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PDZK1 and *GREB1* Are Estrogen-regulated Genes Expressed in Hormone-responsive Breast Cancer[1, 2]

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ABSTRACT

The function of estrogen in breast cancer proliferation and progression is likely to be due to the expression of a repertoire of genes regulated by estrogen receptor (ER). Using suppression subtractive hybridization, we have isolated a set of 14 estrogen-responsive genes that was differentially expressed in MCF7 cells stimulated by β-estradiol as compared with unstimulated cells. Tamoxifen repressed the expression of all 14 estrogen-responsive genes. Thirteen of the genes were induced within 6 h of estrogen treatment, indicating that these were early response genes in the ER-regulated pathway. *PDZK1* and a new gene, *GREB1*, demonstrated a significant correlation with ER phenotype in a panel of breast cancer cell lines. Treatment with cycloheximide indicated that ER directly controls GREB1 expression. Three cDNAs (GREB1a, GREB1b, and GREB1c) were isolated by screening a MCF7 cDNA library. These three cDNAs of GREB1 shared extensive sequences through the open reading frame but had divergent 5′ untranslated regions, indicating the possibility of multiple promoters regulated by β-estradiol. Studies in primary breast cancers showed that PDZK1 and GREB1 were overexpressed in ER-positive breast cancers as compared with ER-negative breast cancers by 19-fold and 3.5-fold, respectively. GREB1 was also induced by β-estradiol in the ER-positive endometrial cell line ECC-1. The pattern of expression suggests a critical role for these two genes in the response of tissues and tumors to β-estradiol.

INTRODUCTION

Increased exposure to estrogen has been correlated with the incidence of breast cancer (1, 2). There is an increased incidence of breast cancer associated with younger age at menarche, late age at first pregnancy, and late age at menopause. All these factors are associated with the duration of the exposure of breast tissue to estrogen (3, 4). In addition, clinical and laboratory studies have demonstrated that breast epithelial cells proliferate in response to estrogen (5). Therefore, it has been postulated that the mitogenic effect of estrogen, combined with inadequate DNA repair, may lead to an accumulation of genetic aberrations culminating in cancer (2). Additional evidence that estrogen is a major adverse factor in breast cancer progression is the observation of regression in tumor growth after ovariectomy and after treatment with antiestrogens like tamoxifen (reviewed in Ref. 6).

Estrogen exerts its effects through a nuclear ligand-activated transcription factor, the ER.[4] An activated hormone-receptor complex transactivates genes by binding to regulatory promoter elements called EREs (7). Two known subtypes of ER exist, ER-α and ER-β, that have distinct tissue and cell patterns of expression (8). In this study, ER specifically refers to ER-α. ER expression is routinely used as a clinical marker to predict the hormone responsiveness of breast tumors (9). Compared with ER-negative breast tumors, a high percentage of ER-positive tumors respond favorably to endocrine treatment, are associated with a better prognosis, and have a well-differentiated phenotype (10–12).

The marked physiological and phenotypic differences between the ER-positive and ER-negative breast tumors have been hypothesized to be due to differences in gene expression between these two tumor types. Studies have demonstrated the differential expression of a number of genes when comparing ER-positive and ER-negative breast carcinoma cell lines (2, 13–16). Although most of the genes found to be differentially expressed in ER-positive cancers were not estradiol responsive, it is likely that estrogen induces the expression of a repertoire of genes controlling cell growth. Using techniques to isolate differentially expressed genes, several estradiol-inducible mRNAs have been identified in ER-positive human breast cancer cell lines. Genes known to be induced by estrogen treatment include *PR* (9), *pS2* (17), *cathepsin D* (18), *HSP27* (heat shock protein 27,000; Ref. 19), *aldolase A*, *dehydrogenase*, *α-tubulin*, and *glyceraldehyde-3-phosphate* (reviewed in Ref. 20). In addition to these genes, a number of estrogen-induced mRNAs of unknown function have been isolated (2, 15).

The repertoire of genes responsible for the physiological effects of estrogen on hormone-responsive breast cancers has not been characterized completely (2). In addition, well-characterized genes such as *pS2* are only expressed in 30–60% of hormone-responsive tumors (21–23), suggesting that their function is not necessary for the normal physiological response to estrogen. Therefore, to more completely understand hormone-responsive breast cancer, there is a pressing need to identify more estrogen-responsive genes and establish their role in tumorigenesis and progression.

In this study, efforts were made to delineate genes that are differentially expressed in response to estradiol treatment in MCF7, an ER-positive, hormone-responsive human breast carcinoma cell line. Using SSH (24), a set of estrogen-responsive genes has been isolated that is also repressed by tamoxifen. Further screening has enabled us to focus on two estrogen-responsive genes that exhibit a distinct correlation with ER expression in breast carcinoma cell lines and primary breast carcinomas. The pattern of expression indicates a critical functional role of these genes in hormone-responsive tissues and cancer.

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[2] The nucleotide sequence data published in this paper have been submitted to the GenBank/European Molecular Biology Laboratory database at the National Center for Biotechnology Information, and accession numbers AF245388, AF245389, and AF245390 have been assigned for GREB1a, GREB1b, and GREB1c, respectively. The cDNA clones generated by SSH have been submitted to the GenBank/European Molecular Biology Laboratory database and assigned the following accession numbers: (*a*) GREB3, accession number BE491961; (*b*) GREB18, accession number BE491962; (*c*) GREB21, accession number BE491963; (*d*) GREB65, accession number BE491964; (*e*) GREB203, accession number BE491965; (*f*) GREB76, accession number BE491966; (*g*) GREB80, accession number BE491967; (*h*) GREB89, accession number BE491968; (*i*) GREB98, accession number BE491969; (*j*) GREB138, accession number BE491970; (*k*) GREB181, accession number BE491971; (*l*) GREB199, accession number BE491972; and (*m*) GREB227, accession number BE491973.

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[4] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; RT-PCR, reverse transcription-polymerase chain reaction; SSH, suppression subtractive hybridization; ERE, estrogen response element; poly(A)$^+$ RNA, polyadenylated RNA; SSPE, saline-sodium phosphate-EDTA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ORF, open reading frame; UTR, untranslated region.

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MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were obtained from Operon Technologies, Inc. (Alameda, CA).

Cell Lines. All cell lines were obtained from American Type Culture Collection (Manassas, VA). Cell lines MCF7, T-47D, BT-20, MDA-MB-231, and HBL-100 were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD); ZR-75-1 and BT-474 were maintained in RPMI 1640 (Life Technologies, Inc.); and MDA-MB-361 was maintained in Leibovitz's L-15 medium (Life Technologies, Inc.). Media were supplemented with 10% FCS (Gemini Bio-Products, Calabasas, CA), 10 units/ml penicillin G (Life Technologies, Inc.), 10 μg/ml streptomycin (Life Technologies, Inc.), and 6 ng/ml bovine insulin (Sigma Chemical Co., St. Louis, MO). All cells were incubated at 37°C in 5% CO_2 except MDA-MB-361 cells, which were maintained in a CO_2-free environment.

