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Identification of Estrogen-Responsive Genes by Complementary Deoxyribonucleic Acid Microarray and Characterization of a Novel Early Estrogen-Induced Gene: *EEIG1*

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Estrogen receptors (ERs) are nuclear transcription factors that regulate gene expression in response to estrogen and estrogen-like compounds. Identification of estrogen-regulated genes in target cells is an essential step toward understanding the molecular mechanisms of estrogen action. Using cDNA microarray examinations, 19 genes were identified as induced by 17β-estradiol in MCF-7 cells, 10 of which have been reported previously to be estrogen responsive or to be linked with ER status. Five known estrogen-regulated genes, E2IG4, IGFBP4, SLC2A1, XBP1 and B4GALT1, and AFG3L1, responded quickly to estrogen treatment. A novel estrogen-responsive gene was identified and named *EEIG1* for early estrogen-induced gene 1. *EEIG1* was clearly induced by 17β-estradiol within 2 h of treatment, and was widely responsive to a group of estrogenic compounds including natural and synthetic estrogens and estrogenic environmental compounds. *EEIG1* was expressed in ER-positive but not in ER-negative breast cancer cell lines. *EEIG1* expression was repressed by antiestrogens 4-OH-tamoxifen and ICI 182,780 but not by protein synthesis inhibitors cycloheximide and puromycin. These results provide evidence that some estrogenic compounds differentially enhance the transcription of estrogen-regulated genes and suggest a role for *EEIG1* in estrogen action. (*Molecular Endocrinology* 18: 402–411, 2004)

ESTROGENS HAVE WIDESPREAD biological actions that are mediated through their ability to bind to estrogen receptors (ERs). ERs are ligand-modulated DNA-binding transcription factors, and they regulate the expression of genes controlling cell growth and differentiation (1). Naturally occurring phytoestrogens and some endocrine-disrupting chemicals (EDCs) mimic some of the actions of endogenously produced estrogens and disrupt estrogen signaling (2, 3). In addition, dietary and environmental agents that can act as estrogens have been linked to the risk for some human disease such as breast cancer, although their true involvement remains to be proven (4).

To elucidate the mechanisms of estrogen function, it is necessary to identify the estrogen target genes. These estrogen targets may serve as biomarkers to monitor EDCs and estrogen-related diseases. Although many estrogen-responsive genes have been characterized, the complete set of estrogen-regulated genes is unknown. DNA microarray technology permits the transcriptional analysis of a large number of genes and gene products simultaneously. Knowledge of gene-expression differences in large numbers of patients with breast cancer will ultimately result in the definition of global gene-expression patterns associated with ER status (5). Microarray studies have helped to define global gene expression data derived from an *in vitro* model to pinpoint novel estrogen-responsive genes of potential clinical relevance (6, 7) and to monitor possible environmental EDCs (8). In this study our goal was to identify genes the expression of which was regulated early in response to 17β-estradiol (E2) treatment in MCF7 cells, which is a hormone-responsive human breast cancer cell line. We also examined which genes were responsive to putative EDCs, because of the potential use of gene-

Abbreviations: Ala, Alachlor; B4GALT1, β 1,4-galactosyltransferase, polypeptide 1; Cho, cholesterol; CHX, cycloheximide; DDT, 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane; DES, diethylstilbestrol; DHT, dihydrotestosterone; E2, 17β-estradiol; E3, estriol; EDCs, endocrine disrupting chemicals; *EEIG1*, early estrogen-induced gene 1; EE2, 17-α-ethinylestradiol; ERs, estrogen receptors; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gen, genistein; HC, 2-hydroxypropyl-β-cyclodextrin; ICI, ICI 182780; IGFBP4, IGF binding protein 4; MC, methyl-β-cyclodextrin; 4NP, 4-nonylphenol; OHT, 4-OH-tamoxifen; 4OP, 4-octylphenol; ORF, open reading frame; Pro, Progesterone; Pur, puromycin; Sit, β-sitosterol; Tes, testosterone; UTR, untranslated region

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expression patterns as a broad screening tool in the analysis of estrogenicity in environmental samples. Using microarrays, we identified seven early estrogen-responsive genes, including one new gene, which we named *EEIG1*. *EEIG1* was responsive to a group of estrogenic chemicals. Moreover, it was only expressed in ER-positive breast cancer cell lines and not in ER-negative breast cancer cell lines. Expression of *EEIG1* was repressed by antiestrogens but not by protein synthesis inhibitors.

RESULTS AND DISCUSSION

Identification of Estrogen-Regulated Genes

Replicate microarray hybridizations were performed using cDNA samples from MCF-7 cells exposed to E2 for 0, 2, 4, 8, 12, 24, and 48 h. Before labeling, the mRNA from E2-exposed cells was first tested for expression of known estrogen-responsive gene PDZK1 (9) using RT-PCR to ensure that the cells had been properly treated (data not shown). After hybridizations of the labeled cDNAs to the cDNA microarrays, a hierarchical clustering revealed 55 up-regulated and 38 down-regulated expressed sequence tag (EST) fragments in MCF-7 cells after exposure to estrogen for 0–48 h (Fig. 1, *left column*). The most highly expressed 25 of the 55 up-regulated EST fragments (Fig. 1, *bar a*, expanded in *right column*) were further analyzed and were found to comprise 19 human genes and two unknown ESTs. In addition, a cluster of genes that responded very quickly to estrogen was identified (Fig. 1, *bar b*).

