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Estrogen-induced transcription of the progesterone receptor gene does not parallel estrogen receptor occupancy

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ABSTRACT **The activation of the silent endogenous progesterone receptor (PR) gene by 17-β-estradiol (E_2) in cells stably transfected with estrogen receptor (ER) was used as a model system to study the mechanism of E_2-induced transcription. The time course of E_2-induced PR transcription rate was determined by nuclear run-on assays. No marked effect on specific PR gene transcription rates was detected at 0 and 1 h of E_2 treatment. After 3 h of E_2 treatment, the PR mRNA synthesis rate increased 2.0- ± 0.2-fold and continued to increase to 3.5- ± 0.4-fold by 24 h as compared with 0 h. The transcription rate increase was followed by PR mRNA accumulation. No PR mRNA was detectable at 0, 1, and 3 h of E_2 treatment. PR mRNA accumulation was detected at 6 h of E_2 treatment and continued to accumulate until 18 h, the longest time point examined. Interestingly, this slow and gradual transcription rate increase of the endogenous PR gene did not parallel binding of E_2 to ER, which was maximized within 30 min. Furthermore, the E_2–ER level was down-regulated to 15% at 3 h as compared with 30 min of E_2 treatment and remained low at 24 h of E_2 exposure. These paradoxical observations indicate that E_2-induced transcription activation is more complicated than just an association of the occupied ER with the transcription machinery.**

The estrogen receptor (ER) is a ligand-activated transcription factor. The location of the unoccupied ER in the nucleus is still unclear, but ER may already be bound to DNA before it associates with 17-β-estradiol (E_2) (1–3). Upon binding E_2, the ER undergoes conformational changes (4–6) and modulates the transcription of target genes (7, 8).

The exact mechanism of E_2-induced transcription activation is not fully understood. The currently accepted model is that the transcriptional complex may be assembled with higher efficiency or may be stabilized by the E_2–ER complex (8, 9). The action of receptors to enhance transcription rates could be caused by simple protein interaction with components of the transcriptional machinery and/or with intermediate proteins. Association of ER with transcription factor IIB and a subset of transcription factor IID complexes without an adapter protein has been demonstrated *in vitro* (10, 11), which implies that ER makes direct protein contacts with components of the transcription machinery (8). If ER activates transcription by a simple mechanism, such that contacts between the ER and the transcriptional machinery are sufficient to enhance transcription, it would be predicted that E_2 occupancy of ER should be closely coupled with the transcription rate of E_2-responsive genes. While this may be true in some cases, it is not always true.

Previously, human ER cDNA was stably expressed in Rat1 cells, which do not express endogenous ER (Rat1+ER) (12). Human ER cDNA contains a point mutation (Gly-400–Val-400). This point mutation causes a 10-fold lower affinity for the ligand but does not affect other functions of the receptor (13). Rat1+ER cells express 20,000–50,000 ERs per cell, which is equivalent to rat uterine ER levels. The ER was functional as determined by ligand binding assays and transcription activation of E_2-responsive reporter plasmids (12). Interestingly, E_2 activated the silent endogenous progesterone receptor (PR) gene at the mRNA level, which was followed by PR protein expression (12).

We have examined the mechanism by which the E_2–ER complex activates transcription of the silent endogenous PR gene in Rat1+ER cells using nuclear run-on assays. This assay measures the rate of transcription, which allows the analysis of transcription independent of mRNA processing (14). Surprisingly, we found that the PR gene transcription rate gradually increased over 24 h of E_2 treatment after an initial lag of 1–3 h. This did not parallel the binding of E_2 to ER, which would activate ER. Furthermore, the E_2–ER level was down-regulated to 15% by 3 h as compared with 30 min of E_2 treatment and was undetectable by 24 h. We propose that E_2-activated transcription is more complicated than mere physical associations with the transcription machinery; rather, the accumulation of catalytic intermediary factors may either directly or indirectly influence transcription over time (Fig. 1).

