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Ligand dependence of estrogen receptor induced changes in chromatin structure

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ABSTRACT

To determine whether the human estrogen receptor requires ligand to bind to its cognate estrogen receptor element (ERE) *in vivo*, we have examined the structure of chromatin at a chromosomally integrated ERE-URA3 reporter gene in yeast, and the influence of ligand bound and ligand free estrogen receptors on that structure. Using indirect end-labelling to map DNaseI and micrococcal nuclease sensitive sites, we found that receptor induced alterations in chromatin structure were completely dependent upon the presence of estradiol. These same alterations in chromatin structure were induced by a truncated estrogen receptor with both TAF-1 and TAF-2 transactivation functions deleted, suggesting that DNA binding *per se* disrupts chromatin structure. These results support models in which the estrogen receptor requires ligand to bind to the ERE *in vivo*.

INTRODUCTION

Steroid hormone receptors are ligand inducible enhancer binding transcription factors (for a review see 1). Although the functions of these receptors are dependent upon the presence of hormone, the precise role of ligand is not clear (for a review see 2). Amongst the transformations that have been attributed to the association of receptor with hormone are: the release of the receptor from heat shock proteins, nuclear localization, dimerization, binding to DNA at the hormone response element, and conformational changes leading to the activation of transcription. In the case of the estrogen receptor (ER), it has been shown that the receptor resides in the nucleus in the presence or absence of estradiol (2, and refs. therein), however, the requirement of ligand for the association of the ER with the estrogen receptor element (ERE) has been a matter of controversy. Gel mobility assays have led to conflicting results (3, 4) that have been attributed to subtle differences in the preparation of receptor extracts. In cases where binding of receptor was ligand dependent (3), it was also found that anti-estrogens can promote DNA binding, suggesting that DNA binding is not sufficient to activate transcription. Presumably, conformational changes in the structure of the receptor, not required for DNA binding *per se*, must occur in order to activate transcription. This interpretation is supported by the recent observation that an ER containing a foreign activation domain, hence an atypical conformation, can activate transcription in response to anti-estrogens (5).

Most *in vivo* studies of steriod receptor binding have focused on the glucocorticoid receptor, where significant hormone dependent changes in chromatin structure have been mapped with nucleotide resolution (6–9). This work has concluded that the effects of hormone addition on chromatin structure are due to the binding of other proteins recruited by the receptor (7, 9), leaving open the question of whether or not the receptor is bound prior to hormone addition. Similar studies with the ER, carried out at lower resolution, have demonstrated estradiol dependent changes in chromatin structure (5, 10), however, these studies were not able to distinguish whether the chromatin structure alterations were the result of estradiol induced binding of the ER to DNA, or to the activation of transcription by a constitutively bound ER. Pham *et al.* (11) have shown that receptor binding to the ERE can affect chromatin structure without activating transcription, but have proposed that the transactivation functions of the receptor facilitate the disruption of chromatin structure. Finally, some alterations in chromatin structure have been observed at estradiol responsive promotors in tissues expressing the ER but not yet exposed to estradiol (10), although it is not known whether these alterations are caused by the presence of the receptor.

The demonstration that the human estrogen receptor (hER) functions in yeast (12) has made it possible to address questions of hER function in a less complex and more manipulable organism. To specifically address whether the ER requires estradiol to interact with the ERE, we have examined the chromatin structure of an estradiol responsive promotor integrated into the genome of *S.cerevisiae*. We show that receptor induced changes in chromatin structure occurring at this promotor are completely dependent upon the addition of hormone to yeast expressing the wild type hER (HEG0). These same changes can

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be induced by a truncated receptor derivative in which the transactivation functions of the ER have been deleted, suggesting that DNA binding *per se* is sufficient to alter chromatin structure and that the binding of hER to DNA is dependent upon ligand *in vivo*.