Ten of the 19 human genes found to be up-regulated by estrogen in this study have previously been reported to be estrogen responsive or to be related to ER status (Fig. 1, identified in *blue*). These known estrogen-regulated genes included trefoil factor 1 (TFF1/pS2) (10, 11), keratin 8 (KRT8) (12), heat shock 60-kDa protein 1 (HSPD1) (11), heat shock 70-kDa protein 8/10 (HSPA8/10) (11), and NM23A protein expressed in nonmetastatic cells 1 (NME1) (11, 13). Five of the known estrogen-regulated genes were found in

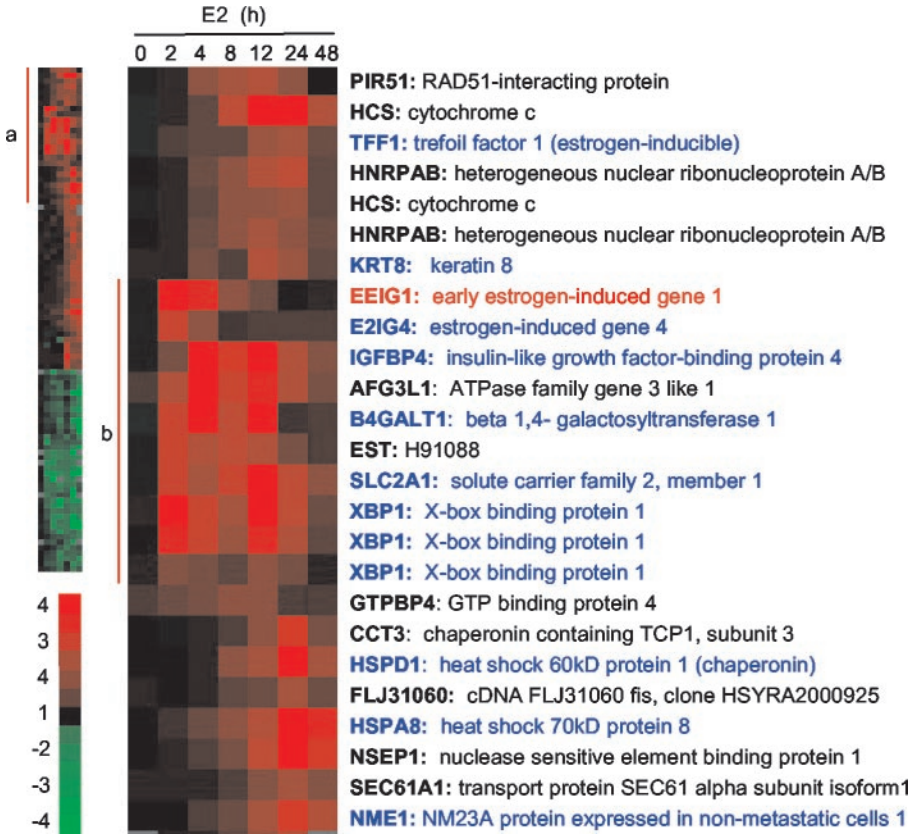

Fig. 1. Microarray Analysis of Estrogen-Regulated Genes in MCF-7

Microarray hybridizations were performed using total RNA from MCF-7 cells exposed to 10 nM E2 for 0–48 h. Hierarchical clustering uncovered a set of 55 up- (*red*) or 38 down- (*green*) regulated ESTs (*left column*). The *color scale* represents the fold change of intensity observed in microarray experiments. The data for the top 25 up-regulated ESTs (*bar a*) were enlarged over a 48-h time course. Of the 25 most highly induced ESTs, 10 are known estrogen-responsive genes (names listed in *blue*), one is a new early estrogen-induced gene (name in *red*), and the remaining include eight unreported estrogen-regulated genes and two ESTs (names listed in *black*). *Bar b* highlights a cluster of early estrogen-induced candidate genes.

the cluster of genes that responded early to E2 (Fig. 1, *bar b*), including estrogen-induced gene 4 (E2IG4) (11), IGF binding protein 4 (IGFBP4) (7), β 1,4-galactosyltransferase, polypeptide 1 (B4GALT1) (14), solute carrier family 2 member 1/glucose transporter 1 (SLC2A1/GLUT1) (15, 16), and three fragments of X-box binding protein 1 (XBP1) (6, 7).

TFF1/pS2 is a well-known estrogen-induced gene in human breast cancer (10). KRT8 was isolated by differential screening, and its different expression between ER-positive breast cancer cell line MCF7 and ER-negative cell line MDA-MB-231 was demonstrated by Northern blot hybridization (12). E2-induced expression of TFF1, E2IG4, NME1, HSPD1, and HSPA8/10 was identified by SAGE and further confirmed by Northern blot hybridization (11). Very recently, estrogen-induced expression of XBP1, IGFBP4, NME1, and B4GALT1 has been reported in microarray experiments (6, 7, 13). Custodia *et al.* (8) found E2 induction of two members of the *Caenorhabditis elegans* HSP70 protein family (Hsp-1 and Hsp-2) using microarrays. The independent confirmation of these known estrogen-responsive genes provides additional support for these previous results and validates our microarray data.

Eight of the 19 candidate genes found to be up-regulated by estrogen in this study have never been shown to be estrogen responsive and may be new targets of estrogen activity (Fig. 1, identified in *black*). Of these genes, cytochrome *c* (HCS) and heterogeneous nuclear ribonucleoprotein A/B (HNRPAB) were induced in MCF-7 after 4–8 h of E2 treatment. Moreover, two fragments corresponding to each of these genes were present on the microarray, and each pair showed the same expression pattern. To confirm that up-regulated genes identified by microarray experiments were truly estrogen regulated, the expression of HCS and HNRPAB was monitored by semiquantitative RT-PCR (Fig. 2). The RT-PCR results and microarray data were consistent.