MATERIALS AND METHODS

Cell Culture Conditions. Rat-1 and Rat1+ER cells were grown in phenol red-free, high-glucose 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 (FBS; HyClone). Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2 and fed every 1–2 days. Before E_2 induction, the cells were washed with Hanks' buffered saline solution (Sigma) and cultured in DMEM supplemented with 10% FBS that was stripped of steroids by treating with acid-activated charcoal-coated dextran three times at 4°C (ST-FBS) (15) for 24–48 h to eliminate any estrogenic source before treatment. Cells were treated with hormones at different times, harvested simultaneously, and subjected to each experiment.

Hormones. All E_2 treatments were done with ST-FBS containing media. E_2 (10–20 nM) was used to maximize the response unless otherwise noted. E_2 was purchased from Sigma. ICI 182,780 (ICI) and monohydroxytamoxifen (MHT) were obtained from Zeneca (Wilmington, DE). All estrogenic compounds were dissolved in 100% ethanol and added to the medium at a $1{:}10^3$ to 10^4 dilution such that the total ethanol concentration was never higher than 0.1%.

Nuclear Run-On Assays. Cells were harvested by trypsinization, washed, and lysed in 0.25% Nonidet P-40 buffer (10 mM Tris·HCl/3 mM $CaCl_2$/2 mM $MgCl_2$/1 mM dithiothreitol/10% glycerol). Nuclei (10^6–10^7) were resuspended in 50 mM

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Abbreviations: PR, progesterone receptor; ER, estrogen receptor; E_2, 17-β-estradiol; MHT, monohydroxytamoxifen.

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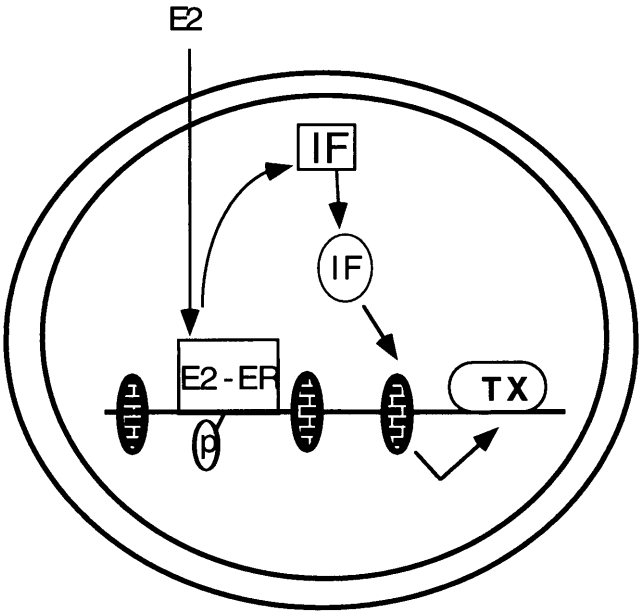

FIG. 1. Proposed model for E_2-induced transcription activation. The activation of the PR gene by ER may involve the activation of catalytic intermediary factors (IF). These IF, once modified due to interaction with the E_2–ER complex may promote PR gene transcription. For details, see Introduction and Discussion. E_2, estrogen; ER, estrogen receptor; E_2–ER, E_2-occupied ER; TX, transcription machinery; , chromatin; , phosphorylation; IF, intermediate factors; , nonactivated IF; , activated IF.