MCF7 cells induced with estradiol were grown under normal conditions as described above until they reached approximately 25% confluence. The media were subsequently changed to phenol red-free MEM (Life Technologies, Inc.) supplemented with 10% charcoal-stripped FCS (Gemini Bio-Products; certified to be β-estradiol free), 10 units/ml penicillin G, 10 μg/ml streptomycin, and 6 ng/ml bovine insulin. MCF7 cells were treated with 1×10^{-8} M water-soluble β-estradiol (Sigma Chemical Co.) and/or 1×10^{-6} M 4-hydroxytamoxifen (Sigma Chemical Co.) for periods of 6–72 h. Cycloheximide (Sigma Chemical Co.) was used at a concentration of 10 μg/ml.

mRNA Isolation. Poly(A)$^+$ RNA was isolated from cell lines using Fast Track Kit 2.0 (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions.

SSH. SSH (24) was performed with the Clontech PCR-Select cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA) as described by the manufacturer. Starting material consisted of 2 μg of poly(A)$^+$ RNA from MCF7 cells grown in the absence of β-estradiol for 5 days and then treated with β-estradiol for 72 h (referred to as the tester) and 2 μg of poly(A)$^+$ RNA from MCF7 cells grown in the absence of β-estradiol for 8 days (referred to as the driver). All PCR products generated using SSH were subcloned into pCR 2.1 vector using the Original TA Cloning Kit (Invitrogen).

Isolation of GREB1 cDNAs. Approximately 1×10^6 plaques from an oligodeoxythymidylic acid and a random-primed MCF7 cDNA library were screened using the 600-bp GREB1 fragment isolated by SSH. The λ phage plaques were grown in *Escherichia coli* XL1-blue MRF′ cells (Stratagene), and phages were transferred to Optitran supported nitrocellulose (Schleicher & Schuell) using the *in situ* plaque hybridization technique (25). Phage lifts were prehybridized and hybridized in a solution containing 50% formamide (v/v), 5× SSPE, 5× Denhardt's solution, 0.1% SDS (v/v), and 100 μg/ml denatured salmon sperm DNA at 42°C. Fifty ng of GREB1 cDNA was ^{32}P-labeled by random priming (Roche Biochemicals) using 50 μCi of [α^{32}P]dCTP (3000 Ci/mmol; Amersham). After a 16-h hybridization, membranes were washed once in 2× SSC/0.1% SDS (v/v) for 20 min at 42°C and twice in 0.2× SSC/0.1% SDS (v/v) for 20 min at 65°C before exposure to Kodak XAR-2 film overnight at −80°C with intensifying screens. The λ phage plaques that hybridized to ^{32}P-labeled 600-bp GREB1 fragment were isolated, and plaques were purified by secondary and tertiary rounds of screening. Isolated plaques were processed to excise the cDNA inserts from the λ vector to yield phagemids containing the inserts of interest in the pBK-CMV vector (Stratagene).

The region of GREB1 cDNA that was not obtained from screening the λ library was obtained by RT-PCR. The region between GREB1 and GREB2 (later determined to be the 3′ end of GREB1 cDNA) was amplified using two separate RT-PCR amplifications. One reaction used the primers 5′-GREB1/2 (5′-TGGACCTGGGATCCTTTGAGAAGGTGGACTTTC-3′) and 3′-GREB1/2 (5′-ACGACGAGGTGACCGACACCTGGACGCTCCTCTG-3′), which generated a DNA fragment of 1565 bp. The second reaction used primers ck5′-GREB1/2 (5′-CCCCAACATTGTGACACTTCACGTGACC-3′) and ck3′-Greb1/2 (5′-CCAAGCAGGAGGAGTTTATCAATCGCAGG-3′), which generated a DNA fragment of 720 bp. Both PCR reactions were cloned and had the identical sequence for the intervening region between GREB1 and GREB2.

Northern Analysis. One μg of mRNA was electrophoresed on a 1% agarose formaldehyde denaturing gel in 1× 4-morpholinepropanesulfonic acid and then transferred to a Nytran membrane (Schleicher & Schuell). Approximately 25–50 ng of each clone in pCR 2.1 or pBK-CMV was ^{32}P-labeled by random priming (Roche Biochemicals). As controls, 25–50 ng of cDNA for pS2, ER, GAPDH, and β-actin were also labeled. Northern blots were prehybridized and hybridized in 50% formamide, 5× Denhardt's solution, 5× SSPE, 1% SDS, and 100 μg/ml denatured salmon sperm DNA at 42°C. Blots were washed in 2× SSC/0.1% SDS (v/v) at 42°C for 20 min, followed by two washes in 0.2× SSC/0.1% SDS (v/v) at 65°C for 20 min each. Northern blots were placed on film with an intensifying screen at −80°C.

DNA Sequence Analysis. Sequencing of the SSH fragments was performed on double-stranded templates using the dideoxynucleotide chain-termination method with [α-^{35}S]dATP (1000 Ci/mmol; Amersham) as a label. Sequencing reactions were carried out using the Sequenase version 2.0 T7 DNA polymerase DNA Sequencing Kits (USB Corporation, Cleveland, OH) with the T7 promoter primer in the pCR 2.1 vector.

The sequence for the GREB1a, GREB1b, and GREB1c cDNAs isolated from the MCF7 cDNA library was determined on both strands using the automated ABI 373 DNA sequencing system with the standard dye terminator AmpliTaq polymerase FS kit by the Stanford University Protein and Nucleic Acid facility (Stanford, CA). T3 and T7 promoter primers and custom sequence-specific primers were used, and all sequences reported were determined by sequencing both strands of DNA. The nucleotide sequence of the cDNA and the protein sequence of GREB1a, GREB1b, and GREB1c were compared against the National Center for Biotechnology Information databases using BLASTN and BLASTP, respectively.

RT-PCR from Primary Tumors. Primary human breast tumor tissue was collected fresh from mastectomy and biopsy specimens (kindly provided by Dr. Helen Feiner, Breast Cancer Resource, New York University Medical Center) and snap frozen in liquid nitrogen. Approximately 0.5 mg of tissue was homogenized, and total RNA was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's recommendations. One μg of RNA from each sample was reverse transcribed using random hexamers with the Advantage RT-for-PCR kit (Clontech) according to the manufacturer's recommendations. The 20-μl reverse transcription sample was diluted to 100 μl with water, and then 2 μl of each sample were analyzed by PCR for GREB1a, GREB1b, GREB1c, PDZK1, ER, and β-actin messages using the Advantage cDNA PCR kit (Clontech) with Advantage KlenTaq Polymerase mixture and gene-specific primers designed across intron/exon junctions. The common 5′ primer for GREB1a, GREB1b, and GREB1c was oGREB-5′ (5′-GGACCAGCTTCAGTCACCTTTCCAGTGGTGGCC-3′). The 3′ primer for GREB1a was oGREB1a-3′ (5′-GGAAGATCTGCTCCAGGTCCTCCATCAAGGGC-3′), the 3′ primer for GREB1b was oGREB1b-3′ (5′-CTCTTTTATGAATTGTCTGGTTTATACGTCCGG-3′), and 3′ primer for GREB1c was oGREB1c-3′ (5′-GGGAGTAAAGCTGGTGCCTGGGGCACAGGTCACG-3′). The fragments generated for GREB1a, GREB1b, and GREB1c were 334, 339, and 325 bp, respectively. Primers for PDZK1 were oPDZ-5′RT (5′-GCTTCTCCTGCGAATTGAGAAGGACACCGAGG-3′) and oPDZ-3′RT (5′-GGTCCAAGTTTGCACACC TCCATTCATCACAGG -3′), which generated a PCR fragment of 331 bp. Primers for ER were oER-1A-long (5′-GTGCCCTACTACCTGGAGAACGAGCCCAGC-3′) and oER-1B-long (5′-AGCATAGTCATTGCACACTGCACAGTAGCG-3′), which generated a 195-bp DNA fragment. Primers for β-actin were oACTIN-5′ (5′-AGCAAGAGAGGCATCCTCACCCTGAAGTACC-3′) and oACTIN-3′ (5′-CAGATTCTCCTTAATGTCACGCACGATTTCCC-3′), which generated a 471-bp DNA fragment. Two-step PCR was used to amplify the genes and was performed on a Perkin-Elmer 9600 DNA thermal cycler as follows: (*a*) 1 cycle of 94°C for 1 min; (*b*) 30 cycles of 94°C for 30 s and 68°C for 3 min; and (*c*) 1 cycle of 68°C for 3 min. PCR samples were then analyzed for GREB1a, GREB1b, GREB1c, PDZK1, ER, and β-actin by electrophoresis on 1.5% agarose gels.