Early Estrogen-Induced Genes

The most highly and early up-regulated gene cluster (Fig. 1, *bar b*) included 10 ESTs corresponding to six known human genes, one gene for a hypothetical hu-

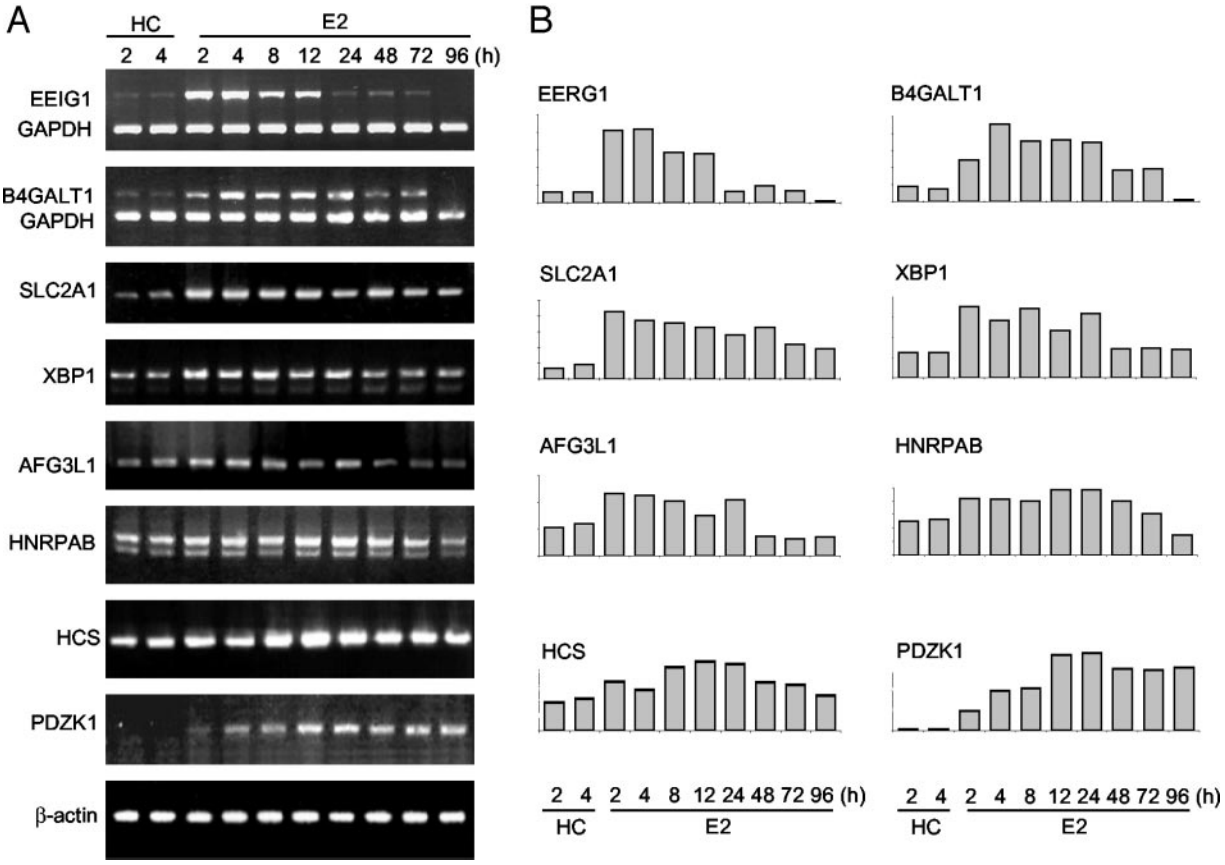

Fig. 2. RT-PCR Verification of Estrogen-Responsive Genes Identified by Microarray Analysis

A, RT-PCR was used to detect the temporal response of seven genes to estrogen stimulation in MCF7 cells grown in the presence of carrier HC for 2 and 4 h, and 10 nM E2 in HC for 2–96 h. The known E2-responsive gene PDZK1 was used as a positive control. β-Actin or GAPDH was used as internal control to confirm approximately equal cDNA templates in all samples. B, *Bar graph* quantifying the intensity of gene expression relative to that of GAPDH or β-actin from the data in panel A over the 96-h time course.

man protein (that we have subsequently called *EEIG1*, see below) and one unknown EST. Of the six known human genes, five (E2IG4, IGFBP4, B4GALT1, SLC2A1/GLUT1, and XBP1) are known to be estrogen regulated, and one corresponded to a known human gene, ATPase family gene 3-like 1 (AFG3L1), which has never previously been shown to be estrogen responsive.

Induction of E2IG4 and IGFBP4 after exposure to E2 for 2–3 h was previously reported (7, 11). We observed a similar time response in our microarray experiments (Fig. 1). Elevated levels of SLC2A1/GLUT1 mRNA were reported in immature rat uteri (15) and monkey brain parenchyma (16) treated with E2. Here, our experimental data showed that SLC2A1was also a target for E2 in a human breast cancer cell line (Figs. 1 and 2). XBP1 is a transcription factor (17) and was shown to be differentially expressed in ERα-positive and -negative human breast cancers (5, 18). In microarray experiments by Finlin *et al*. (6) and Bouras *et al*. (7), XBP1 was also identified as estrogen regulated. Three EST fragments corresponding to XBP1 were printed on our microarrays, and each was found to cluster within the early up-regulated ESTs (Fig. 1). RT-PCR confirmed that XBP1 was estrogen induced as early as 2 h after E2 treatment of MCF-7 cells (Fig. 2).

B4GALT1 belongs to a family of at least six related β-1,4-galactosyltransferases, and its deficiency causes a disease termed congenital disorder of glycosylation type IId (19). B4GALT1 was identified together with PDZK1 and ERα from a human breast cancer cDNA library subtracted from normal tissues (20), which suggests a strong relationship between B4GALT1 and ER status. Very recently, Inoue *et al.* (14) reported that B4GALT1 was induced by estrogen after 72 h of treatment in both MCF-7 and T-47D breast cancer cells but not in other cancer cell lines derived from breast, ovary, endometrium, stomach, brain, renal, or melanoma cells in microarray experiments. Using RT-PCR, we showed that the expression of B4GALT1 increased as early as 2 h after exposure of MCF-7 to E2 (Fig. 2). These data confirm that B4GALT1 is also an early estrogen-regulated gene. Sotiriou *et al.* (21) reported that the expression of B4GALT1 in human breast cancers appeared stronger in patients who responded poorly to chemotherapy compared with those who responded well, suggesting that B4GALT1 might play a role in breast cancer development.

