Regulation of the Estrogen Receptor in MCF-7 Cells by Estradiol

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The role of estradiol in the regulation of its cognate receptor in MCF-7 cells was investigated in this study. After treatment with 10^{-9} M estradiol, the level of receptor protein was measured using an enzymeimmunoassay. By 6 h, the receptor protein declined by about 60% from a level of approximately 3.6 to 1.2 fmol/μg DNA. The level of receptor remained suppressed for 24–48 h. Similar results were obtained with an estrogen receptor (ER) binding assay. The steady state level of ER mRNA was determined by an RNase protection assay. Estrogen treatment resulted in a maximum suppression of mRNA by 6 h. Receptor mRNA remained depressed for 48 h. Transcription run on experiments demonstrated a transient decrease of about 90% in ER transcription after 1 h. By 3–6 h transcription increased approximately 2-fold and remained elevated for at least 48 h. These data suggest that estrogen down-regulates ER mRNA by inhibition of ER gene transcription at early times and by a post-transcriptional effect on receptor mRNA at later times. (Molecular Endocrinology 2: 1157–1162, 1988)

INTRODUCTION

One of the most prevalent of all cancers, breast cancer, is characterized by hormonal control of its growth. Significant levels of estrogen receptor (ER) have been detected in more than 50% of human breast cancers. Approximately 70% of these ER-positive tumors respond to endocrine therapy (1–4), suggesting a correlation between the presence of the ER and the growth of breast tumors. Currently the ER is used to predict those patients who would benefit from hormonal therapy. Estrogens, in common with other steroids, regulate gene expression in target cells through their interaction with specific receptors. These receptors represent a class of transacting regulatory proteins with the ability to bind tightly in the genome to responsive elements. Interaction of specific receptor proteins with the genome results in the activation of responsive genes (see Ref. 5 for review). As a consequence of this interaction there are changes in the synthesis of specific RNAs and proteins involved in the regulation of cell proliferation, differentiation, and physiological function.

In the ER-positive breast cancer cell line MCF-7 (6), estrogens have been shown to be required for optimal cell growth (7, 8). The function of the ER in promoting the growth of breast cancer cells is not completely understood but several lines of evidence have led us and others to suggest that polypeptide growth factors may be common mediators of growth control. The levels of a variety of other mRNAs and proteins are also under estrogen control in these cells (9–14). However, their role in promoting cell growth is unknown.

Since the proliferation and phenotype of mammary cells is determined to a great extent by estrogen, it is important to understand the basis of ER regulation. The concentration of receptor can be substantially altered by cell density, growth rate, and processing after ligand binding (15–19). In MCF-7 cells, it has been shown that estrogen binding to receptor results in the down-regulation of estrogen binding sites (17, 18, 20, 21), how-

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ever, the mechanisms responsible for control are not clearly understood. The recent cloning of the estrogen receptor (22–24) provides the opportunity to investigate, at the molecular level, the regulation of estrogen receptor gene expression.

The purpose of the present study was to determine the mechanism by which estrogen regulates the expression of its cognate receptor in MCF-7 cells. To achieve this goal, the relationship between ER protein concentration and binding capacity, the steady state levels of receptor mRNA, and the level of ER gene transcription must be examined simultaneously.

RESULTS

Effect of Estrogen Treatment on the Level of ER Protein

To determine the level of ER protein, an enzymeimmunoassay (EIA) was employed. The data presented in Fig. 1 show that estrogen treatment, 10^{-9} M, resulted in a decline in total receptor protein by about 60%. Receptor protein declined from a level of approximately 3.64 fmol/μg DNA (422.4 fmol/mg protein) in control cells to approximately 1.2 fmol/μg DNA (205 fmol/mg protein) in cells treated with estradiol. The level of receptor decreased by 6 h and remained depressed for up to 48 h. These data are in good agreement with previous results (17, 18, 20, 21).