MATERIALS AND METHODS

Yeast strains and plasmids

The construction of PL3 (*ura3*-Δ, his3-Δ, leu2-Δ, trp1::*3ERE-URA3*) has been described elsewhere (Pierrat,B., Heery,D., Lemoine,Y., and R.L., submitted). The 2 μ plasmid pYE90, used to express all receptors, as well as the construction of YE90 expressing HEG0 , has been described (Pierrat, *et al.*, submitted). NCDF was made by deleting DNA between the two XhoI sites representing amino acids 287−552 of HEO in the receptor VE11, described in D.M.G., Heery,D, R.L., Lemoine,Y. and P.C. (submitted). Briefly, VE11 contains a short N-terminal leader sequence linked to amino acids 176−595 of the complete estrogen receptor, HEO. Strains were transformed by electroporation as described in Pierrat *et al.* (submitted)

Chromatin preparation and digestion

All experiments shown in this report were carried out at least twice, with identical results each time. Yeast were grown in 500 ml of 0.7% yeast nitrogen base supplemented with 2% glucose, 20 mg/l uracil, 20 mg/l tryptophan, leucine dropout mix (30 mg/l leucine, 30 mg/l isoleucine, 150 mg/l valine) and 100 nM estradiol (where indicated) at 30°C to 10^7-10^8 cells/ml. Cells were harvested in 250 ml bottles and resuspended in 50 mM Tris (pH = 7.5) 30mM DTT at 10^8 cells/ml, transferred to 50 ml centrifuge tubes and incubated for 15 min at 30°C. Cells were then centrifuged and resuspended in 1.1 M sorbitol at 10^9 cells/ml in the same tubes. 1/10 volume of 10 mg/ml zymolase was added and spheroblastification at 30°C was allowed to proceed to about 90% (usually about 15 minutes.). The tubes were then filled to the top (at least 5 volumes) with cold YPD sorbitol (2% bacto-peptone, 1% yeast extract, 2% glucose, 1.1 M sorbitol) supplemented with 100 nM estradiol where appropriate. Tubes were centrifuged gently and resuspended at 2×10^9 cells/ml in cold 'Buffer Y' (15 mM Tris 7.4, 60 mM KCl, 15 mM NaCl, 0.5% Triton-X-100, and 3m MEDTA, stored at 4°C indefinitely) supplemented just prior to use with .75 mM DTT, 5 mM spermidine, 0.15 mM spermine, 10 μg/ml each of the protease inhibitors leupeptin, aprotinin, and pepstatinA, 1 mM PMSF, 5 mM $CaCl_2$ and 100 nM estradiol where appropriate (Note: lower spermidine concentrations were found to give 'half-nucleosome particles' and the addition of magnesium led to the degradation of DNA — D.M.G., unpublished data.). Spheroblasts were lysed by intermittent vortexing while remaining on ice and equal aliquots (150−300 μl) were removed to 1.5 ml eppindorf tubes at 20°C. Micrococcal nuclease or DNaseI was added to the indicated final concentrations and digestion was allowed to proceed for 20 minutes. The reaction was stopped by the addition of an equal volume of 2% SDS, 200 mM Tris (pH = 8.0), 50 mM EDTA, and 200 μg/ml proteinase K, mixing and placing at 65°C for several hours to overnight. Samples were extracted once with phenol:chloroform:isoamyl alchohol 25:24:1 (PCI), once with chloroform:isoamyl alchohol 24:1 (CI), and ammonium acetate was added to 2.5 M. After one hour to overnight at 4°C samples were spun in a microfuge for 15 minutes and the supernatent was transferred (discarding a visible white pellet) to a fresh tube (or two tubes each if necessary) and the DNA precipitated with two volumes of ethanol. After centrifuging and drying, the DNA was resuspended in 300 μl TE (10 mM Tris 8.0, 1mM EDTA) and 100 μg/ml RNaseA and placed at 55°C for several hours to overnight. Samples were then extracted in PCI and CI, incubated in 2.5 M ammonium acetate, centrifuged, and the supernatant precipitated in ethanol as before. The purified DNA pellet was washed in room temperature 70% ethanol, dried and resuspended in 1/3 the original volume (of the chromatin digestion) of TE.

