MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4531–4538
0270-7306/92/104531-08$02.00/0

Vol. 12, No. 10

Examination of the DNA-Binding Ability of Estrogen Receptor in Whole Cells: Implications for Hormone-Independent Transactivation and the Actions of Antiestrogens

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Received 4 May 1992/Returned for modification 24 June 1992/Accepted 24 July 1992

We describe an assay employing the competitive binding of estrogen receptor (ER) with basal transcription factors on a constitutive promoter (cytomegalovirus-hormone response element[s]-chloramphenicol acetyltransferase [CMV-(HRE)$_n$-CAT, containing a hormone response element(s) between the TATA box and the start site of transcription]) to examine the DNA-binding ability of the human ER in whole cells. We used this promoter interference assay to examine the DNA binding of ER in cell lines containing high and low levels of endogenous ER, as well as in CHO cells expressing wild-type and mutant ERs from cotransfected expression vectors. The ER is capable of binding to the promoter interference constructs in the absence of added ligand, and estrogen (estradiol) or antiestrogen (*trans*-hydroxytamoxifen or ICI 164,384) enhances or stabilizes this interaction. The binding of unoccupied ER to reporter gene activation plasmids results in ligand-independent transactivation, presumably due to the TAF-1 function of the receptor. DNA binding of ER in the absence of ligand is observed in cells containing endogenous ER, or expressed ER, and occurs in cells with high or low receptor contents. Although estrogen- and antiestrogen-occupied ER complexes bind to DNA and reduce the template promoter activity, the extent of suppression achieved by ICI-bound ERs is consistently less than that achieved with the other ligands, presumably caused by the fact that ICI rapidly reduces the level of ER in most of the cells examined. However, the ICI-ER complexes that remain are in sufficient quantity to bind to gene activation reporter constructs, and in these cells, ICI still behaves as a pure antagonist of gene transcription and does not activate reporter genes. Hence, obstruction of ER DNA binding or reduction of ER in target cells may contribute to, but cannot fully explain, the pure antagonist character of the antiestrogen ICI 164,384. In addition, DNA binding by the ER alone is clearly not sufficient for ensuring full activation of transcription and argues for an intermediate in the receptor activation of promoters.

The estrogen receptor (ER) mediates gene expression by the hormone estrogen and is primarily a nuclear protein in the presence or absence of ligand (18, 27). It is uncertain whether or not ligand is necessary for DNA binding of the ER protein. Most studies to demonstrate DNA binding have utilized in vitro assays with oligonucleotides containing a consensus estrogen response element (ERE) (5, 12, 19, 21, 22, 31, 37). The prevailing picture is that the ER binds to DNA in the presence or absence of ligand; however, it is not clear what occurs within mammalian cells. These in vitro systems utilize conditions that do not faithfully mimic the nuclear milieu, and studies have shown that ionic conditions affect the ability of ER to bind to DNA at elevated temperatures (5).

The ER contains two transactivation functions. The first, TAF-1, is hormone independent and resides in the amino-terminal A/B region of the ER, and the second, TAF-2, is hormone dependent and is located in the hormone-binding domain (region E) of the ER (36). It has been proposed that the partial agonistic activities of certain antiestrogens originate from the TAF-1 function (4). Also, it has been suggested that the hormone-independent chloramphenicol acetyltransferase (CAT) activity observed for the ER in transfected cells is the result of ER binding to DNA in the absence of ligand (21, 35, 37). Therefore, it is important to determine whether unliganded ER is capable of binding to DNA in mammalian cells and whether this interaction results in hormone-independent transactivation.

Besides raising questions regarding ER occupancy requirements for DNA binding, in vitro studies have also provided conflicting information about the actions of certain antiestrogens (12, 21, 22, 31), compounds capable of potently antagonizing the transcriptionally stimulatory effect of estrogens (4, 12, 21, 23, 25, 30, 36). Only recently have studies tried to examine the DNA-binding abilities of antagonist-ER complexes in intact cells (10, 23, 25, 38). In particular, the actions of the pure antagonist ICI 164,384 (ICI) have been controversial. One study proposes that ICI blocks the dimerization and, subsequently, the in vitro DNA-binding abilities of ER-ICI complexes (12), and a second study demonstrates that ICI causes a rapid reduction in ER protein in target tissues (13). This leaves two questions unanswered. First, does ICI prevent DNA binding of ER in intact cells, and second, does a reduction in the levels of ER protein account for the pure antagonistic nature of ICI?

This study describes an assay for examining ER DNA binding within mammalian cells. It utilizes the principle of competitive binding of ER with basal transcription factors on a constitutive (cytomegalovirus [CMV]) promoter. We used this assay to examine the effect of ligand on DNA binding of the ER in transfected cells and relate it to the hormone-independent activity of the ER. We also examined the effects of ER concentration in the target cell on the binding of ER to DNA and investigated the nature of the antagonism by the pure antiestrogen ICI.