In the EIA, we have found that in the absence of estradiol, the ER is unstable. In order to obtain control values, leupeptin, 2 mM, was added to prevent receptor degradation. In the absence of estradiol and the protease inhibitor, the level of receptor measured was approximately 65% of the value obtained in the presence of the inhibitor. Leupeptin had no significant effect on the level of receptor when estradiol was present.

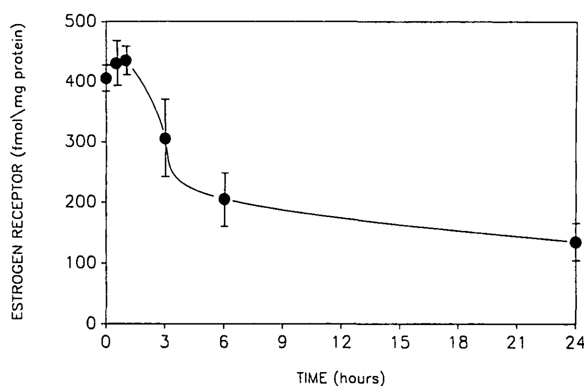

Fig. 1. Effect of Estrogen on the Steady State Level of ER Protein

MCF-7 cells were grown in IMEM medium supplemented with 5% charcoal-treated calf serum. At approximately 80% confluence, the medium was replaced with phenol red-free IMEM containing 5% charcoal-treated calf serum. After 2 days, cells were treated with estradiol, 10^{-9} M, or ethanol for various times. Cells were washed, harvested, and homogenized by sonication. Total estrogen receptor was determined with an EIA kit from Abbott Laboratories using D547 and H222 monoclonal antibodies. Results are presented as femtomoles of ER per mg protein. Each *point* is the mean of several experiments.

Effect of Estrogen Treatment on the Level of Estrogen Binding

To confirm that the decreased level of ER protein, as measured by the EIA, corresponded to a decreased level of estrogen binding sites, a competition assay was employed. The results of the EIA and competition assay are compared in Table 1. In response to estradiol treatment, the number of estrogen binding sites decreased from 3.64 fmol/μg in control cells to 1.24 fmol/μg DNA in 24-h treated cells. The level of binding decreased in a manner similar to the decline in receptor protein. These results demonstrate a down-regulation of the ER by estradiol.

Effect of Estrogen Treatment on the Level of ER mRNA

A highly sensitive RNase protection assay was employed to examine the effects of hormone treatment on the steady state level of ER mRNA. In these experiments, the level of receptor mRNA was normalized to the level of 36B4 mRNA, which is constitutively expressed in the presence of estradiol (14). Figure 2 is a typical autoradiograph of an RNase protection assay showing the effect of estrogen treatment on the level of receptor mRNA. The migration pattern in the control lane is not the result of incomplete probe digestion, as it is not seen in other gels, but may be due to some other idiosyncratic problem such as salt concentration. Changes in ER mRNA were quantified by scanning densitometry and the data graphically presented in Fig. 3 as the ratio of integrated ER to the integrated 36B4 signal. In this study estrogen treatment resulted in a maximum suppression of mRNA by 6 h. After treatment with 10^{-9} M ER mRNA levels decreased to approximately 10% of control values and remained at the suppressed level for up to 48 h. These data demonstrate a close temporal relationship between the level of receptor protein and mRNA. Similar results were obtained if these results were normalized for total DNA

Table 1. Comparison of Estrogen Effect on ER Binding and Protein

	Estrogen Binding (fmol/μg DNA)	ER (fmol/μg DNA)
Control (h)	3.64 ± 1.05 (3)	3.02 ± 0.11 (19)
1	3.82 ± 1.38 (4)	3.67 ± 0.53 (8)
6	1.74 ± 0.58 (3)	1.5 ± 0.41 (6)
24	1.24 ± 0.2 (3)	1.22 ± 0.44 (6)

MCF-7 cells were grown as described in the legend to Fig. 1. Estrogen binding was determined by Scatchard analysis. ER was determined by the EIA. Mean ± *SEM* (n).

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in each sample. A decrease in the level of steroid receptor mRNA in response to its target hormone has, also, been found for the glucocorticoid receptor (25, 26).