After electrophoresis, the samples were transferred onto 0.2 μm Nytran (Schleicher & Schuell) nylon membranes by Southern blotting. Blots were prehybridized and hybridized in 20% formamide, 5× Denhardt's solution, 5× SSPE, 1% SDS (v/v), and 100 μg/ml denatured salmon sperm DNA at 42°C. Southern blots were probed with oligonucleotides that had been end-labeled using [γ-^{32}P]ATP (6000 Ci/mmol; Amersham) and T4 polynucleotide kinase (New England BioLabs). The oligonucleotides were designed internal to the sequence of each gene amplified. The four GREB transcripts were detected using oGREBint (5′-GGCTTGGCCTTGCATATTTTCAGC-3′). Other internal oligonucleotides were oPDZK1/int (5′-CATATGCAGGTTGTGGATCTGG-3′), oERpro-86 (5′-ACCCTGGCGTCGATTAT CTGAATTTGGC-3′), and oActin-int (5′-ATGACCCAGATCATGTTTGAGACC-3′). After 16 h

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of hybridization, the blots were washed sequentially in 2× SSC/0.1% SDS (v/v) at 42°C for 20 min followed by two washes in 2× SSC/0.1% SDS (v/v) at 65°C for 20 min each and then placed on X-ray film with an intensifying screen at for 10 min. Subsequently, the blots were placed on a Kodak Phosphor screen (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software and a Molecular Dynamics PhosphorImager.

RESULTS

Isolating Estrogen-responsive Genes with SSH. SSH performed using mRNA from MCF7 cells grown in the presence and absence of β-estradiol, as a tester and driver, respectively, resulted in the generation of more than 400 putative subtracted clones. Of these, 240 clones were screened using Northern blot analysis of MCF7 cells grown in presence or absence of β-estradiol. Through this process, 15 estrogen-responsive genes were identified. When grown in the presence and absence of β-estradiol, the changes in the levels of gene expression ranged between approximately 4-fold induction to turning on a previously undetectable gene (on/off). The cDNA clones identified as differentially expressed were compared against the European Molecular Biology Laboratory/GenBank databases. Only four of these clones had sequences that matched previously reported genes; however, none of these were known to be estrogen responsive in breast cancer. These known genes include thrombospondin (GenBank accession number X14787), PDZK1 (GenBank accession number AF012281), KIAA0575 from human brain (GenBank accession number AB011147) that is an mRNA coding for a protein of unidentified function, and an immunoglobulin-like gene (GenBank accession number A034198). The remaining 11 genes were deemed novel because their sequence did not match any of the gene sequences reported previously. The novel genes as well as the known genes with undefined function have been designated as GREB (genes regulated by estrogen in breast cancer).

Effect of Tamoxifen on Estrogen-responsive Genes. Tamoxifen is a competitive antagonist of endogenous and exogenous estradiol.

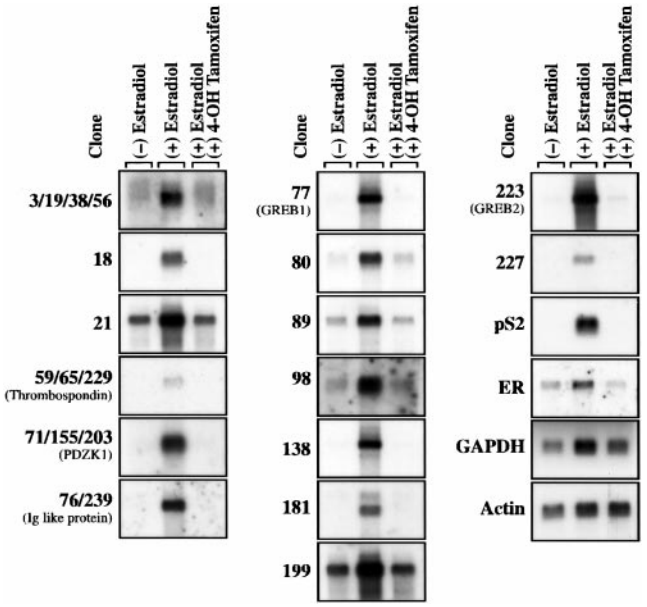

Fig. 1. Northern blots of GREB identified by SSH. Northern blots containing 1 μg of poly(A)+ RNA/lane from MCF7 cells-estradiol MCF7 cells + estradiol, and MCF7 cells + estradiol + tamoxifen were probed with cDNA fragments of genes isolated by SSH. A representative Northern blot for each estrogen-responsive gene identified is shown. Several genes were detected multiple times and are indicated. In addition, pS2 is shown as a control to validate estradiol responsiveness. ER, GAPDH, and β-actin were used as size markers, and the latter two were also used to confirm similar loading of the samples.

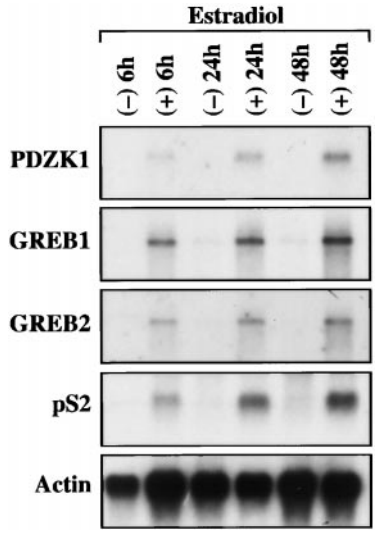

Fig. 2. Kinetics of estradiol response. Northern blots containing 1 μg of poly(A)+ RNA/lane from MCF7 cells grown in the presence and absence of estradiol for 6, 24, or 48 h. As shown, GREB1, GREB2, and PDZK1 respond early to estradiol treatment. The known estradiol-responsive gene, pS2, is shown as a control. Hybridization with β-actin was used to confirm approximately equal loading of poly(A)+ RNA.

