Activation of the Silent Progesterone Receptor Gene by Ectopic Expression of Estrogen Receptors in a Rat Fibroblast Cell Line†

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ABSTRACT: We describe the construction and characterization of a novel estrogen (E2)-responsive cell line, Rat1+ER, which ectopically expresses estrogen receptor (ER). Human ER cDNA was introduced by retrovirus-mediated gene transfer into the Rat1 cell line, which does not express functional ER endogenously. Rat1+ER cells express functional ER based on radioreceptor assays, immunoblotting, and transient transfection experiments using E2-responsive reporter plasmids. The effects of this ectopic ER expression were studied on three endogenous E2-responsive genes, prolactin (PRL), progesterone receptor (PR), and epidermal growth factor receptor (EGFR). PRL, usually expressed in the lactotrophs of the pituitary, is not expressed at all in Rat1+ER cells, with or without E2 addition, and appears to require other factors for expression. In contrast, although PR is not expressed in Rat1 cells, it is induced in Rat1+ER cells upon the addition of E2. This induction appears to occur at the transcriptional level and is insensitive to cycloheximide treatment. This is one of the few examples where the expression of one gene activates an otherwise silent gene. Another contrasting observation is that, although EGFR is basally expressed in Rat1+ER cells, the addition of E2 has no effect. Our studies paint a complicated picture of E2 regulation of endogenous genes: the activation of the PR gene may only require the presence of E2 and ER, whereas EGFR and PRL genes require factors in addition to ER for basal as well as E2-regulated expression.

Steroid hormones play a central role in mammalian development by regulating the expression of a variety of genes (Beato, 1989; Yamamoto, 1985). Estrogen (E2),[1] in particular, is involved in the development of female secondary sex characteristics and is known to have a profound effect on the proliferation and differentiation of E2-responsive tissues, such as the uterus, the pituitary, and the mammary glands (Norman & Litwack, 1987). E2 binds to a specific receptor protein, known as the estrogen receptor (ER), that targets responsive genes through interaction with estrogen response elements (EREs) (Beato, 1989; Evans, 1988; Gorski et al., 1993). Since ER is absolutely required for E2 action, part of the differential effects from E2 can be attributed to the cell-specific expression of the ER. Therefore, cell-specific expression of the steroid receptors is an important regulatory mechanism for bringing about the complex effects of the steroid hormones in higher organisms.

Studies suggest that ER expression is the only factor lacking in E2-nonresponsive cells needed to bring about E2-dependent regulation of transcription (Druege et al., 1986; Seiler-Tuyns et al., 1988). The fact that the expression of developmentally regulated E2-responsive genes correlates with the expression of ER supports this notion (Elbrecht et al., 1984; Evans et al., 1987, 1988; Slabaugh et al., 1982). Furthermore, transient as well as stable expression of ER in a liver cell line that normally expresses very little ER results in greater induction of endogenous E2-responsive genes, consistent with ER being the limiting factor for E2-responsive gene expression (Binder et al., 1990). Finally, the ectopic expression of ER in *Xenopus* oocytes results in the activation of the previously silent E2-regulated yolk protein gene, vitellogenin, raising the possibility that ER may have the ability to activate the pathway for cell-specific differentiation (Knowland et al., 1984; McKenzie et al., 1990).

The expression of ER alone, however, does not confer E2 regulation to all E2-responsive genes. Ovalbumin, which is E2-regulated in an E2-primed chick oviduct, is not expressed at all in any other tissue, including the liver, which has functional ER (Palmiter et al., 1978). Likewise, in mammals, prolactin is expressed and E2-regulated in the pituitary, but not in other E2-responsive tissues (Shull & Gorski, 1986). This tissue-specific differential regulation of responsive genes by E2 indicates that other factors are necessary to bring about the complexity of E2 action in animals.

Finally, the role of ER, if any, in differentiation is unknown. Despite evidence that the expression of developmentally regulated E2-responsive genes correlates with ER expression, the capacity of many E2-regulated genes for expression during development appears to be independent of ER expression. For example, the appearance of DNAse I hypersensitivity, which correlates well with gene expression, for the vitellogenin and prolactin genes is detected before ER expression (Burch & Weintraub, 1983; Durrin & Gorski, 1985; Gross & Garrard, 1988). Furthermore, the activation of the vitellogenin gene in *Xenopus* oocytes requires unusually high amounts of ER, raising the possibility that another factor(s) may normally be required (McKenzie et al., 1990; Watson, 1991). In support of such a hypothesis, a liver-specific factor that facilitates activation of the endogenous vitellogenin gene in oocytes has been reported (Corthésy et al., 1991).

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[1] Abbreviations: E2, estrogen; ER, estrogen receptor; ERE, estrogen response element; PRL, prolactin; PR, progesterone receptor; EGFR, epidermal growth factor receptor; hER, human estrogen receptor; HPT, hygromycin phosphotransferase; SDS, sodium dodecyl sulfate; kb, kilobase; MHT, monohydroxytamoxifen; CAT, chloramphenicol acetyl transferase; RT-PCR, reverse transcriptase–polymerase chain reaction; bp, base pair; ICI, ICI 164 384; DES, diethylstilbestrol.

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These facts taken together suggest that, even though the expression of ER is targeted to specific tissues and cell types during development, additional steps are required to fully program the expression and regulation of E2-responsive genes. The question we wanted to address was the following: What are the effects of ectopic ER expression on cells that normally do not express ER? To this end, we constructed a novel ER-positive cell line derived from an ER-negative, rat embryonic fibroblast-like cell line, Rat1 (Freeman et al., 1970).

We found that ER expression, in the presence of E2, has different effects on the three E2-responsive genes studied: prolactin (PRL), progesterone receptor (PR), and epidermal growth factor receptor (EGFR). Most interestingly, stable ectopic ER expression in Rat1 cells resulted in the activation of a silent PR gene, but had no effect on either the silent PRL gene or the basally expressed EGFR gene.

MATERIALS AND METHODS

Cell Culture Conditions. GH_3 (Tashjian et al., 1968), Rat1 (Freeman et al., 1970), and Rat1+ER cells were grown in phenol red-free, high-glucose Dulbecco's modified Eagle's media [DMEM (Sigma), containing a 1× antibiotic/antimycotic mix (GIBCO), 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, and 0.37% sodium bicarbonate], supplemented with 10% fetal bovine serum (Hyclone). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2 and were fed every 2–4 days. With experiments involving ER protein or E2 induction of a transfected reporter gene (see below), the cells were washed with Hank's buffered saline solution (HBSS, Sigma) and cultured in DMEM supplemented with 10% 3× dextran/charcoal-stripped fetal bovine serum (DFs10; stripped serum prepared as described previously; Horwitz et al., 1976) for 18–24 h prior to analysis or E2 addition. MCF-7 cells (Horwitz et al., 1976) were grown in DMEM with 10% calf serum (Hyclone); this serum contains undetectable levels of E2 as determined by Hyclone. To keep the feeding schedule constant for all of the cells studied, MCF-7 cells were treated exactly as the other cell lines, except that these cells were fed with DMEM containing 10% calf serum instead of DFs10. Both MCF-7 and GH_3 cells were supplemented with insulin (1 μg/mL).

Construction of Retrovirus Vector and Isolation of Rat1+ER. The human ER (hER) cDNA (Green et al., 1986; a gift of Dr. P. Chambon) was cloned into a unique, blunt-ended *Hin*dIII site of pCG(AFVXM)SVhy. pCG(AFVXM)SVhy is a derivative of the retroviral vector, pFVX (Kriegler et al., 1984; kindly provided by Dr. M. Kriegler), which contains the hygromycin phosphotransferase gene (HPT) driven by the SV40 promoter (C. Gélinas, unpublished data). The resultant plasmid, pCG(AFVXM)SVhy+ER, was stably transfected into the ΨAM helper cell line (Cone & Mulligan, 1984; kindly provided by Dr. R. Mulligan) by the $CaPO_4$ coprecipitation/glycerol shock method (Graham & van der Eb, 1973; Parker & Strak, 1979) and selected with 100 μg/mL hygromycin B (Calbiochem) for 14 days. Hygromycin B-resistant clones were pooled and grown to approximately 70–80% confluency in T75 flasks (Costar). Seven milliliters of fresh media were added to these clones 18–24 h prior to removing the conditioned media for infection. One milliliter of conditioned medium from these cells, in the presence of 80 μg/mL polybrene (Sigma), was added directly to 3×10^5 Rat1 cells, which had been plated 18 h previously onto a 60 × 15 mm tissue culture dish (Falcon). The infection was for 4 h at 37 °C, and the cells were incubated with fresh medium overnight. The cells were then split 1:10 into a 150 × 25 mm tissue culture dish (Falcon) and selected with 100 μg/mL hygromycin B for 14–21 days. Clonal lines were established from surviving colonies (designated Rat1+ER.2–8). One monoclonal line was isolated by serial dilution (designated Rat1+ER.1).