Effect of Estrogen Treatment on the Level of ER Gene Transcription

The effects of estrogen on receptor gene transcription were analyzed with a nuclear transcription run-on assay. Transcription run-on assays were performed using nuclei isolated from MCF-7 cells treated with estradiol. Newly synthesized transcripts were hybridized to probes immobilized on nitrocellulose blots. The level of transcription was determined by autoradiography and quantified by scanning densitometry. In this study, the ER probes included exon 1 and pOR3, a probe for the 3′-coding sequence. 36B4 and actin were used as internal constitutive controls and pS2 was employed as positive inducible control. To control for the potential artifacts due to the mitogenic nature of estrogen, 36B4 transcription was used as an internal control and the relative changes in ER transcription were normalized to the signal obtained for 36B4. Similar results were obtained when either pOR3 or exon 1 was used as a probe. There was no significant difference in the results when the data were normalized for the number of nuclei. Due to the homology of the ER with steroid hormone receptors, the progesterone and glucocorticoid receptors were also included to control for cross-hybridization.

The data in Fig. 4 indicate that there is a transient decrease in ER transcription after estrogen treatment.

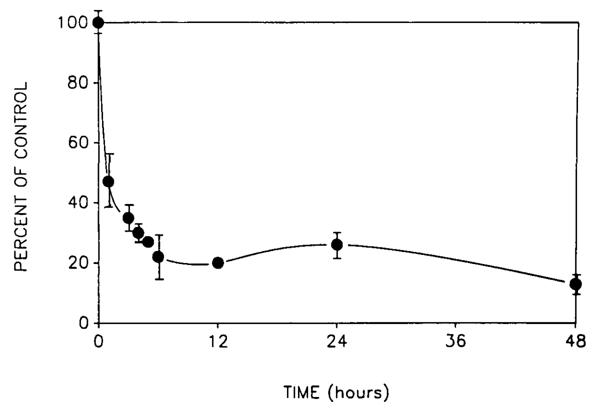

Fig. 3. Effect of Estrogen on the Steady State Level of ER mRNA.

Autoradiographs from the RNase protection assay were quantified by scanning densitometry and the values were expressed as the ratio of the integrated ER signal divided by the integrated 36B4 signal. The results are presented as percent of control. The *points* represent the average of a minimum of three values and in some cases as many as 10 values.

