the expression of the pS2 promoter through both ERE and ERRE in HeLa cells, we sought to determine the transcriptional activity of the ERRs in a more relevant biological context. Three human breast cancer cell lines, one ER-positive (ZR75.1) and two ER-negative (MDA-MB-231 and HS578T), were cotransfected with the pS2 luciferase reporter together with expression vectors for ERα, the three ERR isoforms, or the VP16-ERR chimeras. As shown in Fig. 5, ERα elicited an E_2 response in all three of the cell lines. Note that the lack of response to E_2 in the ER-positive cell line ZR75.1 in the absence of transfected ERα is consistent with previous studies that showed that an episomal pS2 promoter is unresponsive to endogenous ER (28). In agreement with data obtained in HeLa cells, ERRα did not stimulate the activity of the pS2 promoter in all three of the human breast cancer cell lines. However, both ERRβ and γ induced transcription from the pS2 promoter by ~3-fold in ZR75.1 and MDA-MB-231 cells and up to ~10-fold in HS578T cells. In contrast, all three of the VP16-ERR chimeras generated a strong transcriptional response from the pS2 promoter. However, the level of response varied in the three of the cell lines, especially in comparison with the response observed with ERα in the presence of E_2.

We next sought to test whether the ERRs or the VP16-ERR chimeras could modulate the expression of the endogenous *pS2* gene. To do so, we used the ER-negative human breast cancer cell line MDA-MB-231 to avoid interference from the ER. MDA-MB-231 cells express very low basal expression of the *pS2* gene (undetectable by Northern blot analysis) and therefore constitute an ideal vehicle for this type of transient transfection experiments. We used FuGene reagent to transfect a significant proportion (~25%) of MDA-MB-231 cells and expressed ERα; ERRα, β, and γ; or the three VP16-ERR chimeras. Semiquantitative RT-PCR analysis demonstrated that transiently transfected ERα can induce the expression of the *pS2* gene in an E_2-dependent manner (Fig. 6, *Lanes 3* and *4*). As observed previously with the pS2 luciferase reporter, ERRα did not influence

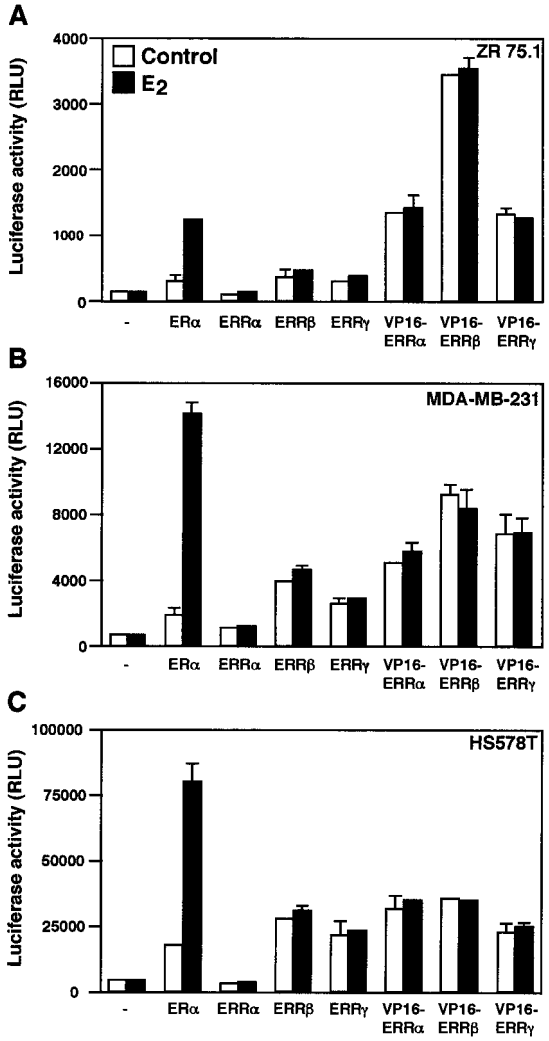

Fig. 5. ERRs activate the pS2 promoter in human breast cancer cells. *A*, ZR75.1 cells were transfected with pS2Luc reporter and expression vector carrying ERα, ERRα, ERRβ, ERRγ, VP16-mERRα, VP16-rERRβ, and VP16-mERRγ. Transfected cells were treated with either 10 nM E_2 or an ethanol vehicle. *B* and *C*, transfections were performed as for *panel A* except that MDA-MB-231 (*B*) or HS578T cells (*C*) were used.