Northern and Southern Blot Analysis. Total RNA was isolated by the method of Chirgwin et al. as previously described (Chirgwin et al., 1979; Kingston et al., 1989). For Northern blot analysis, 20 μg of denatured RNA was fractionated by electrophoresis through a 1% agarose gel containing 3.1% formaldehyde. RNA transfer to Hybond-N (Amersham) and the Northern analysis were done as previously described (Seyfred et al., 1989), except that the prehybridization buffer contained 0.2% sodium dodecyl sulfate (SDS) and 200 μg/mL of denatured, sheared, herring testes DNA (Sigma). An *Eco*RI fragment of the hER expression vector (HEO; Green et al., 1986), which was α-^{32}P-radiolabeled ($\sim 1 \times 10^9$ cpm/μg) by the random primer method (Feinberg & Vogelstein, 1984), was used as the hER probe. The hybridization was carried out at 42 °C using prehybridization buffer that contained (1×10^6)–(1×10^7) cpm/mL of probe. Following the hybridization, the membrane was washed at 65 °C for 30 min in a solution of 1× SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS solution and then in 0.2× SSC and 0.1% SDS. The blots were visualized after exposure to Kodak XAR-5 film at −80 °C using a Du Pont Quanta III intensifying screen. Molecular markers were run with the samples and visualized by ethidium bromide staining of the gel. To control for equal loading of the RNA samples, the blot was stripped and reprobed with the chicken β-actin probe (Cleveland et al., 1980).

Genomic DNA isolation and Southern analysis of 15 μg of *Hin*dIII-digested genomic DNA were performed using methods previously described (Maniatis et al., 1989; Southern, 1975), except that the prehybridization buffer contained 1 mM sodium pyrophosphate. The DNA probe for HPT (see Figure 1A) was α-^{32}P-radiolabeled by a random primer method (specific activity $\sim 1 \times 10^9$ cpm/μg) and used at a concentration of (1×10^6)–(1×10^7) cpm/mL of prehybridization buffer. A 1-kilobase (kb) ladder (Bethesda Research Laboratories) was used as a molecular weight marker.

Western Blot Analysis. Whole cell extracts of MCF-7, Rat1, and Rat1+ER cells were prepared by Dounce homogenization in 10 mM Tris (pH 7.4), 1.5 mM EDTA, 10 mM β-mercaptoethanol, and 3 mM $MgCl_2$ with protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, and 30 μM phenylmethanesulfonyl fluoride) followed by a stepwise salt extraction ([final NaCl] = 0.8 M). Equal amounts of protein from each extract were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. ER was detected using an affinity-purified ER-specific primary antibody (ER 715) generated from a rat ER-specific synthetic peptide [as described in Furlow et al. (1990)] at a 1:500 dilution, goat anti-rabbit alkaline phosphatase (Sigma) at 1:5000, and anti-goat alkaline phosphatase (Sigma) at 1:1000. As a control, ER 715 incubated with an excess amount of synthetic peptide ([final] = 10 μg/mL; ~8000× molar excess) was used as a primary antibody.

Ligand Binding Assays/Scatchard Analysis. ER assays were performed as previously described (Campen et al., 1985), except that confluent 60 × 15 mm tissue culture plates of cells were incubated for 60 min at 37 °C with 0.05–20 nM [^{3}H]E2 (93.4 Ci/mmol; NEN-Du Pont), with or without a 200-fold

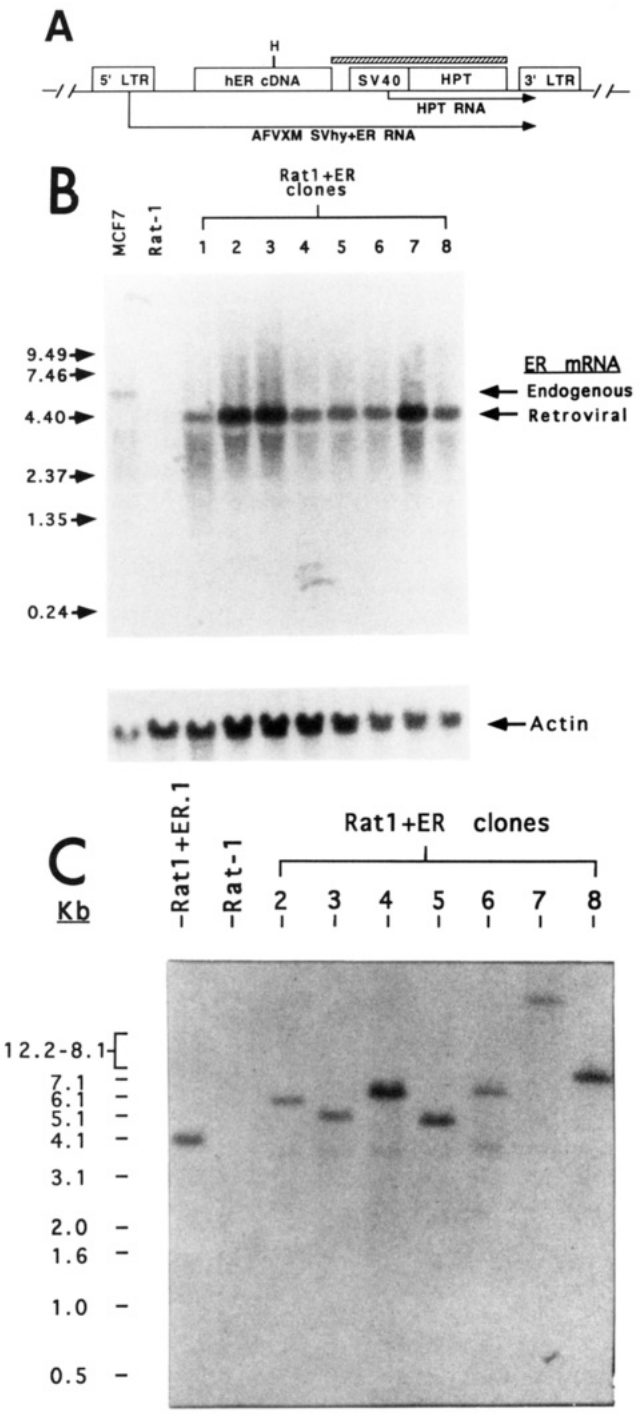

FIGURE 1: Characterization of the integrated provirus in eight clonal lines of Rat1+ER. (A) Schematic representation of the integrated provirus. Human estrogen receptor (hER) cDNA and the hygromycin phosphotransferase (HPT) gene are transcribed from the promoter located within the 5′ long terminal repeat to produce a transcript of ~4.7 kb. A separate SV40 promoter transcribes the HPT gene. Both mRNAs use the identical poly(A)$^+$ signal, located within the 3′ long terminal repeat. The hatched bar corresponds to the HPT probe used for Southern blot analysis; the unique *Hin*dIII (H) restriction site within the provirus is also indicated. (B) Northern blot analysis with the hER probe. Twenty micrograms of total RNA isolated from ER-positive MCF-7, the original Rat1, and eight clonal lines of Rat1+ER cells were analyzed using a radiolabeled hER cDNA probe. The endogenous ER transcript is ~6 kb whereas the retroviral ER transcript is ~4.7 kb. The blot was stripped and rehybridized using chicken β-actin probe. Migration of the molecular weight standards is indicated on the left. (C) Southern blot analysis with the HPT probe. Fifteen micrograms of genomic DNA from Rat1 and eight Rat1+ER clonal lines were digested with *Hin*dIII restriction enzyme and analyzed using a radiolabeled HPT probe. Migration of molecular weight markers is indicated on the left.

molar excess of unlabeled E2. A Burton DNA assay (Burton, 1956) showed that, within a given experiment, each plate had approximately the same amount of DNA. PR assays were performed according to methods previously described (Greco & Gorski, 1989), except that all incubations contained [^{3}H]R5020 (promegesterone; NEN-Du Pont; 72.4 Ci/mmol) and 1 μM unlabeled dexamethasone to minimize R5020 binding to glucocorticoid receptors present in the cells (Strömstedt et al., 1990).

For EGFR binding assays, Rat1+ER cells were grown to confluency in 24-well tissue culture plates (Falcon). The cells were washed with HBSS and incubated in DFs10 for 24 h prior to hormone treatment. The cells were washed with HBSS, and 10 nM E2 or ethanol control prepared in DFs10 was added. After a 24-h incubation at 37 °C, the cells were washed twice with DMEM/0.1% bovine serum albumin. DMEM solutions containing 0.05–10 nM [^{125}I]EGF (7.1×10^5 Ci/mol) with or without a 100-fold excess of unlabeled EGF were added to the cells. After a 3.5-h incubation at 4 °C, an aliquot of medium was sampled for the determination of free (unbound) EGF, and the cells were washed 10 times with DMEM/0.1% bovine serum albumin at 4 °C. The cells were solubilized with 1 mL of 0.5 M NaOH/0.1% Triton X-100 for 10 min at room temperature, and the bound EGF was determined using a γ-counter (Packard Multi-Prias). The data were plotted as saturation binding curves and transformed by the method of Scatchard (1949). Binding parameters were estimated from the LIGAND computer program (BIOSOFT, Inc., Cambridge, U.K.; McPherson, 1985; Munson & Rodbard, 1980).