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MATERIALS AND METHODS

Chemicals and materials. Radioinert 17-β estradiol was obtained from Calbiochem. The antiestrogens ICI 164,384 and *trans*-hydroxytamoxifen (OHT) were provided by Alan Wakeling and ICI Pharmaceuticals (Macclesfield, England). [2,4,5,6-^{3}H]estradiol (90 Ci/mmol) and [dichloroacetyl-1,2-^{14}C]chloramphenicol (50 to 60 Ci/mmol) were from Dupont, NEN Research Products.

Cell culture conditions and transfections. Chinese hamster ovary (CHO) cells were passaged in phenol red-free Dulbecco's modified Eagle medium-Ham's F12 (DME/F12) tissue culture medium supplemented with 10% charcoal-dextran-treated fetal calf serum (CDFCS) (both obtained from Sigma) and were transfected by the $CaPO_4$ coprecipitation method (8) with modifications described in reference 30 with 400 ng of reporter plasmid, 400 ng of pCH110, and 7.2 μg of carrier DNA. ZR-75-1 breast cancer cells (American Type Culture Collection) and GH_4C1 rat pituitary tumor cells (from Patricia Hinkle, University of Rochester Medical School) were maintained in DME/F12 medium with 5% fetal calf serum; MCF-7 K1 and MCF-7 K3 breast cancer cells (9) were maintained in minimal essential medium supplemented with 5% charcoal-treated calf serum (CDCS); MDA-MB-231 breast cancer cells (obtained from EG&G Mason Research Institute) were maintained in minimal essential medium–5% CDCS–5% CDFCS. All media included penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO).

ZR-75-1 and GH_4C1 cells were switched to DME/F12–5% CDCS at least 6 days before transfection and were seeded in 100-mm plates at densities of 3×10^6 and 5×10^6 cells per plate. MCF-7 K1 and K3 and MDA-MB-231 cells were grown in minimal essential medium minus phenol red plus 5% CDCS–5% CDFCS 6 days before transfection and were seeded at 3×10^6, 1.5×10^6, and 2×10^6 cells per 100-mm dish, respectively. All the above cells were given fresh media 30 h after plating and were transfected approximately 12 h later with 2 μg of CMV-hormone response element (HRE)-CAT or ERE-vit-CAT reporter plasmid plus 4 μg of pCH110 (β-galactosidase internal control plasmid) plus 10 μg of carrier DNA. Cells remained in contact with the precipitate for 5 h and were then subjected to a 4-min glycerol shock (20% in Hank's balanced salt solution). Cells were rinsed and given fresh media with or without hormones. Twenty-four hours later, cells were collected, and extracts were prepared by using three cycles of freeze-thaw in 300 to 400 μl of cold 250 mM Tris-HCl (pH 7.5). β-Galactosidase activity was measured (16), and extracts containing equal amounts of β-galactosidase activity were used in CAT assays (14) as described in a previous publication (30).

DNA constructs. All cloning was performed by standard procedures (33). The human ER expression vector pRER (30) is described elsewhere. The CMV-HRE-CAT promoter interference plasmids were constructed by inserting double-stranded oligonucleotides containing a consensus ERE (AGGTCAcagTGACCT) or an ERE containing point mutations (AGATCAcagTGGCCT) into the *Sac*I site of CMV-CAT (see Fig. 1). CMV-CAT was constructed by ligating the CAT gene into the *Sal*I site of the expression vector pCMV-5 (1).

For the transcriptional activation studies, the estrogen-responsive plasmids $(ERE)_2$-TATA-CAT (7, 30) and ERE-vit-CAT (7) were used (provided by D. Shapiro, University of Illinois). $(ERE)_2$-TATA-CAT contains two consensus EREs 38 bp from the TATA box of the *Xenopus* vitellogenin B1 promoter (−42 to +14), and ERE-vit-CAT contains a 618-bp (−596 to +21) 5′-flanking and promoter region of the vitellogenin B1 gene. This construct contains two endogenous imperfect EREs at positions −302 and −334 and contains an exogenous perfect ERE inserted at position −359. The plasmid pCH110 (Pharmacia) was used as an internal control for transfection efficiencies during all experiments.

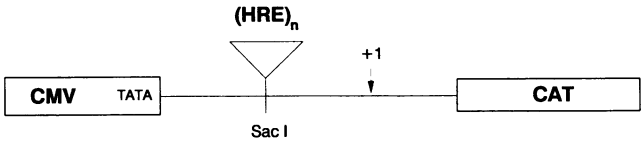

FIG. 1. CMV-HRE-CAT reporter construct. A consensus ERE and a mutant version were inserted into the *Sac*I site of CMV-CAT, which expresses the CAT gene constitutively from the CMV promoter. This site lies between the TATA box and the start site of transcription.

Immunoblots. Whole-cell extracts were prepared from the cells by resuspending the cell pellet from a 100-mm dish of cells in 200 μl of 50 mM Tris-HCl (pH 7.5)–1 mM EDTA–1 mM β-mercaptoethanol–10 mM sodium molybdate–0.5 M NaCl–1% Nonidet P-40–10% glycerol and then incubating on ice for 30 to 40 min and centrifugation at 14,000 × *g*. Extracts were fractionated on polyacrylamide gels under reducing conditions as described previously (20). Proteins were transferred from sodium dodecyl sulfate gels to nitrocellulose and subjected to Western blot (immunoblot) analysis with anti-ER monoclonal antibodies as described previously (15).