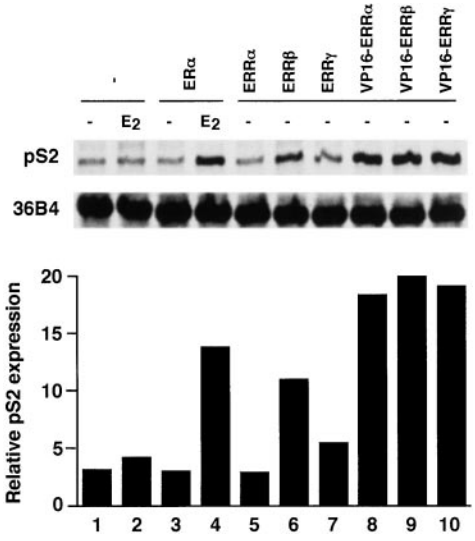

Fig. 6. ERRs regulate the endogenous *pS2* gene. MDA-MB-231 cells were transiently transfected using FuGene with 5 μg of control plasmid pCMX, pCMXhERα, pCMX-ERRs, and pCMXVP16-ERRs expression vectors as indicated. Transfected cells were treated with 10 nM E_2 and harvested 24 h after treatment. Total mRNA was isolated and subjected to semiquantitative RT-PCR analysis for detection of the pS2 transcript and the control 36B4 transcript (see "Materials and Methods"). Level of pS2 transcript relative to the *36B4* gene was determined by quantitation using a Typhoon phosphorimager. A representative of four distinct experiments is shown.

pS2 transcription in this assay. However, significant activation of pS2 expression was observed on transfection with ERRβ and ERRγ expression vectors (Fig. 5, *Lanes 6* and *7*). On the other hand, all three of the VP16-ERR chimeras elicited a strong transcriptional response from the endogenous *pS2* gene (Fig. 6, *Lanes 8* to *10*). These results, which parallel the data obtained with the pS2 luciferase reporter, clearly demonstrate that the ERR isoforms possess the potential to regulate the expression of the *pS2* gene in the context of its own genomic regulatory environment.

Members of the SRC Family Stimulate ERR Transcriptional Activity. ERR isoforms were recently shown to interact with members of the p160 coactivator family in a ligand-independent manner and enhance their constitutive transcriptional activities (12, 29). Therefore, we sought to determine the effect of coexpression of the p160 coactivators on the activity of each ERR isoforms. Remarkably, introduction of SRC-1, GRIP-1, and pCIP resulted in the constitutive activation of the pS2 promoter by ERRα (Fig. 7). The transcriptional activity of ERRβ was also enhanced by all three of the coactivators. In contrast, ERRγ did not respond to the presence of SRC-1, whereas both GRIP-1 and pCIP allowed a stronger transcriptional response from ERRγ. These experiments show that the transcriptional activity of ERR isoforms is dependent on the presence of specific coactivators.

DES Inhibits ERR Transcriptional Activity on the pS2 Promoter. DES has recently been shown to act as a negative effector of ERR transcriptional activity (23). In particular, DES promotes the release of p160 coactivators from ERR isoforms and inhibits their transcriptional activities. Therefore, we tested the action of DES on the transcriptional activity of each ERR isoforms in the presence or absence of GRIP-1. As shown in Fig. 8, DES completely abolished basal or GRIP-1-stimulated ERR responses on the pS2 promoter.