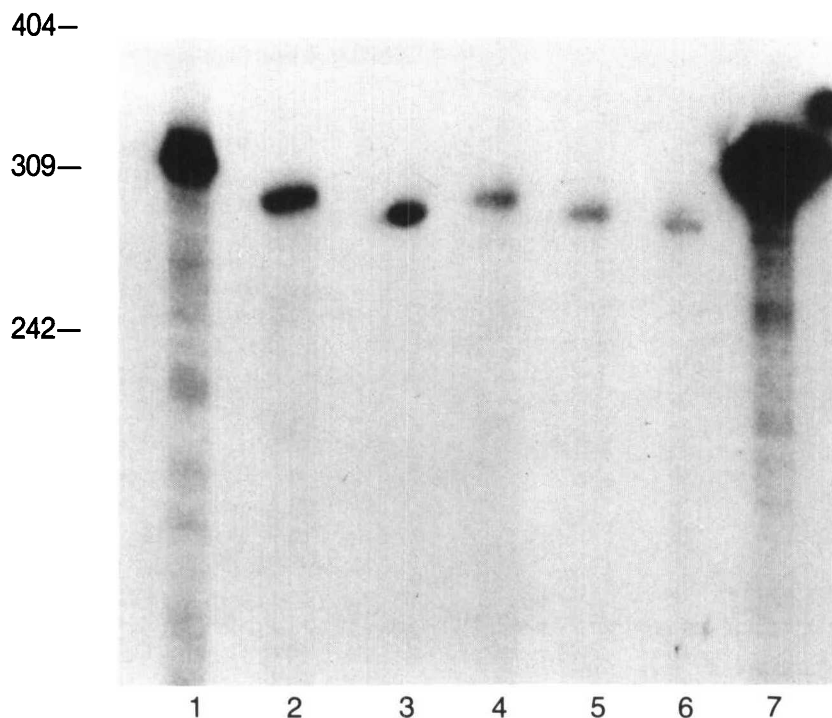

Fig. 2. Effect of Estrogen on ER mRNA

MCF-7 cells were treated as described in the legend to Fig. 1. Total RNA was isolated by homogenization in a buffer containing guanidinum isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion. Sixty micrograms of total RNA were analyzed using the highly sensitive RNase protection assay. A 300 bp fragment of the ER cRNA was protected against RNase A degradation by hybridization of total RNA with ^{32}P-labeled antisense mRNA. After hybridization at 50 C for 12–16 h, total RNA was digested with RNase A. The protected bands were separated on 6% polyacrylamide gel electrophoresis gels and the bands were visualized by autoradiography. Lane 1, Control; 2, 1 h; 3, 3 h; 4, 6 h; 5, 24 h; 6, 48 h; and 7, probe.

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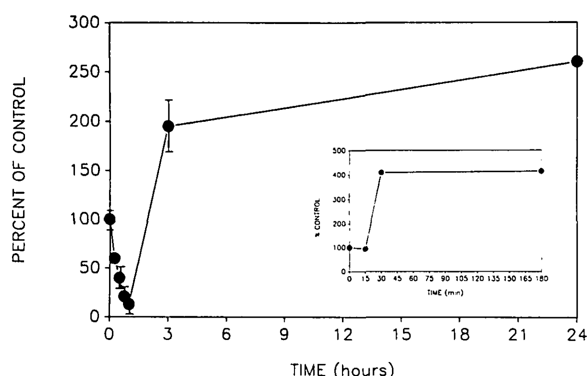

Fig. 4. Effect of Estrogen on ER Gene Transcription

MCF-7 cells were treated as described in the legend to Fig. 1. Nuclei were isolated at the indicated time points by homogenization in 1.5 M sucrose buffer containing 0.1% Brij 58; elongation of nascent transcripts was performed in a reaction buffer containing ^{32}P-UTP. For detection of specific transcripts, the newly synthesized transcripts were isolated and hybridized to filters containing an excess of plasmid DNA. The level of transcription was determined by autoradiography and quantified by scanning densitometry. The level of transcription was expressed as the ratio of the integrated ER signal divided by the integrated 36B4 signal. The results are presented as percent of control. *Inset*; The effect of estrogen on pS2 gene transcription was determined as described above.

By 1 h the level of transcription decreased to approximately 90% of control values. This decrease is not due to a nonspecific toxic response caused by estrogen administration as indicated by the constitutive transcription of 36B4 and a 4-to 5-fold increase in transcription of pS2 by 30 min (Fig. 4 *inset*). After this decrease, transcription increased to a level higher than that observed in control nuclei. This increase is not due to cross-hybridization with progesterone and glucocorticoid receptors. Although estrogen treatment results in a transient suppression of ER transcription, it is improbable that this drop is responsible for the prolonged suppression of ER mRNA because the level of transcription subsequently increases as the level of mRNA decreases. The data suggest the possibility that the predominant mechanism down-regulating ER expression is a posttranscriptional event.

DISCUSSION

Steroid hormones modulate growth and development in eukaryotes by regulating the expression of specific genes. Since the phenotype of mammary cells is determined to a great extent by estrogen, it is important to understand the basis of ER regulation. Cell density, growth rate, and processing after ligand binding (15–21) can substantially alter the cellular concentration of receptor; however, the mechanisms responsible for this control are unknown. The recent cloning of the ER (22–24) provided us with the opportunity to investigate, at the molecular level, the regulation of ER gene expression. The purpose of the present study was to determine the mechanism by which estrogen regulated the expression of its cognate receptor. To achieve this goal, the relationship between ER protein concentration and binding capacity, the steady state levels of receptor mRNA, and the level of ER gene transcription were examined simultaneously. It has recently been reported that dexamethasone treatment results in the suppression of glucocorticoid receptor mRNA in a variety of tissues (25, 26). Although autoregulation of hormone receptor mRNA levels by the homologous ligand has been reported for the glucocorticoid receptor this is the first study, to our knowledge, in which the levels of steroid receptor mRNA and transcription are measured simultaneously.

