Modulation of Estrogen Receptor mRNA Expression by Melatonin in MCF-7 Human Breast Cancer Cells

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Melatonin, the hormonal product of the pineal gland, has been shown to inhibit the development of mammary tumors *in vivo* and the proliferation of MCF-7 human breast cancer cells *in vitro* by mechanisms not yet identified. However, previous studies have demonstrated that melatonin significantly decreased estrogen-binding activity and the expression of immunoreactive estrogen receptor (ER) in MCF-7 breast cancer cells. To determine the mechanism(s) by which melatonin regulates ER expression in MCF-7 cells, the relationship between the level of steady state ER mRNA and the rate of ER gene transcription were examined in response to melatonin. Physiological concentrations of melatonin decreased steady state levels of ER mRNA expression in a dose- and time-specific manner. This decrease was not dependent upon the presence of estrogen since similar decreases in steady state ER mRNA levels were seen in MCF-7 cells cultured in both complete and estrogen-depleted media. The decreased expression of ER mRNA in response to melatonin appears to be directly related to the suppression of transcription of the ER gene. This regulation is independent of the synthesis of new proteins, as cycloheximide was unable to block the melatonin-induced decrease of steady-state ER mRNA levels. The down-regulation of ER by melatonin appears to not be mediated via a direct interaction with the ER and subsequent feedback on its own expression, since melatonin treatment did not alter the transcriptional regulatory ability of the fully activated wild type ER or a constitutively active hormone-binding domain-deleted ER variant. In addition, the stability of the ER transcript was unaffected by melatonin. Thus, it appears that the antiproliferative actions of this pineal indoleamine are mediated, at least in part, through the suppression of the transcription of the ER gene in MCF-7 human breast cancer cells. (Molecular Endocrinology 8: 1681–1690, 1994)

INTRODUCTION

The elucidation of the molecular mechanisms responsible for the hormonal control of cell proliferation in breast cancer has been the object of intense research over the last several decades. Because most breast tumors are initially dependent upon estrogens for continued growth, much of this research has focused on the role of estrogen and the estrogen receptor (ER) in the control of breast cancer gene expression and cell proliferation. Although the ER has a well established predictive value for endocrine responsiveness, prognosis, and survival in breast cancer (1, 2), there are still many aspects of ER regulation that have yet to be identified. MCF-7 human breast cancer cells, like two-thirds of primary breast tumors, express high levels of ER and are responsive to the mitogenic actions of estrogen (3, 4). Therefore, MCF-7 cells provide an excellent model system for the study of the hormonal regulation of ER expression in breast cancer.

The growth of many breast neoplasms as well as breast tumor cell lines is stimulated by estrogen via the induction of a number of growth-stimulatory factors such as transforming growth factor-α (TGFα), insulin-like growth factor I (IGF-I), and the lysosomal protease Cathepsin D (5–7). Recent studies have also found that estrogen induces the expression of the immediate-early protooncogenes, *c-myc* and *c-fos* (8, 9), which are believed to play an important role in cellular proliferation. In addition to the induction of growth-stimulatory factors, there is also evidence that estrogen inhibits the expression of a growth-inhibitory peptide, transforming growth factor-β (TGFβ) (10). Because these growth factors, and probably others, are believed to mediate the mitogenic effects of estrogen, hormones that modulate ER expression may likewise regulate the development and growth of breast cancer.

It has been demonstrated that estrogen induces a rapid down-regulation (within 6 h) of its own receptor in MCF-7 cells (11, 12). In fact, the decrease in ER in response to estrogen treatment has been reported to result from the autologous down-regulation of both ER mRNA and protein levels in MCF-7 cells via a posttranscriptional mechanism (12–13) that causes a rapid destablization of the ER message, which has a normal half-life of approximately 3–4 h (14, 15). In addition, other hormones such as insulin and progesterone (16–18), as well as the phorbol ester 12–0-tetradecanoylphorbol-

13-acetate (TPA) (19), are known to modulate the expression of ER in MCF-7 cells.

ER expression has also been shown to be modulated by melatonin, the hormonal product of the pineal gland (20–22). In addition, we have previously reported that physiological concentrations of this pineal indoleamine inhibit the growth of human breast cancer cells (23). The mechanism(s), however, by which this is accomplished are not yet established. There appears to be an association between the oncostatic effects of melatonin on breast tumor cells and the estrogen-response pathway, since melatonin is able to inhibit the proliferation of estrogen-responsive cell lines, but not estrogen-unresponsive cell lines. In addition to its growth-inhibitory effects, we have also shown that melatonin significantly decreases both estrogen binding activity and the expression of immunoreactive ER in a dose-specific and time-dependent manner (24).

To begin to identify the cellular level at which melatonin exerts its inhibitory effects on cell growth and ER expression (transcriptional, posttranscriptional, translational, or posttranslational), we have examined the steady state levels of ER mRNA as well as ER gene transcription, in response to melatonin. Additionally, we have used the protein synthesis inhibitor, cycloheximide, to determine whether melatonin's down-regulation of ER mRNA is dependent upon the synthesis of new proteins. In these studies we demonstrate that melatonin treatment leads to a rapid reduction in steady state ER mRNA levels in a dose- and time-dependent manner, and that this diminution is due, primarily, to the suppression of ER gene transcription in a process that is not dependent upon the synthesis of new proteins.

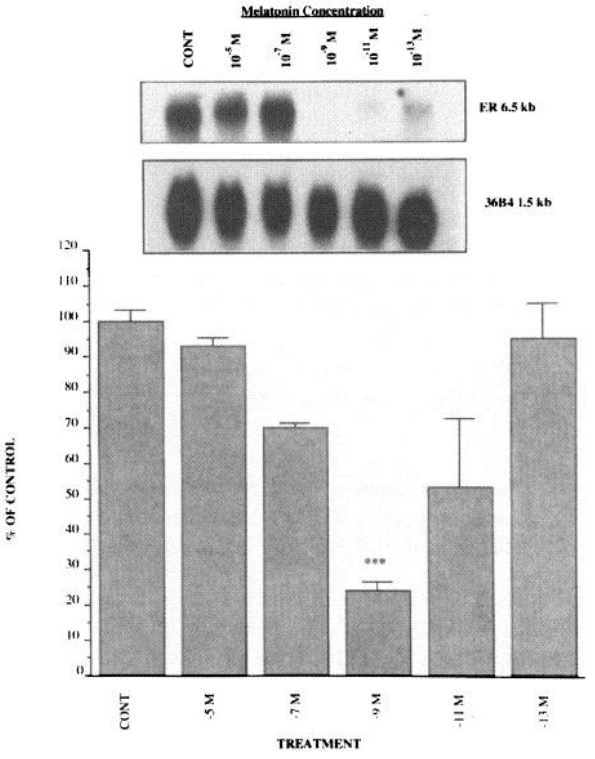

Fig. 1. Dosage Effects of Melatonin on Steady State ER mRNA Expression in MCF-7 Cells Cultured in 10% FBS

MCF-7 cells were incubated with diluent (ethanol 0.001%) or decreasing concentrations of melatonin (10^{-5} M-10^{-13} M) for 48 h. For each concentration examined, 50 μg total RNA were fractionated on a 1% agarose gel and Northern blots conducted as described in *Materials and Methods*. Blots were probed with a ^{32}P-labeled 2.1 kb human ER cDNA and a 0.2 kb 36B4 cDNA to monitor loading. A representative autoradiogram is shown in the *upper portion* of this figure. Autoradiographs from Northern blot analyses were quantified by scanning densitometry and normalization with 36B4 mRNA. Results are presented graphically as percent of control. n = 3 independent experiments, ***, $P < 0.001$.