Naked DNA digestions were done using DNA from undigested samples that were prepared exactly as described above except that they were resuspended in one original volume of 'Naked DNA digestion buffer' (15 mM Tris 7.5, 60 mM KCl, 15 mM NaCl and 1 mM $CaCl_2$, made in advance and stored at 4°C indefinitely) and placed at 20°C. Micrococcal nuclease and DNaseI were added to the indicated concentrations and the reactions allowed to proceed for 10 minutes, at which time they were stopped and incubated at 65°C as with the chromatin digests. Samples were then extracted once in PCI, once in CI, ammonium acetate was added to 2.5 M and the samples were precipitated in 2 volumes of ethanol. After drying and washing with 70% ethanol, samples were resuspended in 1/3 the original volume of TE.

In this manner we noticed no significant sample to sample variations in the amounts of DNA after ethidium bromide staining or hybridization, and exact quantitation was not necessary. The levels of micrococcal nuclease and DNaseI digestion of different preparations of chromatin with the same concentrations of enzyme were practically indistinguishable, and a 160 bp ladder distinguishing 5−7 bands was always observed after micrococcal nuclease digestion. If the ammonium acetate cut was left out of this protocol, naked DNA would not digest with micrococcal nuclease or DNaseI unless diluted by a factor of 5−10. We assume that this step removes some inhibitor of nuclease activity.

DNA analysis

Aliquots of the above samples were tested on 2% agarose gels to match the relative levels of digestion of naked and chromatin digested DNA as well as to verify the quantity and quality of digestion. 10 μl of each sample was then removed and re-digested (where indicated) in a total of 20 μl of New England BioLabs buffer 4 with 10 units each of ApaI and BglII for 8 hours to overnight at 37°C. Samples were migrated in 20 cm long 2% agarose gels until the bromophenol blue dye had migrated 17 cm. DNA size markers were HindIII digested SV40, mixed with the samples to avoid migration artifacts. The migration of markers was determined by autoradiography (after hybridization with nick-translated SV40 DNA). Gels were denatured in 0.4 M NaOH, 0.6 M NaCl and transferred in the same solution to Hybond for 6 hours, filters were rinsed in 2×SSC and dried overnight. The filters were then pre-washed in 50 mM Tris, 1% SDS, and 1 M NaCl for 1 hour, pre-hybridized for 2−3 hours in 10% Dextran sulfate, 1 M NaCl, 1% SDS, and hybridized after the addition of denatured (boiling 5 minutes) probe overnight, all at 65°C. Filters were washed with 4 changes of 2×SSC, 1% SDS at 60°C and exposed to film for 2−4 days with an intensifying screen at −80°C. Probe was stripped from the filters for re-hybridization by boiling for 30 minutes in 0.1×SSC and 1% SDS.

Probes were prepared by mixing 2 μl of purified fragment (about 50 ng) with 1 μl (1 μg) each of two 8 bp oligonucleotides

complementary to the two 3′ ends of the fragment used as a probe, 2 μl of 100 mM Tris 7.5, 50 mM $MgCl_2$, and 8 μl of distilled water. This was then boiled for 5 minutes, chilled immediately on ice, centrifuged for several seconds and the reaction started by the addition of 1 μl of Klenow fragment (10 units/μl) and 5 μl of a mixture containing all four nucleotides with A and C labelled to 800 mCi/mmol at standard nick translation concentrations. This was incubated at 8°C on ice water and the temperature of the bath was allowed to rise to room temperature overnight. In this manner small fragments could be labelled by specific priming from the ends to make full length probes. Probe was purified with an Elutip (Schlieser and Schuell) using the manufacturers protocol and eluted in 400 μl into a tube containing 150 μl of 10% SDS and 150 μl of sonicated salmon sperm DNA (10 mg/ml).

OMPdecase activity

OMPdecase activity was measured according to the method of Wolcott and Ross (13), with crude extracts obtained as previously described (14). Enzymatic activity was expressed in nanomoles of substrate transformed per minute per milligram of protein. Protein content was assayed by the method of Bradford (15).

