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Estradiol-Regulated Expression of the Immunophilins Cyclophilin 40 and FKBP52 in MCF-7 Breast Cancer Cells

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The immunophilins, cyclophilin 40 (CyP40) and FKBP52, are associated with the unactivated estrogen receptor in mutually exclusive heterocomplexes and may differentially modulate receptor activity. We have recently shown that CyP40 and FKBP52 mRNA's are differentially elevated in breast carcinomas compared with normal breast tissue. Other studies suggest that such alterations in the ratio of immunophilins might potentially influence steroid receptor function. Studies were therefore initiated to investigate the influence of estradiol on CyP40 and FKBP52 expression in MCF-7 breast cancer cells. Over a 24-h-treatment period with estradiol, CyP40 and FKBP52 mRNA expression was increased approximately five- and 14-fold, respectively. The corresponding protein levels were also elevated in comparison to controls. The antiestrogen, ICI 182,780, was an antagonist for CyP40 and FKBP52 mRNA induction. Cycloheximide treatment did not inhibit this increased immunophilin expression, suggesting that estradiol-mediated activation is independent of *de novo* protein synthesis. Treatment of MCF-7 cells with estradiol resulted in an increased half-life of both CyP40 and FKBP52 mRNA, as determined by actinomycin D studies. These results suggest that estradiol regulates CyP40 and FKBP52 mRNA expression through both transcriptional and posttranscriptional mechanisms. © 2001 Academic Press

Key Words: **cyclophilin 40; FKBP52; protein expression; mRNA expression; mRNA stability; estrogen receptor; estradiol.**

In the absence of hormone, steroid receptors exist in heteromeric complexes with heat shock protein 90 (hsp90) and a number of accessory proteins (1). These include p23 and one of the three immunophilins, cyclophilin 40 (CyP40), FKBP52 or FKBP51, which bind competitively to hsp90 via conserved tetratricopeptide repeat (TPR) domains (1). One critical function of the receptor-hsp90 heterocomplex is to facilitate the folding of the receptor into a high-affinity steroid binding conformation (1). Despite their similar molecular architecture, the immunophilins have unique features within their TPR domains and adjacent regions, which allow distinct interactions with hsp90 leading to different effects on receptor activity (2, 3). Elevated expression of FKBP51 in the African green monkey results in the incorporation of this immunophilin over its FKBP52 counterpart into glucocorticoid receptor heterocomplexes, affording a receptor with decreased dexamethasone binding affinity and resulting in glucocorticoid resistance (4). There is evidence of a preferential association of FKBP51 with progesterone and glucocorticoid receptors during cell-free assembly reactions and estrogen receptor-hsp90 heterocomplexes assembled in reticulocyte lysate have been shown to contain higher proportions of CyP40 and FKBP52 relative to FKBP51 (2). CyP40 was the most abundant immunophilin observed in association with the unactivated estrogen receptor from bovine uterine cytosol (5, 6). It thus appears that the steroid receptors themselves may, at least in part, dictate the immunophilin composition of their heterocomplexes.

The steroid receptor-associated immunophilins are expressed in all human tissues that have been examined (7). However, some recent studies have suggested that the expression of the immunophilins is affected by hormone treatment. FKBP51 expression in mouse thymocytes was found to be inducible by glucocorticoids (8) and FKBP51 protein levels were elevated by dexamethasone treatment in several glucocorticoid receptor-expressing cells (9). An increase in FKBP52