RESULTS

Concentration-Dependent Regulation of ER mRNA by Melatonin

The steady state levels of ER mRNA in MCF-7 cells were examined by Northern blot analysis after 48 h exposure of cells to increasing concentrations of melatonin (see Figs. 1 and 2). In these experiments the level of ER mRNA was normalized to the level of 36B4 mRNA, a nonestrogen-regulated gene product (25). In all experiments, melatonin treatment produced a dose-dependent suppression of the steady state levels of ER mRNA. As shown in Fig. 1, a physiological concentration of melatonin, 10^{-9} M, significantly ($P < 0.001$ *vs.* control) decreased (76% *vs.* control cells) the steady state levels of ER mRNA in MCF-7 cells cultured in media supplemented with 10% fetal bovine serum (FBS) (complete media). The same concentration of melatonin also significantly ($P < 0.001$) decreased, by 65%, the steady-state ER mRNA levels in MCF-7 cells cultured in estrogen-depleted media [phenol red-free media supplemented with 5% dextran-treated charcoal (DCC)-FBS] (Fig. 2). In all experiments, neither supraphysiological (10^{-5} M and 10^{-7} M) nor subphysiological (10^{-13} M) concentrations of melatonin were effective in suppressing the steady state levels of ER mRNA.

Temporal Regulation of ER mRNA by Melatonin

The time course for melatonin's regulation of ER mRNA levels in MCF-7 cells was examined by Northern blot analysis after exposure of MCF-7 cells to 10^{-9} M melatonin (the most effective concentration of melatonin from dose-response studies) for various times (1 h, 3 h, 6 h, 12 h, 24 h, and 48 h). Changes in ER mRNA were again normalized against 36B4 mRNA by scanning densitometry, and the data are presented graphically in Fig. 3 as the ratio of integrated ER to the 36B4 signal. In all experiments, 10^{-9} M melatonin treatment produced a time-dependent suppression of steady state ER mRNA levels. In cells cultured in complete media, melatonin treatment resulted in significant decreases ($P < 0.001$) in steady state levels of ER mRNA

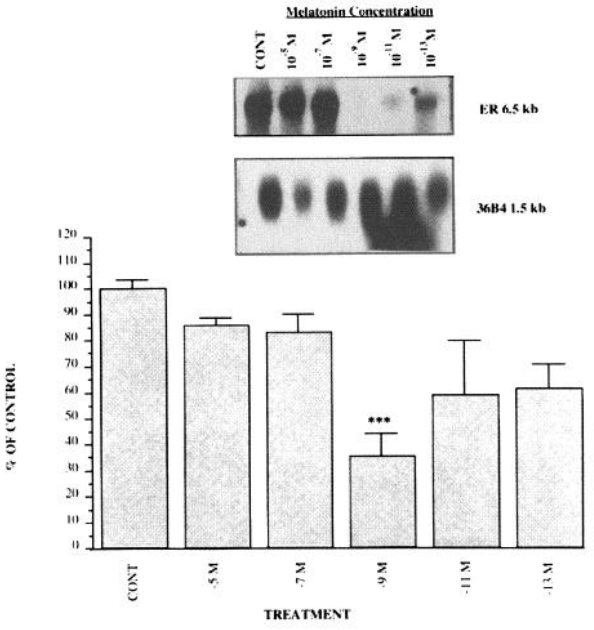

Fig. 2. Dosage Effects of Melatonin on Steady-State ER mRNA Expression in MCF-7 Cells Cultured in 5% DCC-FBS

MCF-7 cells were incubated with diluent (ethanol 0.001%) or decreasing concentrations of melatonin (10^{-5} M-10^{-13} M) for 48 h. For each concentration examined, 50 μg total RNA were fractionated on a 1% agarose gel and Northern blots as described in *Materials and Methods*. Blots were probed with a ^{32}P-labeled 2.1-kb human ER cDNA and a 0.2-kb 36B4 cDNA to monitor loading. A representative autoradiogram is shown in the *upper portion* of this figure. Autoradiographs from Northern blot analyses were quantified by scanning densitometry and normalization with 36B4 mRNA. Results are presented graphically as percent of control. n = 3 independent experiments; ***, $P < 0.001$.

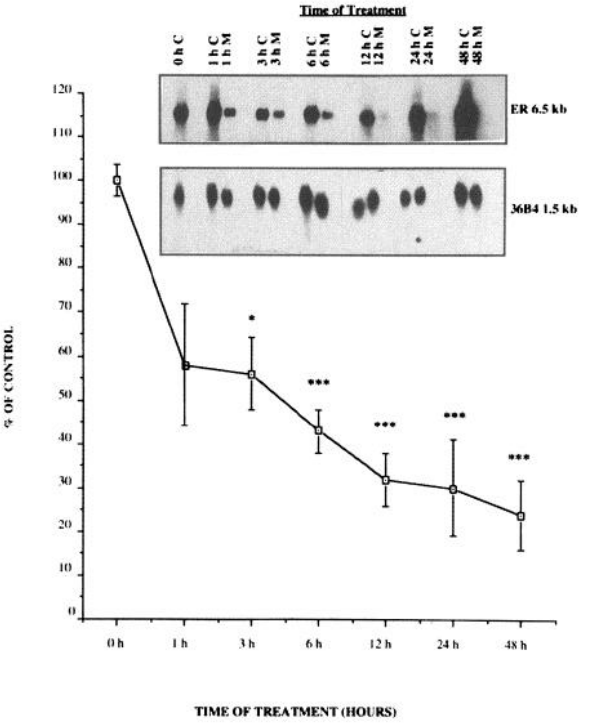

Fig. 3. Time Course of the Effects of Melatonin on Steady State ER mRNA Expression in MCF-7 Cells Cultured in 10% FBS

MCF-7 cells were incubated with diluent (ethanol) or 10^{-9} M melatonin and harvested at the indicated times. For each time point, 50 μg total RNA were fractionated on a 1% agarose gel and blotted as described in *Materials and Methods*. Blots were probed with a ^{32}P-labeled 2.1-kb human ER cDNA and a 0.2-kb 36B4 cDNA to monitor loading. A representative autoradiogram is shown in the *upper portion* of this figure. Autoradiographs from Northern blot analyses were quantified by scanning densitometry and normalization with 36B4 mRNA. Results are presented graphically as percent of control. n = 5 independent experiments; *, $P < 0.05$; ***, $P < 0.001$.

as early as 3 h (44% decrease), with a maximum suppression of ER mRNA levels by 48 h (76% decrease). In cells cultured in estrogen-depleted media, a slightly delayed, but still significant, decrease in response to melatonin treatment was noted in the level of ER mRNA beginning at 6 h (57% decrease), with maximal inhibition occurring at 24 h (78% decrease) (Fig. 4). Significant decreases in steady state ER mRNA levels were maintained through 48 h (66% decrease) without additional melatonin treatment ($P < 0.001$).