AFG3L1 is a human homolog of the yeast ATPase family gene 3-like 1. Its mouse ortholog, Afg3l1, encodes an ATP-dependent zinc metalloprotease that is targeted to the mitochondria. However, AFG3L1 does not seem to be translated in humans (22). In our microarray experiments, AFG3L1 was induced 2 h after E2 treatment in MCF-7 cells. RT-PCR confirmed this E2-regulated expression of AFG3L1 although the effect was not as strong as with B4GALT1 (Fig. 2). This result may provide new evidence for AFG3L1 activity in human breast cancer cells and suggest that AFG3L1 maybe a target of estrogen.

EEIG1 Is a Novel Early Estrogen-Induced Gene

An up-regulated EST corresponding to the gene of human hypothetical protein, LOC90676 (GenBank accession no. XM_033421) was found in the cluster of early E2 responsive genes. We called this gene *EEIG1* for early estrogen-induced gene 1 (Fig. 1, *bar b*, identified in *red*). According to the chromosome 9 working draft sequence (GenBank accession no. NT_029366), *EEIG1* is located at 9q34.13 and consists of eight exons and seven introns. Exon 2 contains the partial 5′-untranslated regions (UTRs) and the ATG initiation codon. The open reading frame (ORF) sequence of 726 bp shares 83.5% identity with its 750-bp mouse homolog (accession no. BC031157). The 242-amino acid sequence of *EEIG1* shares 92% identity with the 255-amino acid sequence in the mouse homolog. No functional information is available for *EEIG1*. To confirm that *EEIG1* is a novel early estrogen-induced gene, its expression in response to estrogen was analyzed by quantitative multiplex RT-PCR using a specific primer pair, EEIG (see Table 1 and Fig. 3 for sequence and location), located at the 5′-UTR of LOC90676 and covering the microarray EST fragment. Detection of *EEIG1* transcripts was clearly observed in MCF-7 cells exposed to E2 compared with the carrier-only controls (Fig. 2). The pattern of gene expression was similar to what we detected in microarray experiments (Fig. 1).

The sequence of the EST fragment on our microarray corresponding to *EEIG1* matched not only with human hypothetical protein LOC90676, but also with human hypothetical protein FLJ00179 (GenBank accession no. AK074108) at their 3′-UTR. FLJ00179 is similar to LOC90676 for the first 580 bp of the ORF and the 3′-UTR. However, FLJ00179 is missing the 5′-UTR, the ATG initiation codon and the first 102 bp of the ORF, and contains an additional untranslated insert of 1149 bp before the last 46 bp of the ORF relative to LOC90676 (Fig. 3A). To determine to which mRNA sequence (LOC90676 or FLJ00179), *EEIG1* corresponded to in our experiments, we designed and synthesized specific primers for both mRNA sequences and tested these with RT-PCR. Amplification of cDNA occurred only in the reactions with the LOC90676-specific primer pairs but not with the FLJ00179-specific primer pairs (Fig. 3B). The LOC90676-specific primers also successfully amplified cDNA from other ER-positive cell lines exposed to E2, but not from ER-negative breast cancer cell lines (Fig. 3C). A new human mRNA AI426465 (GenBank accession no. BC047949) and a mouse mammary tumor mRNA C230093N12Rik clone MGC:38572 (GenBank accession no. NM_153560) are also similar to LOC90676 but not to FLJ00179 (Fig. 3A). These re-

Table 1. List of Primers Used for RT-PCR

Gene	Accession no.	Primer	Sequence (5′ - 3′)	Fragment (bp)
PDZK1	NM_002614	Forward	AAGGTGAAGAAGTCAGGAAGCCGTG	603
		Reverse	TGGCAGACCCCGAATCGCATTTAAG	
SLC2A1	NM_006516	Forward	AGACATGGGTCCACCGCTAT	631
		Reverse	TTCTTCTCCCGCATCATCTG	
XBP1	NM_005080	Forward	AGCTCAGACTGCCAGAGATC	522
		Reverse	TTCCAGCTTGGCTGATGACG	
B4GALT1	NM_001497	Forward	TGGATGTTTGCCTGGTCCGT	400
		Reverse	AGATCAGACACAGCCCCCTA	
ERα	NM_000125	Forward	AGACATGAGAGCTGCCAACC	299
		Reverse	GCCAGGCACATTCTAGAAGG	
AFG3L1	XM_047427	Forward	ATCCACGTTCAGAAGCTGAC	410
		Reverse	CCTTAAATTCACCCCTCACT	
HCS	NM_018947	Forward	CATGGTCTCTTTGGGCGGAA	617
		Reverse	GGTTCTAAGACAGTGAAGCA	
HNRPAB	NM_031266	Forward	AAGTTCCATACTGTCAGTGG	781/643
		Reverse	CACTTCCGTTACAGGAAATG	
β-Actin	NM_001101	Forward	TGACGGGGTCACCCACACTGTGCCCATCTA	661
		Reverse	CTAGAAGCATTTGCGGTGGACGATGGAGGG	
GAPDH	NM_002046	Forward	ACAGTCCATGCCATCACTGCC	266
		Reverse	GCCTGCTTCACCACCTTCTTG	
EEIG1	XM_033421	Forward	TCAGCACTTTCCCCCAAGTC	610
		Reverse	ATTCGTCTGGACACTGTGCG	
LOC1[a]	XM_033421	Forward	CTTGAACCTGGCCGAGTTTG	934
		Reverse	GGTGATCTGAAGGGGAAAGT	
LOC2[a]	XM_033421	Forward	ATGTTCCTGCTCTCTGGAGATCCC	729
		Reverse	TCAATGGCTTTCAATCACAACTGGC	
FLJ1[a]	AK074108	Forward	ATGCCATCGTGGAGAAGATC	563
		Reverse	GCCTTCACTTAGGGCCTAGT	
FLJ2[a]	AK074108	Forward	CGAGATCACAGCAGAGCTAC	601
		Reverse	CATGGATGAGATGTGGCGCA	