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mRNA has been shown to be elicited by serum growth factors (10). In a recent study, we have shown that CyP40 and FKBP52 are universally, but variably expressed in breast carcinomas and breast cancer cell lines and that they are elevated in breast cancer compared with normal breast tissue (11). While the importance of estradiol in determining the overall accumulation of these immunophilins is not clear at this stage, isolation and sequence analysis of the CyP40 promoter revealed the presence of estrogen response element (ERE) half sites and an imperfect ERE in the proximal promoter region (12). The close association of CyP40 and FKBP52 with the estrogen receptor and the presence of potential estrogen responsive regulatory elements in the CyP40 promoter suggested the possibility that estrogen may have an effect on the expression of these immunophilins. Moreover, altered ratios of CyP40 and FKBP52, as seen in breast cancer compared with normal breast tissue (11), may have functional effects on the estrogen receptor which could contribute to hormonal resistance in breast cancer. Therefore, this study was initiated as a preliminary investigation into the influence of estradiol on CyP40 and FKBP52 expression in MCF-7 breast cancer cells. Here, we describe, for the first time, the upregulation by estradiol of steady-state CyP40 and FKBP52 mRNA and protein expression in MCF-7 cells and show that the increased message levels are a possible consequence of both the direct effect of estradiol on transcription as well as increased mRNA stability of the two immunophilins.

MATERIALS AND METHODS

Materials. 17β-estradiol, cycloheximide, and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO). The antiestrogen, ICI 182,780, was a generous gift from Dr. Alan Wakeling, ICI Pharmaceuticals (Macclesfield, UK). α-^{32}P dCTP was purchased from Du-Pont (Sydney, Australia). Fetal bovine serum, charcoal stripped fetal bovine serum, and DMEM were purchased from Trace Biosciences (Sydney, Australia). The recombinant plasmid containing human FKBP52 cDNA (13) was obtained from Dr. D. A. Peattie, Vertex Pharmaceuticals (Cambridge, MA). The mouse monoclonal antibody, KM1166, directed against CyP40 (14), was a kind gift from Dr. Joh-E Ikeda (Tokoi University School of Medicine, Japan) and the mouse monoclonal antibody, Hi52c, directed against FKBP52 (7), was obtained from Dr. David Smith (Mayo Clinic, Scottsdale, AZ).

Cell culture and treatments. MCF-7 breast cancer cells were maintained in DMEM medium supplemented with 5% fetal bovine serum, 22.7 mM $NaHCO_3$ (Sigma), and 20 mM Hepes (Sigma) at 37°C in a humidified incubator containing 5% CO_2. For estradiol treatment, MCF-7 cells were grown to 70–80% confluence, washed twice in Versene (0.02% EDTA, 0.9% NaCl, pH 7.4) and cultured in phenol red-free medium containing charcoal-stripped fetal bovine serum for 3 days. The cells were washed twice in Versene, trypsinized, and split into 21 individual 90 mm diameter petri dishes with the phenol red-free medium. After a 48 h exposure to 10^{-8} M ICI 182,780, the cells (approximately 70% confluent) were washed in Versene, and fresh phenol red-free medium was added with 10^{-8} M estradiol in ethanol. The cells were harvested for RNA at different time intervals (0, 1, 3, 5, 10, 15, 24 h) in triplicate. For cycloheximide treatment, the cells were grown to 70% confluency and treated with ethanol alone, 10^{-8} M estradiol in ethanol, and 20 and 50 μM cycloheximide with and without 10^{-8} M estradiol. The cells were pretreated with cycloheximide for 1 h before the addition of estradiol. The cells were harvested for RNA after 8 h of treatment with either vehicle or estradiol. For actinomycin D studies, MCF-7 cells were grown in phenol red-free medium containing charcoal-stripped fetal bovine serum for 4–5 days before trypsinisation and distribution among 30 individual 90 mm diameter petri dishes with the phenol red-free medium. The cells were subjected to 10^{-8} M ICI 182,780 for 48 h, washed in Versene, and fresh phenol red-free medium added. The petri dishes were then divided into two groups, one of which was treated with ethanol, while the other was dosed with 10^{-8} M estradiol overnight. Cells from both groups were further treated with ethanol alone or with 5 μg/ml of actinomycin D and harvested for RNA after 4, 8, 12, 16 h in triplicate.