Tris·HCl (pH 8.3), 40% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and frozen in liquid N_2. The nuclei were thawed, mixed with 5× transcription buffer (25 mM Tris·HCl, pH 8.0/12.5 mM $MgCl_2$/750 mM KCl/1.25 mM of each GTP, ATP, and CTP/2.5 mM dithiothreitol), and 100 μCi (1 Ci = 37 GBq) of ^{32}P-labeled UTP followed by incubation for 20 min at 30°C. Nuclei were then digested with ribonuclease-free DNase I (Boehringer Mannheim) for 10 min at 30°C. The nuclei were lysed and further digested with proteinase K. The ^{32}P-labeled RNA was extracted with phenol/chloroform and ethanol precipitated. Free nucleotides were removed through a ribonuclease-free G-50 spin column (Boehringer Mannheim). This RNA was dissolved in 10 mM TES (pH 7.4), 0.2% SDS, 10 mM EDTA, and 100 μg yeast tRNA at 1–10 × 10^6 cpm/ml, and hybridized at 65°C for 48–60 h to 5 μg of denatured plasmid DNA that had been immobilized on a nitrocellulose membrane. After hybridization the filters were washed consecutively with 2× SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0)/0.1% SDS and 0.2× SSC/0.1% SDS, washed again with 2× SSC/50 μg/ml RNase A to remove any unhybridized RNA, subjected to autoradiography or phosphorimaging and quantified by PhosphorImager (Molecular Dynamics).

The PR and control plasmids that were included to measure PR gene transcription rates in Rat1+ER cells were as follows. The rat PR cDNA [rPR2; a gift of Kelly E. Mayo, Northwestern University, Chicago (16)] includes 65 base pairs at the end of exon 1, the entire DNA binding domain, the hormone binding domain, and the 3′ untranslated regions. The plasmid used for prolactin (PRL) was a 3.6 kb genomic clone that includes exon 1 and exon 2 (17). The PRL gene, which is not expressed in Rat-1 and Rat1+ER cells with or without E_2 (12), was used as a background control. CHOB (18), a cDNA of ribosomal protein S2 that is not affected by E_2, was used as an internal standard (16). The PR gene transcription rates were normalized to the transcription rate of CHOB after subtracting backgrounds. The results were expressed as fold-changes relative to the control values.

E_2 Whole Cell Uptake Assays. E_2 binding assays were performed as previously reported with minor modifications (19). Cells were cultured in 60 × 15 mm Petri dishes to near confluency as described above and bound with 10 nM [^{3}H]–E_2 (95.3 Ci/mmol, DuPont/NEN) for the indicated time points. Addition of 100-fold excess of unlabeled E_2 was used to determine nonspecific binding. Cells were then washed twice with 3 ml of cold phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/1.4 mM KH_2PO_4, pH 7.4) with 0.1% bovine serum albumin and washed once again with 3 ml of Hanks' buffered saline solution/5 mM EDTA/0.1% methylcellulose. Cells were harvested by trypsinization and centrifugation at 1000 × *g*. After three washes with 1 ml of Hanks' buffered saline solution/5 mM EDTA/0.1% methylcellulose, cells were resuspended in 700 μl of ethanol and incubated for 30 min at room temperature. Five hundred milliliters of the extracts was added to 4 ml Ready-Safe scintillation cocktail (Beckman) and quantified by scintillation counting. Specific binding was calculated as total minus nonspecific.

RNA Isolation and Reverse Transcription (RT)–PCR Assays. Rat-1 and Rat1+ER cells were treated for the indicated times as described in Figs. 4 and 5 with or without E_2 (10 nM), MHT (10 nM), or ICI (10 nM). Total RNA was prepared as described previously (20). RNA concentration was measured by absorbence at 260 nm. Total RNA (2.5 μg) was reverse transcribed in reaction buffer of 50 mM KCl, 10 mM Tris·HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 5.25 mM $MgCl_2$, 32 units of RNasin (Promega), 100 pmol of random hexamer (Pharmacia), 1 mM each dNTP, and 8 units of avian myeloblastosis virus reverse transcriptase (Promega) at 42°C for 75 min. Aliquots of RT reactions were added to the final reaction mixture of 50 mM KCl, 5.0 mM $MgCl_2$, 10 mM Tris·HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 500 ng of PCR primers, 2 mM each dNTP, and 5 units of *Taq* DNA polymerase (Promega). The PCR reactions were amplified for 35 cycles (94°C for 30 s; 60°C for 30 s; and 72°C for 30 s) using a DNA thermo cycler (model 480, Perkin–Elmer/Cetus). The sequence of the ER primers were ERa (5′-GAGATCCTGATGATTGGTCT-3′) and ERb (5′-CATCTCCAGCAGCAGGTCAT-3′) (21). PR primers were PRa (5′-CCCACAGGAGTTTGTCAAGCTC-3′) and PRb (5′-TAACTTCAGACATCATTCCGC-3′) (12). Ribosomal protein L19 (RPL19) primers were RPL19a (5′-CTGAAGGTCAAAGGGAATGTG-3′) and RPL19b (5′-GGACAGAGTCTTGATGATCTC-3′) (22). The expected size of amplicons for ER, PR, and RPL 19 are 477, 327, and 194 bp, respectively. Reaction products were resolved on 2.5% agarose gels and visualized by ethidium bromide staining.

