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Antiestrogen can establish nonproductive receptor complexes and alter chromatin structure at target enhancers

(estrogen receptor/DNA binding/transactivation)

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ABSTRACT **We describe in this report experiments *in vivo* that demonstrate that antiestrogens promote DNA binding of the estrogen receptor without efficiently inducing transcription. When the receptor is modified to carry a foreign unregulated transactivation domain, transcription can be induced efficiently by both estrogen and antiestrogens. Under apparent saturation conditions, antihormone–receptor complexes binding to responsive enhancer elements elicit only a low level of transcription. In addition, we show that both estrogen and an antiestrogen, nafoxidine, effect very similar alterations in chromatin structure at a responsive promoter. These results indicate that *in vivo* steroid receptor action can be regulated subsequent to the DNA binding step, by regulating interactions with the target transcriptional machinery. In this regard, antihormones can function by establishing receptor–DNA complexes that are transcriptionally nonproductive.**

Steroid hormone receptors are members of a superfamily of transcription factors whose functions are regulated directly by hormonal ligands (for reviews, see refs. 1–3). The receptors regulate transcription upon binding to cognate steroid responsive elements located in the vicinity of target genes. The binding of ligand is required for receptor function, although the precise role of the hormone is still unclear.

A considerable amount of evidence exists that suggests that the DNA binding activity of steroid hormone receptors is regulated by hormone. *In vivo* footprints and a DNase I-sensitive chromatin structure at steroid responsive elements appear only after hormone addition (4–7). In addition, hormone-dependent DNA binding has been reported *in vitro* (8–11). However, other reports suggest that hormone-free receptor can bind DNA (12–14). An explanation for these conflicting results is that in the absence of hormone the receptor is prevented from binding to DNA due to its association with heat shock proteins, which may mask the DNA binding domain of the receptor (3, 15, 16). Accordingly, there is some evidence that antihormones, ligands that inhibit receptor function, can trap the receptor in a non-DNA-binding form (16–21).

Whether the regulation of the receptors occurs solely at the DNA-binding level has been unclear. There have been reports that various antihormones can stimulate DNA binding of their respective receptors *in vitro* (8–11), suggesting an important regulatory step subsequent to DNA binding. However, in many cases these same antihormones in other studies have been found to interfere with the activation of the receptor to a DNA-binding form (16, 19, 20). A possible reason for these inconsistencies may be due to the fact that *in vitro* receptor–DNA binding experiments are highly dependent on receptor extraction conditions, which can allow the dissociation of inhibitory proteins normally required to prevent DNA binding *in vivo*. Receptor-interference experiments *in vivo* support the hypothesis that antihormones can induce DNA binding of the receptors (22, 23). However, the precise significance of these experiments is difficult to assess since the inhibition of transcription observed may be due to squelching or formation of nonfunctional heterodimers (for a review, see ref. 24). In fact, the transactivation domains of the estrogen receptor (ER) have recently been shown to be able to efficiently squelch itself as well as the acidic activators of GAL4 and herpes simplex viral protein 16 (VP16) (25). To date, it has not been shown directly that antihormone–receptor complexes can bind DNA efficiently in intact cells.

To further elucidate the mechanisms of receptor regulation, we have examined how antiestrogens regulate the function of the ER *in vivo*, in mammalian cells and in yeast. In this report, we demonstrate that the observed inhibition of ER activity by antiestrogens is not due to a decreased efficiency in promoting DNA binding in cells. Furthermore, both estrogen and an antiestrogen can induce similar alterations in chromatin structure at a responsive promoter. Our results indicate that ligands of the ER can differentially regulate gene transactivation by inducing distinct allosteric (or covalently modified) receptor–DNA complexes.

MATERIALS AND METHODS

Construction of Plasmids. The ER and ER–VP16 mammalian expression plasmids (26) and the ERE–tk–CAT reporter plasmid (27) were constructed as described. The yeast reporter plasmid YRPE2 was constructed from pLG670Z (28) by inserting a 75-base-pair oligonucleotide [containing two estrogen responsive elements (EREs)] into the *Xho* I site upstream of the CYC1 (iso-1-cytochrome *c*) promoter. The yeast ER expression plasmid YEPE10 was originally constructed using the original mutant ER clone (ref. 29, in pGEM-35hER, from G. Greene, University of Chicago). pGEM-35hER was digested with *Bam*HI and *Hin*dIII and ligated to a linker that encoded the last six amino acids of ubiquitin (30) and that contained also the *Afl* II and *Nco* I restriction sites. The *Eco*RI site of the resulting plasmid was then converted to a *Kpn* I site. The *Afl* II–*Kpn* I fragment of this plasmid was inserted into the cognate sites of YEPV1 (31), yielding the plasmid YEPE2. Finally, the human ER point mutation was corrected by replacing the *Asp* I–*Sma* I fragment of YEPE2 with that of the wild-type human ER cDNA. The yeast ER–VP16 expression plasmid was constructed by inserting the *Not* I fragment encoding the VP16 acidic activating region (from the mammalian ER–VP16 expression plasmid) into the *Not* I site of YEPE10.