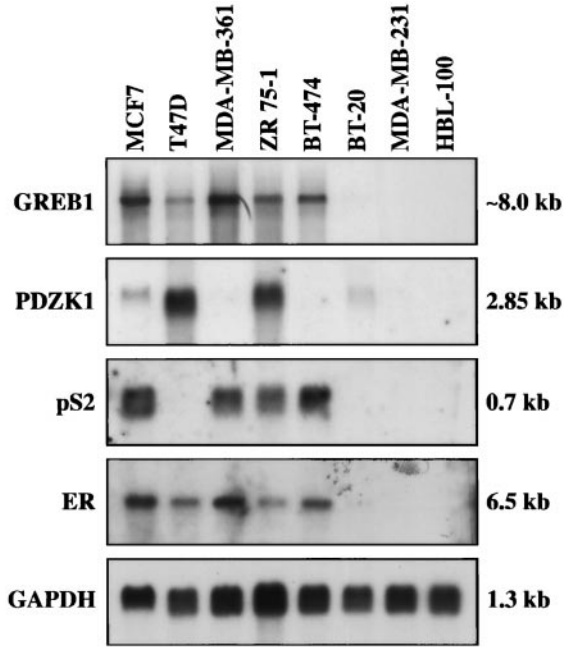

Fig. 3. Expression of GREB1 and PDZK1 in breast cancer cell lines. Northern blots demonstrating the gene expression pattern of GREB1 and PDZK1 compared with pS2 and ER in a panel of breast carcinoma cell lines. *Lanes 1–5*, cell lines that express high levels of ER (MCF7, T-47D, MDA-MB-361, ZR-75-1, and BT-474); *Lane 6*, BT-20, a cell line that expresses very low levels of ER; and *Lanes 7* and *8*, ER-negative cell lines (MDA-MB-231 and HBL-100). GAPDH was used as a control to confirm similar loading of poly(A)+ RNA.

Tamoxifen acts as an inhibitor of estrogen-induced responses and also inhibits the effect of estradiol on cell proliferation and on the regulation of specific genes. Thus, the effect of tamoxifen on the 15 estrogen-responsive genes was examined. Northern blot analysis of MCF7 cells grown in the absence or presence of β-estradiol and in the presence of β-estradiol and tamoxifen indicated that all of the 15 genes that were induced by β-estradiol were repressed by tamoxifen (Fig. 1). Expression of the estradiol-responsive gene *pS2* was also found to be repressed by tamoxifen, as has been reported previously (26).

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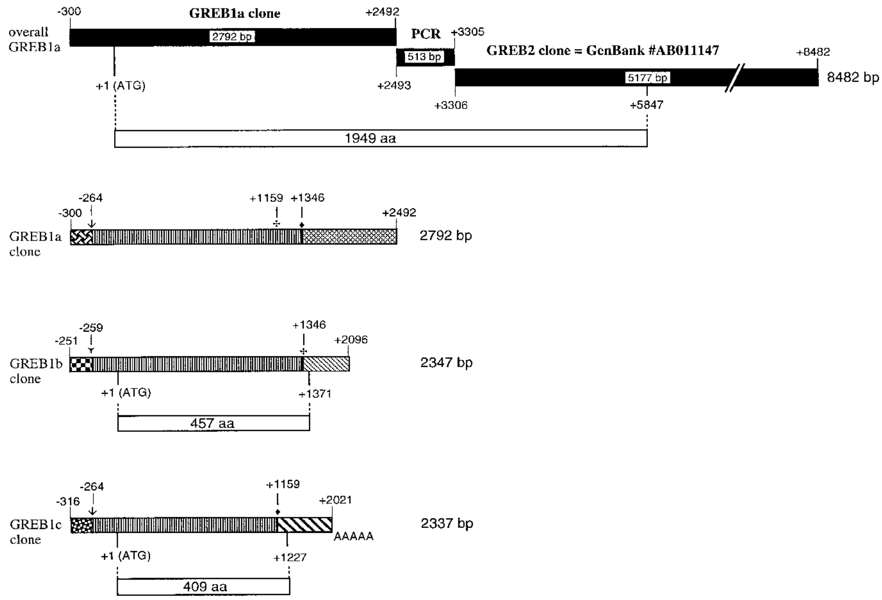
A



B

Sequence of GREB1a protein

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   1  MGNSYAGQLK TTRFEEVLHN SIEASLRSNN LVPRPIFSQL YLEAEQQLAA LEGGSRVDNE EEEEGEGGL  ETNGPPNPFQ LHPLPEGCCT TDGFCQAGKD  100
 101  LRLVSISNEP MDVPAGFLLV GVKSPSLPDH LLVCAVDKRF LPDDNGHNAL LGFSGNCVGC GKKGFCYFTE FSNHINLKLT TQPKKQKHLK YYLVRNAQGT  200
 201  LTKGPLICWK GSEFRSRQIP ASTCSSSLFP ALESTAAFPS EPVPGTNPSI LMGAQQAGPA SDHPSLNAAM GPAVFNGKDS PKCQQLAKNN LLALPRPSAL  300
 301  GILSNSGPPK KRHKGWSPES PSAPDGGCPQ GGGNRAKYES AGMSCVPQVG LVGPASVTFP VVASGEPVSV PDNLLKICKA KPVIFKGHGN FPYLCGNLND  400
 401  VVVSPLLYTC YQNSQSVSRA YEQYGASAIQ PISEEMQLLL TVYYLVQLAA DQVPLMEDLE QIFLRSWRES HLTEIRQYQQ APPQPFPPAP SAAAPVTSAQ  500
 501  LPWLASLAAS SCNDSVHVIE CAYSLAEGLS EMFRLLVEGK LAKTNYVVII CACRSAAIDS CIAVTGKYQA RILSESLLTP AEYQKEVNYE LVTGKVDSLG  600
 601  AFFSTLCPEG DIDILLDKFH QENQGHISSS LAASSVTKAA SLDVSGTPVC TSYNLEPHSI RPFQLAVAQK LLSHVCSIAD SSTQNLDLGS FEKVDFLICI  700
 701  PPSEVTYQQT LLHVWHSGVL LELGLKKEHM TKQRVEQYVL KLDTEAQTKF KAFLQNSFQN PHTLFVLIHD HAHWDLVSST VHNLYSQSDP SVGLVDRLLN  800
 801  CREVKEAPNI VTLHVTSFPY ALQTQHTLSS PYNEIHWPAS CSNGVDLYHE NKKYFGLSEF IESTLSGHSL PLLRYDSSFE AMVTALGKRF PRLHSAVIRT  900
 901  FVLVQHYAAA LMAVSGLPQM KNYTSVETLE ITQNLLNSPK QCPCGHGLMV LLRVPCSPLA VVAYERLAHV RARLALEEHF EIILGSPSSG VTVGKHFVKQ 1000
1001  LRMWQKIEDV EWRPQTYLEL EGLPCILIFS GMDPHGESLP RSLRYCDLRL INSSCLVRTA LEQELGLAAY FVSNEVPLEK GARNEALESD AEKLSSTDNE 1100
1101  DEELGTEGST SEKRSPMKRE RSRSHDSASS SLSSKASGSA LGGESSAQPT ALPQGEHARS PQPRGPAEEG RAPGEKQRPR ASQGPPSAIS RHSPGPTPQP 1200
1201  DCSLRTGQRS VQVSVTSSCS QLSSSSGSSS SSVAPAAGTW VLQASQCSLT KACRQPPIVF LPKLVYDMVV STDSSGLPKA ASLLPSPSVM WASSFRPLLS 1300
1301  KTMTSTEQSL YYRQWTVPRP SHMDYGNRAE GRVDGFHPRR LLLSGPPQIG KTGAYLQFLS VLSRMLVRLT EVDVYDEEEI NINLREESDW HYLQLSDPWP 1400
1401  DLELFKKLPF DYIIHDPKYE DASLICSHYQ GIKSEDRGMS RKPEDLYVRR QTARMRLSKY AAYNTYHHCE QCHQYMGFHP RYQLYESTLH AFAFSYSMLG 1500
1501  EEIQLHFIIP KSKEHHFVFS QPGGQLESMR LPLVTDKSHE YIKSPTFTPT TGRHEHGLFN LYHAMDGASH LHVLVVKEYE MAIYKKYWPN HIMLVLPSIF 1600
1601  NSAGVGAAHF LIKELSYHNL ELERNRQEEL GIKPQDIWPF IVISDDSCVM WNVVDVNSAG ERSREFSWSE RNVSLKHIMQ HIEAAPDIMH YALLGLRKWS 1700
1701  SKTRASEVQE PFSRCHVHNF IILNVDLTQN VQYNQNRFLC DDVDFNLRVH SAGLLLCRFN RFSVMKKQIV VGGHRSFHIT SKVSDNSAAV VPAQYICAPD 1800
1801  SKHTFLAAPA QLLLEKFLQH HSHLFFPLSL KNHDHPVLSV DCYLNLGSQI SVCYVSSRPH SLNISCSDLL FSGLLLYLCD SFVGASFLKK FHFLKGATLC 1900
1901  VICQDRSSLR QTVVRLELED EWQFRLRDEF QTANAREDRP LFFLTGRHIZ                                                 1949
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C