RNA isolation and Northern analysis. Tissue culture cells were washed in ice-cold PBS and total RNA was extracted by guanidinium isothiocyanate and phenol-chloroform treatment followed by isopropanol precipitation. Northern blot analysis was performed as previously described (11). Briefly, total RNA (30 μg) was electrophoresed through a 1% denaturing agarose gel and then transferred by overnight capillary blotting to a Zeta Probe GT membrane (Bio-Rad, Hercules, CA). Membranes were probed initially for CyP40 and then, sequentially after stripping, for FKBP52 and 18S rRNA. mRNA signals were quantitated by densitometric scanning using ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

Western analysis. MCF-7 cells grown in 175 cm^2 flasks, were treated with ethanol or 10^{-8} M estradiol for 24 or 36 h and were trypsinized, resuspended in PBS buffer, and counted for cell number using a haemocytometer. Appropriate volumes of each suspension containing 10^7 cells were pelleted and harvested for total protein in 0.5 ml of sample buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 20% glycerol, pH 6.8). Western analysis was performed as described previously (11). Briefly, protein in equal volumes of extracts derived from each treatment regimen were separated on a SDS-12.5% polyacrylamide gel and blotted overnight at 4°C onto nitrocellulose membranes. CyP40 and FKBP52 proteins were immunodetected using as primary antibody, 1:10,000 dilutions of mouse monoclonal antibody, KM1166, directed against human CyP40 or of mouse monoclonal antibody, Hi52c, directed against human FKBP52. Anti-mouse antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Sydney, Australia) was used at 1:10,000 dilution as secondary antibody.

RESULTS

Estrogen-induced increase in CyP40 and FKBP52 steady-state mRNA expression. We investigated the influence of estradiol stimulation on CyP40 and FKBP52 mRNA expression in the estrogen receptor-positive breast cancer cell line, MCF-7. To increase the sensitivity of the response, MCF-7 cells cultured in phenol red-free medium were growth arrested with the pure antiestrogen ICI 182,780 and then rescued by the addition of estradiol. Northern analysis of total RNA from harvested cells was performed and CyP40 and FKBP52 mRNA levels were standardized against 18S RNA. Figure 1A shows that over a 24 h treatment period CyP40 and FKBP52 mRNA expression was increased approximately five- and 14-fold respectively, relative to the zero time point control. CyP40 and FKBP52 protein levels were also elevated after a 24 h treatment with estradiol and remained elevated over a

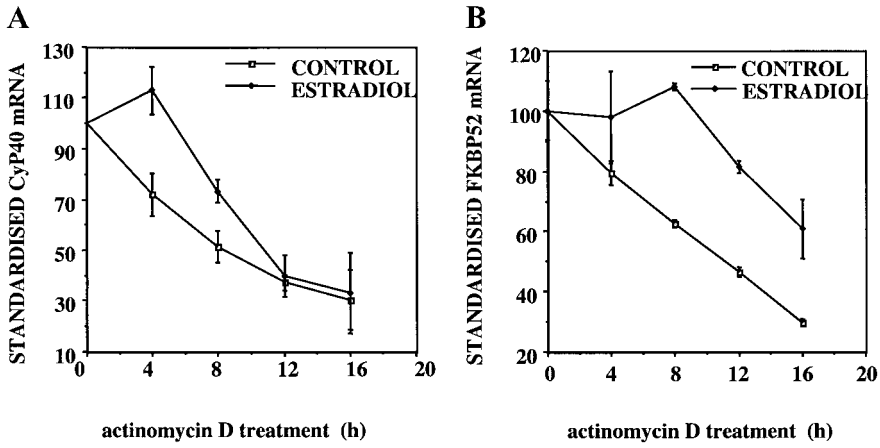

FIG. 1. Estradiol-induced increase in CyP40 and FKBP52 mRNA and protein levels. (A) Upper panels, Northern blot analysis of total RNA (30 μg) extracted from MCF-7 cells cultured in phenol red-free medium and exposed to 10^{-8} M estradiol for the indicated times and hybridized sequentially with CyP40, FKBP52, and 18S rRNA DNA probes. Lower panels, densitometric quantitation of Northern blots above, shown in histogram form. (B) Western blot analysis of total protein extracted from equal numbers (10^7) of MCF-7 cells treated with ethanol or estradiol for 24 and 36 h and probed for CyP40 and FKBP52.

further 12 h period (Fig. 1B). Both results confirmed the estradiol-induced stimulation of CyP40 and FKBP52 gene expression.