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Abbreviations: ER, estrogen receptor; ERE, estrogen responsive element; VP16, herpes simplex viral protein 16; CAT, chloramphenicol acetyltransferase.

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Antiestrogens. Tamoxifen and nafoxidine were from Sigma. ICI 164,384 was obtained from ICI Pharmaceuticals, and LY156 and LY117 were from Lilly.

Transient Transfection Assays. Transient transfections into CV-1 cells and chloramphenicol acetyltransferase (CAT) assays were performed exactly as described (26).

Assay of ER Function in *Saccharomyces cerevisiae*. The yeast expression plasmids and reporter plasmid were transformed into protease-deficient yeast BJ3505 (obtained from E. Jones, Carnegie–Mellon University) by using the lithium transformation method (32). To assay for transcriptional activity, the cells were grown in selective medium and induced with hormone for 4 hr. The cells were then harvested and LacZ activity was assayed as described (31).

Western Blot Analysis. Protein samples used in the Western blot were obtained from yeast cells transformed with YEPE10 and YRPE2 and induced with nafoxidine for 4 hr as described above. The cells were then harvested and lysed by glass-bead homogenization in Z buffer (31) with 0.3 M NaCl. Western blot analysis was performed as described (31) except that a human ER monoclonal antibody (D75, a gift from G. Greene) was used.

Chromatin Structure Analysis. Yeast cells transformed with YEPE10 and YRPE2 were grown in selective medium to an optical density (at 600 nm) of ≈0.4. The culture was then divided into three parts and induced with 0.6 μM estradiol or 2 μM nafoxidine or not induced. After 4 hr of induction at 30°C, the cells were harvested and converted to spheroplasts as described (33), except that the azide treatment step was omitted. The spheroplasts were lysed in 15 mM Tris·HCl, pH 7.5/3 mM $MgCl_2$/0.5 mM $CaCl_2$/20 mM NaCl. DNase I was then added to the lysate at various concentrations and incubated for time intervals as specified in Fig. 4. After DNase I digestion, the DNA was purified as described (34). The purified DNA was digested to completion with *Cla* I, electrophoresed on a 1.5% agarose gel, and transferred onto a GeneScreen*Plus* membrane. The blot was probed with a 400-base-pair *Cla* I–*Hpa* I fragment from the *lacZ* gene by using published procedures (35).

RESULTS

The ER is thought to possess two independent nonacidic transactivation domains, one in the N-terminal region and another within the hormone binding domain (30). The hormone binding domain alone is able to confer ligand responsiveness (22, 36), whereas the N-terminal domain may modulate transactivation and other receptor functions. If ligand functioned solely by regulating the endogenous transactivation regions of the ER, it follows that the addition of a strong constitutive transactivation function onto the ER may create a receptor molecule having transcriptional activity even when bound to an estrogen antagonist. To evaluate this possibility, we utilized a chimeric receptor in which the acidic activator of the herpes simplex virus protein VP16 was inserted into the N-terminal region of the ER (26). This chimera has been shown to be 10-fold more potent than ER in activating transcription (26).

The activity of native and chimeric ER in response to hormone was assayed by cotransfection with a CAT reporter into mammalian CV-1 cells. The target promoter consists of EREs linked to a thymidine kinase promoter. As shown in Fig. 1 *A* and *B*, both ER and ER–VP16 require hormone to activate transcription. However, whereas the ER is not induced by the triphenylethylene antiestrogen nafoxidine in these cells, ER–VP16 activity can be induced by nafoxidine to a similar extent as with estradiol. The dose–response curve for nafoxidine is shifted compared to that for estradiol most likely because the ER has a 20–50 times lower affinity for nafoxidine (37). Since ER–VP16 has a powerful transactivating region that functions