RESULTS

Time Course of E_2-Induced PR Gene Transcription. Rat1+ER cells were treated with E_2 for 0, 1, 2, 3, 5, 6, 8, and 24 h, and the E_2-induced transcription of the endogenous PR gene was determined by nuclear run-on assay (Fig. 2*A*). E_2-induced PR gene transcription was normalized to the transcription rate of CHOB, which is not affected by E_2 (16). The quantified results represent an average of four independent experiments (Fig. 2*B*). The specific PR gene transcription rate was negligible at 0 h. There was still no marked effect on PR gene transcription rate at 1 h of E_2 treatment. After 3 h of E_2 treatment, PR mRNA synthesis rate increased 2.0- ± 0.2-fold and continued to increase to 3.5- ± 0.4-fold by 24 h as compared with 0 h (Fig. 2*B*). It was surprising that the estrogen stimulation of transcription was not an immediate but rather a gradual process that occurred only after a 1–3 h lag.

Time Course of E_2 Binding in Rat1+ER Cells. If the E_2 binding in Rat1+ER cells is slow and increases over time, it would result in gradual transcription activation as in our transcription data. We therefore examined the kinetics of [^{3}H]E_2 (10 nM) binding by whole cell uptake in Rat1+ER cells

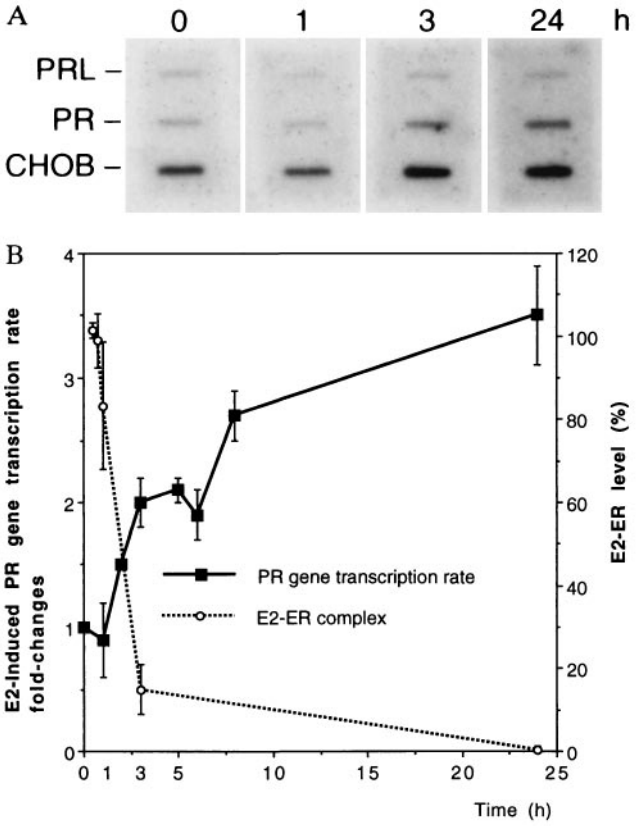

FIG. 2. Time course of PR mRNA transcription rate after E_2 treatment. Rat1+ER cells were treated with E_2 (10 nM) for the indicated times and the nuclei were isolated. Nuclei were incubated with ^{32}P-UTP and other ribonucleotides to label nascent transcripts from engaged RNA polymerases. The steady-state number of RNA polymerases, i.e., transcription rate, was determined by hybridizing ^{32}P-labeled primary transcripts to the plasmids