ERRα Is Expressed in Breast Cancer Cell Lines. We next sought to determine the pattern of expression of ERR isoforms in human breast cancer cell lines and tumors. Total RNA was extracted from a panel of human breast cancer cell lines and analyzed by Northern blotting. As shown in Fig. 9, expression of ERRα was detected in all of the human beast cancer cell lines as well as the normal mammary epithelial MCF-10a and MCF-12 cell lines. The expression of ERRβ and γ was not detected using that technique.

Specific Inhibition of Breast Cancer Cell Proliferation by DES. It has been demonstrated previously that pharmacological concentrations of estrogens, especially DES, inhibit human breast cancer cell growth via a mechanism that is apparently different from that of antiestrogens (30). We decided to conduct similar experiments with the cell lines used here (the ER-negative MDA-MB-231 and ER-positive MCF-7 cell lines) and concentrations of estrogens that should be stimulatory at a low does and inhibitory at a pharmacological dose. As observed in Fig. 10, both DES and E_2 at 10 nM stimulated MCF-7 cell proliferation but, as expected, had no effect on MDA-MB-231 cell proliferation. In contrast, when used at 3 μM, DES but not E_2 inhibited the proliferation of both cell lines suggesting the existence of a DES-dependent ER-independent mechanism for this effect.

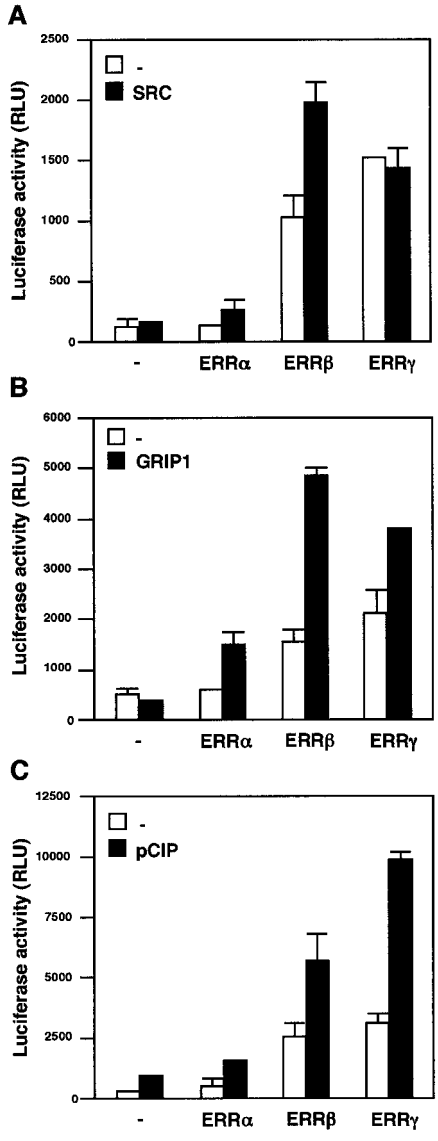

Fig. 7. Coactivators enhance the transcriptional activity of ERRs. *A*, Cos-1 cells were cotransfected with pS2Luc reporter and expression plasmids encoding ERRα, ERRβ, and ERRγ in the presence or the absence of 0.4 μg pCMXSRC-1. The cells were harvested 24 h after transfection. *B* and *C*, similar to *panel A* except pCMXGRIP1 (*B*) or pCMX-pCIP (*C*) were used. *bars*, $\pm$ SE.