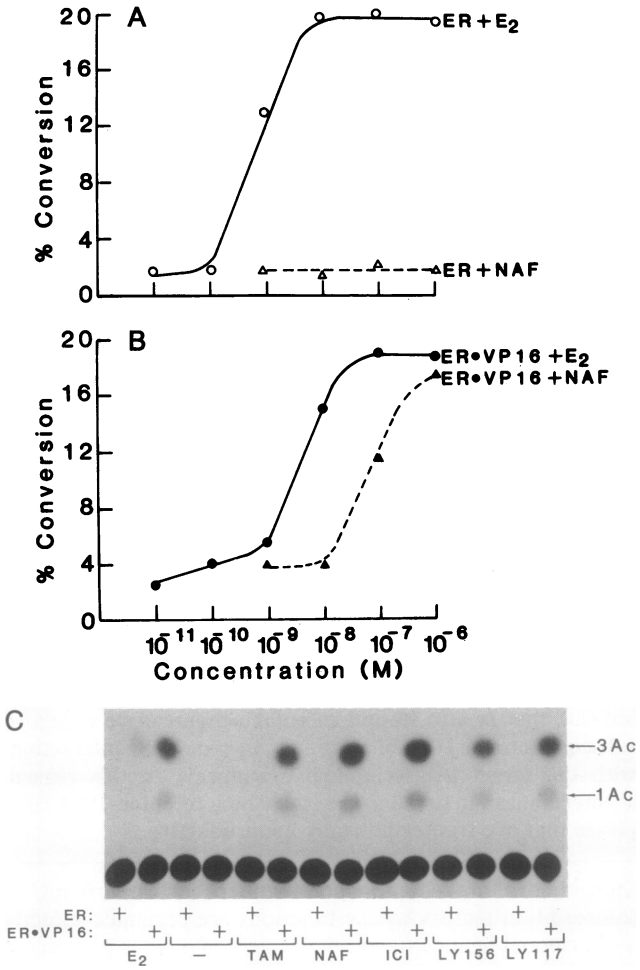

FIG. 1. ER–VP16 chimeras are inducible by both estrogen and antiestrogens in mammalian cells. (*A* and *B*) Stimulation of CAT activity by the ER and ER–VP16 exposed to various concentrations of estradiol (E_2) and nafoxidine (NAF). The CAT activity shown was obtained using 20 μg (*B*) or 100 μg (*A*) of protein from cellular extracts with assay incubation times of 2 and 4 hr, respectively. (*C*) Induction of CAT expression by ER and ER–VP16 in the absence (−) or presence (+) of 10 nM estradiol (E_2) or an antiestrogen at 0.1 μM: tamoxifen (TAM), nafoxidine (NAF), ICI 164,384 (ICI), LY156758 (LY156), and LY117018 (LY117). The same amount of protein (20 μg) was assayed in all lanes. Ac, acetyl group.

independent of ligand, it is likely that, in the absence of hormone, ER–VP16 is inactive because it is prevented from reaching its target element. These data then imply that both estradiol and nafoxidine are able to stimulate DNA binding. However, only the agonist can promote transactivation by native ER. It is interesting to note that all of the antiestrogens examined can induce transcription of ER–VP16 (Fig. 1*C*), suggesting that their functions are mechanistically similar.

Unexpectedly, the original cloned human ER (used in constructing the ER–VP16 chimera) was recently found to possess a point mutation in the hormone binding domain, which reduced the affinity of the receptor for hormone but appears to have negligible effects on other aspects of receptor function (38). When the activity of the wild-type human ER clone was examined in transient transfection, it was found to have a high level of constitutive activity. Apparently, the greater hormone binding affinity of the wild-type ER causes it to be activated by remaining traces of estrogenic substances in phenol-red-free charcoal-stripped mammalian cell culture medium (38). Therefore, to study further the ligand-dependent activity of the ER, we turned to a simpler eukaryotic system, *Saccharomyces cerevisiae* (bakers' yeast),

where ER function had been successfully reconstituted (39, 40). Yeast can be grown in defined synthetic medium, and thus the activity of ER in yeast is fully dependent on added hormone (ref. 38; also see below).