Transient Transfection Assays. A pERE15 plasmid (Klock et al., 1987; a gift from Dr. G. Klock) was transiently transfected by the $CaPO_4$ precipitation method (Graham & van der Eb, 1973) as described in Kingston and Sheen (1989) (see Figure 4). The cells were incubated in DFs10 for 18–24 h, and 15 nM E2, 15 nM monohydroxytamoxifen (MHT), or ethanol was added as control. The cells were then incubated for an additional 24 h prior to harvesting. All other transfections with HEO, pERE15, and +698/+729-TK-CAT (Savouret et al., 1991; a gift from Dr. J. F. Savouret), as well as 2.4 PRLCAT and RSV-Pit-1 (Iverson et al., 1990; Maurer & Notides, 1987; both gifts from Dr. R. Maurer), were performed by electroporation. Briefly, the cells were grown to near confluency in one or two T75 flasks and fed with fresh media 24 h prior to harvest for transfection. The cell pellet was resuspendend in 1× phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4) at a concentration of 1×10^7 cells/mL. Ethanol-precipitated DNA resuspended in 100 μL of 10 mM Tris Cl (pH 8.0), and 1 mM EDTA and 800 μL of resuspended cells were combined and directly transferred to a Bio-Rad gene pulser cuvette (0.4-cm electrode gap). The electroporation was carried out using a Bio-Rad gene pulser apparatus with capacitance extender at 960 μF and 400 (for Rat1 cells) or 450 V (for Rat1+ER cells). The cells were immediately transferred to 2 mL of fresh medium and incubated at room temperature for 5–10 min. The cells were aliquoted to T75 flasks or 60- and/or 100-mm Petri dishes and incubated overnight at 37 °C prior to the addition of DFs10.

Chloramphenicol acetyl transferase (CAT) enzymatic assays were performed by methods previously described (Gorman et al., 1982; Kingston & Sheen, 1989). An aliquot of the extract (25–50 μg of protein) was incubated in the presence of 0.53 mM acetyl CoA and 1 μL of 60 mCi/mmol [*dichloroacetyl*-1,2-$^{14}C_2$]chloramphenicol (New England Nu-

clear) for 1 h at 37 °C. The acetylated chloramphenicol (Ac-CAM) was separated from the nonacetylated CAM by thin-layer chromatography in a chamber containing 18:2 (v/v) chloroform/methanol and visualized by autoradiography or quantified by scintillation counting.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis. The RT reaction was done using 5 μg of total RNA in a 20-μL total reaction as previously described (Park & Mayo, 1991). A 1–5-μL aliquot or the entire RT reaction was added to the PCR reaction mixture (preheated to 95 °C), which contained 20 mM Tris Cl (pH 8.4), 50 mM KCl, 0.1 mg/mL bovine serum albumin, 250–500 ng/μL PCR primers, 2.5 units of Taq DNA polymerase (Promega), and the appropriate concentrations of $MgCl_2$ (see below). The final volume of the PCR reaction mixture was adjusted to 100 μL with diethyl pyrocarbonate-treated H_2O. The optimal $MgCl_2$ concentrations for each set of primers were determined to be 5 mM for the PR primers, 8 mM for the PRL primers, and 1.5 mM for the ribosomal protein L19 (RPL19) primers (data not shown). The PCR reaction was overlaid with mineral oil (Perkin-Elmer Cetus) and amplified for 30 cycles using a Perkin-Elmer Cetus Model 480 DNA thermal cycler. The cycling parameters were the following: denaturation at 95 °C for 30 s, renaturation at 65 °C for 30 s, and polymerization at 72 °C for 30 s. Prior to the start of the cycle, the reaction was heated to 95 °C for 1 min, and at the end of the cycle, the polymerization was extended for 7 min at 72 °C. The number of cycles used was in the linear amplification range (data not shown). Products from the reaction were analyzed by 2% agarose gel electrophoresis. In some experiments (i.e., Figure 12B), a trace amount of [α-^{32}P]dATP was added to the PCR reaction, and the PCR product was analyzed as previously described (Park & Mayo, 1991).

The primers for the rat PR and RPL19 used in these studies were previously described (Park & Mayo, 1991). The primers for rat PRL were 5′-CTGAAGACAAGGAACAAGCCCA-3′ (located within exon III) and 5′-TCAGGAACT-TGAGATAATTGTC-3′ (located within exon V). The expected amplified products for PR, RPL19, and PRL are 325, 194, and 369 base pairs (bp), respectively. The amplified 369-bp product derived from the PRL primers was confirmed to be part of rat PRL cDNA by Southern blot analysis using radiolabeled rat PRL probe (data not shown).

Cycloheximide Studies. One hour prior to the addition of E2, the cells, grown to confluency in T75 flasks, were washed with HBSS, and 10 μM cycloheximide (Sigma) in DFs10 was added to the cells. DFs10 without cycloheximide was added to the control flasks. One hour later, 5 nM E2 or ethanol control was added to both sets of flasks. Following an additional 12 h, the cells were harvested and total RNA was prepared and analyzed by RT-PCR as described above. Protein synthesis in both E2-treated and control cells was inhibited >94% as assayed by trichloroacetic acid precipitation of proteins labeled with [^{35}S]methionine (data not shown).

RESULTS

Construction of Rat1+ER Cells. hER cDNA (Green et al., 1986) was first cloned into a Moloney murine leukemia virus-based retroviral vector plasmid (Figure 1A) as described in the Materials and Methods section. The resultant plasmid, pCG(AFVXM)SVhy+ER, was stably transfected into an amphotropic helper cell line, ΨAM (Cone & Mulligan, 1984), by $CaPO_4$ coprecipitation. The cells were selected with hygromycin B, and cells that survived the selection after 14 days were pooled and used to generate the virus for infecting the Rat1 cells. From three separate infections, 14 surviving colonies were isolated, 13 of which appeared to bind E2 specifically (data not shown). Eight clones were further characterized (see below), and a monoclonal line was produced from one of the clones (clone 1, Rat1+ER.1), which was used for most of these studies.

Characterization of hER Expressed in Rat1 (Rat1+ER) Cells. In order to confirm that the isolated clonal lines of Rat1+ER expressed ER as a result of the retroviral infection, Northern analysis was performed using an ER-specific probe. If the provirus had correctly integrated into the host chromosome, the viral ER transcript should be ~4.7 kb, in contrast with the ~6-kb endogenous ER RNA (see Figure 1A). The viral construct also produced another transcript, the HPT-specific mRNA, separately transcribed from the SV40 early promoter, which was not detectable with an ER-specific probe (Figure 1A; Kaneko et al., 1993). Total RNA prepared from seven clonal (clones 2–8; see Materials and Methods) and one monoclonal (clone 1) Rat1+ER lines, Rat1, and the ER-positive breast cancer cell line MCF-7 was analyzed using the radiolabeled ER cDNA fragment as a probe (Figure 1B). The result from this Northern blot analysis shows that ER-specific mRNA of ~4.7 kb is present in all Rat1+ER lines but not in the Rat1 cells. The specificity of the ER probe is indicated by the detection of the endogenous ~6-kb ER transcript in RNA prepared from MCF-7 cells.

Southern blot analysis of eight Rat1+ER clones was performed in order to confirm that the recombinant retrovirus had integrated into the host chromosome and that each clonal line was derived from an independent integration event. Genomic DNA prepared from all eight Rat1+ER lines as well as the parental Rat1 were digested with restriction endonuclease *Hin*dIII and separated in a 0.7% agarose gel, and Southern analysis was performed (Figure 1C). Since *Hin*dIII cuts once within the provirus (in ER cDNA), a probe specific for HPT should detect only one of the fragments generated by the *Hin*dIII digest. All eight clones showed a distinct band of different size that hybridized to the probe, indicating that the clones were generated from eight independent integration events. The fact that all eight clones integrated at distinct sites in the host chromosome was confirmed by independent Southern analysis using DNA digested with another restriction endonuclease, *Eco*RI (data not shown).

The presence of ER protein in Rat1+ER cells was then confirmed by Western blot analysis. Whole cell extracts from Rat1+ER.1, Rat1, and MCF-7 cells were analyzed for the presence of ER protein using the polyclonal antibody ER 715 generated against a rat ER-specific synthetic peptide, which reacts with the hER (Furlow et al., 1990). Western blot analysis using this antibody showed that both Rat1+ER and MCF-7 extracts contained an ER protein of approximately 65 kDa, the size of the mature ER protein; conversely, no ER was detected in the Rat1 extract (Figure 2A). No ER-specific band was detected in any of the extracts when ER 715 was blocked by an excess amount of synthetic peptide (Figure 2B). These results suggest that Rat1+ER expresses ER protein of the appropriate size and is recognized by an ER-specific antibody.