Effect of Cycloheximide on Melatonin Modulation of Steady State ER mRNA Levels

To determine whether the synthesis of new proteins is necessary for the suppression of steady-state ER mRNA levels by melatonin, studies were conducted using the protein synthesis inhibitor, cycloheximide. Figure 5 is a typical autoradiograph showing the effect of melatonin, cycloheximide, or both on steady state levels of ER mRNA. Changes in ER mRNA levels were quantified by scanning densitometry, and the data are graphically presented in Fig. 5 as the ratio of integrated ER to the 36B4 signal. In this study, melatonin treatment resulted in a maximal suppression (61% decrease) of ER mRNA by 24 h ($P < 0.001$). Cycloheximide alone had no significant effect on steady state ER mRNA levels and clearly did not abolish the suppressive effect of melatonin (71% decrease). The concentration of cycloheximide (10 μg/ml) used in these studies inhibited protein synthesis by greater than 95% (data not shown).

Effect of Melatonin on ER Transcriptional Regulatory Activity

A yeast expression system was employed to determine whether melatonin could modulate the transcriptional regulatory activity of the ER on β-galactosidase expression (see Figs. 6 and 7). In the presence of 10^{-9} M estradiol (Fig. 6), the wild type ER showed maximal transcription activity (2976 Miller units), whereas minimal activity was noted in the absence of estradiol or in the presence of the pure antiestrogen ICI 164,384 (600 Miller units). Under these conditions, melatonin did not

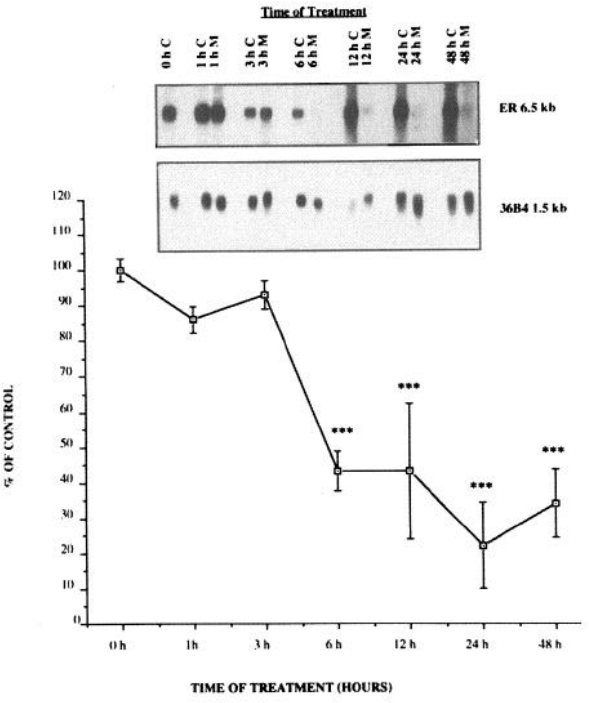

Fig. 4. Time Course of the Effects of Melatonin on Steady-State ER mRNA Expression in MCF-7 Cells Cultured in 5% DCC-FBS

MCF-7 cells were incubated with diluent (ethanol) or 10^{-9} M melatonin and harvested at the indicated times. For each time point, 50 μg total RNA were fractionated on a 1% agarose gel and blotted as described in *Materials and Methods*. Blots were probed with a ^{32}P-labeled 2.1-kb human ER cDNA and a 0.2-kb 36B4 cDNA to monitor loading. A representative autoradiogram is shown in the *upper portion* of this figure. Autoradiographs from Northern blot analyses were quantified by scanning densitometry and normalization with 36B4 mRNA. Results are presented graphically as percent of control. n = 3 independent experiments; ***, $P < 0.001$.

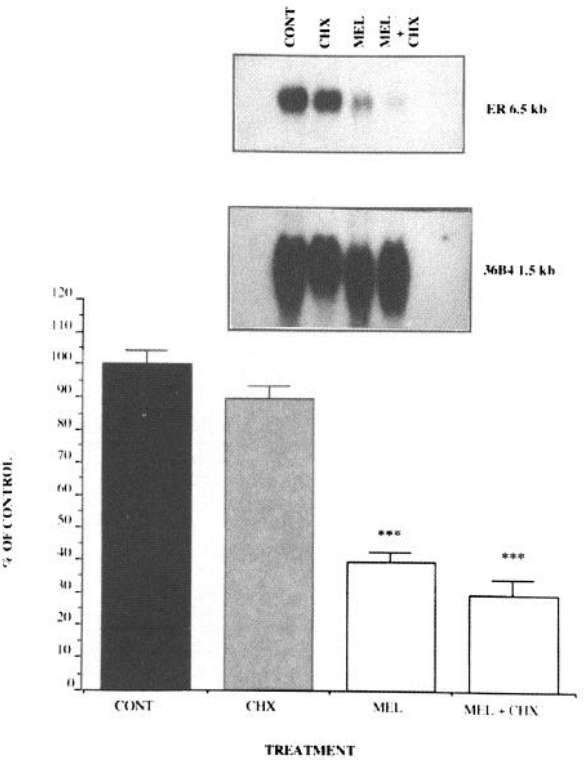

Fig. 5. The Effect of Melatonin (MEL) and Cycloheximide (CHX) on the Steady State Level of ER mRNA

MCF-7 cells were grown in phenol red-free medium containing 5% DCC-FBS for 5 days and treated with melatonin (10^{-9} M) and/or cyclohexamide (10 μg/ml) for 24 h. Total RNA was isolated and analyzed using Northern blot analysis. Fifty micrograms of total RNA were fractionated on a 1% agarose gel and blotted as described in *Materials and Methods*. Blots were probed with a [^{32}P]-labeled 2.1-kb human ER cDNA and a 0.2-kb 36B4 cDNA to monitor loading. A representative autoradiogram is shown in the *upper portion* of this figure. Autoradiographs from Northern blot analyses were quantified by scanning densitometry and normalization with 36B4 mRNA. Results are presented graphically as percent of control. n = 5 independent experiments; ***, $P < 0.001$.

inhibit the ability of the fully activated ER to induce β-galactosidase gene expression. Similarly, melatonin had no inhibitory effects on the transcriptional modulatory activity of the constitutively active, hormone-binding domain-deficient exon 5 deletion ER variant (Fig. 7).