To determine that the induction of CyP40 and FKBP52 expression by estradiol was receptor-mediated, we tested the ability of ICI 182,780 to negate the observed estrogenic effect. MCF-7 cells were treated with estradiol or antiestrogen, either alone or in combination, over a 24 h period. Figure 2 shows that ICI 182,780 effectively inhibited the estradiol-induced expression of both immunophilins. The result is consistent with a role for the estrogen receptor in the regulation of steady-state CyP40 and

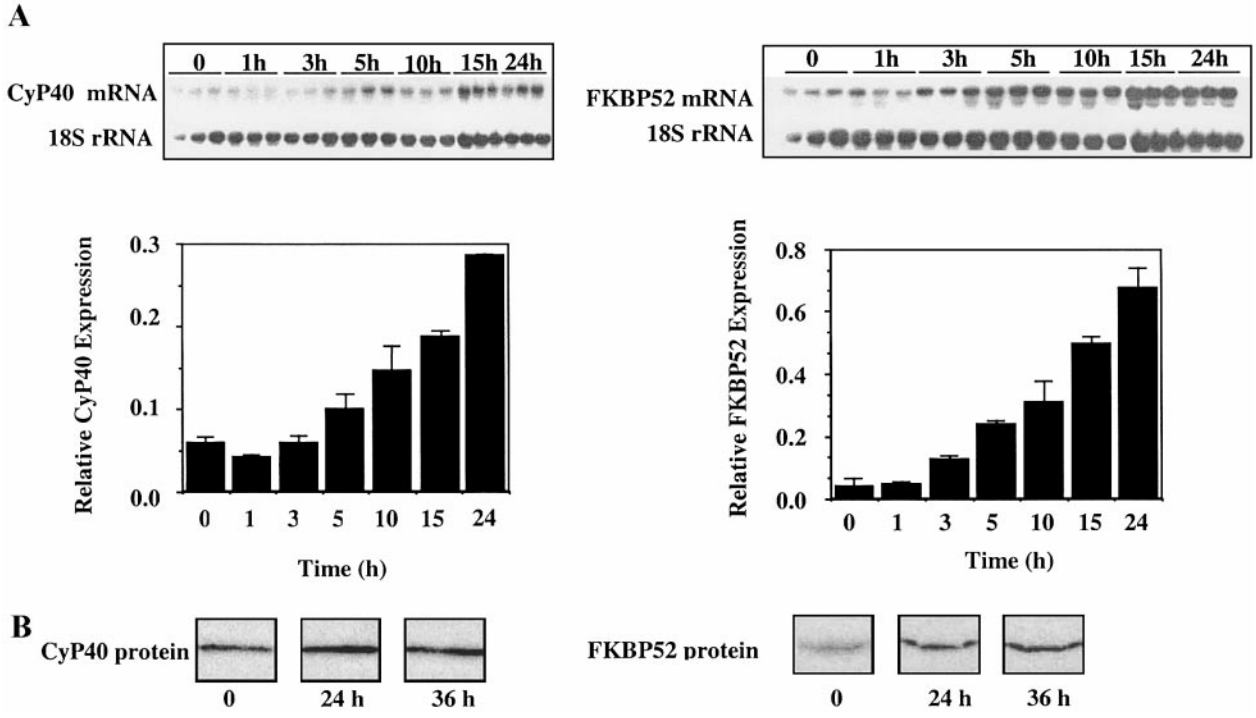

FIG. 2. Influence of ICI 182,780 on estradiol-induced expression of CyP40 and FKBP52 mRNA. Upper panels, Northern blot analysis of total RNA (30 μg) extracted from MCF-7 cells cultured under phenol red-free conditions and treated with vehicle alone, 10^{-8} M estradiol, 10^{-7} M ICI 182,780, or both together for 24 h. Blots were hybridized sequentially with CyP40, FKBP52, and 18S rRNA DNA probes. Lower panels, densitometric quantitation of Northern blots above, shown in histogram form.