immobilized on the membrane. (*A*) One representative nuclear run-on blot is shown with each plasmid designated. For detailed description of plasmids, see *Materials and Methods*. Time periods for E_2 treatments are labeled at the top. (*B*) The blots were quantified by PhosphorImager. PR gene transcription rates were normalized to those of CHOB after subtracting backgrounds. The results were expressed as fold-changes relative to the control value. The quantified results represent an average of four independent experiments and are shown on the right, vertical axis. The E_2–ER level was measured by whole cell uptake using the same E_2 concentration, as shown in Fig. 3*A*. The data are overlaid on the left, *y* axis as a dashed line.

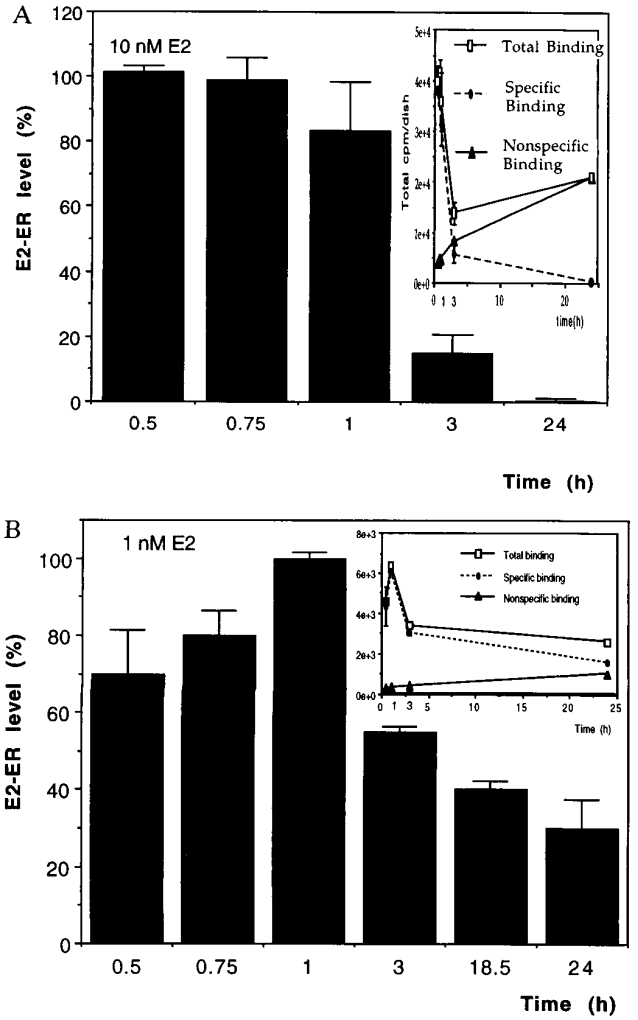

FIG. 3. Time course of E_2 binding after E_2 treatment. (*A*) Rat1+ER cells were incubated with 10 nM [^{3}H]E_2 for the indicated times. Total binding was measured from incubations with 10 nM [^{3}H]E_2, and nonspecific binding was measured from incubations with 10 nM [^{3}H]E_2 plus a 100-fold excess of unlabeled E_2. Specific binding was calculated by subtracting the nonspecific counts from the total counts. The E_2–ER level is expressed as the percent change of specific binding relative to the binding at 30 min of E_2 treatment. The initial E_2 binding curve for one experiment is shown in the inset. (*B*) The results from whole cell [^{3}H]E_2 uptake assays using 1 nM [^{3}H]E_2 are shown. Specific binding is expressed as percentage change relative to the binding at 1 h of E_2 treatment. The inset shows the raw binding data from one experiment. The results represent an average of two experiments.