Functional Analysis of ER Expressed in Rat1+ER.1 Cells. Two important functional features of ER are the following: (i) It specifically binds to E2 with high affinity. (ii) It activates transcription in the presence of E2. E2 binding studies were done by intact cell uptake of [^{3}H]E2 in the presence or absence

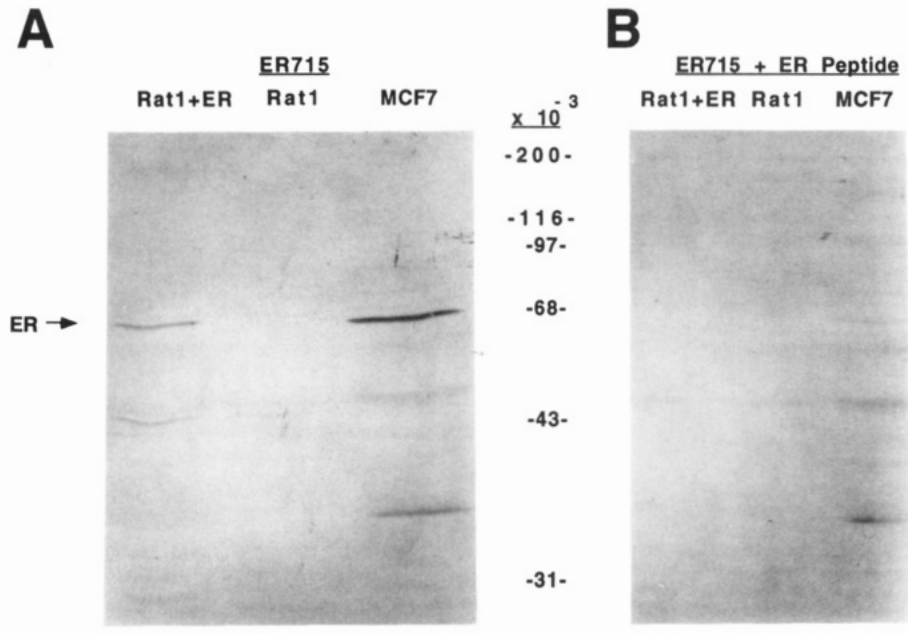

FIGURE 2: Western blot analysis using the ER antibody ER 715. Whole cell extracts prepared from MCF-7, Rat1, and Rat1+ER cells were immunostained with a 1:500 dilution of either ER 715 alone (A) or ER 715 that was blocked with ~8000-fold molar excess of synthetic peptide (B). A 65-kDa ER protein was observed in extracts prepared from both MCF-7 and Rat1+ER cells when ER 715 alone was used for immunostaining.

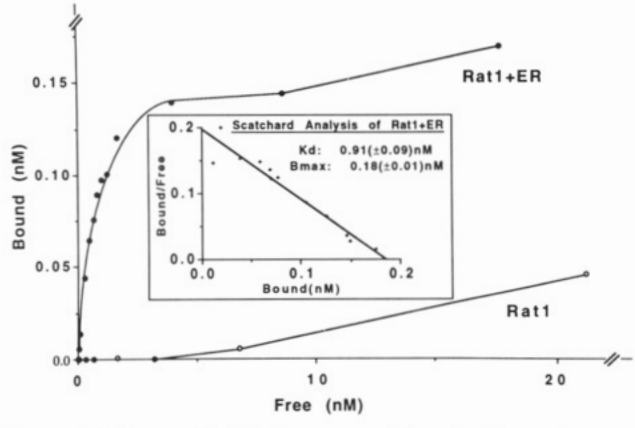

FIGURE 3: E2 saturation binding assay and Scatchard analysis. Rat-1 and Rat1+ER cells were grown to confluency and incubated with solutions containing increasing concentrations of [^{3}H]E2 alone or [^{3}H]E2 with a 200-fold excess of unlabeled E2. Specific binding was determined by subtracting the nonspecific counts ([^{3}H]E2 and excess unlabeled E2) from the total counts ([^{3}H]E2 alone). The binding data were analyzed using the LIGAND computer program. The inset shows the binding data obtained for Rat1+ER cells replotted by the method of Scatchard (1949).

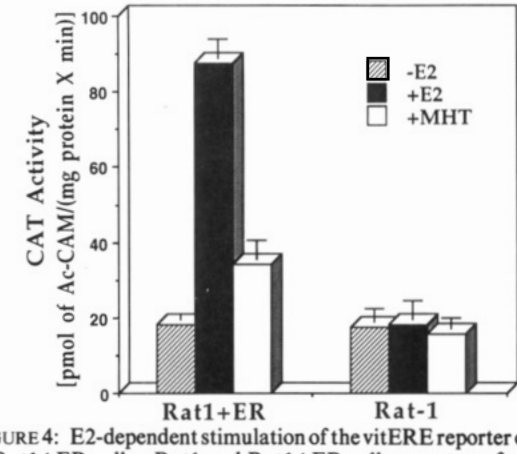

FIGURE 4: E2-dependent stimulation of the vitERE reporter construct in Rat1+ER cells. Rat1 and Rat1+ER cells were transfected with pERE15 (see Materials and Methods) using the $CaPO_4$ coprecipitation method. The cells were treated with ethanol control (EtOH), 15 nM estradiol (E2), or 15 nM monohydroxytamoxifen (MHT) for 24 h before harvesting. The CAT activity was analyzed by incubating 25–50 μg of whole cell extracts with [^{14}C]chloramphenicol for 1 h at 37 °C, and the products were separated by thin-layer chromatography. The acetylated chloramphenicol was quantified by scintillation counting, and the results presented are the average of two independent experiments.

of a 200-fold excess of unlabeled E2 in order to estimate nonspecific binding (Campen et al., 1985). When specific [^{3}H]E2 binding was plotted against increasing concentrations of E2, Rat1+ER cells showed saturable E2 binding. However, no such high-affinity binding was obtained with Rat1 cells (Figure 3). When the binding data for Rat1+ER cells are replotted as a Scatchard plot (Scatchard, 1949), the ER in Rat1+ER cells appears to bind E2 at a single site with an equilibrium dissociation constant (K_d) of 0.9 ± 0.09 nM (Figure 3, insert). The K_d value obtained is consistent with the finding of Tora et al. that the ER cDNA in HEO contains a point mutation in the steroid binding domain; this mutation results in the receptor with an affinity of ~1 nM, as compared with the normal hER which has a K_d of ~0.1 nM at 37 °C (Tora et al., 1989a). The ER in Rat1+ER cells, however, does bind E2 specifically and with relatively high affinity. This particular clone of Rat1+ER expresses approximately 50 000 receptors per cell, while other clones express 20 000–50 000 receptors per cell (data not shown).

To test for E2-dependent transcriptional activity of the ER in Rat1+ER.1 cells, a pERE15 reporter construct, containing the ERE from the *Xenopus* vitellogenin A2 gene (vitERE), was used for transient transfection assays (Klock et al., 1987). It was previously demonstrated that E2 induces CAT expression from pERE15 when this plasmid is transiently transfected into MCF-7 cells (Klock et al., 1987). When pERE15 was transiently transfected into Rat1+ER cells and assayed for CAT enzymatic activity, E2 stimulated CAT expression over that of the basal level (Figure 4). Such an E2-dependent increase in CAT expression was not detected in Rat1 cells. The fact that we observed a slight induction of CAT activity with the estrogen antagonist, MHT, is

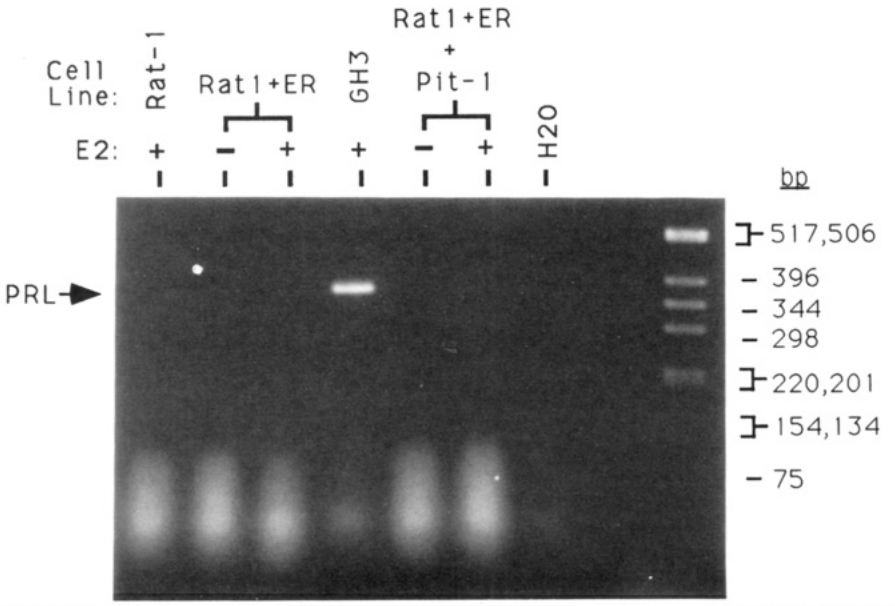

FIGURE 5: RT-PCR assay of total RNA using PRL-specific primers. Five micrograms of total RNA prepared from E2-treated Rat1, GH_3, and Rat1+ER, as well as ethanol-treated Rat1+ER cells, were reverse-transcribed with avian myeloblastosis virus reverse transcriptase. One-fourth of the RT reaction was amplified for 30 cycles using PRL-specific primers, and the amplified product was analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Five micrograms of total RNA prepared from E2- or ethanol-treated Rat1+ER.1 cells transiently transfected with a Pit-1 expression vector (RSV-Pit-1) were also reverse-transcribed and amplified. The expected 369-bp amplified product was observed only with RNA prepared from GH_3 cells. The negative control (H_2O lane) for PCR was done using a parallel RT reaction without added RNA. Molecular weight markers are shown on the right.

consistent with the observation made by Berry et al., who concluded that MHT can act as a partial agonist in a cell-type and promoter-specific manner (Berry et al., 1990). However, because increasing the concentration of MHT in the presence of E2 blocked full induction by E2 (data not shown), the ER expressed in Rat1+ER cells appears to be transcriptionally functional in an E2-dependent manner. Therefore, by all assays used, Rat1+ER cells express functional hER protein.