RESULTS

Promoter activity of constructs containing multiple EREs. An ER-dependent promoter interference reporter plasmid was constructed by inserting consensus EREs or a mutated version of an ERE (EREmut) into the *Sac*I site that lies between the TATA box and the start site of transcription of the vector CMV-CAT (Fig. 1). Binding of ER at that position should block the assembly of the transcription initiation complex and hence reduce the expression of the CAT gene. The effect of inserting oligonucleotides into the CMV-CAT vector on CAT expression was examined in cells lacking any measurable ER. The constructs containing one and two wild-type EREs and a construct containing two mutated EREs expressed CAT activity to levels of 107 to 90% of CMV-CAT lacking any inserted DNA sequences when transfected into ER-negative breast carcinoma MDA-MB-231 cells and CHO cells (Fig. 2). However, a construct which contains four EREs displayed a significant reduction in the expression of CAT activity in both cell types (Fig. 2), indicating a disruption in promoter activity or mRNA translation.

Suppression of CAT activity is dependent on functional ER. The functionality of the CMV-ERE-CAT constructs were verified by cotransfection of the CMV-$(ERE)_2$-CAT and CMV-$(EREmut)_2$-CAT constructs with the expression vectors for the wild-type ER and previously identified ER DNA-binding mutants into CHO cells. Cotransfection of the CMV-$(ERE)_2$-CAT construct with increasing amounts of the ER expression vector pRER, which expresses the human ER from the Rous sarcoma virus promoter, leads to a dose-dependent suppression of CAT activity (Fig. 3A). CAT activity was reduced in the presence or absence of added estradiol, although liganded receptor appears to be more

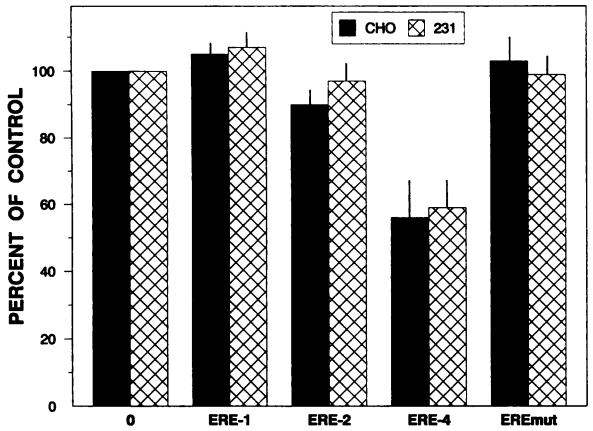

FIG. 2. Activity of the CMV-HRE-CAT constructs in cell lines containing exceedingly low or undetectable levels of ER. MDA-MB-231 and CHO cells were transfected by the calcium phosphate coprecipitation method as described in the Materials and Methods with the indicated CMV-HRE-CAT constructs. The level of CAT activity in cells transfected with CMV-CAT was set at 100% and represents the control value. The values are the means and standard errors of the means of three to six determinations.

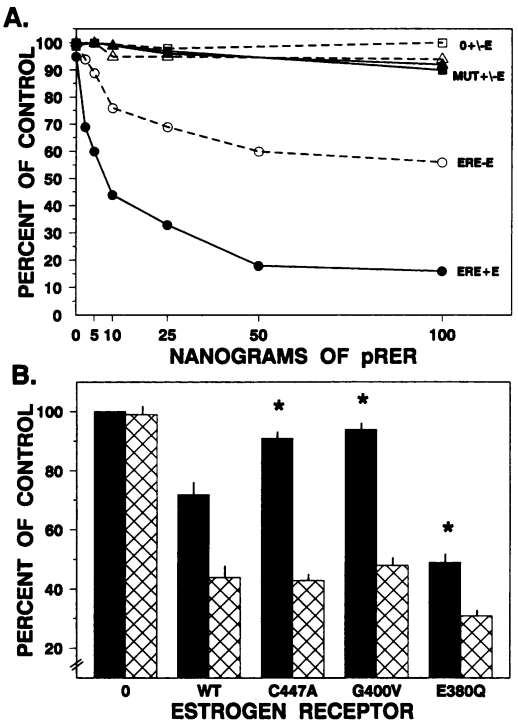

FIG. 3. Examination of the DNA-binding abilities of ER expressed in CHO cells. (A) CHO cells were cotransfected with 400 ng of the CMV-CAT construct containing no inserts (0, squares), two EREs (ERE, circles), and two mutated EREs (EREmut, triangles) with increasing amounts of the ER expression vector pRER. After transfection, the cells were treated with control vehicle (open symbols) or with 10 nM estradiol (filled symbols) for 24 h. Control values were determined as the CAT activity in cells transfected with each CMV-HRE-CAT construct in the absence of pRER or ligand and were set at 100%. (B) Ability of previously identified ER DNA-binding mutants to suppress CMV-$(ERE)_2$-CAT promoter activity was examined in CHO cells. The CMV-$(ERE)_2$-CAT reporter construct was cotransfected with 25 ng of the expression vector coding for the ER mutants indicated on the x axis. Cells were treated with 10 nM estradiol for 24 h (▩) or not treated (■), and CAT activities were measured. Results are the means and standard errors of the means of four to six determinations. *, $P < 0.05$ versus wild type by Student's t test.

effective. Increasing amounts of pRER had minimal effects on the CAT expression from CMV-CAT or CMV-$(ERE\text{-}mut)_2$-CAT, indicating that the suppression of CAT activity was dependent on ER-binding sites.