DISCUSSION

The close functional kinship between members of the ER and ERR families led us to investigate whether ERR isoforms could regulate a classic estrogen-dependent pathway in breast cancer cells, namely the breast cancer marker gene *pS2*. The results of our study not only demonstrate that all three of the ERR isoforms can directly regulate *pS2* gene expression in breast cancer cells but, surprisingly, that this effect is mediated in part through an ERRE within the pS2 promoter sequence. The observation that the pS2 ERRE also plays a role in ERα-mediated response places the ERRs at an unique position in the control of estrogenic signaling pathways and reinforces the notion that these orphan nuclear receptors may play a more important role than anticipated previously in estrogen physiology (22). Whereas the ER and ERR isoforms share a highly conserved DNA binding domain, a property that allows for the recognition of common response elements and the ability to regulate common target genes, important functional differences also exist between members of the two subfamilies and receptor isoforms. ERα and β are inactive in the absence of hormonal signal, whereas ERR isoforms display significant constitutive transcriptional activity as both repressors and activators (2, 12, 14–17, 23, 29, 31). In particular, the activity of the ERR isoforms as activators or repressors appears to be dependent on the relative level of coactivator proteins and culture conditions when assayed in transient transfection experiments. Therefore, it is difficult at present to precisely delineate the exact role played by the ERR isoforms in pS2 expression.

Our expression study suggests that ERRα is the major isoform expressed in breast cancer cell lines (Fig. 9). ERRα transcriptional activity is particularly low in the absence of coactivator protein and could therefore interfere with ER signaling. On the other hand, ERRα activity has been found to be stimulated by a charcoal treatment-removable compound present in specific batches of sera (14), suggesting that ERRα may exert a ligand-dependent positive influence on pS2 expression in a more relevant physiological context. In addition, a significant number of human breast tumors display amplification of the *AIB1/pCIP* coactivator gene (32, 33), which could contribute to the enhancement of ERRα activity in cancer cells. Taken together, these observations suggest that ERRα could play an important role in regulating estrogenic pathways in breast tumors. Therefore, it will be crucial to assess the relative levels of expression of ERR isoforms and coactivator proteins during breast cancer progression, especially during the transition from a hormone-dependent to a hormone-independent status. In addition, we have detected expression of both ERRα and ERRβ isoforms during all stages of mammary gland development in the mouse,[4] suggesting that both isoforms may play a role in mammary gland physiology.

We have recently demonstrated that the transcriptional activity of ERRs and ERs can be controlled by at least one common synthetic ligand, DES, which indicates that their ligand-binding pockets share common functional determinants (23). More importantly is the demonstration that DES has opposite action on ERs and ERRs: agonistic on ERs and antagonistic on ERRs. It is well known that treatment with pharmacological doses of DES is as effective as tamoxifen therapy for the treatment of breast cancer but that the substantial incidence of side effects prevents a wide usage of DES in a clinical setting (34–36). However, no clear biological explanation and molecular mechanism

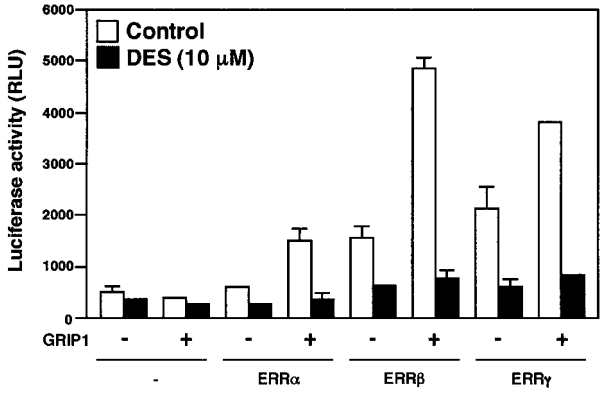

Fig. 8. DES inhibits the transcriptional activity of ERRs and coactivating function of GRIP1. The pS2Luc reporter plasmid was transfected into Cos-1 cells with expression vectors for ERRα, ERRβ, and ERRγ with or without mGRIP1 expression plasmid. After transfection, cells were treated with 10 μM DES. Cells were harvested 24 h after treatment. *bars*, $\pm$ SE.