to compare it to the fold change in PR gene transcription rate after the same time period of E_2 treatment (Fig. 2*B*). Nonspecific binding was determined by addition of 100-fold excess of unlabeled E_2. Specific binding was calculated as total binding minus nonspecific binding. Within 30 min of E_2 incubation with the cells, E_2 binding was maximized (Fig. 3*A*). Therefore, the 1–3 h lag in PR gene transcription activation was not due to the lack of E_2 binding during this period. In addition, the transcription rate of ER was down-regulated maximally within 1 h, confirming that the E_2–ER complex was functional within 1 h of E_2 treatment (23). Furthermore, while the rate of PR gene transcription was continuously increased from 3–24 h, the E_2–ER levels decreased to 83 ± 15% at 1 h and 15 ± 6% by 3 h, as compared with 30 min of E_2 treatment, and was undetectable after 24 h of exposure to E_2 (Fig. 3*A*).

Since 1 μM of E_2 was used to determine nonspecific binding, the high concentration may have been harmful to the cells. Therefore, we performed identical experiments with 1 nM [^{3}H]E_2 (50% ER occupancy) to measure total binding and 100 nM E_2 to determine nonspecific binding. E_2 binding was 70 ± 12% at 30 min, down-regulated to 55 ± 2% at 3 h, and 30 ± 8% by 24 h of E_2 treatment as compared with 1 h (Fig. 3*B*). The overall pattern of binding was similar, although as expected, the binding was slower and the loss of E_2 binding was less dramatic than that at 10 nM E_2.

Time Course of PR mRNA Accumulation. The long lag in E_2-induced PR transcription rate prompted us to examine the time course for E_2 induction of PR mRNA accumulation in Rat1+ER cells. Total RNA prepared from each treatment as described below was analyzed for steady-state PR mRNA levels using RT-PCR assays. As an internal control, constitutively expressed RPL19 mRNA was coamplified in each sample (16). PR and ER primers are specific for the hormone binding domains and span two and three introns, respectively (21). These primers can discriminate any signal from contaminating genomic DNA in RNA samples since they span intron

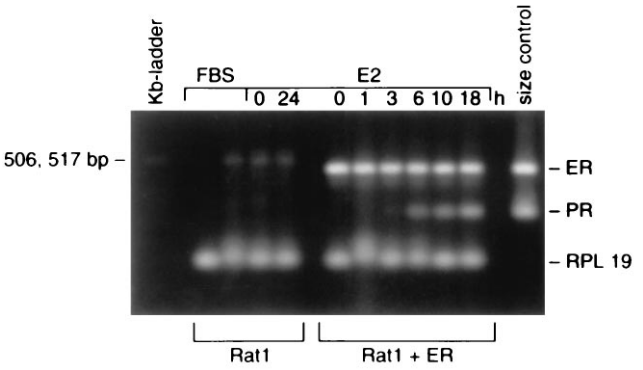

FIG. 4. Time-course of PR mRNA accumulation after E_2 treatment. An agarose gel of the amplified RT-PCR products is shown. Rat1 cells were treated with medium containing 10% FBS without E_2 in duplicate, 10% ST-FBS without E_2, or 10% ST-FBS with E_2 for 24 h. Rat1+ER cells were treated with medium containing 10% ST-FBS with 10 nM E_2 for the indicated times. Total RNA was isolated and reverse-transcribed. Each RT reaction was PCR amplified with ER primers, PR primers, and RPL19 primers. PCR products were analyzed on a 2.5% agarose gel and visualized by ethidium bromide staining. Each amplicon with the correct, expected size is designated. Treatment conditions are indicated at the top. Total RNAs from Rat1 and Rat1+ER cells are labeled at the bottom. DNA amplified from the ER and PR cDNA is run in parallel as a size marker in addition to a 1-kb ladder.

junctions. Amplification from genomic DNA would result in larger and different size fragments.