To further verify the assay conditions, we examined the ability of ER DNA-binding mutants to suppress CAT from the CMV-$(ERE)_2$-CAT construct in CHO cells. Two ER mutants, G400V (19, 37) and C447A (32), that show hormone-dependent DNA binding in vitro at elevated temperatures were examined. The two ER mutants displayed a much reduced ability to suppress CAT expression from the CMV-$(ERE)_2$-CAT construct in the absence of ligand compared with the wild-type ER (Fig. 3B); however, both were able to suppress the promoter to equal levels in the presence of ligand. An ER mutant which demonstrates higher ligand-independent transactivation ability than the wild-type ER (E380Q [24a]) was also examined. As shown in Fig. 3B, the E380Q mutant ER demonstrates higher ligand-independent suppression of CAT activity as well as a greater effectiveness than the wild-type ER in the presence of ligand (Fig. 3B), suggesting that its greater ligand-independent transcriptional activity may derive from a greater DNA-binding ability.

The amounts of pRER needed to suppress CAT activity from the promoter interference plasmid were similar to those necessary to activate transcription from an estrogen-responsive plasmid containing two consensus EREs (Fig. 4; under both assay conditions, half-maximal plasmid amounts were between 2.5 and 5 ng of pRER plate), indicating that promoter interference occurs at ER concentrations that are functional within the cells. This and the lack of reduction of CAT activity expressed from the CMV-CAT constructs that do not contain EREs (Fig. 3A) indicate that the reduction in CAT activity is not attributable to a squelching phenomenon which has previously been shown to occur with steroid receptors (24) and can be seen to occur in Fig. 4 with pRER amounts greater than 25 ng per plate in the presence of hormone. A reporter plasmid containing a minimal promoter that almost exclusively measures TAF-2 function in CHO cells (ERE-TATA-CAT [unpublished data]) failed to show an increase in CAT activity in cells transfected with increasing amounts of pRER in the absence of added ligand (Fig. 4A), suggesting that the DNA binding of unliganded ER to the promoter interference constructs is not attributable to contaminating steroids in the culture medium. In further support of this, the promoter interference constructs were suppressed in cells treated with control vehicle to a level of approximately 50% of that observed in cells treated with ligand. Therefore, if the DNA binding observed in vehicle-treated cells is the result of contaminating steroids in the culture medium, one would predict that 50% of the ERs would be occupied by ligand and would result in the induction of the reporter construct to 50% of that observed in estradiol-treated cells. When a reporter plasmid with a more complex promoter capable of measuring both TAF-1 and TAF-2 functions was used (ERE-vit-CAT [unpublished data]), an increase in CAT activity was observed in the absence of added ligand (Fig. 4B). Since the increase in CAT activity was not observed in the absence of added ligand with an ER whose TAF-1 function was eliminated by the

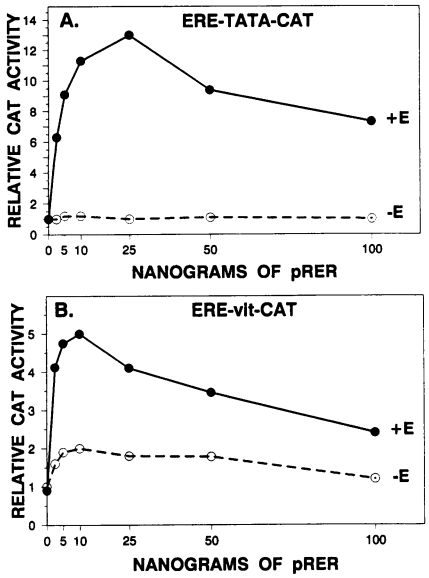

FIG. 4. Examination of the transactivation abilities of ER in cells transfected with two reporter plasmids. (A) CHO cells were cotransfected with increasing quantities of pRER (the ER expression vector) and 400 ng of ERE-TATA-CAT, a minimal promoter which measures TAF-2 function of the ER. Cells were treated with (●) 10 nM estradiol for 24 h or not treated (○). CAT activity is presented as relative CAT activity compared with that of cells transfected with the reporter plasmid in the absence of pRER or ligand. (B) Cells were treated as described above, except that 400 ng of ERE-vit-CAT, a complex promoter capable of measuring TAF-1 and TAF-2 functions of ER, was used.