Effect of Melatonin Treatment on the Level of ER Gene Transcription

The effect of melatonin on ER gene transcription was analyzed to determine whether the decrease in ER mRNA steady state levels produced by melatonin was the result of a decreased rate of transcription of the ER gene. To measure the rate of trasncription of the ER gene, nuclear transcription run-on assays were performed using intact nuclei isolated from MCF-7 cells treated with 10^{-9} M melatonin or diluent. Newly synthesized radiolabeled transcripts were hybridized to either ER or β-actin cDNAs immobilized on nitrocellulose membranes. The level of transcription was determined by autoradiography and quantified by scanning densitometry. Transcription of β-actin was used as an internal control, and the relative changes in ER transcription were normalized to the signal obtained for β-actin. No significant differences in the results were observed when the data were normalized for the number of nuclei. As shown in Fig. 8, the transcriptional rate of the ER gene in MCF-7 human breast cancer cells was significantly reduced ($P < 0.001$) by melatonin (59% decrease) after just 4 h of treatment and continued to be suppressed through 12 h of melatonin exposure (53% decrease). This decrease in ER gene transcription is not due to a nonspecific toxic response caused by the administration of melatonin as indicated by the constitutive transcription of β-actin.

DISCUSSION

Estrogens, via their cognate receptor, modulate the development and growth of breast tumors by regulating

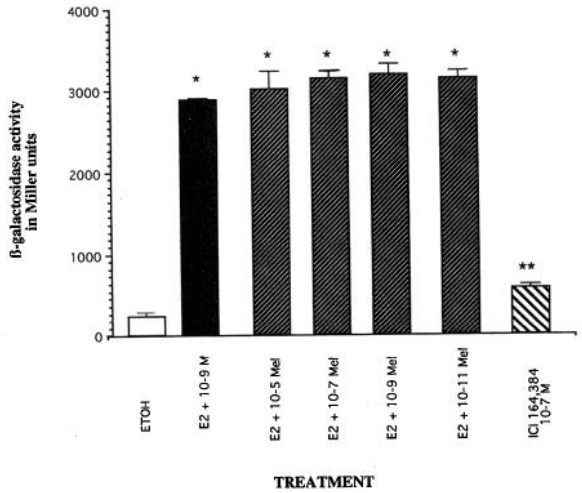

Fig. 6. Effect of Melatonin on ER Transcriptional Regulatory Activity in Yeast

Functional analysis of the ability of melatonin to inhibit the activation of a β-galactosidase reporter gene in yeast cells expressing wild type (YEPE 10) ER grown in medium containing 25 μM $CuSO_4$ in the presence or absence of estradiol (10^{-9} M). β-Galactosidase activity is expressed in Miller units. n = 6; *, $P \leq 0.001$ *vs.* ETOH control; **, $P \leq 0.001$ *vs.* E_2 and E_2 + Mel-treated cells.

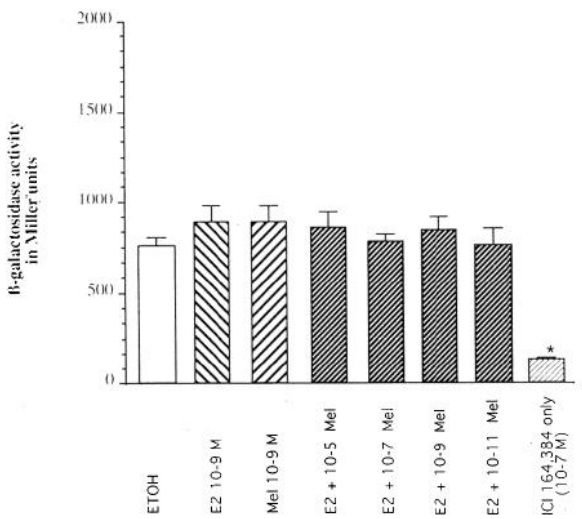

Fig. 7. Effect of Melatonin on Variant ER Transcriptional Regulatory Activity in Yeast

Functional analysis of the ability of melatonin to inhibit the activation of a β-galactosidase reporter gene in yeast cells expressing a hormone-binding domain deletion variant ER (YEPE Δ5) grown in medium containing 25 μM $CuSO_4$ in the presence or absence of estradiol (10^{-9} M). β-Galactosidase activity is expressed in Miller units. n = 6; *, $P \leq 0.001$ *vs.* ETOH control, E_2 and E_2 + Mel-treated cells.

the expression of specific genes. In addition to regulating the expression of specific genes, estrogens also modulate the expression of their own receptor (11). Unfortunately, the major mechanism(s) controlling ER expression are currently not well understood. However,

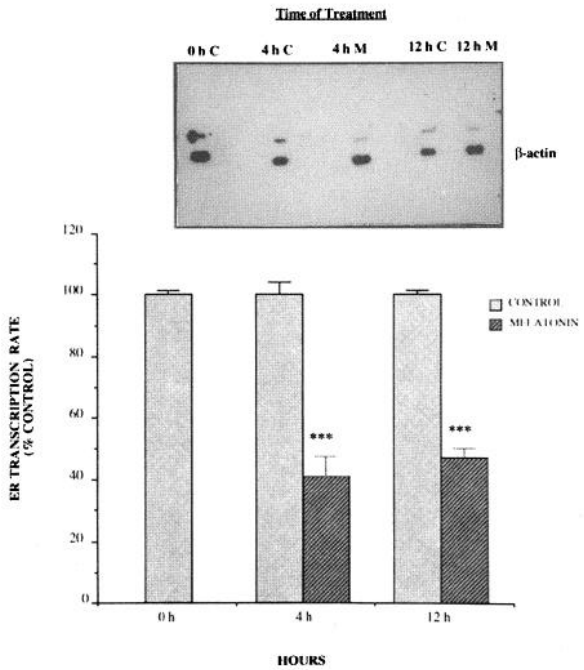

Fig. 8. Effect of Melatonin on ER Gene Transcription

MCF-7 cells were grown in phenol red-free medium containing 5% DCC-FBS for 5 days and treated with diluent (C) or 10^{-9} M melatonin (M). Cells were harvested, and nuclei isolated at 4 h or 12 h. Transcript elongation was performed in the presence of [^{32}P]UTP, and the ^{32}P-labeled run-on mRNA transcripts were hybridized to cDNAs bound to nitrocellulose membranes containing 20 μg human ER cDNA and 10 μg chicken β-actin cDNA. A representative autoradiogram is shown in the *upper portion* of this figure. 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 β-actin signal. The results are presented as percent of control. n = 3; ***, $P < 0.001$.

it is known that factors such as cell density, growth rate, processing after ligand-binding, and even other hormones (insulin, PRL) can substantially alter the cellular concentration of receptor (16, 17, 26–28). Since the phenotype of breast epithelial cells as well as breast tumor cells is modulated to a great extent by estrogen, it is important to understand the basis of ER regulation. The purpose of the present study was to begin to clarify possible mechanism(s) by which melatonin might modulate the expression of the estrogen receptor in MCF-7 human breast cancer cells. To begin to understand how melatonin modulates ER expression, the relationship between the steady-state levels of ER mRNA and the level of ER gene transcription in response to melatonin treatment was examined. Additionally, cycloheximide, an inhibitor of protein synthesis, was used to determine whether the decrease in steady state ER mRNA levels was dependent upon the induction of other proteins. A yeast transcriptional assay system was employed to determine whether melatonin interacts directly with the ER, and whether this might down-regulate ER mRNA expression. The results presented

here demonstrate that treatment of MCF-7 human breast cancer cells with melatonin resulted in a dose- and time-dependent diminution in the steady state levels of ER mRNA. This decrease in ER mRNA levels closely paralleled the time course of suppression of immunoreactive ER and estrogen-binding activity we previously reported (24).