Endogenous PRL and EGFR Genes Are Not Affected by ER Expression in Rat1 Cells. PRL is an E2-responsive gene that is expressed principally in a subset of ER-positive cells, which includes the lactotrophs of the pituitary (Shull & Gorski, 1986). PRL gene expression, therefore, appears to require factors other than the ER to be activated. Consistent with this hypothesis, preliminary Northern analysis using a PRL cDNA probe failed to detect any PRL transcripts in Rat1 or Rat1+ER cells (data not shown). Furthermore, a more sensitive RT-PCR assay using PRL-specific primers indicated that the endogenous PRL was not expressed in Rat1+ER cells even upon E2 addition, since the expected amplified product of 369 bp is present in RNA isolated from the E2-treated ER-positive pituitary cell line, GH_3 (Tashjian et al., 1968), but not in Rat1 or Rat1+ER cells (Figure 5). It appears, then, that the expression of ER alone in an E2-nonresponsive cell line is insufficient for PRL expression. These observations are consistent with reports in the literature and suggest that ER alone cannot trigger PRL expression.

A pituitary-specific transcription factor, Pit-1 (GHF-1), has been implicated as playing a major role in the activation of PRL and subsequent regulation by E2 (Day et al., 1990; Simmons et al., 1990). In order to test whether transient expression of Pit-1 in Rat1+ER cells resulted in endogenous PRL expression, Pit-1 cDNA was introduced into Rat1+ER cells by electroporation. Total RNA was assayed for PRL expression by RT-PCR (Figure 5; Iverson et al., 1990). RT-PCR showed that transient expression of Pit-1 into Rat1+ER did not result in the activation of endogenous PRL, even in the presence of E2.

However, to confirm an earlier report that Pit-1 was the only factor in Rat1 cells (besides ER) required for E2 regulation of a transiently introduced 5′ PRL regulatory region, preliminary experiments were performed in which a Pit-1 expression vector was cotransfected with the 2.4 PRLCAT reporter construct into Rat1+ER.1 cells (Day et al., 1990; Iverson et al., 1990; Maurer & Notides, 1987). This construct contains ~2.4 kb of 5′ flanking, as well as 39 bp of the transcribed sequences of the rat PRL gene, linked to the CAT reporter gene. In the absence of Pit-1, the CAT activity was barely detectable in either the presence or absence of E2 (Figure 6). When Pit-1 was cointroduced with 2.4 PRLCAT, there was an increase in basal CAT expression that was further augmented by the addition of E2 (Figure 6). These results are consistent with reports in the literature (Day et al., 1990; Maurer & Notides, 1987; Simmons et al., 1990). Therefore, although the expression of ER and Pit-1 was sufficient for E2 regulation of 2.4 PRLCAT, activation of the endogenous PRL depends on one or more as yet unidentified factors.

It has been reported that EGFR in the rat uterus is up-regulated by E2 at both the protein and mRNA levels (Lingham et al., 1988; Mukku & Stancel, 1985). Since EGFR is basally expressed in Rat1 cells, it was of interest to examine whether the expression of ER in these cells allowed E2 regulation of EGFR (Magun et al., 1980). To our surprise, E2 had no effect on the level of EGFR protein as assayed by [^{125}I]EGF binding assays (Figure 7), where the B_{max} values for the untreated and E2-treated Rat1+ER cells were 9.99 ± 0.6 and 9.31 ± 0.4 pM, respectively. Furthermore, E2 had no effect on the K_d of the EGFR; these K_d values (0.20 ± 0.02 nM with E2 treatment and 0.18 ± 0.02 nM without E2 treatment) are consistent with reports in the literature (Mukku & Stancel, 1985). Therefore, even though EGFR is expressed at basal levels in Rat1+ER cells, the expression of ER does not confer E2 regulation on the EGFR protein.

Stable Ectopic ER Expression Results in the Induction of the PR Protein. PR, another member of the steroid hormone receptor superfamily, in contrast to PRL, is an E2-responsive

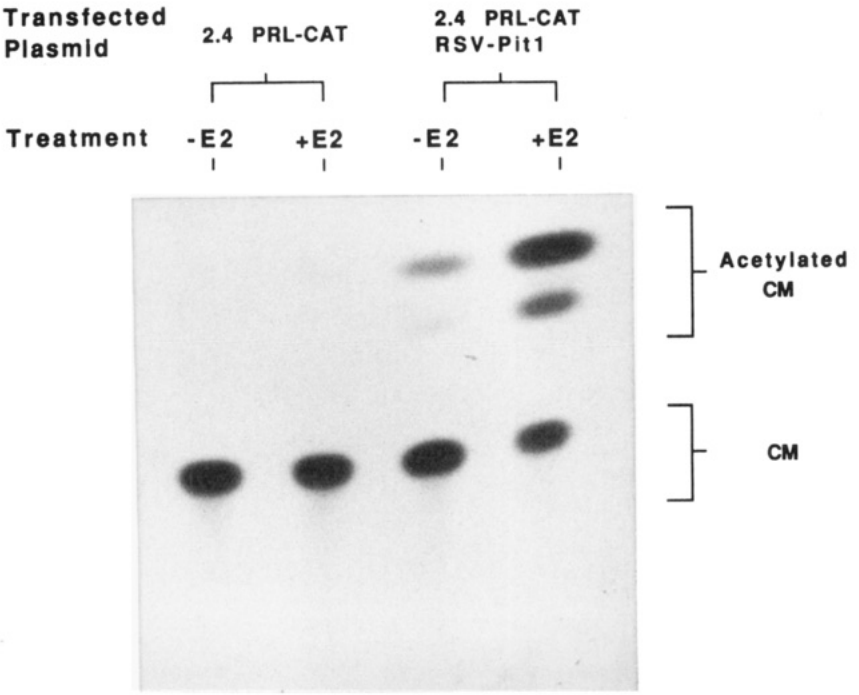

FIGURE 6: Pit-1-dependent E2 stimulation of CAT activity from a 2.4 PRL-CAT plasmid in Rat1+ER cells. Rat1+ER cells were transfected with 2.4 PRL-CAT alone (left two lanes) or with 2.4 PRL-CAT and RSV-Pit-1 (right two lanes) and treated with either 10 nM E2 or 10 nM ethanol control. CAT assays were performed as described in Figure 4.

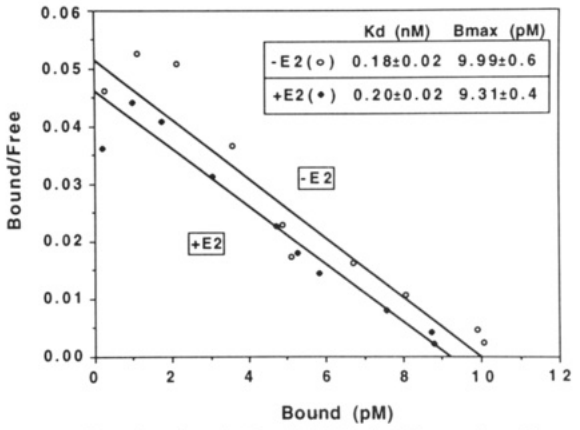

FIGURE 7: Scatchard analysis of EGFR in E2- or ethanol-treated Rat1+ER cells. Rat1+ER cells were treated with either 10 nM E2 or 10 nM ethanol for 24 h and were incubated with increasing concentrations of [^{125}I]EGF in either the presence or absence of a 100-fold molar excess of unlabeled EGF at 4 °C. Specific counts (bound) and free [^{125}I]EGF counts were quantified using a γ counter, and the data were analyzed using the LIGAND computer program.