Rat-1 cells, which do not contain ER, were grown with or without E_2 (10 nM) in media supplemented with ST-FBS or FBS for 24 h. Under no conditions were ER mRNA and PR mRNA expressed in Rat-1 cells. The lack of ER and PR signals were not due to RNA degradation since RPL19 was amplified. Rat1+ER cells were treated with E_2 (10 nM) for 0, 1, 3, 6, 10, and 18 h. ER mRNA was constitutively expressed in Rat1+ER cells. No PR mRNA was detected at 0, 1, or 3 h of E_2 treatment in Rat1+ER cells. PR mRNA accumulation was detected at 6 h of E_2 treatment and continued to accumulate until 18 h, the latest time studied in this experiment (Fig. 4).

These data showed that the increase in the PR gene transcription rate was followed by PR mRNA processing, which resulted in mRNA accumulation. The appearance of PR mRNA was not apparent until 6 h of E_2 treatment, which is consistent with the observed initial time lag in PR gene transcription (Fig. 2). Previously reported down-regulation of ER mRNA (24) was not observed in these RT-PCR assays because of the saturation of the signal of the relatively abundant ER mRNA due to the exponential PCR amplification.

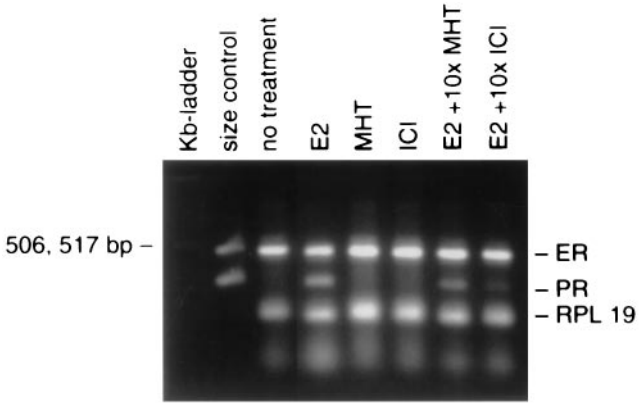

FIG. 5. ICI effects on PR mRNA accumulation in Rat1+ER cells. An agarose gel showing the amplified RT-PCR products. Rat1+ER cells were treated with E_2 (10 nM), MHT (10 nM), ICI (10 nM), E_2 with 10× MHT (100 nM), or E_2 with 10× ICI (100 nM) for 24 h. Total RNA was isolated and subjected to RT-PCR assays. Samples are indicated at the top. Each amplicon with the correct, expected size is designated. DNA amplified from the ER and PR cDNAs is run in parallel as a size control in addition to a 1-kb ladder.

ICI Competition. The competition of ICI, a pure antiestrogen, on E_2-induced PR gene expression was examined in Rat1+ER cells (Fig. 5). As described above, RPL19 and ER mRNAs were coamplified as controls. No PR mRNA was detected without the addition of exogenous E_2. PR mRNA was expressed at 24 h of E_2 treatment. Cotreatment with 10× excess ICI plus E_2 blocked PR mRNA expression. ICI competition of E_2 showed that PR gene activation is through ER. However, MHT had no effect alone and failed to compete with E_2. This may be due to incomplete competition or partial agonistic effects of MHT in Rat1+ER cells as observed (12).