[a] LOC1 and LOC2 are specific primers designed for ORF LOC90676. FLJ1 and FLJ2 are the specific primers designed to cover two separate parts of ORF FLJ00179 (see Fig 3).

sults confirm the identity of *EEIG1* as the gene for hypothetical protein LOC90676, and that *EEIG1* is a novel early estrogen-induced gene**.**

Effect of E2 Concentration on *EEIG1* Expression

To determine the dose response and the lowest concentration of E2 that results in a detectable response, MCF-7 cells were exposed to different concentrations of E2, ranging from 10^{-7} nM to 100 nM for 4 h. Expression of B4GALT1, *EEIG1*, and PDZK1detected by RT-PCR demonstrated a dose-dependent response in these experiments (Fig. 4A). Induction of gene expression was clearly detectable down to 10^{-3} nM E2. The highest levels of expression were detected at 10 nM E2, which was the working concentration used in this study.

Gene Expression Patterns in Response to Putative EDCs

To understand the response of B4GALT1, *EEIG1*, and PDZK1 to other estrogenic chemicals, MCF-7 cells were exposed to 10 selected compounds including natural estrogens, synthetic estrogens, xenoestrogen, phytoestrogens, and pesticides. The expression of B4GALT1, *EEIG1*, and PDZK1 under these conditions was evaluated by RT-PCR. Increased expression of *EEIG1*, B4GALT1, and PDZK1 was observed when cells were exposed to natural, synthetic, and xenoestrogens, but was less evident with phytoestrogens or pesticides (Fig. 4B). Because many chemicals released into the environment potentially disrupt the endocrine system in wildlife and in humans, these results have many implications for the connection between chemicals in the environment and cancer or other ecosystem or human health effects (23–25). B4GALT1, *EEIG1*, and other responsive genes could be used as indicators of exposure to exogenous estrogenic compounds such as possible environmentally relevant endocrine disruptors. Moreover, the response to individual putative estrogenic compounds was different for the different genes, PDZK1, B4GALT1, and *EEIG1* (Fig. 4B), indicating that gene-expression patterns might be specific for each compound or group of compounds. These patterns of gene expression may provide a means to identify the source of estrogenic activity in complex environmental samples.