3' end of GREB1b protein

```
1345  GCA CAT CAA ATA CGA AAT CCG GAC GTA TAA  1374
 449  A   H   Q   I   R   N   P   D   V   Z    457
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3' end of GREB1c protein

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1156  AAA GTA TTT GTA AAT GGT GCT ACC CAA ATG GTA GCC CTT GGT CCG GCA GAA CCT GCA TCG CCC CGG AGC TTA TGA  1230
 386  K   V   F   V   N   G   A   T   Q   M   V   A   L   G   P   A   E   P   A   S   P   R   S   L   Z    409
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alternate 5' ends of GREB

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5'-AAATAAAGTTTTTTCAATGGAAGGCTTGCAGCTCTTGAGGACCTGCCAAATGGAAGAAGGACAGAGACCTGGAGCCCTATGGAAAGTTCTGACACCATGTGTGGAAGGACATGGCTTTTAACACGTGTGGTGACTGGAGTAGCTG-3'  GREB1a
5'-GAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAA-------------CTG-3'  GREB1b
5'-GAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAAGTGAGTTCAGTAGCTG-3'  GREB1d
5'-GAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAAGTGAGTTCAGTAGCTG-3'  GREB1b(i)
5'-GAGCAGCAGTGTGTTCATGGAGAAAGAATCAAAGCGCACATCTTGTACTTTGATGCCCATAGGAAGGGCTCTCCTCTGGCCCCTCTGGCTTTGTTTGGAGCAGAAAACAACAAAGTGAGTTCAGTAGCTG-3'  GREB1b(ii)
5'-GCATTACGCGCCCCACGCATCCTCTTCCATCCCCAGGCACAGATCAAAGGCGCAGCCCAGGAGGCGGGAGCCCCTGCACACTTTCCACCTCTGCTGGGCTTAGCCTCTTGGCTGGTTGGTCTGTGGAGTGCCTGAAGTGACCAGCTTTTTGTAAGTAGCTG-3'  GREB1c
5'-GTTACTTATATAACTTCCTGTAATTGCCCGGCAGTAGCTG-3'  GREB1e
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In uterus and bone, tamoxifen is known to be a partial agonist of estradiol. Therefore, we investigated whether tamoxifen treatment could up-regulate genes in breast cancer cells compared with estrogen deprivation. SSH was performed using mRNA from MCF7 cells grown under strict estrogen deprivation as the driver, whereas the tester sample comprised mRNA from MCF7 cells grown in the presence of β-estradiol and tamoxifen. Using Northern blot analysis, 126 putative subtracted clones were examined. However, none of the clones analyzed were found to be significantly up-regulated by tamoxifen (data not shown). This result is consistent with the hypothesis that tamoxifen acts as an antiestrogen in breast tissue and that the pattern of expression resulting from estrogen withdrawal is identical to the pattern of expression induced by tamoxifen treatment.

Time-Course Study of Gene Induction by β-Estradiol. To study the temporal response to estrogen stimulation, a kinetic study of gene induction of the 15 estrogen-responsive genes was undertaken in which the expression of the genes at different time points after the addition of β-estradiol was examined. MCF7 cells grown in estrogen-free media for 5 days were then supplemented with β-estradiol (1×10^{-8} M) for 6, 24, or 48 h before harvesting. A similar set of MCF7 cells grown in the absence of β-estradiol was used as a control. Northern blot analysis revealed that 14 of the 15 genes examined responded early to β-estradiol treatment because gene expression was apparent within 6 h of treatment. These results suggest that these genes are direct targets of activated ER. Gene expression was detected after 48 h for the one gene that did not respond early. Fig. 2 shows representative examples of early responders to the β-estradiol treatment.

***GREB1* and *GREB2* Are the Same Gene.** The pattern of expression and size of the mRNAs suggested that *GREB1* and *GREB2* might be the same gene. As noted above, the sequence of GREB2 matched an expressed sequence tag, KIAA0575 from human brain (GenBank accession number AB011147), whereas GREB1 had no match in the GenBank database. More recently, a mouse gene was reported that had homology to both GREB1 and GREB2. We were unable to obtain a cDNA clone from the λ cDNA library that spanned the region between these two clones (see below). Therefore, RT-PCR was used to clone the region not represented by the λ clones. Two separate PCR reactions were performed using primers in the 3′ end of GREB1 and the 5′ end of GREB2. Based on the size of the mouse gene, these PCR reactions generated fragments of the expected size. Both PCR products were cloned and sequenced to determine the region between GREB1 and GREB2, thus confirming that these two clones are from the same mRNA.

Expression of Estrogen-responsive Genes on a Panel of Breast Carcinoma Cell Lines. We wanted to focus attention on genes whose pattern of expression correlated with ER expression in breast cancer cell lines. To correlate the estrogen responsiveness of the genes to ER expression in human breast carcinomas, we examined the expression of the 14 estrogen-responsive genes in a panel of breast cancer cell lines. The cDNA fragments isolated during SSH were used as probes on Northern blots containing six ER-positive and two ER-negative human breast carcinoma cell lines. Six of the 14 genes examined (*PDZK1*, *GREB1*, and *clones 3*, *21*, *76*, and *138*) were expressed in some or all of the ER-positive breast carcinoma cell lines and absent in all of the ER-negative breast carcinoma cell lines. Fig. 3 shows the results of the two estrogen-responsive genes, *GREB1* and *PDZK1*, with the best correlation to ER expression. The expression pattern of pS2 is shown for comparison. In addition to exhibiting an on/off response to β-estradiol stimulation, GREB1 was expressed in all five of the strongly ER-positive cell lines and in none of the two ER-negative breast carcinoma cell lines or in BT-20, which has low levels of ER. In addition, GREB1 is induced by estradiol in ECC-1, an endometrial carcinoma cell line (data not shown). As indicated in Fig. 3, GREB1 shows a better correlation with ER than is seen with *pS2*, a gene that is often used as a marker for hormone-responsive breast cancer. *PDZK1*, which has been associated with breast cancer but has not been related to hormone responsiveness, was detected in three of the five ER-positive breast cancers cell lines as well as in BT-20. Because the expression pattern of these two genes had the best correlation with ER expression in this panel of cell lines, we chose to focus additional experiments on these two genes.