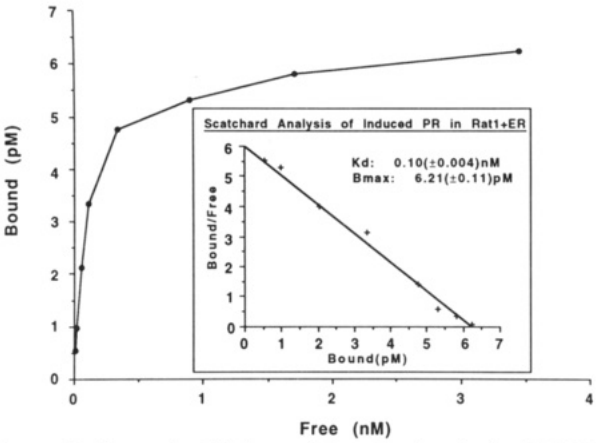

FIGURE 8: Saturation binding and Scatchard analysis of R5020 to E2-treated Rat1+ER cells. Whole cell extracts prepared from E2-treated Rat1+ER cells were incubated with increasing concentrations of [^{3}H]R5020 with or without a 100-fold molar excess of unlabeled R5020. The binding data for the E2-treated Rat1+ER cells were plotted by the method of Scatchard, and the parameters were estimated by the LIGAND computer program.

gene that is expressed in many E2-responsive cells. In fact, it appears that E2, in addition to progesterone itself, is the primary regulator of PR expression (Clarke & Sutherland, 1990). Therefore, we examined whether or not ER expression is sufficient for activating the silent PR gene.

Initial binding experiments, using the progesterone agonist [^{3}H]R5020, indicated that PR protein was being expressed in E2-treated Rat1+ER cells (data not shown). To confirm that the specific binding detected in E2-treated Rat1+ER cells was due to *bona fide* PR, which binds R5020 with high affinity, saturation ligand binding analysis was done. These studies demonstrate that the specific binding of R5020 detected in E2-treated Rat1+ER cells is saturable and that the component responsible for specific binding in E2-treated Rat1+ER has a K_d of 0.1 ± 0.004 nM, indicative of a high-affinity PR (Figure 8; Greco & Gorski, 1989). Furthermore, this PR induction in Rat1+ER.1 cells followed the same time course and E2 dose dependence as those previously reported for the MCF-7 cell line (Nardulli, 1988; K. J. Kaneko, unpublished data).

E2 Induces PR in Rat1+ER Cells at the mRNA Level in the Absence of Protein Synthesis. The studies on PR to this point used ligand binding assays, leaving the mechanism of E2 induction of PR in Rat1+ER.1 cells unclear. It is conceivable, for example, that the addition of E2 converts nonbinding PR already present in Rat1+ER.1 cells to PR that is able to bind R5020. To test whether the PR induction by E2 in Rat1+ER.1 cells occurs at the mRNA level, we performed the RT-PCR assay using rat PR primers (Figure 9A; Park & Mayo, 1991). Total RNA from GH_3 cells, which express PR (data not shown; Roos et al., 1980), exhibited an amplified DNA fragment of 325 bp, consistent with observations of the rat uterus (Park & Mayo, 1991). Furthermore,

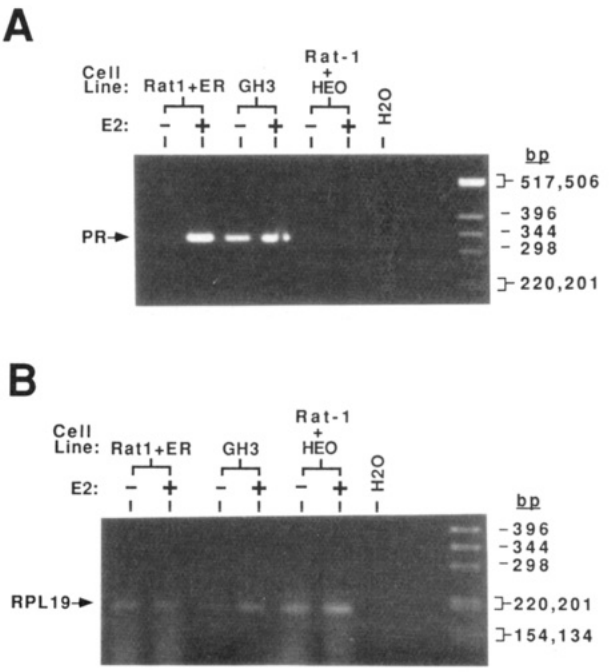

FIGURE 9: RT-PCR assay of total RNA using PR and RPL19 primers. Total RNA prepared from E2- or ethanol-treated Rat1+ER and GH_3 cells was reverse-transcribed and PCR amplified as described in Figure 6, with the exception of using PR (A) or RPL19 (B) primers. (A) An RT-PCR assay using PR primers shows that a 325-bp amplified fragment was present in GH_3 as well as in E2-induced Rat1+ER cells. To test whether transient expression of ER in Rat1 cells also resulted in the induction of the endogenous PR, Rat1 cells were transiently transfected with HEO plasmid (see Materials and Methods). When the total RNA prepared from E2- or ethanol-treated cells was analyzed for the presence of PR mRNA using PR primers, PR-specific PCR product was not observed. (B) A PCR assay of the same RT reaction used in A, except that RPL19, instead of PR, primers were included in the assay. The 194-bp amplified products were present in all samples, and they suggest that, at least qualitatively, the amount of RNA and the extent of RT were approximately similar among the samples. No amplified product was observed when no RNA was added to the RT reaction (H_2O lanes).

the same amplified DNA fragment was present in E2-induced Rat1+ER.1 cells. Although no amplified bands were detected in the water control (Figure 9A) or in the E2-treated Rat1 cells (data not shown), we consistently observed a small amount of amplified fragment in E2-untreated Rat1+ER.1 cells (see below). The RT-PCR of the same RT reactions using RPL19 (Park & Mayo, 1991) showed that, qualitatively, similar amounts of RNA were present in all samples (Figure 9B). These observations suggest that the specific PR binding detected in E2-treated Rat1+ER was due to an increase in the mRNA for the PR protein.

To examine whether stable ER expression was required for PR gene activation, an hER expression vector (HEO; Green et al., 1986) was transiently transfected into Rat1 cells, and the total RNA was analyzed for PR expression by RT-PCR (Figure 9A). Again, in order to monitor the success of the transfection, pERE15 (see Figure 4) was cotransfected with HEO and assayed for CAT expression. Such control studies show that transcriptionally functional ER is expressed in Rat1 cells that are transiently transfected with HEO (data not shown). Interestingly, PR expression is not detected in the transfected Rat1 cells, even in the presence of E2. Therefore, transient transfection of ER in Rat1 does not result in PR expression at detectable levels.

The observed increase in the steady-state level of PR mRNA could be due to a secondary effect of E2, where E2 may induce the synthesis of an unknown factor that, in turn, activates the PR gene. To examine this possibility, E2-dependent PR activation in Rat1+ER.1 cells was examined under conditions where the majority of protein synthesis was inhibited. Rat1+ER.1 cells were induced with E2 in either the presence or absence of the protein synthesis inhibitor cycloheximide, and total RNA prepared from these cells was analyzed for PR induction using RT-PCR (Figure 10). PR was still induced in the presence of cycloheximide under conditions where >94% protein synthesis was inhibited (data not shown), providing strong evidence that E2 directly activates the PR gene in Rat1+ER.1 cells.

Finally, in order to rule out the possibility that the PR gene was activated serendipitously by fortuitous provirus integration, other clonal lines of Rat1+ER were examined for PR expression by RT-PCR. In all other clonal lines, PR expression was activated in the presence of E2 (Figure 11). These

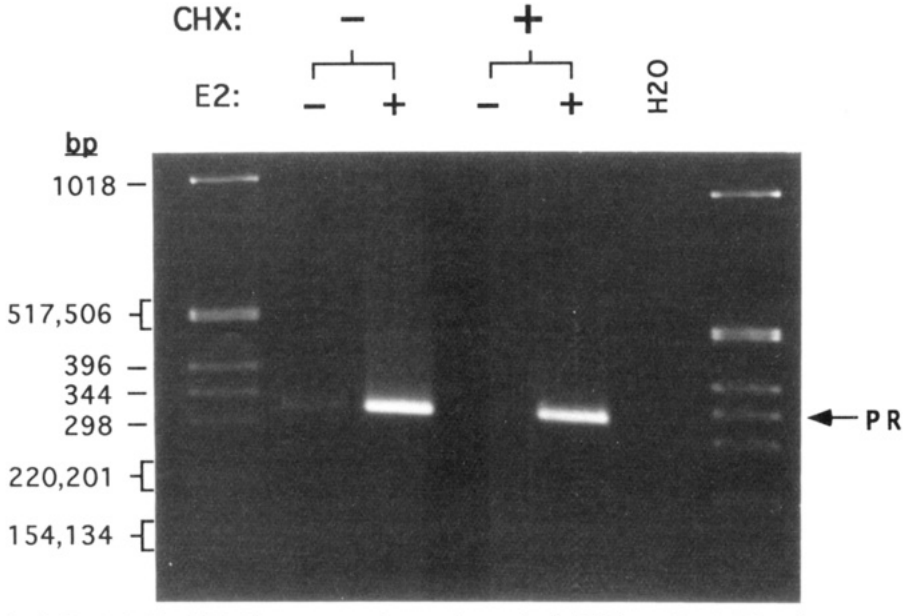

FIGURE 10: PR induction in Rat1+ER cells in the presence of a protein synthesis inhibitor. Rat1+ER cells were treated with or without 10 μM protein synthesis inhibitor cycloheximide (CHX) and incubated with 5 nM E2 or ethanol control. After 24 h, total RNA was prepared and the induction of PR mRNA was analyzed by RT-PCR as described in the legend for Figure 9A.