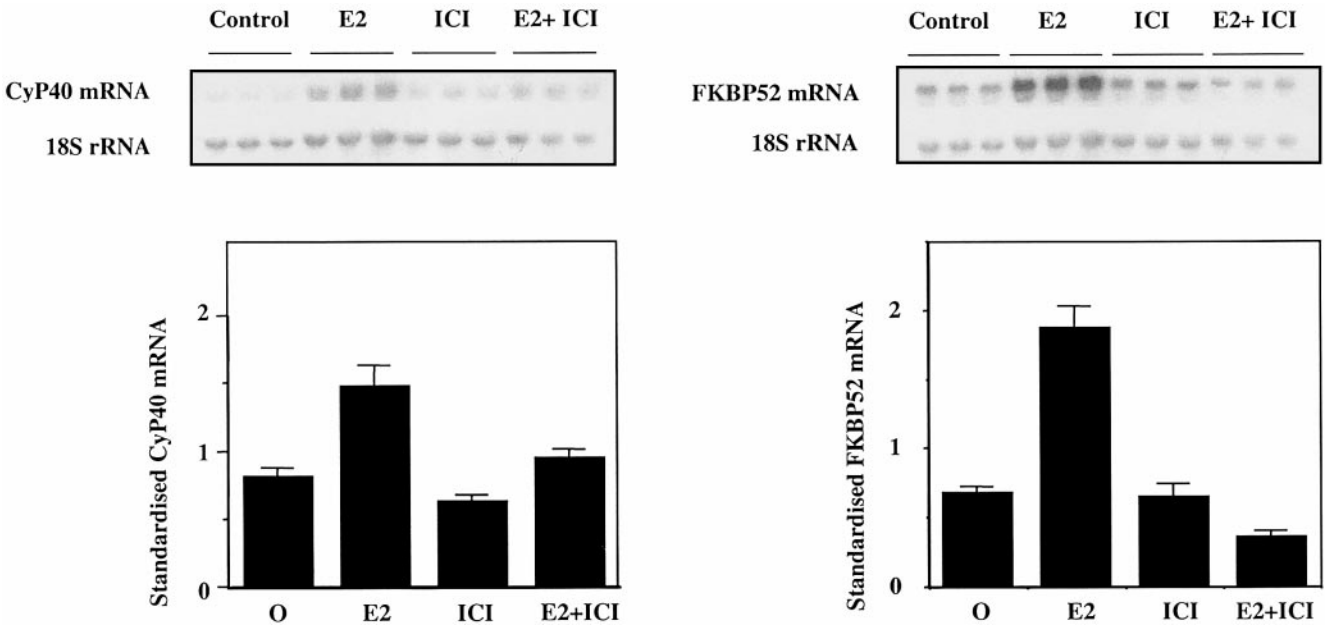

FIG. 3. Lack of inhibition by cycloheximide in the estradiol-induced expression of CyP40 and FKBP52 mRNA. Upper panels, Northern blot analysis of total RNA (30 μg) extracted from MCF-7 cells cultured in phenol red-free conditions and treated with either vehicle alone (−) or 10^{-8} M estradiol for 8 h in the absence or presence of 20 or 50 μM cycloheximide (CHX). Blots were hybridized sequentially with CyP40, FKBP52, and 18S rRNA DNA probes. Lower panels, densitometric quantitation of Northern blots above, shown in histogram form.

FKBP52 mRNA levels in receptor-positive breast cancer cells.