DISCUSSION

The mechanism by which the E_2–ER complex induces transcription is largely unknown. However, the prevailing model suggests that simple protein-protein interactions of the E_2–ER complex with the transcription machinery lead to transcription activation (8, 10, 11). According to this simple model, the rate of transcription would have a close temporal relationship with ligand binding. This was the case in a cell-free transcription system in which purified chicken PR was used (25). We have analyzed in intact cells the kinetics of E_2-activated transcription of the endogenous PR gene by a direct quantitative method. To our surprise, E_2 activation of PR gene transcription was a gradual process with an initial 1–3 h lag, followed by PR mRNA accumulation after a 3–6 h lag. This did not parallel ER occupancy as measured by whole cell E_2 uptake. We propose that E_2–ER regulates some genes by activating catalytic intermediary factors that in turn modify some component of the transcription machinery or chromatin structure. Accumulation of those products may then enhance transcription over time (Fig. 1). We do not know the identities of these intermediates or their substrates. However, the activation of PR gene transcription by E_2 is cycloheximide-insensitive in Rat1+ER cells as well as MCF-7 cells (12, 26), suggesting that these intermediates are either nonproteins or preexisting proteins. It was recently reported that the p300/CBP protein, which acts as a corepressor in the action of the viral oncogene EA1, may be involved in transcription activation by nuclear receptors (27) Yang *et al.* (28) demonstrated that the p300/CBP protein has an associated protein, Raf/CB, crucial for transcription activation, which has histone acetylase activity. It can be speculated that chromatin acetylation or a similar reaction could be a model for the mechanism of slow response to estrogen.

This slow mode of transcription activation by E_2 has been also observed in other systems. Spelsberg *et al.* (29) have also noted the time lag between ligand bound to nuclear receptor and the accumulation of certain mRNAs. They suggested a cascade model in which early genes have products that in turn regulate later genes. Our data, however, show that transcription changes lag even though no intermediary step requiring protein synthesis can be detected. Studies in which specific mRNA accumulation is the end point do not provide appropriate information about transcription because they fail to account for the marked effect of RNA turnover on the time course of induced accumulation (30).

Our group previously observed that PRL gene transcription rate also increased slowly over several hours for both the endogenous gene as well as stably transformed minichromosomes containing 5′ flanking regulatory regions of the PRL gene in E_2-treated rat pituitary-derived GH_3 cells (31). Similarly, the E_2-induced transcription rate of the vitellogenin gene in *Xenopus* liver was maximized only after 4 days of E_2

treatment (32). The silent vitellogenin gene in males and immature females of chicken can be activated by exogenous E_2 treatment. During primary E_2 induction, accumulation of vitellogenin mRNA was increased markedly only after a lag of 4–6 h, independent of protein synthesis. Subsequent estrogen administration to pretreated chickens induced expression of the gene with no lag (33–35). Thus, cellular components may have been altered after the first exposure to E_2 such that the subsequent E_2-induced transcription was more efficient (34). The ovalbumin gene in chicken oviduct was activated with a 2 h lag and gradually increased over 4–5 days (36), which was considerably slower than nuclear E_2–ER binding and conalbumin (or transferrin) gene transcription (14). This probably is due to protein synthesis being required for ovalbumin gene transcription by E_2 (37).

The existence of mediators of steroid hormone receptor action has been proposed by others (38, 39). The thyroid hormone receptor has been shown to interact with ATPases, implying that ATP hydrolysis is involved in transcription activation (40). SWI-SNF proteins, which remodel chromatin (41), were shown to be required for ER to activate transcription in *Saccharomyces cerevisiae* (42). Proteins (160 and 140 kDa) that exhibit E_2-dependent binding to the ER have been identified (43, 44). However, the physiological significance of these accessory proteins is not known.

Furthermore, there are many *in vivo* observations that cannot be easily explained with the simple protein interaction model. Only estrogenic ligands are thought to activate ER. However, there is evidence suggesting that ER can be activated via phosphorylation pathways (45). These effects were not mediated solely through the phosphorylation of the ER (46). The two signaling pathways may communicate via common intermediates that can be induced by either pathway (46). *In vivo* genomic footprinting on the tyrosine aminotransferase gene suggests that the glucocorticoid receptor may bind to its recognition sequence only briefly since a different protein factor was found over the glucocorticoid receptor binding sites (47). It has been proposed that transient associations of the occupied glucocorticoid receptor with DNA initiate subsequent events, including chromatin remodeling and recruitment of other factors, which leads to transcription activation (47, 48).

Our transcription rate studies of the endogenous PR gene reveal that E_2-induced gene transcription is uncoupled from ER occupancy. We suggest that E_2-induced transcription is more complicated than is commonly assumed and may require the accumulation of catalytic mediators.

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