Cloning GREB1 cDNA. The 600-bp GREB1 cDNA clone isolated through SSH was used as a probe to screen a random primed MCF7 cDNA library. Six positive clones were isolated. Sequencing of the two largest clones revealed two cDNAs of 2792 and 2347 bases with different 5′ and 3′ ends (Fig. 4*A*). These were designated as GREB1a and GREB1b, respectively. Screening of an oligodeoxythymidylic acid-primed MCF7 cDNA library yielded a third clone, GREB1c, which had a poly(A) tail and also had 5′ and 3′ ends that were divergent from those of GREB1a and GREB1b (Fig. 4*A*). The three clones were sequenced on both strands and analyzed. Comparison of the sequences with the European Molecular Biology Laboratory/GenBank databases using the BLASTN and BLASTP DNA analysis programs did not yield any significant similarity with known sequences in the database.

The longest cDNA of GREB1a is shown in Fig. 4*A*. Using a combination of our λ clones, the GenBank sequence of AB011147, and the PCR-generated region between the two, the longest cDNA of *GREB1* gene was obtained (see Fig. 4*A*). The GREB1a mRNA is 8482 bases long [not including the poly(A)+ tail], which agrees with the size obtained by Northern blot. This mRNA is predicted to encode a protein of 1949 amino acids, which is shown in Fig. 4*B*. However, the alternate transcripts from this gene may encode smaller proteins with variable COOH termini. Comparison of GREB1a, GREB1b, and GREB1c sequences using the Clustal V sequence alignment program (27) revealed that the 5′ UTR was dissimilar for all three cDNAs until 264 bases upstream of the putative start site of the longest ORF predicted by DNA Strider version 1.2 (28), as exhibited in Fig. 4, *A–C*. GREB1b differed slightly in that it had a deletion of 13 bases prior to the beginning of the sequence that matched for all three of the cDNAs. Other shorter clones [GREB1b(i), 1b(ii), and 1d] with this same 5′ end did not contain this deletion (Fig. 4*C*). Sequence homology between GREB1a and GREB1b continues for 1346 nucleotides (indicated by ⌘) downstream of the putative start site beyond which the predicted ORF of GREB1b continues for an additional 28 nucle-

Fig. 4. Structure and nucleotide and amino acid sequence for GREB1 transcripts. *A*, schematic diagram of GREB1a cDNA determined from cDNA cloning, RT-PCR, and the published sequence of AB011147. The predicted protein of the longest clone is 1949 amino acids. *Below* is schematic of GREB1a, GREB1b, and GREB1c transcript cDNAs obtained from library screening. *B*, predicted amino acid sequence of GREB1a protein. Using DNA Strider 1.2 (28), the GREB1a transcript is predicted to have a 300-bp 5′ UTR preceding the initiation site of the putative ORF. Sequence homology between GREB1a and GREB1b and between GREB1a and GREB1c is indicated using the symbols ⌘ and ♦, respectively. Within the homologous region of the three transcripts, GREB1c contains a single nucleotide change from GREB1a and GREB1b, the location of which is indicated with by ●. *C*, the nucleotide sequences of the different 5′ and 3′ ends for various transcripts of GREB1 are shown. The sequences of the alternate 3′ ends of GREB1b and GREB1c with the putative ORFs of GREB1b and GREB1c continue for an additional 28 and 71 bp, respectively, beyond the sequence homologous with GREB1a. A 729-bp 3′ UTR was present in the GREB1b transcript, whereas the GREB1c transcript contained a 790-bp 3′ UTR ending with a polyadenylated tail. For the alternate 5′ end of GREB1, the beginning of sequence identity between GREB1a and GREB1c is indicated by ↑. Although other transcripts also begin sequence homology at ↑, GREB1b contained a 13-bp deletion prior to the region of sequence homology with other transcripts indicated by ⅄.

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Fig. 5. Verification of differential splicing of GREB1. RT-PCR was used to verify the structure of the different GREB1 transcripts isolated from the MCF7 cDNA library. For each transcript, four sets of RT-PCR reactions were performed. We used a 5′ primer common to all transcripts and unique 3′ primers that were designed against unique regions for GREB1a, GREB1b, GREB1c, and GREB1d. Templates for the four reactions are as follows: cDNA control, reverse transcribed poly(A)+ RNA, poly(A)+ RNA without reverse transcriptase, and zero template.

otides. Divergence of sequence homology between GREB1a and GREB1c occurs after 1159 nucleotides (indicated by ♦). The predicted stop codon for the GREB1c ORF is 71 nucleotides beyond the sequence divergence from GREB1a. GREB1b and GREB1c had putative ORFs of 457 and 409 amino acids, respectively. In addition, both their 3′ untranslated sequences were different from each other and from that of GREB1a (Fig. 4*D*). Within the region that was homologous, GREB1c had a single-nucleotide change from an A to a C as compared with GREB1a and GREB1b. On translation, this alteration would result in a conservative amino acid change from asparagine (N) to threonine (T), as indicated with by ● in Fig. 4*B*.

To establish that GREB1a, GREB1b, and GREB1c were genuine transcripts and not chimeras, Northern blot analysis and RT-PCR were performed. In addition, GREB1d, a transcript of 1 kb that was isolated during the cloning process, was included for comparison. GREB1d had been sequenced from both ends, and analysis revealed that sequences at the 3′ end of this clone matched those of a rRNA gene, whereas the 5′ end was unique and matched GREB1b. This seemed to imply that GREB1d could be a chimera. Northern blot analysis using GREB1a, GREB1b, and GREB1c cDNAs as probes confirmed differential expression of these transcripts. However, Northern blot analysis using GREB1d showed hybridization to the expected 8-kb band as well as to a 4.2-kb band that was not differentially expressed with β-estradiol treatment (data not shown). Additionally, RT-PCR was performed on the four clones using 5′ primers that were common to all four transcripts and 3′ primers that were designed against the unique 3′ region of each clone. As indicated in Fig. 5, a single band of the expected size could be seen for GREB1a, GREB1b, and GREB1c in the control lanes (with cDNA from each clone) and also in the lanes containing RNA from MCF7 cells grown in the presence of β-estradiol that had been reverse transcribed. Conversely, for GREB1d, which was believed to be a chimera, no expression of the gene was visible in the lane with reverse-transcribed MCF7 mRNA + β-estradiol, confirming that it was chimeric. In all cases, gene amplification was not visible in the negative control lanes (MCF7 mRNA without reverse transcriptase; Fig. 5, *Lane 3* of each set). These data prove that the cDNAs of GREB1a, GREB1b, and GREB1c represent the expression of different mRNAs with divergent 5′ untranslated exons.