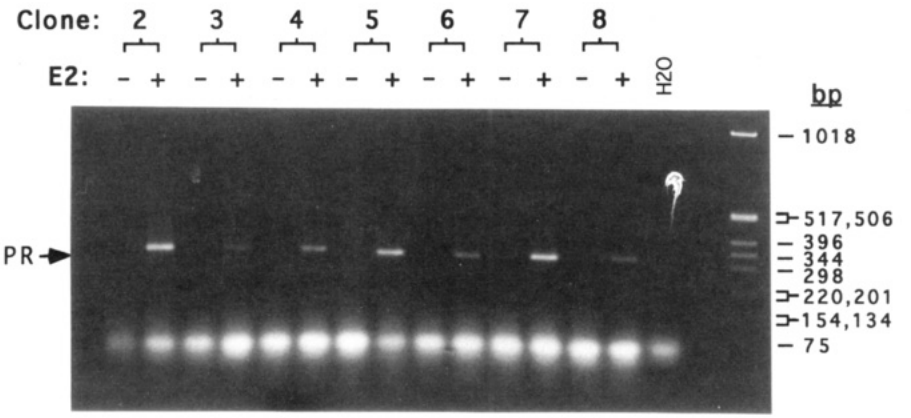

FIGURE 11: RT-PCR assay of other clonal lines of Rat1+ER cells using PR primers. Other clonal lines of Rat1+ER were treated with 10 nM E2 or ethanol for 24 h, and the total RNA prepared from those samples was analyzed for the presence of PR mRNA by RT-PCR as described in the legend for Figure 9A.

observations suggest that stable ER expression in Rat1 cells results in the direct activation of a silent PR gene.

MHT, but Not ICI 164,384, Is a Partial Agonist for PR in Rat1+ER Cells. Two commonly used E2 antagonists are MHT and ICI 164,384 (ICI) (Jordan & Murphy, 1990; Jordan & Tate, 1981; Wakeling & Bowler, 1988b). Whereas ICI is a complete antagonist, MHT has partial estrogen agonist activities in some tissues (Berry et al., 1990; Wakeling & Bowler, 1988a). Since a partial agonistic activity of MHT had been observed in PR expression (Savouret et al., 1991; Wakeling & Bowler, 1988a), we examined whether MHT produced such effects on the PR expressed in Rat1+ER cells.

In order to examine the effect of MHT in Rat1+ER cells, we initially performed transient transfection studies using the reporter construct containing the ERE from the PR gene (+698/+729-TK-CAT; Savouret et al., 1991). This ERE mediates not only the E2 regulation of a heterologous *tk* promoter linked to a CAT reporter gene but also the partial agonist activity of MHT not observed with ICI (Savouret et al., 1991). When +698/+729-TK-CAT was transiently transfected into Rat1+ER.1 cells, E2, but not ICI, induced CAT activity (Figure 12A). Furthermore, consistent with the observations made by Savouret et al., MHT partially induced CAT activity in Rat1+ER.1 cells.

To examine whether MHT had partial agonistic activity with the endogenous PR gene, Rat1+ER.1 cells were treated with an E2 agonist, such as diethylstilbestrol (DES), MHT, ICI, or ethanol vehicle, and total RNA was examined for PR expression by RT-PCR (Figure 12B). In cell culture systems, no differences are observed between E2 and DES in inducing PR expression (Campen et al., 1985). As seen with E2, DES induced PR expression in Rat1+ER cells, while ICI appeared to act as a complete antagonist. Interestingly, MHT does appear to be a partial agonist for the PR gene in Rat1+ER.1 cells, consistent with what is observed in normal E2-responsive cells. Therefore, these observations are consistent with the fact that ER expression, at least in Rat1 cells, results in the conversion of an ER-negative cell to an E2 target cell.

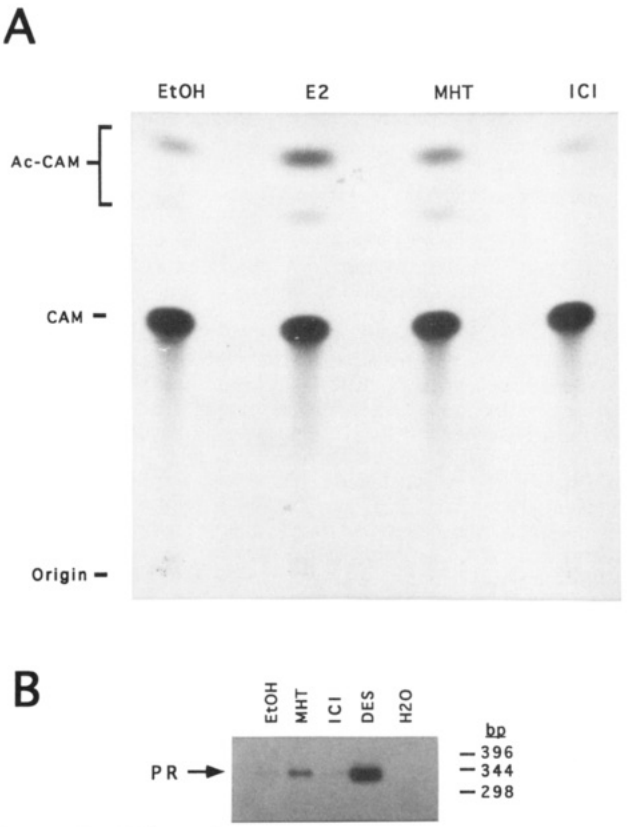

FIGURE 12: Effects of E2 antagonists on Rat1+ER cells. (A) Rat1+ER cells were transfected by electroporation with +698/+729-TK-CAT (see text), which includes the putative ERE for the PR cloned 5′ of the *tk* promoter-CAT gene. The transfected cells were aliquoted equally into four plates and treated with ethanol control (EtOH), 10 nM E2, 100 nM MHT, or 100 nM ICI for 24 h prior to harvest. Twenty-five micrograms of the whole cell extracts were assayed as described for Figure 4, except that the autoradiography of the thin-layer chromatograph is shown. (B) Confluent Rat1+ER cells were treated with either EtOH, 15 nM MHT, 100 nM ICI 164,384, or 10 nM DES, an estrogen agonist, for 24 h, and the total RNA was prepared and analyzed for PR mRNA using the RT-PCR assay. In this assay, the PCR reaction contained all 20 μL of the RT reaction and a trace amount of [α-^{32}P]dATP (0.2 μCi/100 μL of PCR reaction), and the amplified products were analyzed by 5% polyacrylamide gel electrophoresis and autoradiography. Molecular weight markers are indicated on the right.

DISCUSSION

Understanding E2 action becomes more complicated because, in various ER-positive cells, different E2-responsive genes are affected by E2. The underlying theme appears to be that many endogenous E2-responsive genes cannot be activated simply by ER expression. To gain insight into how E2 and ER differentially regulate various E2-responsive genes, we first constructed a novel cell line that stably expressed a functional ER from a normally ER-negative cell line and studied E2 regulation of transiently transfected E2-responsive reporter constructs as well as endogenous E2-responsive genes.

Stable, but Not Transient, Expression of ER Results in the Activation of a Silent PR Gene. Since PR is expressed

and regulated by E2 in many ER-positive cells, we hypothesized that the PR gene may be activated by the mere expression of ER. Indeed, when E2 was added to Rat1+ER cells, significant induction of PR was observed. This induction occurs at both protein and mRNA levels as a result of direct activation by E2. Furthermore, a *bona fide* functional PR is induced since the Scatchard analysis revealed the K_d of this protein to be approximately 0.1 nM, consistent with what has been reported in the literature (Greco & Gorski, 1989). Finally, since all eight Rat1+ER clones examined show PR induction, PR activation most likely is not due to fortuitous isolation of a variant population of cells that exhibits this activity.

Our observations of PR induction in Rat1+ER cells is one of the few examples cited in the literature where the expression of one gene can activate an otherwise silent locus. In recent years, genes that singly alter the phenotypes of the cells in which they are expressed have been isolated (Davis et al., 1987; Nambu et al., 1991). In one particular case, the expression of MyoD results in the expression of otherwise silent muscle-specific genes and the alteration of the phenotype from a nonmuscle cell to a muscle cell (Davis et al., 1987; Weintraub et al., 1989, 1991).