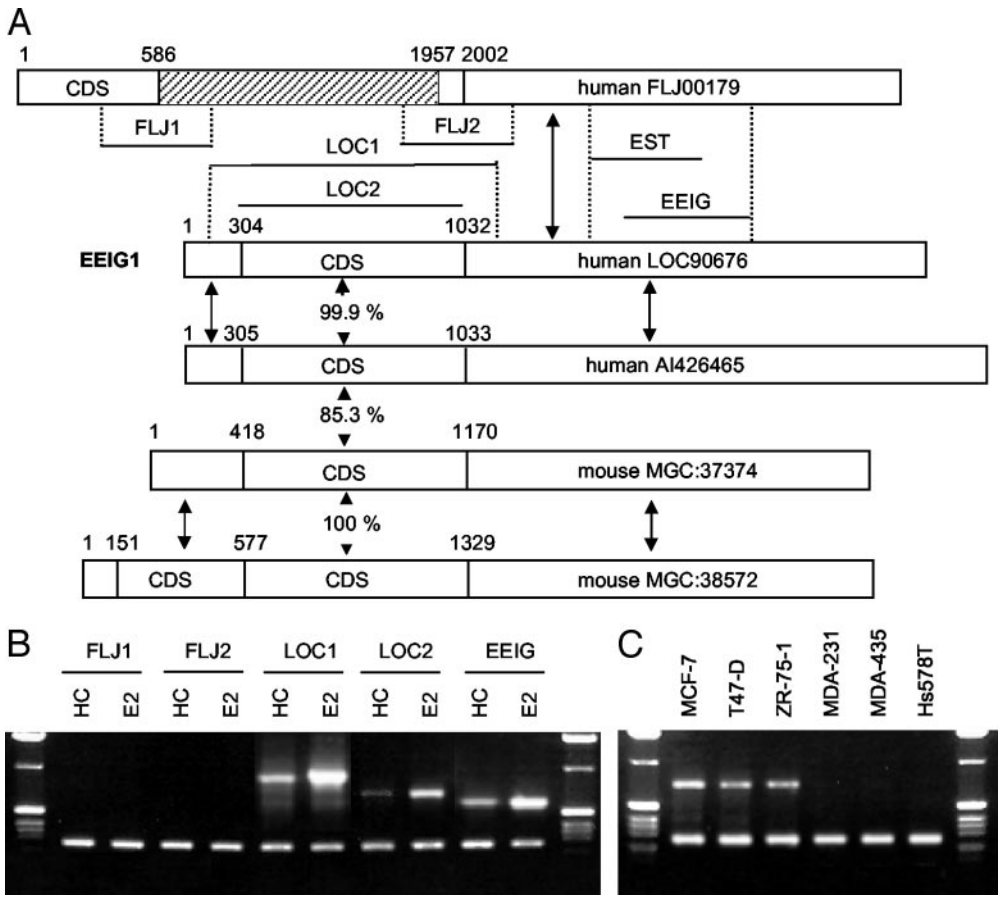

Fig. 3. Confirmation of *EEIG1* Gene Structure

A, Comparison of mRNA sequences of human hypothetical protein FLJ00179 (GenBank accession no. AK074108), *EEIG1* (human hypothetical protein LOC90676, GenBank accession no. XM_033421), human hypothetical protein AI426465 (GenBank accession no. BC047949), and mouse hypothetical protein C230093N12Rik clones MGC:37374 (GenBank accession no. BC031157), and MGC:38572 (GenBank accession no. NM_153560). *Arrows* point to essentially identical sequence fragments, and the identical homology between the coding regions (CDS) are shown. Positions of the EST and PCR-amplified fragments are indicated by *dotted lines*. The primers and fragment sizes are listed Table 1. The CDS are *boxed*, and the untranslated insert of 1149 bp in FLJ00179 is *shaded*. B, Identification of *EEIG1* structure by RT-PCR. Amplification of cDNA occurred only in the reactions with the LOC90676-specific primer pairs LOC1 and LOC2 but not with the FLJ00179-specific primer pairs FLJ1 and FLJ2, and expression was observed only with E2-treated MCF7 cells, and not with HC-treated control cells. C, Expression of *EEIG1* in ER-positive (MCF7, T-47D, and ZR-75-1) but not in ER-negative (MDA-MB-231, MDA-MB-43, and Hs578T) breast cancer cell lines in the presence of 10 nM E2 using LOC90676-specific primer pair LOC2. The *lower band* in each RT-PCR result is GAPDH used as internal control to confirm approximately equal cDNA templates in all samples.

Expression of *EEIG1* and PDZK1 appeared to be specifically related to the presence of estrogenic compounds, because these genes were not significantly induced in MCF-7 cells exposed to progesterone, to the androgens testosterone (Tes) or dihydrotestosterone (DHT), or to cholesterol (Cho), a biochemical precursor to steroid hormones (Fig. 4C). These results suggest that genes such as *EEIG1* and PDZK1 are specifically responsive only to estrogenic compounds in MCF-7 cells.

EEIG1 Expression Depends on ER But Not on New Protein Synthesis

Tamoxifen (OHT) acts as an inhibitor of estrogen-induced responses and inhibits the effect of E2 both on cell proliferation and on the regulation of specific genes. For example, expression of the E2-responsive gene PDZK1 was found to be repressed by OHT (6). ICI 182,780 (ICI) is also widely used as an antiestrogen (14). Therefore, we compared the expression of *EEIG1* and PDZK1 in the presence and absence of OHT and ICI to determine whether the expression of *EEIG1* depends on ER activity, as is the case with PDZK1. In our experiments, OHT and ICI significantly blocked the E2-induced expression of these two genes (Fig. 4C). The inhibitive effect of OHT and ICI further confirms specific E2-induced expression of *EEIG1*. Furthermore, we observed that the E2-mediated induction of *EEIG1* and PDZK1 was not prevented by pretreatment of the cell with two protein synthesis inhibitors, cyclo-