Cycloheximide does not inhibit immunophilin expression in the presence of estradiol. The requirement of newly synthesized protein for the increase in steady-state CyP40 and FKBP52 mRNA levels was explored by the use of the protein synthesis inhibitor, cycloheximide. MCF-7 cells, cultured in phenol red-free medium, were pretreated with cycloheximide (20 and 50 μM) for 1 h prior to estradiol stimulation for a further 8 h. Figure 3 shows that over the treatment period estradiol increased CyP40 and FKBP52 mRNA expression over basal levels. Cycloheximide alone was found to increase levels of CyP40 and FKBP52 mRNA. Importantly, however, cycloheximide did not inhibit CyP40 and FKBP52 stimulation by estradiol. Together, hormone and cycloheximide produced an enhanced response over that induced by either agent alone, suggesting that the estradiol-induced increase in steady-state CyP40 and FKBP52 mRNA levels occurs without the requirement for *de novo* protein synthesis.

Estradiol effects on CyP40 and FKBP52 mRNA stability in MCF-7 cells. The estradiol-mediated increase in steady-state CyP40 and FKBP52 mRNA levels could result from increased transcription, RNA stabilization or a combination of these events. Therefore, the possibility that the increase in steady-state mRNA levels was a result of altered message stability was examined. MCF-7 cells were cultured in phenol red-free medium and treated with either ethanol vehicle or 10^{-8} M estradiol for 15 h. Following actinomycin D (5 mg/ml) treatment, total RNA was isolated at 0, 4, 8, 12, and 16 h and analysed by Northern blotting. The half-life of CyP40 and FKBP52 mRNA was compared in vehicle and estradiol-treated cells. The graphical representation of the data (Fig. 4) shows that the half-life of CyP40 mRNA in vehicle-treated cells was nearly 8 h, while in estradiol-treated cells it was extended to 11.5 h. By comparison, the half-life of FKBP52 mRNA in vehicle-treated cells was 11 h, and this was increased to more than 16 h in estradiol-treated cells. The time-treatment interaction was highly significant as determined by two-way analysis of variance (P = 0.0125 and P = 0.0003 for CyP40 and FKBP52, respectively). In the presence of estradiol, both CyP40 and FKBP52 mRNA were initially stabilized, FKBP52 mRNA for a longer period (at least 8 h) than CyP40 mRNA (Fig. 4). Rapid rates of degradation of CyP40 mRNA after 4 h saw mRNA levels decrease to levels of control cells by 12 h. On the other hand, FKBP52 mRNA levels were elevated in the presence of estradiol compared to that of control at each of the time points examined after exposure to actinomycin D.

DISCUSSION

The essential aim of this study was to investigate the influence of estradiol on the expression of the two estrogen receptor-associated immunophilins, CyP40 and