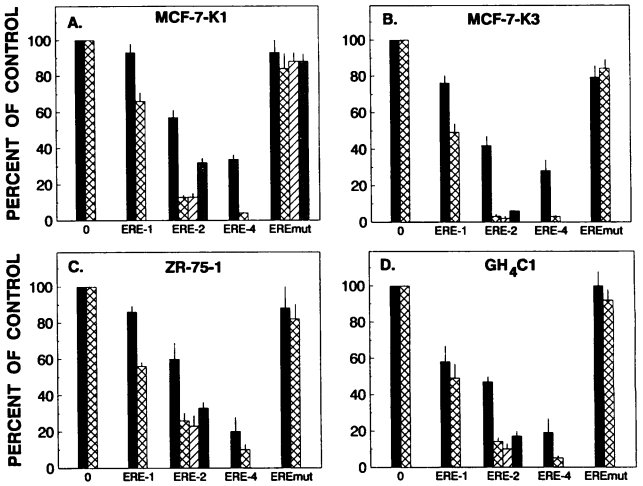

FIG. 5. Examination of the DNA-binding abilities of ER in cell lines expressing high and low levels of endogenous ER. MCF-7-K1 (A), MCF-7-K3 (B), ZR-75-1 (C), and GH_4C1 (D) cells were transfected with 2 μg of the CMV-HRE-CAT construct indicated on the *x* axis. Cells were treated with control vehicle (■), 10 nM estradiol (▩), 10^{-6} M OHT (▨), or 10^{-6} M ICI (▩) for 24 h. Data are presented as percentage of control, with control being the level of CAT activity of the CMV-CAT construct (lacking any EREs) in each particular cell line. Values are the means and standard errors of the means of three determinations.

deletion of the A/B region of the ER (data not shown), this ligand-independent transactivation may be attributable to the TAF-1 function of the ER. The ERE-vit-CAT construct used in these studies contains an inserted consensus ERE and two imperfect EREs of the vitellogenin gene in its 5′ regulatory region and differs considerably from the promoters in the ERE-TATA-CAT and promoter interference constructs. However, we feel that the differences in the ER-binding sites between these constructs are negligible, since the amount of transfected ER expression plasmid necessary to give a half-maximal response was similar for all three constructs (see above and Fig. 3A and 4).

These studies verify that the suppression of CAT activity expressed from the CMV-ERE-CAT reporter templates is dependent on ER and functional ERE sequences and is not ligand dependent, although ligand increases the extent of suppression by ER. Also, ER DNA-binding mutants that demonstrate ligand-dependent DNA binding in vitro are much less effective at suppressing the promoter constructs in the absence of ligand in whole cells, and an ER mutant that shows higher constitutive transcriptional activity was more effective than the wild-type ER at suppression of the promoter construct in the absence of ligand.

Examination of the DNA binding state of ER in cells expressing high and low levels of ER. The CMV-HRE-CAT constructs were transfected into human breast cancer cells that contain unusually high levels of ER (MCF-7-K1 and MCF-7-K3), as well as into a breast cancer cell line (ZR-75-1) and a rat pituitary tumor cell line (GH_4C1) that contain low levels of ER. The ER contents of these cells were measured by a whole-cell hormone binding assay (31) after transfection of the CMV-$(ERE)_2$-CAT construct and were found to be (in sites per cell, mean ± standard deviation) 153,236 ± 10,061 (MCF-7-K1); 260,298 ± 21,113 (MCF-7-K3); 11,271 ± 2,305 (ZR-75-1); and 8,413 ± 1,821 (GH_4C1). The MCF-7 cell subline MCF-7-K3 (9) contains about twice as much ER as its parental line MCF-7-K1.

The activities of the various CMV-HRE-CAT constructs in these cells are presented in Fig. 5. In cells containing either high (Fig. 5A and B) or low (Fig. 5C and D) levels of ER, there was a decrease in promoter activity that was dependent on the number of inserted EREs. Direct comparison of the ERE-4 constructs with the others is difficult, however, because this construct was less active in cell lines with undetectable levels of ER (Fig. 2). The construct containing two mutated EREs showed no promoter interference activity, similar to the construct lacking any inserted oligonucleotides (ca. 100% of control activity). Also, cotransfection of CMV-$(ERE)_2$-CAT with a fivefold excess of carrier DNA containing two EREs reduced the magnitude of repression of the promoter in the cell types examined (data not shown). These results indicate that the repression is specific for ERE sequences and, most likely, specific for the binding of ER.

The promoters containing functional ERE sequences were suppressed in cells that were treated with control vehicle alone, indicating that unliganded ER is capable of repressing promoter function, presumably through binding to DNA in cells in the unliganded form. It is unlikely that DNA binding of ER is the result of the activation of ER by serum components in the culture medium, because MCF-7 K1 cells transfected under serum concentrations ranging from 10 to 0.3% displayed the same magnitude of suppression of the CMV-$(ERE)_2$-CAT construct (data not shown). Clearly, if serum components were responsible for the ligand-independent DNA binding observed in these cells, reduction of the serum content in the culture medium by 30-fold should have