Our studies found that treatment of MCF-7 cells with a physiological concentration of melatonin (10^{-9} M) caused a rapid reduction in the steady state levels of ER mRNA. This effect of melatonin on ER mRNA levels was not dependent upon, or significantly affected by, the presence of estrogens since similar decreases in steady state ER mRNA levels were noted in cells cultured in either complete medium or in estrogen-depleted medium. In these studies the suppressive effect of melatonin on ER mRNA levels is limited to a very narrow window of physiological concentrations as neither supraphysiological (10^{-8} to 10^{-6} M) nor subphysiological concentrations (10^{-12} M) of melatonin affect overall ER expression.

This window of effectiveness is quite puzzling and may be a key to our understanding of melatonin's action. One possible explanation for this observation is that supraphysiological concentrations of melatonin may rapidly down-regulate the recently cloned melatonin receptor (29), thus suppressing signal transduction events necessary for ER down-regulation normally induced by physiological concentrations of melatonin. It is also possible that supraphysiological levels of melatonin, independent of receptor binding, might modulate other signal transduction events necessary for the down-regulation of ER.

The simple suppressive effect of melatonin on ER mRNA expression is to some degree in contrast with our earlier findings that supraphysiological concentrations of melatonin were able to enhance ER protein levels. This discrepancy may suggest that, at physiological concentrations, melatonin modulates ER mRNA expression, while supraphysiological concentrations may work via alternative mechanisms to modulate ER protein synthesis and/or turnover. To understand whether the effect of melatonin on ER mRNA levels is dependent upon the synthesis of new proteins, we incubated MCF-7 cells with melatonin and the protein synthesis inhibitor, cycloheximide. Since cycloheximide was unable to prevent the melatonin-induced decrease in ER mRNA expression, we conclude that the steady state levels of the ER message are largely regulated at the transcriptional or posttranscriptional level rather than via a posttranslational mechanism involving the synthesis of new proteins.

To further clarify whether melatonin interacts directly with the ER to mediate ER mRNA expression, we performed a series of studies in a yeast transcriptional assay system. Our studies clearly show that melatonin does not inhibit the transcriptional regulatory capacity of the fully activated (estrogen-bound) ER. Melatonin also did not effect the transcriptional regulatory activity of the constitutively active exon 5-deleted ER variant in which the hormone-binding domain is truncated. This information, combined with our earlier report (24) that melatonin does not compete with and displace estradiol from the ER, suggests that melatonin's effects on ER mRNA expression are not mediated via a direct interaction with any particular domain of the ER. It is, however, still a possibility that melatonin may act via other mechanisms, including its own putative receptor (29), to modulate ER activity (*i.e.* receptor phosphorylation) that might lead to altered ER function and ultimately decreased ER gene expression. Additional studies are currently underway to address such possibilities.

The potential role of melatonin as a transcriptional regulator of the ER gene was suggested, given that the reported half-life of the ER transcript is just 3–5 h (15) and our data showed that melatonin decreased ER mRNA levels by approximately 50% after just 6 h and continued to reduce ER mRNA levels to a minimum after 24 to 48 h. Since our earlier studies (24) demonstrated that melatonin neither affects the affinity of the ER for estrogen, nor does it appear to bind to the ER, the possibility that the down-regulation of the steady state levels of ER mRNA was a consequence of decreased ER gene transcription was investigated employing nuclear transcription run-on experiments. Melatonin was able to significantly suppress the transcription of the ER gene within 4 h of treatment. The greater than 50% reduction in transcription of the ER gene at this early time point is sufficient to account for the significant decrease in the steady state levels of ER mRNA seen after 6 h of treatment. It may be important to note that this was not a complete blockade of ER gene transcription but was only a general slowing of the transcription of this gene. These studies demonstrate that the decrease in steady state levels of ER mRNA upon melatonin treatment is mediated, at least in part, by a melatonin-induced suppression of ER gene transcription.

Previous reports have demonstrated that decreases in ER levels seen in MCF-7 cells in response to estrogen treatment result from an autologous down-regulation of ER mRNA and protein (11, 12). In fact, the phenomenon of negative autoregulation of steroid hormone receptor mRNA is not uncommon. Similar decreases in steroid receptor mRNA levels in response to their cognate hormone have also been found for the glucocorticoid (30), progesterone (31), and androgen (32) receptors. Additionally, it has been reported that the phorbol ester TPA induces marked decreases in ER protein levels, binding capacity, and steady-state mRNA levels (18). However, these investigators reported that the TPA-mediated decrease in ER mRNA was not accompanied by a significant decrease in gene transcription, but rather was principally due to posttranscriptional destabilization of the ER mRNA (19). Indeed, it also appears that the predominant mechanism by which estrogen regulates the expression of its own receptor is via posttranscriptional destabilization of ER mRNA (14). Using the RNA synthesis inhibitor, actinomycin D (32), we failed to find any effect of melatonin on the stability

of the ER transcript. Therefore, even though the levels of ER mRNA expression are often regulated through modulation of the stability of the message by various agents including its cognate ligand, melatonin's modulation of ER mRNA expression appears to be mediated primarily at the transcriptional level.

In an earlier study (24) and in the present study we have shown (in a yeast expression assay system) that melatonin neither inhibits the transcriptional regulatory activity of the activated wild type ER nor the constitutively active Δ5 variant ER that has its hormone-binding domain deleted. However, our current data also show that this indoleamine suppresses the transcription of the ER gene. Our general conclusion, therefore, is that melatonin's actions on ER gene transcription are likely mediated via two possible pathways. First, melatonin may inhibit the ER gene transcription machinery through its interaction with the membrane-associated, putative melatonin receptor (29) and the subsequent activation of signal transduction pathways. Second, melatonin, being highly lipophilic, may diffuse into the nucleus to directly bind to promoter sequences and inhibit transcription of the ER gene. Other possible scenarios may involve the binding to and sequestering of key transcriptional regulatory factors or the induction of important negative regulatory transcription factors, mediated possibly through interaction with a nuclear melatonin binding protein (33). Currently, studies are underway in our laboratory to characterize the promoter elements in the 5′-upstream regulatory region of the ER gene. Additional studies using band-shift analysis will identify transcriptional regulatory proteins that interact with these upstream regulatory elements. Once the basic organization of the promoter region of the ER gene has been defined and key transcriptional regulatory proteins have been identified and characterized, further studies into the exact mechanism by which melatonin modulates ER gene transcription will be possible.