The results presented herein demonstrate that treatment of MCF-7 cells with estrogen resulted in the down-regulation of the ER. The decline in receptor protein to a new steady state level accompanied a parallel decrease in the level of receptor mRNA. In contrast to the effect on protein and mRNA, estrogen treatment resulted in a transient decrease in ER transcription followed by an enhanced level of expression. The drop in receptor transcription appeared to be a specific response since the transcription of 36B4 and actin were not affected by hormone treatment and transcription of pS2 increased in response to estrogen. Although estrogen treatment resulted in a transient decrease in ER gene transcription, it is improbable that this decrease is responsible for the suppression of receptor mRNA. It suggests that the predominant mechanism regulating ER expression is a posttranscriptional event. These results are similar to the effects of estrogen seen in other systems (27). Estrogen treatment of *Xenopus laevis* results in the suppression of albumin gene expression through a complex interaction of transcriptional inhibition and posttranscriptional suppression of albumin mRNA.

It has been proposed that nuclear processing is responsible for the new steady state level of ER seen in MCF-7 cells after estrogen administration. Processing of ER complexes after nuclear translocation is measured as a decrease in competible ^{3}H-estradiol binding in 0.6 M KCl nuclear extracts (17). The marked loss of nuclear estrogen binding is completed by 6 h and does not return to control values in the presence of estrogen. The new steady state level of ER may be due to one of several factors including a decreased half-life of receptor or decreased synthesis of receptor protein. Previous studies report a rapid turnover of ER with a half-life of 2.5–4.5 h in the presence or absence of estrogen (28, 29). Our results suggest that the decreased synthesis of receptor protein plays a role in the down-regulation of receptors. Although our data do not discount nuclear processing as an early event in ER regulation they do suggest that the new steady state level of receptor is largely determined by suppression of ER mRNA.

The monoclonal ER-EIA assay has recently been introduced as a method for determining ER concentra-

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tion. However, there is some controversy as to its accuracy and sensitivity when measuring the ER content in patient samples (30–33). The reason for these differences are not known but may be due to endogenous hormones and proteins that nonspecifically bind estrogens in the binding assay. In this study the concentrations of receptor determined using either the ER-EIA or a binding assay were found to be approximately the same.

In conclusion, the present study demonstrates that estrogen regulates its cognate receptor through suppression of receptor mRNA. The data suggest that the level of mRNA is regulated almost exclusively by a posttranscriptional event. It is possible that estrogen alters mRNA stability, however, estrogen may effect other processing events such as splicing or nuclear transport. Further studies will be necessary to determine the nature of the posttranscriptional event and the role of the ER.

MATERIALS AND METHODS

Tissue Culture

Monolayer cultures of MCF-7 breast cancer cells were grown in Improved Minimal Essential Medium (IMEM) supplemented with 5% (vol/vol) charcoal-treated calf serum. The calf serum was pretreated with sulfatase and dextran-coated charcoal to remove endogenous steroids. When the cells were 80% confluent the medium was replaced with phenol red-free IMEM (34) containing 5% charcoal-treated calf serum. After 2 days in these conditions, estradiol, 10^{-9} M, was added and cells were harvested at the times indicated.

ER Assay

For analysis of ER protein levels MCF-7 cells were cultured and treated as described above. The level of receptor protein was assayed using an EIA kit from Abbott Laboratories (North Chicago, IL) containing monoclonal antibodies, D547 and H222. To obtain total ER, the cells were homogenized by sonication in a high salt buffer (10 mM TRIS, 1.5 mM EDTA, 5 mM Na_2MoO_4, 0.4 M KCl, 1 mM monothioglycerol, with and without 2 mM leupeptin). The homogenate was incubated on ice for 30 min and centrifuged at 100,000 × *g* for 1 h at 4 C. Aliquots of the total extracts were then analyzed according to the manufacturer's instructions.