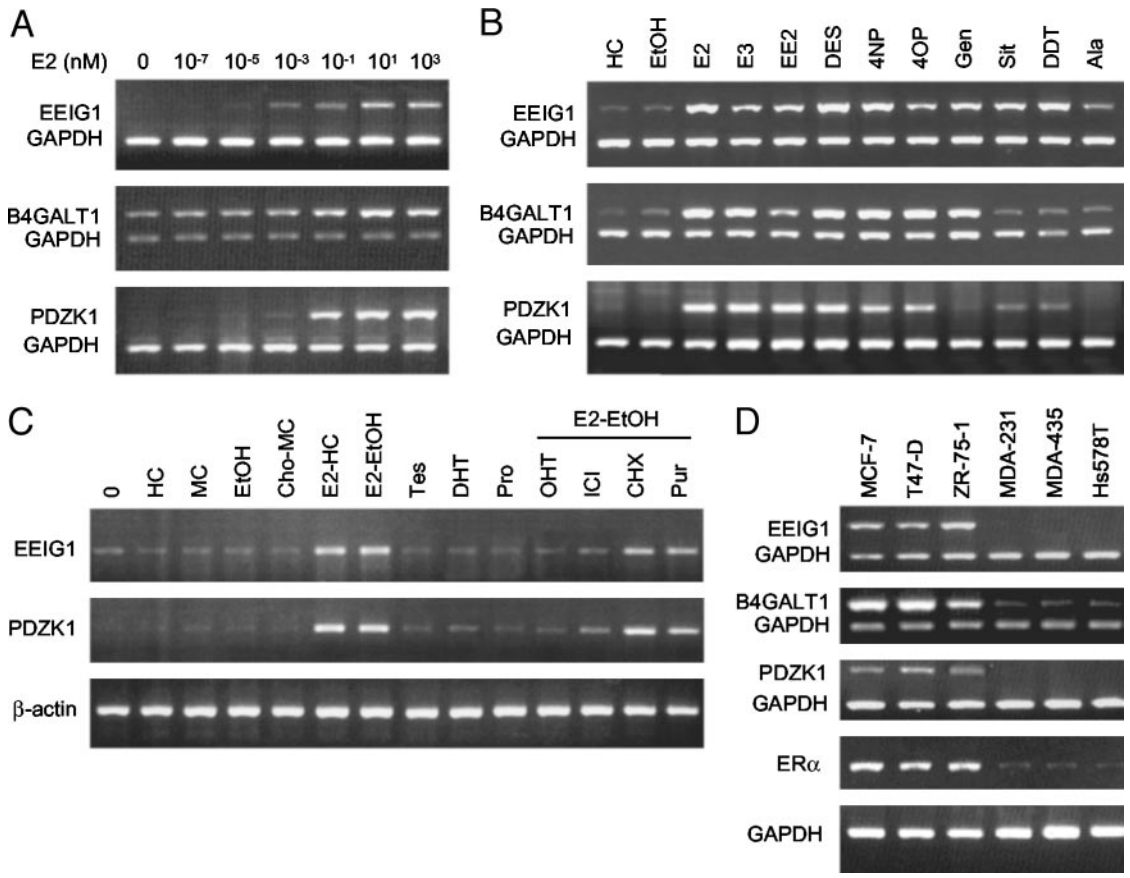

Fig. 4. RT-PCR Comparison of *EEIG1*, B4GALT1, and PDZK1 Expression

RT-PCR was used to measure *EEIG1* and B4GALT1 expression in response to different compounds and in different cell lines. The known E2-responsive gene PDZK1 was used as a positive control. GAPDH or β-actin was used as control to confirm approximately equal cDNA templates in all samples. A, Dose-dependent increase in B4GALT1 and *EEIG1* expression in MCF7 cells exposed to E2 at different concentrations from 10^{-7} nM to 100 nM for 4 h. B, Expression of B4GALT1, *EEIG1*, and PDZK1 in the presence of different estrogenic compounds including natural estrogens E2 (10 nM) and E3 (100 nM), synthetic estrogens EE2 (8 nM) and DES (4 nM), xenoestrogens 4NP (10 μM) and 4OP (10 μM), phytoestrogens Sit (100 μM) and Gen (100 μM), and pesticides DDT (1 μM) and Ala (10 μM) in MCF-7 cells exposed for 4 h in comparison with control cells exposed only to HC and EtOH. C, *EEIG1* and PDZK1 expression in MCF-7 cells exposed for 4 h to various treatments: time zero 0; carrier controls (HC, MC, and EtOH); 10 nM cholesterol in MC (Cho-MC); 10 nM E2 in either HC (E2-HC) or in ethanol (E2-EtOH); 10 nM androgens (Tes or DHT); and 10 nM Pro. To test the effects of estrogen antagonists, MCF-7 cells were treated with 10 nM E2 and either 100 nM OHT or 50 nM ICI for 4 h. To check the effects of protein synthesis inhibitors, MCF-7 cells were pretreated with either 50 μM of CHX or Pur for 1 h and then cultured in the presence of 10 nM E2 for 4 h. D, E2-induced expression of *EEIG1*, B4GALT1, and PDZK1 in a variety of breast carcinoma cell lines. Lanes 1–3, ER-positive cell lines MCF7, T-47D, and ZR-75-1; lanes 4–6, ER-negative cell lines MDA-MB-231, MDA-MB-43, and Hs578T. ERα was used to confirm the presence of the ER in these cell lines.

heximide (CHX) and puromycin (Pur) (26, 27) (Fig. 4C). These results suggest that these genes are directly regulated by E2.

EEIG1 and B4GALT1 Expression Is Related to Cell ER Status

The microarray data and RT-PCR analysis revealed that both B4GALT1 and *EEIG1* responded early to E2 treatment. This early effect may be expected to be more reproducible and indicative that these genes may be direct targets of activated ER. We therefore focused additional experiments on these two genes. To investigate the correlation of the estrogen responsiveness of the B4GALT1 and *EEIG1* to ER status in human breast carcinomas, we examined the expression after exposure to E2 of these two genes in three ER-positive and three ER-negative human breast carcinoma cell lines by RT-PCR. B4GALT1 and *EEIG1* were expressed in ER-positive cell lines MCF-7, T47-D, and ZR-75–1 but were not expressed in ER-negative breast carcinoma cell lines MDA-MB-231, MDA-MB-435, and Hs578T (Fig. 4D). The expression patterns of ERα and PDZK1 under the same conditions are shown for comparison. The selective expression in ER-positive cell lines for B4GALT1 and *EEIG1*

shows their expression is dependent on the presence of a functional ER. The pattern of expression suggests an important role for these two genes in the physiological response of breast tumors to estrogen.

In summary, using microarray and RT-PCR techniques, 19 estrogen-regulated genes were confirmed in the human breast cancer cell line MCF-7. Seven of these genes, including a newly identified human gene *EEIG1*, were induced by E2 within 2 h. We also found that some estrogenic compounds and environmental contaminants differentially enhance the transcription of estrogen-regulated genes, suggesting that assays of multiple gene expression profiles could be useful in identifying potential endocrine-disrupting chemicals.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO) except where noted. The chemicals used in this study included natural estrogens: E2 and estriol (E3); synthetic estrogens: 17-α-ethinylestradiol (EE2) and diethylstilbestrol (DES); xeno-estrogens: 4-nonylphenol (4NP) and 4-octylphenol (4OP); phyto-estrogens: β-sitosterol (Sit) and genistein (Gen); pesticides: 1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane (DDT) and alachlor (Ala); androgens: Tes and DHT; progesterone (Pro); cholesterol (Cho); antiestrogens: OHT and ICI (Tocris Cookson, Inc., Ellisville, MO); protein synthesis inhibitors: CHX and Pur; and solvent and carriers: ethanol (EtOH, solvent for most of the endocrine compounds), 2-hydroxypropyl-β-cyclodextrin (HC, carrier for water-soluble E2), and methyl-β-cyclodextrin (MC, carrier for water-soluble Cho). Cells treated with only EtOH, HC, or MC were used as controls for different endocrine compounds. Cell culture media and supplements and microarray hybridization and RT-PCR reagents were products of Invitrogen (Carlsbad, CA) except where noted.

Cell Lines and Cell Culture

Human breast cancer cell lines including ER-positive cell lines MCF-7, T47-D, and ZR-75–1 and ER-negative cell lines MDA-MB-231, MDA-MB-435, and Hs578T were obtained from American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI 1640 medium supplemented by 10% fetal bovine serum, penicillin, and streptomycin and incubated at 37 C in 5% CO_2. Before estrogen exposure, the cells were transferred into phenol red-free RPMI 1640 medium supplemented with 10% charcoal/dextran treated (hormone-free) fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and subsequently grown for 6 d. The cells were then challenged with estrogen or estrogen-like compounds at specific concentrations for different time intervals as described below.

Experiments with Estrogen

To study the temporal response to estrogen stimulation, MCF7 cells were first grown in estrogen-free medium for 6 d, after which the medium was amended with 10 nM water-soluble E2 in HC. In microarray experiments, a water-soluble E2 bound to HC was used to treat the cells, rather than E2 in ethanol, to avoid possible interactions between ethanol and ER signaling (28), A similar set of MCF7 cells amended with an equal volume of HC but without E2 was used as a control. Cells were harvested from both the estrogen-treated samples and the control samples 2, 4, 8, 12, 24, 48, 72, or 94 h after exposure. To compare the different responses in cell lines with and without ER, six different human breast cancer cell lines were grown in the absence of estrogen for 6 d and then were exposed to 10 nM E2 for 4 h. To determine the effects of concentration, MCF7 cells were exposed to E2 concentrations of 0 (carrier only) 10^{-7}, 10^{-5}, 10^{-3}, 10^{-1}, 10, and 10^{3} nM. Cells were harvested after 4 h exposure to E2.