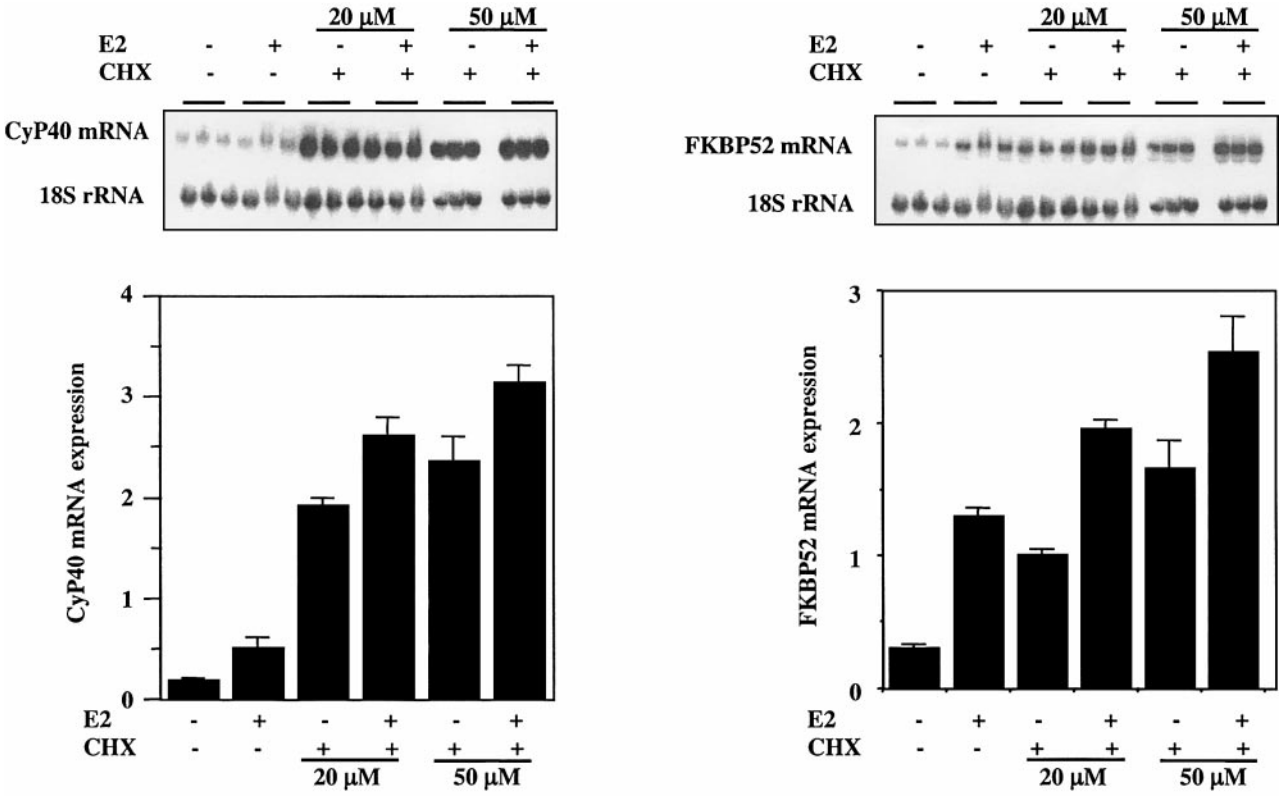

FIG. 4. Estradiol effects on CyP40 and FKBP52 mRNA stability in MCF-7 cells. MCF-7 cells cultured under phenol red-free conditions were treated with either ethanol vehicle or 10^{-8} M estradiol for 15 h. Following actinomycin D (5 mg/ml) treatment, total RNA was isolated at 0, 4, 8, 12, and 16 h and Northern blot analysis performed using CyP40, FKBP52, and 18S rRNA DNA probes. For both control and estradiol treated cells, standardized CyP40 and FKBP52 mRNA levels were expressed relative to that at time zero of actinomycin D treatment.

FKBP52. Our study clearly shows that in MCF-7 cells there is a progressive accumulation of CyP40 and FKBP52 mRNA and protein over a 24 h period following exposure to estradiol. Inhibition of the stimulatory effect of estradiol on CyP40 and FKBP52 mRNA expression by the pure antiestrogen ICI 182,780, supports a role for the estrogen receptor in the upregulation of both immunophilins by estradiol. Both transcripts were induced in the presence of the protein synthesis inhibitor, cycloheximide, alone. A similar effect of cycloheximide has been shown in studies related to other genes and is hypothesized to be due to the inhibition of the cellular levels of degrading proteins (15). However, estradiol treatment of cycloheximide-treated cells produced a further increase in CyP40 and FKBP52 mRNA levels, suggesting that estrogen activation is independent of new protein synthesis.