The wild-type human ER was expressed in yeast using a ubiquitin fusion construct driven by the copper-inducible yeast metallothionein (CUP1) promoter (Fig. 2*A*). As shown in Fig. 2*B*, the basal level expression of the ER is sufficient to activate transcription of a reporter plasmid (YRPE2) that contains EREs inserted upstream of the yeast CYC1 promoter fused to the *lacZ* reporter gene. LacZ activity is highly inducible by estrogen, by greater than 200-fold, with half-maximal activity at 0.5 nM estradiol. The antiestrogen nafoxidine is a competitive inhibitor of estradiol activity (Fig. 2*B*). Nafoxidine itself has weak agonistic activity in yeast. At 2 μM, a saturating concentration, the activity of nafoxidine is $\approx$15% of that obtained with estradiol. This is in general agreement with the results in animals in which nafoxidine has been shown to be a partial agonist (10%+ activity) (41, 42). Although the inhibition of estradiol activity by nafoxidine is relatively inefficient, it is competitive in nature. When 2 μM nafoxidine is used, at low levels of estradiol (from 1 to 5 nM) the inhibition of transcription is as great as 70%. When the estradiol concentration is increased, transcription is essentially restored. The reason that much higher levels of nafoxidine are required to inhibit estradiol is primarily a result of the known lower affinity of the ER for nafoxidine compared to estradiol (37). In short, ER function in yeast reflects its function in mammalian cells, indicating conserved regulatory mechanisms.

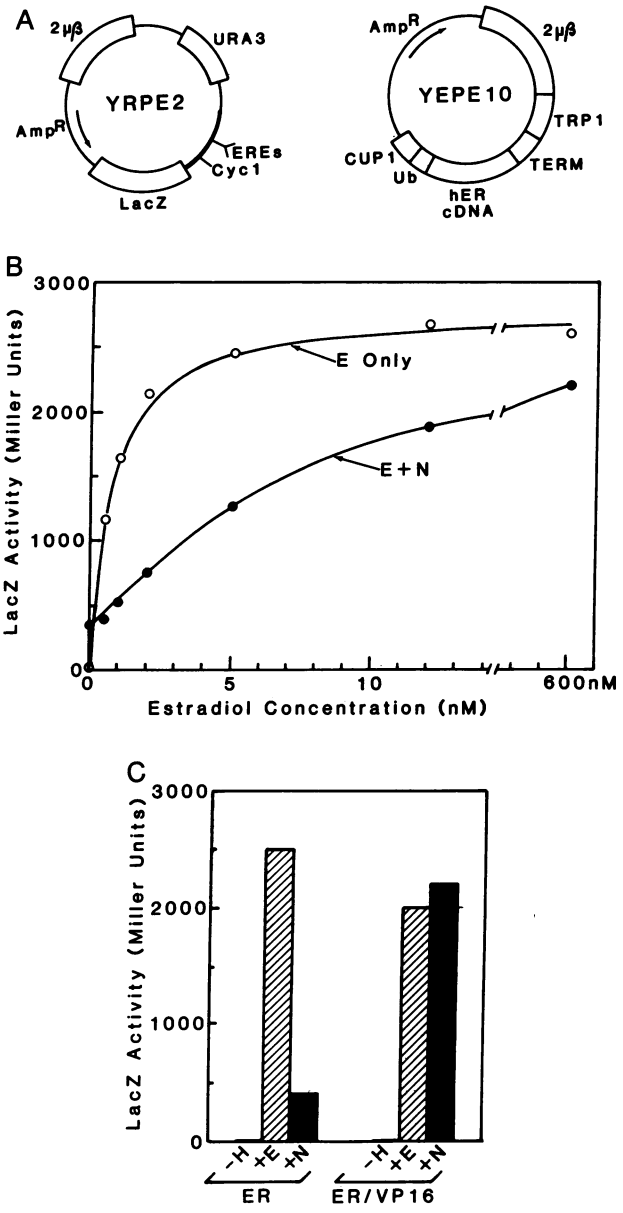

FIG. 2. Hormone and antihormone regulate ER function in *S. cerevisiae*. (*A*) Diagram of the 2-μm expression and reporter plasmids used in these studies. The reporter (YRPE2) contains two EREs inserted upstream of the CYC1 promoter. The expression plasmid (YEPE10) contains the wild-type human ER cDNA (hER) fused to ubiquitin and driven by the CUP1 promoter. (*B*) Induction of *lacZ* expression at various estradiol concentrations with estradiol (E) alone or with estradiol plus a constant concentration (2 μM) of nafoxidine (N). (*C*) Comparison of the transcriptional activity of ER and ER–VP16 in the presence of 0.6 μM estradiol (bars +E), 2 μM nafoxidine (bars +N), or without added hormone (bars −H).