Experiments with Putative EDCs

To study the effects of different endocrine compounds or possible EDCs, MCF7 cells that were pregrown in estrogen-free medium for 6 d were amended with either 10 nM E2, 100 nM E3, 8 nM EE2, 4 nM DES, 10 μM 4NP, 10 μM 4OP, 100 μM Gen, 100 μM Sit, 1 μM DDT or 10 μM Ala; or 10 nM Cho, Tes, DHT, and Pro. These chemicals are relatively water insoluble. In these experiments, E2 was solubilized in HC, Cho was solubilized in MC, and all other chemicals were dissolved in ethanol. Concentrations of putative environmental EDCs were chosen based on their relative potencies in cell proliferation or ERα binding in MCF-7 cells compared with E2 (29) and concentrations used in other studies (9, 11, 14). Cells were harvested after 4 h exposure. MCF7 cells amended with an equal volume of carrier (HC, MC, or ethanol) were used as controls in these experiments.

Experiments with Antiestrogens and Protein Synthesis Inhibitors

To understand the effects of estrogen antagonists on the E2-induced gene expression, MCF-7 cells were treated with 10 nM E2 and either antiestrogens OHT (100 nM) or ICI (50 nM) for 4 h. To determine whether E2-induced gene expression depended on new protein synthesis, MCF-7 cells were pretreated for 1 h with 50 μM of the protein synthesis inhibitors CHX or Pur, and then cultured in the presence of 10 nM E2 for 4 h.

RNA Preparation

All cell pellets were immediately frozen in liquid nitrogen and stored at −70 C. QIAshredder spin columns (QIAGEN, Valencia, CA) were used to homogenize the samples. Total RNA was extracted from cell pellets using the QIAGEN RNeasy kit according to the manufacturer's instructions, and then frozen at −70 C until used.

Microarray Hybridizations

For comparative microarray hybridization, cDNAs were synthesized from E2-treated test RNA or HC reference RNA and labeled according to the manufacturer's protocols of the Microarray Centre at the Ontario Cancer Institute, Toronto, Canada (http://www.microarray.ca/). Briefly, 10 μg of total RNA were reverse transcribed with Superscript II reverse transcriptase, AncT primer (5′-T_{20}VN-3′) and aminoallyl-dUTP (Sigma) at 42 C for 2 h. cDNA from test and reference samples were labeled with Cy5 and Cy3 dyes, respectively (Amersham Pharmacia Biotech, Arlington Heights, IL) at room temperature for 1 h, following recommended protocols of the Microarray Centre. The two separately labeled cDNA probes from test and reference samples were purified and combined, and then hybridized to a pair of cDNA microarray slides containing 19,008 characterized and unknown human ESTs. Hybridization was conducted at 37 C overnight in DIG Easy Hyb solution (Roche Clinical Laboratories, Indianapolis, IN). The slides were washed in 0.1× standard saline citrate and 0.1% sodium dodecyl sulfate at 50 C and then scanned using

a GenePix 4000 scanner (Axon Instruments, Inc., Union City, CA). Microarray image analysis was performed using Axon's GenePix Pro 3.0.

Data Analysis

The data files produced by GenePix Pro 3.0 were further analyzed using in-house software developed by Goryachev *et al.* (30). This DOS-based software was used to normalize Cy5 and Cy3 channels by setting the average ratio of Cy5: Cy3 equal to 1 (assuming the method of constant majority), and to calculate normalized expression ratios as test sample divided by control sample for each EST on the microarray. The software was also used to combine normalized data files from several data sets and to compare and average results obtained from replicate microarray experiments. To minimize false-positive results, replicate DNA microarray experiments (two to four arrays per experimental condition) were performed. Only those genes that had similar expression levels in at least two independent experiments and showed at least a 2-fold increase or decrease in expression were selected as estrogen-regulated candidate genes for further analysis. Hierarchical clustering was performed on all time course experiments, starting from normalized average data for candidate genes for each time point, using Cluster and TreeView software (http://rana.lbl.gov/EisenSoftware.htm).

RT-PCR

RT-PCR was used to ensure that the cells were responding to estrogen and to confirm that the genes identified by cDNA microarray screening were indeed estrogen responsive. RT-PCR was conducted according to the protocol of Invitrogen with slight modifications. Briefly, total RNA (10 μg) was reverse transcribed with the oligo(dT)$_{20}$ primer described above in a total reaction volume of 40 μl. PCR was carried out using 1 μl of reverse transcription product for amplification of target genes in a total reaction volume of 25 μl. PCR was performed using a GeneAmp PCR System 2400 (PerkinElmer Corp., Norwalk, CT). In semiquantitative RT-PCR, target genes and the reference gene β-actin were amplified from the same sample in individual reactions. The PCR conditions were as follows: 94 C for 30 sec, 57 C for 30 sec, and 72 C for 1 min repeated over 25 cycles. In quantitative multiplex RT-PCR, target genes and the reference gene GAPDH were amplified simultaneously in a single reaction. In this case, 30 PCR cycles were used. PCR products were visualized by electrophoresis on 1.2% agarose gels. The reference genes β-actin and GAPDH were used to confirm the success of RT reaction and to verify that an approximately equal cDNA template was added to each reaction. The sequences of the primers and their amplified cDNA fragments sizes are listed in Table 1. To confirm that the cells were indeed responding to added estrogen, PDZK1, a gene known to be expressed in response to estrogen (9), was used as a biomarker.

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