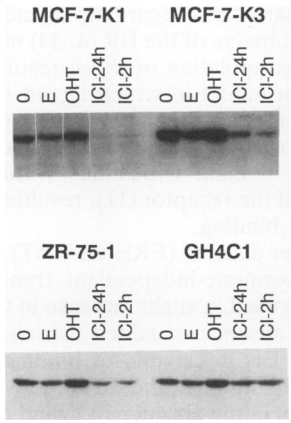

FIG. 6. Western blot analysis of extracts from transfected cells treated as described in the legend to Fig. 5. Whole-cell extracts were prepared from transfected cells treated with or without the indicated ligands for 24 h and from cells treated with ICI for 2 h. Proteins were fractionated and transferred to nitrocellulose and then subjected to Western blotting with the ER-specific monoclonal antibody H222. Relative ER contents were estimated by densitometry of appropriately exposed autoradiograms and were found to be as follows. ZR-75-1 cells: 0, 100%; E, 60%; OHT, 198%; ICI-24h, 28%; ICI-2h, 20%. GH_4C1 cells: 0, 100%; E, 83%; OHT, 200%; ICI-24h, 77%; ICI-2h, 70%. MCF-7 K1 cells: 0, 100%; E, 58%; OHT, 189%; ICI-24h, 10%; ICI-2h, 10%. MCF-7 K3 cells: 0, 100%; E, 52%; OHT, 135%; ICI-24h, 13%, ICI-2h, 8%.

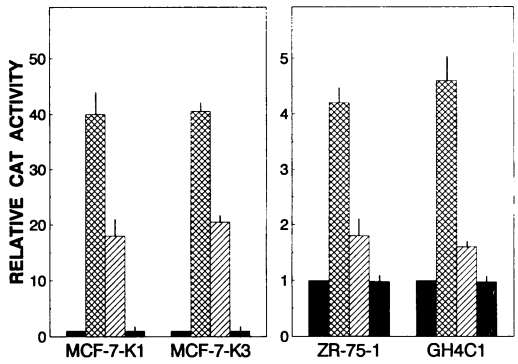

FIG. 7. Examination of the antagonistic nature of two antiestrogens in transfected cells. Cells were transfected with 2 μg of ERE-vit-CAT expression vector and treated as described in the legend to Fig. 5. After 24 h of ligand exposure, CAT activity in extracts prepared from the cell lines indicated on the *x* axis was examined. Data are expressed as relative CAT activity compared with the control, which was set as the CAT activity in transfected cells treated with control vehicle. Values are the means and standard errors of the means of three to four determinations.

had a significant effect. The magnitude of suppression was, however, enhanced by treatment with ligand, suggesting that liganded ER forms a more stable ER-DNA complex. Since promoter function was reduced in cells containing either high or low levels of ER, the DNA binding observed in transfected cells is not an artifact of the overexpression of ER to superphysiological levels. Apparently, in this system, ER content does not affect the ability of unliganded receptor to bind to DNA, and ligand enhances ER-DNA interaction within the cell.

Antiestrogen-ER complexes bind to DNA in cells. Cells exposed to the antiestrogen OHT displayed a reduction in CMV-$(ERE)_2$-CAT promoter activity similar to that of cells treated with estradiol (Fig. 5), indicating that OHT-ER complexes can bind to DNA. Similarly, cells treated with the pure antagonist ICI 164,384 (ICI) showed a decrease in promoter activity of the CMV-$(ERE)_2$-CAT construct, indicating that ER-ICI complexes are capable of binding to DNA within the cell. While ICI treatment reduced the promoter activity in these cell lines well below the level of no added hormone, the extent of suppression was slightly, but consistently, less than that of cells treated with estradiol or OHT (Fig. 5).

The apparent reduced ability of the ICI-ER complexes to suppress promoter activity compared with estradiol- or OHT-treated cells most likely stems from the reduction of ER content in cells exposed to ICI. Western blots of extracts prepared from transfected cells after treatment with ligand show that ER levels in the cells treated with ICI were reduced by as quickly as 2 h and remained low for the 24-h period examined (Fig. 6) in several cell types. This phenomenon has been reported previously in mouse uterus (13). In contrast to the reduction in ER level seen upon cell exposure to ICI, ER content showed modest increases after OHT exposure, with slight decreases after estradiol exposure (Fig. 6). The reduction of ER upon exposure to estradiol has been observed previously in breast cancer cells (3, 29). Of the four cell lines examined, GH_4C1 cells showed the least alteration in ER content in response to the ligands.

Transfection of quantities of an estrogen-responsive reporter plasmid (ERE-vit-CAT, capable of measuring the TAF-1 activity of ER) equal to those of the CMV-HRE-CAT constructs used in the promoter interference assays shows that ICI behaves as a pure antagonist while OHT behaves as a partial agonist and estradiol is a strong stimulator of reporter gene transcription (Fig. 7). Hence, even though ICI-ER complexes and OHT- and estradiol-ER complexes bind to DNA and the quantities of receptor in these cells are sufficient to bind to the CMV-$(ERE)_2$-CAT promoter construct and are presumably abundant enough to bind to the transactivation reporter plasmid, ICI still behaves as a pure antiestrogen, evoking no increase in ERE-vit-CAT transactivation.