In yeast, the ER–VP16 chimera (constructed from the wild-type human ER clone) is equally inducible by both estradiol and nafoxidine (Fig. 2*C*), supporting the mammalian transfection results described earlier. Although basal level expression of ER–VP16 results in the same level of transcription of *lacZ* compared to that obtained with ER, the ER–VP16 chimera is actually much more active than the ER, since the steady-state concentration of ER–VP16 is approximately 100 times lower than that of ER (data not shown).

A regulatable ER expression system in yeast presents us with another way to test the hypothesis that the observed antagonistic function of antiestrogens is due to interference with the DNA binding of the ER (43), resulting in only a small percentage of the target sites being bound by ER. If this hypothesis were correct, it should then be possible to increase the saturation of EREs *in vivo* by increasing the total amount of ER in the cell, thereby leading to a higher level of transcriptional activity. In our CUP1 promoter ER expression system, there is a substantial basal level of expression, but we can further induce ER expression by adding copper to the growth medium. ER levels can be increased approximately 6- to 10-fold from the basal level, as detected in a Western immunoblot (Fig. 3*A*). The induced ER is active in DNA binding, as determined by a mobility-shift assay (data not shown). However, even when ER expression is increased at least 6-fold, the amount of *lacZ* transcription in the presence of either estradiol or nafoxidine remains unchanged (Fig. 3*B*). These results suggest that even under basal level expression of ER the target sites are maximally occupied in the presence of either estradiol or nafoxidine. Consequently, the lower amount of transcription in the presence of nafoxidine is likely to be due to the intrinsically lower transactivation potential of the bound ER.

If antiestrogens can induce DNA binding of ER *in vivo*, a significant question is whether they would alter the DNase I sensitivity of the chromatin template at target responsive elements. It is known that the action of agonist ligands of steroid receptors is correlated with specific DNase I hypersensitive sites in chromatin (5–7). And, in one case, an antiglucocorticoid, dexamethasone 21-mesylate, was found to suppress the hormone-induced DNase I hypersensitive site over the mouse mammary tumor virus promoter (44). However, whether less active or inactive antihormone–receptor complexes that interact with responsive elements can induce similar alterations in chromatin structure is not known.

By using indirect end labeling, it is possible to examine directly alterations in DNase I sensitivity over the ERE–CYC1–lacZ reporter construct (Fig. 4*A*). As shown in Fig. 4*B*, both estradiol and nafoxidine can induce alterations in chromatin structure. Most notably, the promoter region becomes hypersensitive to DNase I. The hypersensitive sites are highly reproducible and are clearly not the result of artifacts in DNase I digestion. The same DNase I hypersen-

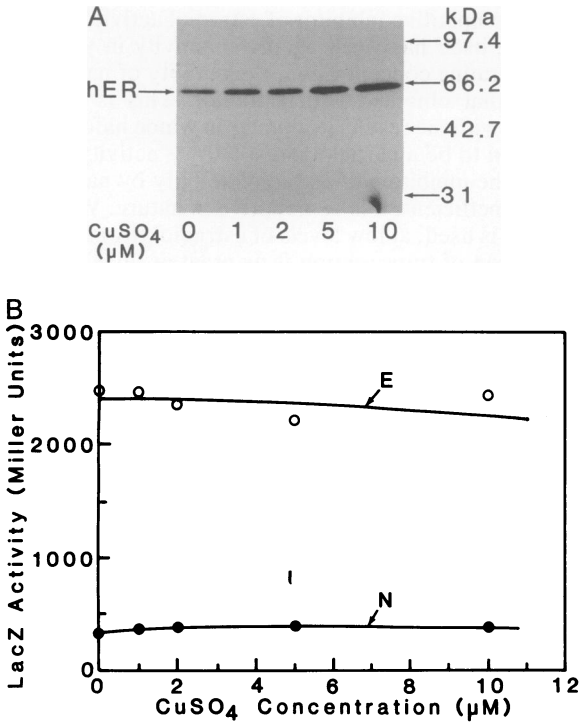

FIG. 3. Effect of the level of ER expression on transcriptional regulation by estradiol and nafoxidine. (*A*) Western blot analysis showing the levels of human ER (hER) expressed as a function of $CuSO_4$ concentration. Protein samples used in this blot were obtained from cells exposed to 2 μM nafoxidine for 4 hr. (*B*) Effect of $CuSO_4$ on the expression of *lacZ* in the presence of 0.6 μM estradiol (E) or 2 μM nafoxidine (N).