To measure estrogen binding sites, cells were incubated with ^{3}H-estradiol under uncompeted and competed conditions as previously described (35). Specific binding activity was calculated as the difference between total and noncompetible ^{3}H-estradiol binding. Binding constants and estrogen receptor concentrations were obtained by the method of Scatchard (36). The results were expressed as femtomoles of ER per mg protein or μg DNA.

Plasmids

The clone, pOR-300, was constructed by subcloning a 300 base pair (bp) restriction fragment of pOR3 (22, 24) into the pGem4 polylinker region using the restriction enzymes, *Pst*I and *Eco*RI. The genomic clone, corresponding to exon 1, Q7 (MP unpublished results), is a 3 kilobases (kb) *Eco*RI-*Sal*I fragment subcloned into Bluescribe M13+ (Stratagene, La Jolla, CA). The human progesterone receptor, A.16 (gift of Andree Krust), contains 1.2 kb *Hin*dIII-*Eco*RI fragment and the human glucocorticoid receptor, HG3 (gift of Vijay Kumar, INSERM, CNRS, Strasbourg, France), contains a 1.6 kb *Eco*RI fragment. The clones, pS2 (37) and 36B4 (14), used as controls, were described previously. The clone p36B4 was constructed by subcloning a 220 bp fragment of 36B4 into the *Pst*I restriction site of the pGem4 polylinker.

Measurement of Cellular ER mRNA Levels

Total cellular RNA was extracted from MCF-7 cells by homogenization in a 4 M guanidine isothiocyanate lysing buffer containing 5 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% sarkosyl. After centrifugation through a 5.7 M CsCl pad at 100,000 × *g* for 16 h at 20 C (Beckman SW40 rotor), ER mRNA was determined by a RNase protection assay. For this analysis, homogeneously ^{32}P-labeled antisense molecules (cRNA) were synthesized *in vitro* from pOR-300 and p36B4 using T7 polymerase. Sixty micrograms of total RNA were hybridized for 12–16 h to the radiolabeled cRNA. AFter a 30-min digestion at 25 C with RNase A, ^{32}P-labeled cRNA probes protected by total RNA were separated by electrophoresis on 6% polyacrylamide gels. The bands were visualized by autoradiography and quantified by optical densitometry. Similar results were obtained when the data were normalized for DNA content.

Isolation of Nuclei

MCF-7 cells were harvested and resuspended in 5 ml 1.5 M sucrose buffer plus 0.1% Brij 58 (38). The cells were then homogenized with 10 strokes in a dounce homogenizer using pestle A. The homogenate was diluted to 15 ml with 1.5 M sucrose and centrifugated at 10,000 rpm for 20 min at 4 C. The nuclear pellet was resuspended in 0.5 ml nuclei storage buffer [20 mM HEPES, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM phenylmethylsulfonylfluoride, 50% glycerol). The concentration of nulcei was determined by diluting a portion in 0.4% (wt/vol) trypan blue and counting the number of nuclei. Nuclei were stored at −70 C until the transcription elongation assay was performed.

Transcription Elongation Assay and Isolation of RNA for Hybridization

The nuclear transcription run-on assay was performed with a procedure previously described (38). Briefly, nuclei were isolated at various times after administration of estradiol and incubated with ^{32}P-UTP and unlabeled ATP, CTP, and GTP. The radiolabeled RNA transcripts were isolated and hybridized to an excess of denatured plasmid DNA immobilized on a nitrocellulose filters. The denatured plasmid used for the detection of specific transcripts were exon 1, pOR 3, progesterone receptor, glucocorticoid receptor, 36B4, pS 2, and pBR 322. Autoradiographs were analyzed by densitometry, and the background was subtracted. These results were normalized for the number of nuclei or by comparison to the transcriptional level of 36B4 or actin.

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