DISCUSSION

A critical step in the transactivation process of nuclear receptors is the transformation of the receptor to a DNA-binding state. The ER is different from other steroid receptors in that the ER is capable of binding to DNA in the absence of ligand in in vitro DNA binding assays (5, 12, 21, 22, 31, 37), and examination of an ER mutant (C447A) which displays hormone-dependent DNA binding in vitro, as well as in whole cells at 37°C, suggests that the difference in the ligand modulation of DNA binding between the ER and other steroid receptors has a significant effect on the function of the ER (32).

We utilized a promoter interference assay based on the same principle applied by others to study the interaction of the *lac* repressor with DNA in cells (17) to provide evidence that the ER is capable of binding to DNA within whole cells in the absence of ligand and that ligand enhances or stabilizes the interaction of the ER with DNA response elements. This was observed in CHO cells cotransfected with an ER expression vector (pRER) and in human and rat cell lines containing endogenous ER. The repression was dependent on the insertion of functional EREs. We did find that insertion of four EREs into the promoter construct led to a

reduction in CAT activity expressed in cells lacking functional levels of ER. It is possible that the four EREs form a low-affinity binding site for a cellular inhibitory protein capable of binding to multimerized binding sites. However, we feel this is unlikely, because in all the cells examined (CHO and MDA-MB-231, Fig. 2; COS-1, data not shown), the relative level of CAT enzyme expressed from this construct was the same. It is reasonable to believe that these three very distinct cell types would express this putative inhibitory factor at different levels, leading to a different magnitude of supression of the construct. We feel that insertion of more than two EREs may mechanistically disrupt transcription and/or translation, which may be a consequence of the ERE's palindromic nature. Insertion of two EREs into this construct between the start site of transcription and the start codon greatly impaired the expression of CAT activity in ER-deficient cell lines (data not shown), while inserting an equal number further upstream had minimal effects (Fig. 2). The result of inserting multiple EREs into the reporter construct displays a positional effect, and if the suppression of these constructs in ER-deficient cell lines was due to a inhibitory cellular protein binding to the construct, we would predict that the position of the inserted oligonucleotide would have a minimal effect on the expression of CAT enzyme from these constructs.

We also demonstrate that the suppression of the $(ERE)_2$-CAT construct is dependent on ER by using wild-type ER and DNA binding mutants. We strongly believe that ER is acting alone on the reporter interference constructs. While it cannot be absolutely ruled out that the ER may form a complex with an inhibitory cellular factor to suppress the CMV-$(ERE)_2$-CAT construct, we feel it is highly unlikely, because we would expect that this complex would similarly form on the ERE_2-TATA-CAT reporter plasmid, resulting in the impairment of ER action on this construct.

It is unlikely that the DNA binding of ER observed in the absence of added estradiol is the result of residual estrogens in the culture media, because similar quantities of cotransfected ER expression vector failed to elicit any CAT activity from an estrogen-responsive reporter plasmid that measures almost exclusively the hormone-dependent transactivation function of ER (TAF-2) in CHO cells (i.e., ERE-TATA-CAT), and reduction of the serum content in the culture medium by 30-fold had no effect on the DNA binding observed in vehicle-treated cells. How ligand is capable of enhancing the suppression of these constructs by the ER is not clear. It is unlikely that ligand acts to alter the fraction of the ER in the nucleus, because unlike the glucocorticoid receptor, the ER is nuclear in the absence of ligand (18, 27). Ligand may, however, dissociate inhibitory factors from the ER within the cells, such as heat shock proteins (for a review, see reference 6). Our data provide evidence that at least 50% of the ERs are not bound by these inhibitory proteins, and since heat shock proteins are far more abundant in cells than is ER, we see no reason why all the ERs would not be bound by heat shock proteins and therefore prevented from binding DNA in the absence of ligand. Our data suggest that the ER is not bound by hsp90 in whole cells (similar to the in vitro form that sediments as a 4S to 5S molecule on sucrose gradients) and that the interaction of ERs with heat shock proteins may represent an in vitro artifact. One study, however, demonstrated diminished steroid receptor responsiveness of promoters in heat shock protein-deficient yeast cells (26), but this effect has yet to attributed to the interaction of these proteins with the ER in vivo. We also feel that the differences observed in yeast versus mammalian cells regarding ligand specificity and transactivation domains of the ER (4, 34) may warrant some caution in the extrapolation of those results to mammalian cells. We favor a model in which ligand stabilizes the ER protein within the cells (with the noted exception of ICI 164,384 [see below]) or stabilizes ER dimerization through the dimerization domain which lies within the hormone-binding domain of the receptor (11), resulting in an increased stability of DNA binding.

When a reporter plasmid (ERE-vit-CAT) that is capable of measuring the hormone-independent transactivation function (TAF-1) was used, a slight increase in CAT activity was observed in the absence of added estradiol. These results suggest that the ER is capable of binding to DNA in the unoccupied state and that the CAT activity observed on certain promoters in the absence of ligand most likely stems from the activity of the TAF-1 function of ER bound to DNA in the unliganded form. This is supported by our experiments with an ER mutant lacking the TAF-1 region, which failed to show an increase in CAT activity from this construct in the absence of ligand. A similar result has been seen for the mouse ER (21). The consequences of this are far reaching. The data suggest that unliganded ER is capable of modulating gene transcription by binding to promoter regions. The response observed would depend on the promoter and cell type. Promoters such as those in the ERE-vit-CAT construct would be weakly stimulated by unliganded ER, while the ovalbumin promoter, which has been shown to be strongly stimulated by the TAF-1 function of ER in chicken fibroblasts (4), might display greater induction by the unliganded ER. Alternatively, unliganded ER bound to promoters that are unresponsive to the TAF-1 function of the ER could lead to the repression of the promoter by interfering with other *trans* factors, leading to the control of promoters by a mechanism not previously studied for ER.