The expression of ER, however, may not activate PR in all cells. It has been reported, for example, that when ER is stably expressed in HeLa cells, PR does not appear to be activated (Maminta et al., 1991; Touitou et al., 1990). Furthermore, PR is not induced when E2 is added to Chinese hamster ovary cells that overexpress ER (ERC cells) (Kushner et al., 1990; K. J. Kaneko, C. Ying, & J. Gorski, unpublished data). Since elements that confer cell-specific gene repression have been identified in the regulatory regions of some E2-responsive genes (Baniahmad et al., 1990; Gaub et al., 1987; Jackson et al., 1992; Schweers et al., 1990), the presence of a negative factor that represses PR activation in HeLa and ERC cells cannot be ruled out. Therefore, the possibility remains that, in an embryonically derived cell line such as Rat1, a factor(s) required for PR activation (other than ER) is present or that a factor(s) that maintains the repression of the PR gene is absent.

Finally, transient expression of ER in Rat1 cells does not appear to result in PR activation. The reasons for this are not known. One possibility may be that only small populations of cells actually express the transfected gene in transient transfections; therefore, the levels of PR expression are not detectable under the RT-PCR conditions used. It has also been suggested that DNA replication may be required for modulation of gene expression in some cases (Enver et al., 1988; Miller & Nasmyth, 1984). If such a mechanism is required to activate previously silent genes, the transient transfection conditions used may not allow activation of the PR gene.

The partial agonist activity of MHT observed with PR in some E2-responsive systems was also seen in Rat1+ER cells. Our observations of MHT effects in transient transfection experiments are consistent with those of Berry et al. (1990), but not of Sarouret et al. (1991). However, the fact that the agonist activity of MHT is cell-type-dependent may partially explain the differential effects of vitellogenin and PR EREs reported by Savrouret et al. (1991). It is interesting to note that efficient MHT agonist activity was observed in cells, similar to Rat1+ER cells, that are embryonic fibroblast in origin (Berry et al., 1990).

Interestingly, in some Rat1+ER clonal lines (1 and 7), a very small basal level of PR mRNA was detected using RT-PCR (Figures 9–11; data not shown). In at least one of the clones (Rat1+ER.1), this basal level (detected by RT-PCR) was not due to E2 contamination, since the E2 antagonist, ICI, did not lower the level of expression (Figure 12B). We do not know the nature or the significance of this basal expression. In neither Rat1 nor untreated Rat1+ER cells could we detect PR that bound [^{3}H]R5020 in a saturable manner, indicating that if PR protein capable of binding R5020 is expressed in untreated Rat1+ER cells, the levels are below the limit of detection (data not shown). However, in many cases, basal level expression for PR is observed in E2-responsive systems (see Figure 9, E2-untreated GH_3 cells; Greco & Gorski, 1989). Since other mechanisms can modulate PR expression, as observed by using different cell culture conditions (K. J. Kaneko & J. Gorski, unpublished results; Greco & Gorski, 1989; Aronica & Katzenellenbogen, 1991; Katzenellenbogen et al., 1987), we cannot rule out the possibility that an ER-independent mechanism controls the basal level of PR expression. What is clear is that this low level of PR expression is not required for the E2 induction of the PR gene in Rat1+ER cells since those clones that do not express basal level PR mRNA also show PR induction (Figure 11).

Stable ER Expression Does Not Activate the Silent PRL Gene or Allow E2 Regulation of EGFR. PRL is a lactotroph-specific E2-responsive gene that has multiple functions, including promoting lactation in females. Using mice as a model system, Slabaugh et al. established that E2 stimulation is required for the initial induction of PRL and that a functional ER may be the limiting factor in PRL expression (Slabaugh et al., 1982). This implication of ER regulation for PRL expression led to the examination of whether PRL is activated by ectopic ER expression in nontarget cells.

Our observations of Rat1+ER cells suggest that ER expression, however, is clearly not the only prerequisite for PRL expression. This lack of PRL expression may be attributed to Rat1+ER missing another factor that either activates PRL and/or allows E2 regulation of the gene. Consistent with this view, several laboratories (using transient transfection studies) reported that a cell-specific transcriptional factor, Pit-1, is required for basal as well as E2 activation of reporter genes linked to PRL regulatory sequences (Day et al., 1990; Simmons et al., 1990). Indeed, coexpression of Pit-1 in Rat1+ER cells was required for the activation and E2 regulation of the transiently transfected 5′ regulatory region of the PRL gene (Figure 6).

However, transient expression of Pit-1 was not sufficient to activate the endogenous PRL gene in the Rat1+ER cells. The failure to detect endogenous PRL expression could be due to the limit of sensitivity by RT-PCR, as discussed in the preceding section. Since Rat1 cells that stably expressed Pit-1 also failed to show activation of endogenous PRL expression, the activation of the silent PRL gene in Rat1 cells most likely requires a factor in addition to Pit-1 and ER (K. J. Kaneko, R. Maurer, & J. Gorski, unpublished data). These observations are consistent with those seen with the vitellogenin gene. Seiler-Tuyns et al. observed that, while transient expression of ER activated a transiently expressed reporter gene containing vitERE, it could not activate the endogenous vitellogenin gene (Seiler-Tuyns et al., 1988). Similarly, our observations suggest that, although all of the factors necessary for E2 regulation for PRL were present in our transient transfection studies (i.e., ER and Pit-1), the endogenous gene remained silent in ER-positive, PRL-negative cells. The activity of Pit-1 in the developmental regulation of PRL may be subordinate to another PRL-specific factor(s) that allows both Pit-1 and ER access to their respective regulatory

elements. The activation of PRL, then, may result from events proposed by both Simmons et al. and Dollé et al., in that the activation of PRL by Pit-1 may be preceded by expression of another factor that allows Pit-1 to regulate PRL (Dollé et al., 1990; Simmons et al., 1990).

The expression of at least two other factors in addition to ER, therefore, may be required for E2 regulation of PRL. A factor may be necessary for converting the chromatin structure of these genes to a state in which ER and Pit-1 can gain access to their respective target elements. Durrin and Gorski have shown, for example, that PRL hypersensitive sites, present in lactotrophs but not in liver, are present early in development and are not induced by E2 (Durrin & Gorski, 1985). Therefore, the PRL regulatory region may contain as yet unidentified *cis*-acting elements similar to those of the locus-activating region of the β-globin genes. Such elements may require factors other than ER and Pit-1 to be activated. In addition, Pit-1 may be required for efficient ER binding to the ERE(s) located in the regulatory region of PRL. Day et al. have indicated that the mutation of the imperfect palindromic PRL ERE to a perfect palindrome can circumvent the need of Pit-1 for E2 regulation (Day et al., 1990). Since the addition of Pit-1 did not augment the E2-induced expression from the mutant perfect palindromic PRL ERE, one possible role for Pit-1 in PRL expression is to facilitate the efficient binding of ER to its element. Furthermore, the observation that the various distinct EREs located upstream of the chicken vitellogenin II gene show different *in vivo* footprinting patterns implicates the existence of auxiliary factors that facilitate interactions between ER and the various EREs (Philipsen et al., 1988).

Finally, since EGFR was shown to be E2-regulated in the uterus, we examined whether the constitutively expressed EGFR in Rat1 cells could be regulated by E2 upon ectopic expression of ER. For some E2-responsive genes that are constitutively expressed, such as cathepsin D and *myc*, the mere expression of ER is sufficient for E2 regulation, at least in HeLa cells (Maminta et al., 1991; Touitou et al., 1990). The chromatin structure of basally expressed genes may be such that their regulatory sites are accessible to transcription factors. Surprisingly, E2 appeared to have no effect on the level of EGFR protein in Rat1+ER cells. Since different E2 effects on EGFR are observed in the breast tumor cell line MCF-7 and the rat uterus (Lee et al., 1989; Lingham et al., 1988), this result is consistent with the hypothesis that E2 regulation of EGFR may be complex and, perhaps, cell-specific. The differential cell-specific effects on EGFR by E2 may be achieved through regulating the appropriate concentrations of other transcription factors, perhaps factors such as fos/jun, such that the activation or repression of the E2-responsive gene by ER may be modulated. Indeed, since jun and fos have been shown to inhibit ER activity in certain cell types, concentrations of these factors in Rat1 may be such that E2 activation of EGFR is not observed (Tzukerman et al., 1991). Conversely, the possibility that an essential component(s) in E2 regulation of EGFR is present in the uterus but not in Rat1 cells cannot be ruled out.

Since E2 regulates its responsive genes differentially in a cell-specific manner, it is not surprising that the effects of stable ectopic ER expression in an ER-negative cell line on endogenous genes are complex. Most interestingly, stable ER expression leads to the activation of a silent PR gene in Rat1 cells. This suggests that, since the expressions of PR and ER are closely correlated, ER may be one of the necessary components involved in activating PR. In contrast, such ER expression has no effect on either the PRL or the EGFR gene. These observations indicate that the overall effects of E2 on E2-responsive genes may require components in addition to ER; one possible role of these accessory factors may be to alter the chromatin assembly of target genes such that the ER can gain access to its ERE.

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