Taken together, these data show that melatonin rapidly and dramatically down-regulates the steady state levels of ER mRNA in MCF-7 human breast tumor cells by decreasing the transcriptional rate of the ER gene. Since the expression of various growth-regulatory factors such as TGFα, TGFβ, IGF-I, and Cathepsin D are modulated by estrogen, it is possible, and even likely, that melatonin, through its suppression of ER expression, will modulate the down-stream expression of these growth factors, and thus, to some degree, the proliferation of these breast tumor cells. These studies provide a rational explanation for the rapid and dramatic decline in the cellular ER mRNA and thus receptor protein levels upon melatonin treatment, and although they do not definitively delineate melatonin's mechanism of action, they do begin to unravel the mystery of melatonin's action in these cells. Maybe more importantly, these studies highlight the cross-talk between different hormone receptor systems, and underscore the complexity of the hormone-response system in breast cancer cells.

MATERIALS AND METHODS

Materials

Melatonin (*N*-acetyl-5-methoxytryptamine), 17 β-estradiol, penicillin, streptomycin, actinomycin D, and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). [^{32}P]uridine-5′-triphosphate (UTP) [3000 Ci/mmol], [^{3}H]L-methionine (194 mCi/mmol), and [^{32}P]2-deoxycytidine-5′-triphosphate (dCTP) [800 Ci/mmol] were obtained from DuPont-New England Nuclear (Boston, MA). The random primer DNA labeling kit was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose filters were purchased from Schleicher & Schuell, Inc. (Keene, NH), while Hybond nylon membranes were obtained from Amersham Corp. (Arlington Heights, IL). Dulbecco's modified Eagles medium (DMEM) with or without phenol red was obtained from Irvine Scientific (Santa Ana, CA), while FBS and methionine-deficient DMEM were purchased from GIBCO Laboratories (Grand Island, NY).

Cell Culture

MCF-7 human breast cancer cells were kindly provided by the late Dr. William McGuire (University of Texas at San Antonio). MCF-7 cells (passage 187–210) are routinely propagated in 75 cm^2 (T75) Corning tissue culture flasks containing DMEM supplemented with 10% FBS, 50 mM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 nM bovine insulin at 37 C in a humidified 5% CO_2 atmosphere. In experiments examining the effects of melatonin on MCF-7 cells in estrogen-depleted media, phenol red-free DMEM was supplemented with 5% FBS treated with DCC (DCC-FBS) to remove endogenous estrogens. The concentration of melatonin in the FBS before dilution in DMEM was 20 pg/ml as measured by RIA. After charcoal stripping, to remove endogenous estrogens, the concentration of melatonin fell below the detectable limits of the assay. One week before the start of each experiment, cells were harvested from stock plates containing DMEM supplemented with 10% FBS and seeded at a density of 3×10^6 cells/150 cm^2 flask (T150) in either medium supplemented with 10% FBS or phenol red-free DMEM containing 5% DCC-FBS and allowed to grow for 5 days (three fourths confluency). While cells were still subconfluent, the medium was replaced with fresh estrogen-deficient medium and melatonin was added directly from a 1000-fold stock solution in ethanol, stored at 4 C, and protected from light, to achieve the desired concentrations (10^{-5} M, 10^{-7} M, 10^{-9} M, 10^{-11} M, 10^{-13} M). Time course experiments were conducted under the same culture conditions as described above using the most effective concentration of melatonin determined in the dose-response studies (10^{-9} M). Semiconfluent monolayers of MCF-7 cells were exposed to 10^{-9} M melatonin and harvested at various times (1 h, 3 h, 6 h, 12 h, 24 h, and 48 h).

Cycloheximide and Actinomycin D Studies

To assess whether the down-regulation of ER by melatonin is dependent upon protein synthesis, studies were conducted with cycloheximide, a protein synthesis inhibitor. MCF-7 cells were treated with either 10^{-9} M melatonin, 10 μg/ml cycloheximide, or both for 24 h. To determine the half-life of the ER mRNA, MCF-7 cells treated with melatonin (10^{-9} M) for 6 h were incubated for various times (0 h, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h) with actinomycin D (5 μg/ml). Control cells were treated with vehicle (0.001% ethanol) and actinomycin D (5 μg/ml). Monolayers of treated cells were harvested in a solution of 0.25% EDTA in PBS by scraping with a rubber policeman. After centrifugation, cell pellets to be used for Northern blot analysis were snap frozen in liquid nitrogen and stored at −70 C until total cellular RNA was isolated.

Radioactive Labeling of Protein

Protein synthesis was evaluated in MCF-7 cells in the presence or absence of 10 μg/ml cycloheximide by measurement of uptake of [^{3}H]methionine into trichloroacetic acid (TCA)-precipitable proteins as described previously (34). Briefly, 1×10^6 cells/25 cm^2 flask were seeded in DMEM containing 10% FBS. After 2 days, the cells were switched to DMEM supplemented with 5% FBS and allowed to grow for 5 days (three fourths confluency). The cells were then incubated for 2 h in methionine-free DMEM supplemented with 5% FBS which had been dialyzed with two changes of PBS to remove amino acids. Labeling was then performed in the presence of 25 μCi/ml [^{3}H] methionine (194 mCi/mmol) with or without cycloheximide for 3 h or 6 h. The incubation was terminated by placing the cells at 4 C, and the cell monolayer was then lysed by the addition of 1 ml 0.1 N NaOH. The protein samples were heated at 37 C for 20 min before precipitation by the addition of 1 ml cold 10% TCA. Samples were then chilled on ice for 15 min, collected by filtration under vacuum onto 0.45-μm HA filters (Millipore, Milford, MA), washed with 5% TCA and dried, and their radioactivity was counted.