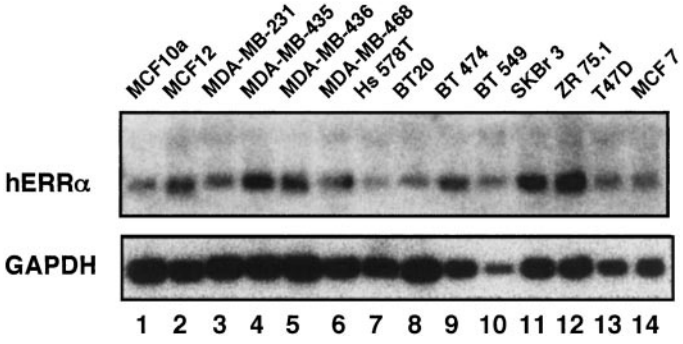

Fig. 9. Expression of ERR isoforms in human breast cancer cell lines. Total RNA was extracted from normal breast cells (MCF-10a and MCF-12a) and a number of breast cancer cell lines. Northern blotting analysis was carried out using a 322-bp human ERRα cDNA fragment as probe. A human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probe was used to monitor loading.

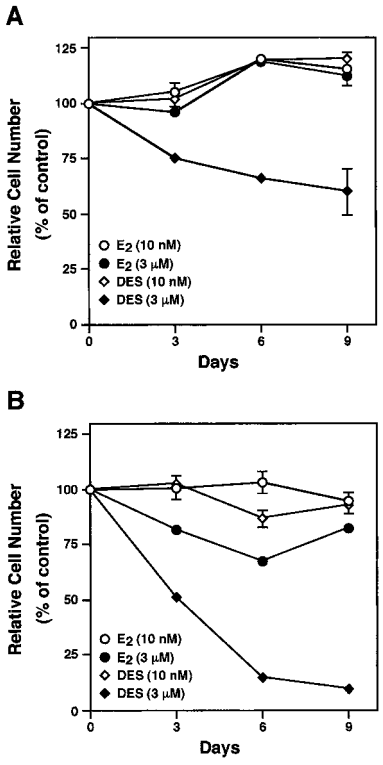

Fig. 10. DES inhibits human breast cancer cell proliferation. MCF-7 cells (*A*) and MDA-MB-231 cells (*B*) were treated with 10 nM and 3 μM E_2 or DES, respectively. Cell proliferation rates were measured by a colorimetric assay as described in "Materials and Methods." Values are expressed as percentage of control and represent the means of three independent experiments; *bars*, $\pm$ SE.

[4] G. Charhour and V. Giguère, unpublished observation.

have been provided for the inhibitory action of DES on breast cancer cell proliferation and the role that the ERs might play in that process (30, 37). The observation that the constitutive activity of the ERR isoforms on the *pS2* gene can be abrogated by DES (Fig. 8) suggests that the inhibitory action of DES on ERR transcriptional activity may be an element of its therapeutic efficacy. The demonstration that DES can inhibit the proliferation of both ER-positive and ER-negative cells also support this concept (Fig. 10 and Ref. 30). Whereas future investigations with ERR-specific inhibitors will be necessary to validate this hypothesis, the observation that ERRα is expressed in breast cancer cell lines and control a marker gene linked to the estrogenic signaling pathway and that a therapeutically relevant ligand downregulates this activity opens new research avenues on the role of ERRα and its potential ligands in breast cancer etiology and treatment.

The orphan nuclear receptors ERRα, β, and γ and the classic nuclear receptors ERα and β can now be considered, functionally, as an extended family. These receptors share common target genes (this study and Ref. 22) and a pharmacologically relevant synthetic ligand (23). These findings indicate that careful consideration should be given to all members of this extended family of nuclear receptors when physiological and pathological pathways linked to estrogens are being investigated. The identification of a natural hormone associated with ERR signaling and/or the development of specific ERR ligands, combined with the use of genetic models with null alleles of ER and ERR genes and functional genomics tools, will be essential to assign precise functions to each receptor isoform and to evaluate the extent of transcriptional cross-talk between the two receptor subfamilies.

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Transcriptional Regulation of the Estrogen-inducible pS2 Breast Cancer Marker Gene by the ERR Family of Orphan Nuclear Receptors

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