For the actinomycin D studies, treatment of MCF-7 cells with estradiol resulted in an initial stabilisation of CyP40 mRNA levels followed by a rapid decline to control levels within 12 h, while FKBP52 mRNA was stabilized for a longer period than that for CyP40 mRNA and continued to remain elevated above control levels even after 16 h. This clearly demonstrates a role for estradiol in the post-transcriptional regulation of CyP40 and FKBP52 mRNA resulting in increased message stability. The increased stabilization of FKBP52 compared with CyP40 mRNA might help to explain why the FKBP52:CyP40 protein ratio is generally much higher in MCF-7 and other breast cancer cell lines containing the estrogen receptor than in those breast cancer cell lines not containing a functional receptor (11). There are several points in RNA metabolism at which steroid hormones might act to alter the steady state levels of mRNA. Hormones not only modulate transcriptional activity, but have also been found to regulate steps from polyadenylation (16) to post-translational modification of the regulated proteins (17). In the chick oviduct, estrogen has been shown to increase the stability of ovalbumin and conalbumin mRNA (18) and the administration of estradiol to male *Xenopus laevis* induces both the transcription of the vitellogenin genes and the stabilization of vitellogenin mRNA against cytoplasmic degradation (19). Recently, estradiol has been shown to alter the rate of degradation of the peptidylglycine α-amidating monooxygenase (PAM) primary transcript, leading to a decrease in cytoplasmic PAM mRNA (20).

Our studies with cycloheximide and the estrogen receptor antagonist ICI 182,780 suggest that estradiol could have a direct effect on CyP40 and FKBP52 transcription rates via classical pathways involving the receptor. It is possible that a combination of increased transcription as well as increased message stability in the presence of estradiol is responsible for increased steady-state CyP40 and FKBP52 mRNA and protein levels.

Despite a similar trend of estradiol upregulation of both immunophilins, there is an emerging picture of differences in CyP40 and FKBP52 expression in MCF-7 cells. At the protein level, expression of FKBP52 is 35-fold greater than that of CyP40 in MCF-7 cells cultured under normal conditions in the presence of phenol red (11). Under these conditions, phenol red functions as a weak estrogen to stimulate MCF-7 cell proliferation (22). Our results suggest that such an estrogenic stimulus might also favor FKBP52 over CyP40 expression leading to the preferential incorporation of FKBP52 into estrogen receptor heterocomplexes and other steroid receptor heterocomplexes, generally. In this regard, it is of interest that FKBP52 appears to be exclusively isolated with estrogen receptor complexes immunopurified from MCF-7 cells (23). We have recently determined that GA-binding protein (GABP), an Ets transcription factor (21), is a key regulator of CyP40 basal expression, but does not appear to have a regulatory role in the expression of FKBP52 (12). We have also noted that in MCF-7 cells, the peptide hormone, EGF, elicited increases of two- to threefold in both CyP40 and FKBP52 mRNA expression, but IGF-1 stimulated only FKBP52 expression (unpublished results). Different mechanisms of regulation of CyP40 and FKBP52 genes might therefore contribute to varied expression and hence differential incorporation of the immunophilins into steroid receptor heterocomplexes.

The upregulation of CyP40 and FKBP52 that we have observed in response to the mitogenic influences of estradiol in MCF-7 breast cancer cells is consistent with a possible wider role for these immunophilins in cell proliferation. Increased expression of FKBP52 in the late G_1 phase during renewed cell growth has led to proposals that the immunophilin may be required for cell progression (10). Deletion of the *Saccharomyces cerevisiae* cyclophilin, Cpr7, a yeast homolog of CyP40 originally identified through its interaction with the transcriptional regulator Responsible, results in a slow growth phenotype (24). Furthermore, evidence of CyP40 involvement in c-Myb growth regulatory pathways (25) and the recent identification of CyP40 as one of few genes to be upregulated in peroxisome proliferator-induced liver cancer (26), are consistent with a mitogenic role for the cyclophilin.

In conclusion, we have shown that estradiol upregulates CyP40 and FKBP52 mRNA and protein expression through the estrogen receptor. Increased message stability of the immunophilins in the presence of estradiol may be an important mechanism of estradiol regulation of the two immunophilins. The possibility of a direct transcriptional effect of estradiol on CyP40 expression via putative EREs observed in the CyP40 proximal promoter region is currently being investigated in our laboratory. Finally, our results suggest that estradiol may contribute significantly to the regulation of immunophilin expression in hormone-dependent cells and may impact on the role of these immunophilins in steroid receptor assembly and function.

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