RNA Isolation and Northern Blot Analysis

Isolation of total cellular RNA from frozen cell pellets was performed using the guanidine thiocyanate-phenol-chloroform procedure (35). The resulting RNA pellet was precipitated in ethanol and resuspended in RNase-free water. The concentration of RNA was determined spectrophotometrically by measuring absorbance at 260 nm, and RNA integrity was assessed by agarose gel electrophoresis. Fifty micrograms per sample of total RNA were heat denatured at 85 C for 15 min and electrophoretically separated on a 1% denaturing agarose gel containing 2.2 mol/liter formaldehyde, and transferred to Hybond membrane (Amersham) by capillary action in 20 × sodium citrate (SSC) (1× SSC = 0.15 M NaCl, 0.0125 M sodium citrate, pH 7.0) overnight. RNA was fixed to the membrane by UV-cross-linking followed by baking at 80 C for 2 h. The membranes were prehybridized for 12 h in a solution containing 5× SSC, 10% sodium-dextran-sulfate (SDS), 0.1% Ficoll, 0.1% BSA, 0.2% polyvinylpyrrolidone, 20 mM sodium phosphate, 0.2% SDS, 200 μg salmon sperm DNA, and 50% formamide at 42 C. The membranes were then hybridized at 42 C in the same solution with the full length coding region of the human ER cDNA probe [2.1 kilobases (kb)], kindly provided by Dr. Geoffrey Greene (36), labeled with [^{32}P]dCTP by nick translation (37). After hybridization, the membranes were washed once with 2× SSC, 0.2% SDS at room temperature for 3 h followed by two washes at 65 C with 0.1× SSC containing 0.2% SDS for 12 min. The membranes were exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) at −70 C for 1–2 days. The transfer and loading of RNA were monitored by staining the gel with ethidium bromide both before and after transfer, and by reprobing of the blot with a 36B4 cDNA (0.2 kb), a non-estrogen-regulated gene. The resultant autoradiographs were analyzed by a GS300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). The size of the ER transcript was determined by comparison to an RNA ladder (0.36–9.49 kb; Novagen, Madison, WI) run on an adjacent lane and stained with ethidium bromide (10 μg/ml).

Yeast Expression Assay

The YEPE 10 expression vector containing wild type ER cDNA and the truncated YEPE Δ5 ER cDNA were used as previously described (38). The YEPE Δ5 cDNA contains a deletion of exon 5 that results in a frame-shift mutation and the presence of a stop codon just inside exon 6. When transcribed and translated, this variant cDNA gives rise to a truncated ER lacking the hormone-binding domain that is unable to bind estradiol, but is able to constitutively activate estrogen-responsive genes (39). The YEPE 10 and YEPE Δ5 constructs were transformed into a protease-deficient strain of *S. cerevisiae* (BJ3505). These yeast cells were also cotransformed with an estrogen-responsive reporter construct, YEPE 2, which contains two copies of the vitellogenin A2 estrogen response element within the proximal promoter elements of the yeast isochrome C promoter, fused to the β-galactosidase gene. This construct was used as an ER-inducible marker. Yeast cells were grown overnight at 30 C in 5 ml minimal media supplemented with 2% glucose. An additional 5 ml media were added along with 25 μM $CuSO_4$, and the cells were grown overnight. The absorbance of the cells was determined at 595 nm, and the cells were seeded at a density of 0.5 A/ml, supplemented with 25 μM $CuSO_4$ in the presence or absence of 17β-estradiol (10^{-9} M), melatonin (10^{-10-11} M), and/or the antiestrogen ICI 164,384 (10^{-7} M), and allowed to grow for an additional 4 h at 30 C. β-Galactosidase activity was measured as previously described (24). Briefly, protein extract (2–40 μg protein) was preincubated for 10 min at 30 C before a 30-min incubation with 200 μl o-nitrophenyl β-D-galactopyranoside (4 mg/ml). The reaction was stopped by the addition of 1 M sodium carbonate (500 μl) and the absorbance measured at 420 nm. Enzyme activity was expressed as Miller units.

Isolation of Nuclei

Briefly, MCF-7 cells, after exposure to either 10^{-9} M melatonin or vehicle (ethanol) for either 4 or 12 h, were harvested and resuspended in 5 ml 1.5 M sucrose buffer plus 0.1% Brij 58 (40). 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 centrifuged 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 dithiothreitol, 0.124 mM phenylmethylsulfonylfluoride, 50% glycerol). The concentration of nuclei was determined by diluting an aliquot 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.

Transcriptional Elongation Assay

The effect of melatonin on the rate of ER mRNA transcription was examined using a nuclear run-on transcription assay as described previously (41). A 500 μl sample containing approximately 1×10^8 nuclei was incubated with 50 μCi [^{32}P]UTP (3000 Ci/mmol; New England Nuclear) and unlabeled ATP, CTP, and GTP (1.6 mM each) in a transcription reaction buffer [50 mM HEPES (pH 7.8), 6 mM $MgCl_2$, 4 mM $MnCl_2$, 6 mM spermidine, 10 mM dithiothreitol, and 0.4 M ammonium sulfate] at 26 C for 1 h. The reaction was terminated by digestion with 50 μg/ml DNase I (DPRF, Worthington, Freehold, NJ) in the presence of 20 mM $CaCl_2$ and 100 μg/ml yeast tRNA for 30 min at 26 C. The reaction mixture was deproteinized by digestion with 100 μg/ml proteinase K in the presence of 1.5 mM EDTA and 0.1% (vol/vol) SDS for 30 min at 42 C. The radiolabeled nascent mRNA transcripts were precipitated from the aqueous phase with 5% TCA at 4 C for 15 min, and the precipitate was collected on 0.45 μm HA filters (Millipore) by vacuum filtration and washed with 3% TCA. RNA was then eluted from the filters by incubation at 65 C for 10 min in 100 μl 10× SET [0.1 M Tris (pH 7.5), 0.05 M EDTA, 10% SDS] and recovered by precipitation at −20 C with an additional 30 μg yeast tRNA, 180 mM sodium acetate, and 2.5 vol ethanol. The radiolabeled RNA transcripts were isolated and hybridized to an excess of denatured plasmid DNA immobilized on nitrocellulose filters. The denatured plasmids (10–20 ng) used for the detection of specific transcripts were ER pOR8 and β-actin. After hybridization, the filters were washed with four changes of 2× saline-sodium phosphate-EDTA (SSPE), 0.1% SDS for 5 min at 25 C, and subsequently washed with four changes of 1× SSPE, 1.0% SDS for 30 min at 65 C. Filters were exposed for 5–7 days to Kodak XAR film at −70 C, autoradi-

ographs were analyzed by scanning densitometry, and the background was subtracted. These results were normalized for the number of nuclei or by comparison to the transcriptional level of β-actin. Transcription rate values for ER were then expressed as percentages of control values.

Statistical Analysis

The data are presented as the mean ± SEM. Statistical significance was determined by two-way analysis of variance followed by the Newman-Kuels post hoc test.