sitive sites are induced by estradiol and nafoxidine. These hypersensitive sites occur adjacent to the EREs; the actual ER binding sites are not hypersensitive. We believe that the interaction of the ER with the ERE alters chromatin structure, resulting in DNase I hypersensitivity, while the ERE itself is protected from DNase I by the bound ER. If nafoxidine-bound ER were to alter chromatin structure without occupying the ERE, one should observe a hypersensitive region without internal protection. The magnitude of the hypersensitivity induced by estradiol or nafoxidine is very similar, suggesting that chromatin structure alterations occur on the same percentage of the minichromosomes inside the cell. Again, this result suggests that the antihormone does not interfere with ER binding to its cognate ERE *in vivo*. However, the actual pattern of the hypersensitivity is slightly different and is perhaps a reflection of differences in the level of transcription. For reasons that are not clear, the transcribed region becomes less DNase I sensitive with active transcription. The ER has no effect on chromatin structure in the absence of hormone (Fig. 4*B*, compare lanes -H to -ER), consistent with the fact that hormone is required for DNA binding.

DISCUSSION

The transcriptional activation function of steroid hormone receptors is directly regulated by hormonal ligands, although exactly how this is achieved is not entirely clear. The hormone is not absolutely required for the receptor to bind to DNA (12–14) or to activate transcription *in vitro* (14). It has therefore been suggested that the role of the hormonal ligand is to overcome the effects of inhibitory factors that are complexed with the receptors *in vivo* but that readily dissociate during receptor isolation. To identify receptor functions under direct hormonal control, a useful strategy is to examine how receptor functions are affected in the presence of antihormones. In this

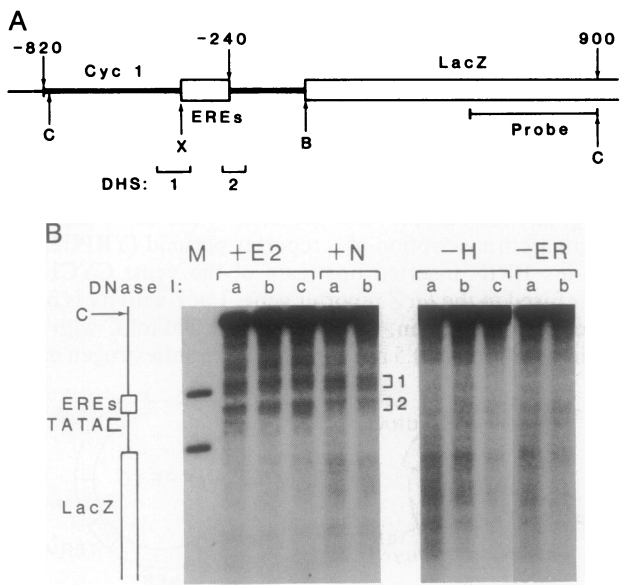

FIG. 4. Alterations in chromatin structure induced by estrogen and antiestrogen. (*A*) Diagram of the ERE–CYC1–*lacZ* reporter used in chromatin structure analysis. C, *Cla* I; X, *Xho* I, B, *Bam*HI. The probe used in Southern blot hybridization is a 400-base-pair *Cla* I–*Hpa* I fragment of *lacZ* sequences. DNase I hypersensitive sites (DHS) induced by hormone are shown. It corresponds to the data shown in *B*. (*B*) DNase I sensitivity of the chromatin analysed by indirect end labeling. The hypersensitive sites are bracketed and labeled 1 and 2. Shown are the DNase I sensitivity of the chromatin from cells not treated with hormone (lanes −H) and cells treated with nafoxidine (lanes +N) or estradiol (lanes E_2). Also shown are samples from cells that were not transformed with the expression plasmid (lanes −ER). DNase I digestion were performed at 600 units/ml for 6 min (lanes a), 300 units/ml for 6 min (lanes b), and 300 units/ml for 3 min (lanes c). The marker lane shows the position of the *Xho* I site (upper band) and the *Bam*HI site (lower band).

report, we present several lines of evidence that show that *in vivo* antiestrogens can promote DNA binding of the wild-type ER without efficiently inducing transactivation. This indicates that the activity of the ER can be regulated subsequent to DNA binding and reveals a requirement of hormone to potentiate the transactivation domains *in vivo*.