GREB1 Is a Primary Target for ER. To determine whether GREB1 and PDZK1 are primary targets for ER regulation, MCF7 cells were exposed to β-estradiol in the presence or absence of cycloheximide. As seen in Fig. 6, GREB1 is expressed in the presence of cycloheximide. The slight increase over estradiol alone is likely the result of stabilization of mRNA known to occur with cycloheximide treatment. PDZK1, however, is repressed by cycloheximide, indicating the need for new protein synthesis for expression. This finding demonstrates that PDZK1 is a secondary target for estradiol induction and that the PDZK1 promoter is not likely to be dependent on an ERE. The *pS2* gene is expressed in the presence of cycloheximide, although the level of expression is slightly reduced. This result suggests that there may be additional mechanisms increasing expression of pS2 that require the synthesis of new protein. The result with actin confirms the abundance of mRNA in each lane.

Expression in Primary Breast Cancers. To understand the biological significance of the estrogen-induced breast cancer genes isolated in this study, the expression of GREB1 and PDZK1 was evaluated in primary breast cancers by RT-PCR. Primers for this experiment were designed using sequence information from the cloned GREB1a and from the published sequence of PDZK1 (GenBank accession number NM_002614).

Preliminary experiments were performed in the absence of reverse transcriptase to verify that the PCR amplification was due to reverse transcription of mRNA present in the tumor samples and not due to contamination with genomic DNA in the total RNA extract. Qualitative analysis of the expression of the three genes is presented in Fig. 7*A*. As the figure indicates, GREB1 and PDZK1 were predominantly present in ER-positive breast tumors, with only a few ER-negative tumors exhibiting gene expression above background levels. Semiquantitative analysis was performed by determining signal intensity using phosphorimaging. Values were normalized against β-actin to determine the relative expression of the different genes analyzed in this study. As indicated in Fig. 7*B*, GREB1 expression was 3.5-fold greater in ER-positive compared with ER-negative breast tumors. Similarly, *PDZK1* gene expression was 19-fold greater in ER-positive breast tumors than in ER-negative breast tumors.

DISCUSSION

Estrogen has profound effects on the growth and differentiation of hormone-responsive tissues (29, 30). In ER-positive breast cancer, estrogen acts as a mitogen, and antiestrogens such as tamoxifen are the main form of chemotherapy (31–33). Despite the importance of estrogen function, our understanding of the molecular details controlling physiological responses to estrogen is incomplete. The *pS2* gene is one of the most extensively studied estrogen-responsive genes (17). However, *pS2* is not expressed in all ER-positive breast carcinoma cell lines (see Fig. 3) and is only expressed in 30–60% of ER-positive tumors (21–23). In addition, pS2 null/null mice have hyperplasia of gastric mucosa but do not appear to have altered responses to estrogen (34). More recently, attention has been focused on cyclin D1 as an important mediator of the response of tumors to estrogen (35). *PR* (9), *HSP27* (19), and *TGF-β* (36) are other genes induced by estrogen in hormone-responsive breast cancer that have been studied extensively. Although several physiological functions have been ascribed to these genes, the expression of the known estradiol-regulated genes fails to adequately explain the effects of estrogen in hormone-responsive tumors. For this reason, we have undertaken a study to identify additional genes induced in hormone-responsive tumors in response to estradiol treatment. Specifically, we were interested in identifying genes that were induced by estradiol and repressed by tamoxifen and that demonstrated a high degree of correlation with ER expression in breast cancer cell lines and primary tumors. Two genes were identified—*PDZK1* and *GREB1*—that fulfill these criteria.

Several methods have been described to identify estrogen-regulated genes. Early research studying estrogen-induced gene expression used techniques such as double isotope protein labeling of induced proteins followed by analysis on SDS-PAGE (14). The *pS2* gene had been identified using the technique of differential library screening (17). This technique is useful for identifying abundant genes but does not allow isolation of scarce mRNAs (15). SSH has been recently de-

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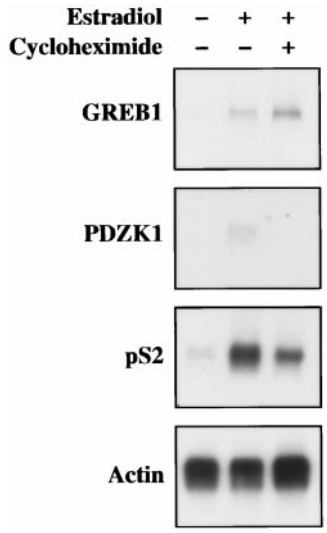

Fig. 6. GREB1 is a primary target for ER. Expression of GREB1 and PDZK1 was examined after exposure to estrogen in the presence or absence of cycloheximide. MCF7 cells were grown in the absence of β-estradiol or in the presence of β-estradiol for 24 h without or with cycloheximide. Northern blots were probed with GREB1, PDZK1, pS2, or β-actin.

scribed for the identification of differentially expressed genes and has several advantages over older methods (24). SSH allows isolation of differentially expressed mRNAs of low abundance with a false positive rate of 30–80% (15, 37). SSH has not previously been applied to the isolation of estradiol-induced genes, and earlier studies suggested that there are many more estradiol-regulated genes than had been reported previously (15). For these reasons, applying this new technique to identify estradiol-responsive genes was likely to result in the isolation of genes critical to hormone response.