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REFERENCES

1. DeSombre ER, Greene G, Jensen EV 1980 Estrogen receptors and the hormone dependence of breast cancer. In: Brennan MJ, McGrath CM, Rich MA (eds) Breast Cancer: New Concepts in Etiology and Control. Liss, New York, pp 69–87
2. Edwards DP, Chamness GC, McGuire WL 1979 Estrogen and progesterone receptors in breast cancer. Biochim Biophys Acta 560:457–486
3. Lippman ME, Bolan G, Huff K 1976 The effects of estrogen and antiestrogen on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res 36:4595–4601
4. Darbe P, Yates J, Curtis S, King RJB 1983 Effects of estradiol on human breast cancer cells in culture. Cancer Res 43:349–354
5. Bates SE, Davidson NE, Valverius EM, Dickson RB, Freter CE, Tam JP, Kudlow JE, Lippman ME, Salomon DS 1988 Expression of transforming growth factor alpha and its mRNA in human breast cancer: its regulation by estrogen and possible functional significance. Mol Endocrinol 2:543–555
6. Yee D, Paik S, Lebovic GS, Marcus RR, Favoni RE, Cullen KJ, Lippman ME, Rosen N 1989 Analysis of insulin-like growth factor I gene expression in malignancy: evidence for a paracrine role in human breast cancer. Mol Endocrinol 3:506–517
7. Westley B, Rochefort H 1980 A secreted glycoprotein induced by estrogen in human breast cancer cell lines. Cell 20:352–362
8. Dubik D, Shiu R 1988 Transcriptional regulation of *c-myc* oncogene expression in hormone-responsive human breast cancer cells. J Biol Chem 263:12705–12708
9. Weisz A, Bresciani F 1988 Estrogen induces expression of *c-fos* and *c-myc* protooncogenes in rat uterus. Mol Endocrinol 2:816–824
10. Knabbe C, Lippman ME, Wakefield LM, Flanders KC, Kasid A, Derynck R, Dickson RB 1987 Evidence that transfroming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48:417–428
11. Eckert RL, Katzenellenbogen BS 1982 Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. Cancer Res 42:139–144
12. Horwitz KB, McGuire WL 1978 Nuclear mechanisms of estrogen action: effects of estradiol and antiestrogens on estrogen receptors and nuclear receptor processing. J Biol Chem 253:8185–8191
13. Read LD, Greene GL, Katzenellenbogen BS 1989 Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Mol Endocrinol 3:295–304
14. Saceda M, Lippman ME, Lindsey RK, Puente M, Martin MB 1989 Role of an estrogen receptor-dependent mechanism in the regulation of estrogen receptor mRNA in MCF-7 cells. Mol Endocrinol 3:1782–1787
15. Eckert RL, Mullick A, Rorke EA, Katzenellenbogen BS 1984 Estrogen receptor synthesis and turnover in MCF-7 breast cancer cells measured by a density shift technique. Endocrinology 114:629–637
16. Shafie SM, Gibson SL, Hilf R 1977 Effects of insulin and estrogen on hormone binding in the R3220AC mammary adenocarcinoma. Cancer Res 37:4641–4649
17. Shafie S, Brooks SC 1977 Effect of prolactin on growth and estrogen receptor level in human breast cancer cells (MCF-7). Cancer Res 37:729–799
18. Horwitz KB, McGuire WL 1978 Estrogen control of progesterone receptor in human breast cancer: correlations with nuclear processing of estrogen receptors. J Biol Chem 253:2223–2228
19. Saceda M, Knabbe C, Dickson RB, Lippman ME, Bronzert D, Lindsey RK, Gottardis MM, Martin MB 1991 Post-transcriptional destabilization of estrogen receptor mRNA in MCF-7 cells by 12-O-tetradecanoylphorbol-13-acetate. J Biol Chem 266:17809–17814
20. Danforth DN, Tamarkin L, Lippman ME 1983 Melatonin increases oestrogen receptor binding activity of human breast cancer cells. Nature 305:323–305
21. Roy EJ, Wilson MA 1981 Diurnal rhythm of cytoplasmic estrogen receptors in the rat brain in the absence of circulating estrogens. Science 213:1525–1527
22. Lawson NO, Wee B, Blask DE, Castles CG, Spriggs LL, Hill SM 1992 Modulation of hypothalamic estrogen receptors by melatonin in female LSH/SsLak golden hamsters. J Biol Reprod 47:1082–1090
23. Hill SM, Spriggs LL, Simons MA, Muraoka H, Blask DE 1992 The growth inhibitory action of melatonin on human breast cancer cells is linked to the estrogen response system. Cancer Lett 8:6121–6126
24. Molis TM, Walters MR, Hill SM 1993 Melatonin modulation of estrogen receptor expression in MCF-7 human breast cancer cells. Int J Oncol 3:687–694
25. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P 1982 Cloning of cDNA sequences of hormone-regulated genes from MCF-7 human breast cancer cell line. Nucleic Acid Res 10:7895–7903
26. Jakesz R, Smith CA, Aitken S, Huff KK, Schuette W, Shackney S, Lippman ME 1984 Influence of cell proliferation and cell cycle phase on expression of estrogen receptor in MCF-7 breast cancer cells. Cancer Res 44:619–625
27. Howitz KB, McGuire WL 1978 Actinomycin D prevents nuclear processing of estrogen receptor. J Biol Chem 255:9699–9705
28. Nawata H, Chong MT, Bronzert D, Lippman ME 1981 Estradiol-independent growth of a subline of MCF-7 human breast cancer cells in culture. J Biol Chem 256:6895–6902
29. Episawa T, Karne S, Lerner MR, Repper SM 1994 Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores. Proc Natl Acad Sci USA 91:6133–6137
30. Okert S, Poellinger L, Dong Y, Gustafsson J-A 1986

Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. Proc Natl Acad Sci USA 83:5899–5903
31. Read LD, Snider CE, Miller JS, Greene GL, Katzenellenbogen BS 1988 Ligand-modulated regulation of progesterone receptor messenger ribonucleic acid and protein in human breast cancer cell lines. Mol Endocrinol 2:263–271
32. Wolf DA, Herzingert T, Hermeking H, Blaschke D, Horz W 1993 Transcriptional and posttranscriptional regulation of human androgen receptor expression by androgen. Mol Endocrinol 7:924–936
33. Burns DM, Cos C, Blask DE, Demonstration and partial characterization of 2-iodomelatonin binding sites in experimental breast cancer. Program of the 72nd Annual Meeting of The Endocrine Society, Atlanta, GA 1990 (Abstract 151), p 62
34. Levenson R, Housman D 1979 Developmental program of murine erythroleukemia cells. J Cell Biol 82:715–725
35. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. Anal Biochem 162:156–159
36. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J 1986 Sequence and expression of human estrogen receptor complementary DNA. Science 231:1150–1154
37. Feinberg AP, Vogelstein BA 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13
38. McDonnell DP, Pike JW, Drutz DJ, Butt TR, O'Malley BW 1989 Reconstitution of the vitamen D-responsive osteoclasin transcription unit in *Saccharomyces cerevisiae*. Mol Cell Biol 9:3517–3523
39. Castles CG, Fuqua SAW, Klotz DM, Hill SM 1993 Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast tumor cell line. Cancer Res 53:5934–5939
40. Martin MB, Riegel AT, Schoenberg DR 1986 Differential induction of vitellogenin gene transcription and total transcriptional activity by estrogen in *Xenopus laevis* liver. J Biol Chem 261:2355–2361
41. Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M, Martin MB 1986 Regulation of estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol 2:1157–1162