To examine whether the transactivation functions of the ER are normally under direct hormonal control, we characterized the activity of an ER chimera containing a strong unregulated transactivator. When the acidic region of VP16 is fused to the N-terminal region of the ER, the resulting chimera is still hormone-regulated, although it can be activated by what are normally estrogen antagonists. This illustrates dramatically that both estrogen and antiestrogens can induce conformational changes that allow the ER to bind DNA *in vivo*, although only the agonist promotes a conformation of wild-type ER that induces gene transactivation. Moreover, we have shown this to occur both in mammalian cells and in yeast cells. An important consideration germane to the interpretation of the experiment is whether the insertion of the VP16 acidic transactivator has altered the structure of the hormone binding domain such that the ligand–receptor interactions becomes fundamentally altered. We believe that this is highly unlikely because hormone binding assays do not reveal significant differences in ligand binding affinities between the ER and ER–VP16 *in vitro* (unpublished data). Also, the dose–response curve for estradiol is comparable between the ER and ER–VP16, in view of the limits of the transient transfection assay.

All of the antiestrogens examined can activate ER–VP16 efficiently. Thus the steroidal antiestrogen ICI 164,384 (45), thought to be a pure antagonist, appears to be able to induce

DNA binding as efficiently as the triphenylethylene antiestrogens tamoxifen or nafoxidine, partial agonists in most cell types (36). These results suggest that these antagonists differ primarily in the extent to which they allow ER to interact conformationally with the transcriptional machinery.

Fawell *et al.* (46) reported evidence *in vitro* that suggests that the antiestrogen ICI 164,384 can inhibit DNA binding by interfering with receptor dimerization. Although this may appear to contradict our results, we believe that the two observations can be reconciled. One possibility is that the VP16 transactivation domain may have a dimerization function that has not been identified. However, a more likely explanation is that the dimerization function associated with the hormone binding domain (47) is not absolutely required for DNA binding, although it may enhance the binding affinity. It is possible that the concentration of ER in transiently transfected cells is sufficiently high to overcome the decrease in affinity resulting from an inhibition of dimerization. In fact, it has been shown that an ER derivative in which the hormone binding domain (and dimerization motif) has been deleted has transcriptional activity and, indeed, even full transcriptional activity in some cell types (30, 36). Finally, it should be noted that our result with ICI 164,384 is in agreement with that of Martinez and Wahli (11) who have shown that this antiestrogen can stimulate the binding of ER to vitellogenin EREs *in vitro*.

The observations stemming from the ER–VP16 experiments are confirmed by further experiments performed in yeast. We chose yeast as a model experimental system because it allows us to conveniently examine the interactions of the ER with more natural chromatin templates (the 2-μm minichromosome), in contrast to transient transfection of mammalian cells, and to probe directly the interaction of ER with chromatin. We have shown in the yeast system that under conditions in which target EREs appear to be saturated with receptor bound to estrogen or antiestrogen, antiestrogen–receptor complexes elicit a lower level of transcription. An alternative interpretation of this experiment is that some component other than the DNA target is actually saturated. However, this appears unlikely since the level of *lacZ* expression induced by the antiestrogen is only 15% of that obtainable with estrogen and, therefore, should not saturate the transcription/translation machinery. In the presence of the antiestrogen nafoxidine, the interactions of the ER with the chromatin template induce DNase I sensitivity surrounding the EREs while ER binding protects the EREs themselves from DNase I. Estradiol and nafoxidine induce similar patterns of DNase I sensitivity, suggesting similar interactions of the receptor with the chromatin template.

Although yeast is not a natural system for the ER, our data clearly indicate that the regulation of the ER in yeast reflects its regulation in animal systems. This strongly suggests that conserved mechanisms are involved and that an understanding of how the ER functions in yeast should prove useful in understanding how it regulates gene expression in animal cells.

The results presented in this report offer an explanation for how antiestrogens devoid of agonistic activity can exert estrogen-independent effects on cell growth and proliferation. By forming nonproductive receptor complexes at target gene promoters, antiestrogens may interfere with basal-level transcription. Finally, our results may have relevance to the future design of pharmacologic agonists or antagonists of steroid hormones. For example, if we accept the principle that ER can bind DNA in a transcriptionally less active or inactive form, it appears conceivable that prospective antagonists could trap the receptor in a variety of allosteric forms that interact with the transcriptional machinery to various degrees.

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