Four of the estrogen-induced genes identified in this study were genes that had been reported previously. These genes include thrombospondin, PDZK1, a mRNA coding for an unidentified protein (KIAA0575) from human brain (herein termed GREB2), and an immunoglobulin-like gene. None of these had previously been identified as being responsive to estradiol. Thrombospondin is a matrix-bound adhesive glycoprotein that is present in a variety of cells, including mammary epithelial cells and osteoblasts (38). It has been shown to play a role in tumor angiogenesis and tumor cell proliferation (39, 40). A study of human osteoblast-like cells exhibited colocalization of thrombospondin with transforming growth factor βI and insulin-like growth factor-I. These proteins were expressed in the extracellular matrix and were modulated by β-estradiol (41). However, in another study, thrombospondin was not found to be responsive to estrogen in ER-positive MCF7 breast carcinoma cells (40). Our study established that although thrombospondin did not correlate with ER in a panel of breast carcinoma cell lines, there was an approximately 4-fold up-regulation of expression in MCF7 cells in response to β-estradiol treatment.

One of the genes induced by estrogen identified in these studies was *PDZK1*. The PDZ domain was named for three proteins in which the domain was first recognized: (*a*) post-synaptic density protein PSD-95 (42); (*b*) *Drosophila* disc large tumor suppressor *dlg* (43); and (*c*) the tight junction-associated protein ZO-1 (44). Proteins containing this domain have been reported to be involved in organizing proteins at the cell membrane (45) and are also involved in linking transmembrane proteins to the actin cytoskeleton (46). Through these interactions, PDZ domain proteins regu-

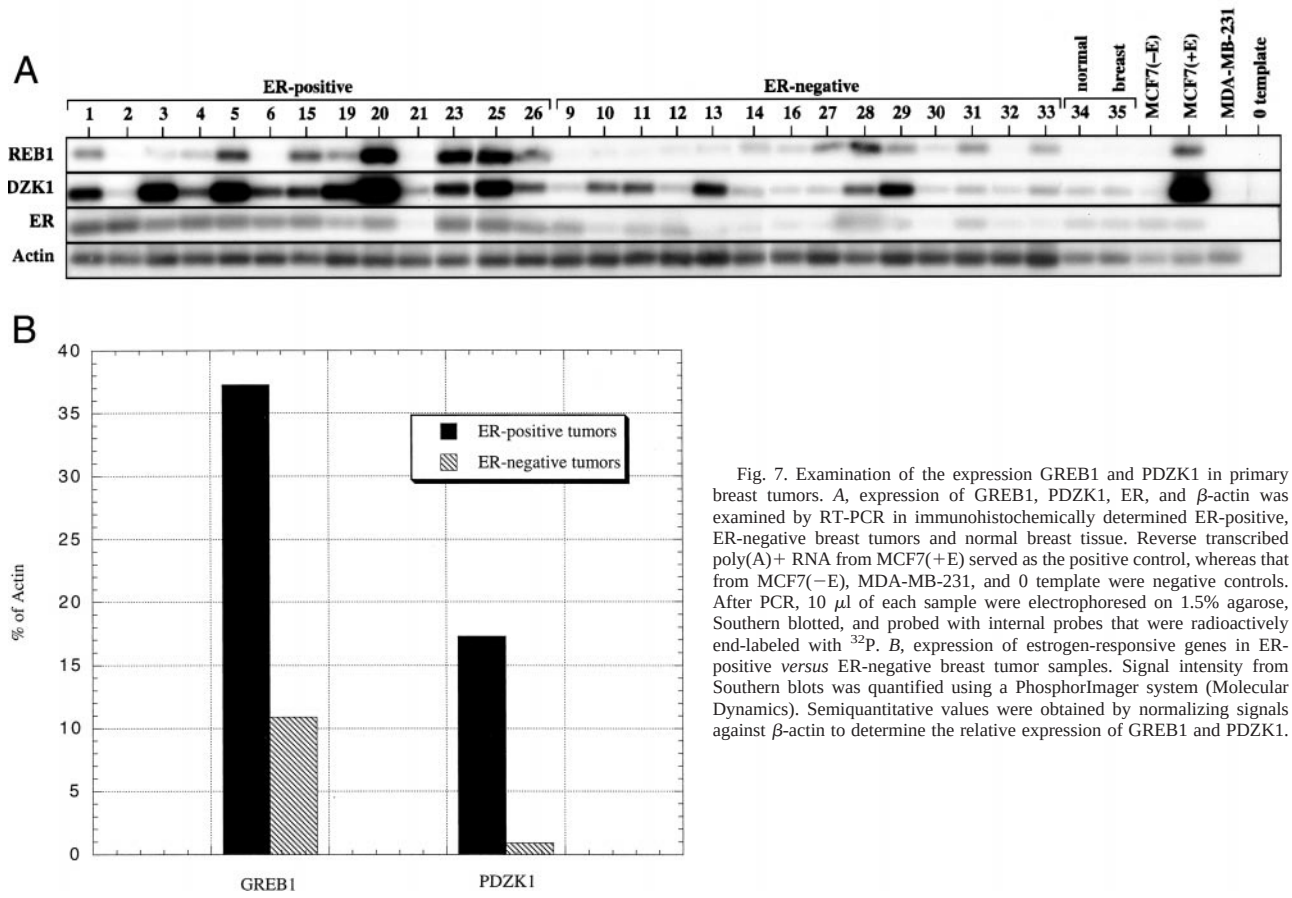

Fig. 7. Examination of the expression GREB1 and PDZK1 in primary breast tumors. *A*, expression of GREB1, PDZK1, ER, and β-actin was examined by RT-PCR in immunohistochemically determined ER-positive, ER-negative breast tumors and normal breast tissue. Reverse transcribed poly(A)+ RNA from MCF7(+E) served as the positive control, whereas that from MCF7(−E), MDA-MB-231, and 0 template were negative controls. After PCR, 10 μl of each sample were electrophoresed on 1.5% agarose, Southern blotted, and probed with internal probes that were radioactively end-labeled with ^{32}P. *B*, expression of estrogen-responsive genes in ER-positive *versus* ER-negative breast tumor samples. Signal intensity from Southern blots was quantified using a PhosphorImager system (Molecular Dynamics). Semiquantitative values were obtained by normalizing signals against β-actin to determine the relative expression of GREB1 and PDZK1.

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late a diverse set of cell functions including control of signal transduction, determination of cell polarity, cell differentiation (47), and ion transport (48). PDZK1 was first isolated in a yeast two-hybrid screen designed to identify proteins interacting with MAP17, a membrane-associated protein involved in regulation of cell proliferation (49). Subsequently, PDZK1 has been reported to interact with cMOAT(MRP2), which is a canalicular organic anion transporter associated with multidrug resistance (50). Expression of PDZK1 is limited to epithelial cells, and overexpression of this protein has been reported in a variety of carcinomas (49). PDZK1 contains four PDZ domains and could interact with a number of proteins simultaneously, thereby coordinating the interplay of multiple proteins at the cell membrane. The demonstration that PDZK1 is induced by estradiol in hormone-responsive breast cancer provides an important mechanism to explain membrane alterations such as formation of microvilli that occur with estrogen treatment (51). It has recently been reported that estrogen induces expression of Na^+-H^+ exchanger regulatory factor (NHE-RF), the human homologue of the Na^+/K^+ exchange regulatory factor (52). These results provide important clues related to the physiological effects of estrogen on cell membrane alterations.

The *GREB1* gene demonstrated a striking correlation with ER expression in a panel of breast cancer cell lines. Expression of GREB1 was similarly associated with ER expression in primary breast cancers, although the correlation was not perfect. However, cancer specimens obtained from patients are not controlled for ligand exposure. Because expression of these genes is dependent on estrogen, it might be expected that tumors resected from postmenopausal women might not demonstrate expression of estrogen-induced genes. In addition, the use of estrogens and newer antiestrogens such as raloxifene may alter patterns of gene expression. However, these results do establish that these estrogen-regulated genes are expressed in primary breast tumors.

The structure of GREB1 is complex in that there are at least three different noncoding 5′ exons. The expression of each of these transcripts is estrogen regulated, suggesting the presence of multiple estrogen-inducible promoters. The occurrence of multiple estrogen-inducible promoters is not unique. The *PR* gene has been shown to have multiple estrogen-regulated promoters that are controlled by distinct EREs. In the case of PR, these different transcripts encode two distinct PRs, PR A and B, which have different transcriptional activation ability (53). The different 5′ exons for GREB1 are noncoding, and each transcript uses the same initiation codon. However, there are various splicing patterns involving the 3′ end of the gene that could result in proteins with different COOH termini. Recently, the mouse homologue of this gene was identified, but no function has been determined (54). Although the function of this gene remains unknown, the pattern of expression and regulation by estrogen implies an important function in hormone-responsive breast cancer.

In conclusion, SSH was used to identify estradiol-regulated genes in hormone-responsive breast cancer. Of the 14 genes identified, 13 were induced early (6 h), and all were repressed with tamoxifen. In addition, SSH was used to compare the pattern of gene expression in the absence of estrogen and in the presence of estrogen together with tamoxifen. No significant differences were detected, suggesting that tamoxifen is a pure antiestrogen in breast tissue. These results indicate that the only effect that tamoxifen has on gene expression occurs through its interaction with ER. Two estrogen-regulated genes were identified: *PDZK1* and *GREB1*. The pattern of expression of these genes suggests an important role for these proteins in the physiological response of tumors to estrogen.

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