We observed binding of ER to DNA within whole cells in the absence of ligand in cells expressing both high and low levels of ER. This contrasts with a study done on yeast cells, which suggested that the apparent ligand-independent DNA-binding and transactivation functions of ER in transfected cells were due to overexpression of the ER to superphysiological levels (23). Examination of the DNA-binding abilities of ER in cells expressing both higher (MCF-7-K3) and lower (ZR-75-1 and GH_4C1) levels of ER than those expressed in the yeast cells used in that study demonstrated that ER concentration did not have an effect on the DNA-binding state of the ER. The differences in these two studies may reflect differences in the transformation of the ER to the DNA-binding state in the two organisms or differences in the function of the two ER transactivation domains in yeast and mammalian cells. In mammalian cells, the hormone-dependent transactivation function (TAF-2) is dominant, while TAF-2 is quite inactive in yeast cells and most of the transactivation function resides in the hormone-independent transactivation function, TAF-1 (4).

Taken together, these studies present evidence for an intermediate in the activation of the ER and suggest that DNA binding alone is not sufficient for the full activation of gene transcription. ER bound to DNA is capable of weakly activating promoters through its TAF-1 function and can be activated further through its TAF-2 function by ligand or by ligand-independent pathways such as protein kinase-mediated pathways. Ligand-independent DNA binding may be a prelude to the regulation of transcription by estrogen and other steroid receptors through protein kinase-mediated pathways (2, 28).

ER occupied with antiestrogen or estrogen is capable of binding to ERE DNA in whole cells. ER-ICI complexes bound to DNA, as did ER occupied by estradiol and OHT. Studies with a chimeric ER that had the acidic activation domain of VP-16 fused to the amino terminus of the human ER confirm these results. However, the investigators speculated that the DNA binding of the VP-16–ER chimera occupied with ICI might be due to possible dimerization functions in the VP-16 portion of the molecule (25). Our results clarify this point and indicate that the results of Pham et al. (25) are not an artifact of the chimeric ER.

Treatment of cells with ICI caused a rapid reduction in the levels of cellular ER. The magnitude of the reduction was dependent on cell type. GH_4C1 cells treated with estradiol or ICI showed little difference in ER levels, while MCF-7 cells showed large differences. However, the ER levels remaining in all cells examined after ICI exposure were sufficient to markedly suppress promoter activity of the CMV-$(ERE)_2$-CAT construct, and transfection of similar quantities of a transactivation reporter plasmid (ERE-vit-CAT) showed that ICI failed to activate transcription, despite the fact that there should be enough ER in these cells to bind to the ERE-vit-CAT construct. This point is underscored in transfected GH_4C1 cells, in which ICI treatment led to a decline in ER levels similar to that in cells treated with estradiol, yet ICI still acted as a pure antagonist. These findings suggest that (i) ICI-ER complexes are capable of binding to DNA in whole cells, and (ii) the reduction in ER levels caused by ICI may not fully explain the pure antagonistic nature of this ligand. In addition, the antiestrogen OHT-occupied ERs suppressed promoter activity as effectively as did estradiol-occupied ERs, yet these OHT-occupied ERs gave only partial activation of the ERE-vit-CAT reporter template, indicating that ER binding to ERE DNA alone is not sufficient to ensure full receptor activity.

In conclusion, we demonstrated that ER is capable of binding to DNA templates in whole cells in the presence or absence of ligand and that estrogen and antiestrogen are capable of enhancing or stabilizing this interaction. The binding of unoccupied ER to DNA was observed in cells expressing high or low levels of receptor, and the binding of unliganded ER causes hormone-independent transactivation of a reporter plasmid, presumably through the TAF-1 function in the amino terminus of the ER. Also, ER-ICI complexes are capable of binding to DNA, and the reduction in ER levels caused by ICI treatment may not fully explain the purely antagonistic nature of this ligand-ER complex. Differences in the interaction of antiestrogen-ER and unoccupied ER complexes with components of the preinitiation complex may explain the ineffectiveness of these ERs in activating transcription despite binding to DNA response elements.

ACKNOWLEDGMENTS

We are grateful to G. Greene for providing the ER cDNA λOR8, to D. W. Russell for providing the expression vector pCMV-5, and to D. Shapiro for providing us with the reporter plasmids. We also thank Abbott Laboratories for the ER monoclonal antibody H222.

This work was supported by Public Health Service grant CA18119 to B.S.K. and by a predoctoral fellowship to J.C.R. (Public Health Service grant GM07283) from the National Institutes of Health.

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