RESULTS

Estradiol dependent DNase I hypersensitive sites near the ERE

Yeast strain PL3 (Pierrat,B., Heery,D., Lemoine,Y., and R.L., submitted) contains an ER responsive reporter gene consisting of the entire yeast URA3 gene and its promotor, with the UAS sequences responsible for both basal and activated transcription replaced by three ERE's. This reporter gene has been integrated at the TRP1 locus of PL3, in which the natural URA3 gene has been deleted, and the measurement of OMPdecase activity, the product of the URA3 gene, has been shown to provide a sensitive measure of ER-mediated activation. URA3 expression in PL3 expressing the ER has been shown to be strictly estradiol dependent (Pierrat,B., Heery,D., Lemoine,Y., and R.L., submitted). Since each cell contains a single copy of the reporter gene at the same chromosomal location with no basal rate of transcription, this system provides several advantages over existing lacZ based plasmidic reporter systems that often result in a high basal level of expression (12) and cell to cell variability in copy number (16). In addition, positioned nucleosomes in the vicinity of the URA3 and TRP1 genes have been mapped (17), whereas others have reported that mapping the positions of nucleosomes on yeast promotor-lacZ fusion plasmids is hindered by the presence of the bacterial lacZ sequence on which nucleosomes may not be phased at regular intervals (18).

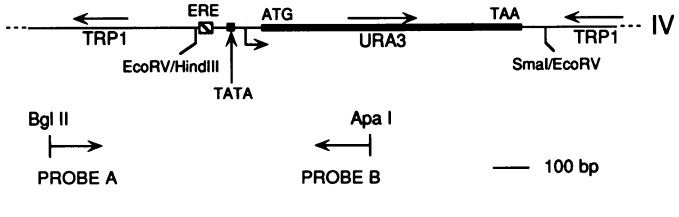


Figure 1. Structure of the ERE-URA3 reporter. A HindIII-SmaI DNA fragment containing the URA3 coding sequence fused to an ERE-URA3 hybrid promotor was cloned into the EcoRV site of TRP1 in a reverse orientation (arrows over structural genes indicate the orientation of the open reading frames). The TRP1 gene disrupted by the ERE-URA3 gene was introduced back into a ura3 strain by gene replacement at the TRP1 locus located on chromosome IV. The bent arrow indicates the transcription initiation region of URA3. The probes A and B used in Southern blot hybridization are a 237bp BglII-HindIII fragment of TRP1 and a 190 bp ApaI-EcoRV fragment of URA3, respectively.

The structure of the PL3 genome at the TRP1 locus is schematized in Figure 1. The positions of DNaseI and micrococcal nuclease cutting sites were determined relative to the *ApaI* and *BglII* sites shown in the figure using the indirect end labeling procedure (19, 20). The *ApaI/BglII* restriction fragment conveniently places the ERE's at a position roughly equidistant from the ends. Thus, by restricting genomic DNA simultaneously with both enzymes and hybridizing the resulting Southern blots sequentially with probeA and probeB (shown in Figure 1), we were able to map the positions of cutting sites to within 20 bp, with the most accurate positions at the centrally located ERE's.

We began by determining whether a DNaseI hypersensitive site was present at the position of the three ERE's in PL3, and whether the sensitivity of DNA at this site was affected by the presence of ligand activated or inactivated HEG0 expressed from a multicopy plasmid. DNaseI digests chromatin most efficiently where large windows of exposed DNA allow access of the enzyme to both strands of the DNA, where it must make complimentary single stranded nicks in order to detect a cut in non-denaturing agarose gels (20). This often occurs at important regulatory sites where the interaction of other proteins has prevented the formation of nucleosomes (21). Chromatin isolated from PL3 cells containing no HEG0, or containing HEG0 and grown in the presence or absence of estradiol, was digested with limiting concentrations of DNaseI and the cutting sites were mapped as described (Figure 2). In the absence of estradiol, there was no detectable effect of HEG0 expression on chromatin prepared from PL3. However, when estradiol was added to cultures of PL3 expressing HEG0, DNase I hypersensitive sites were induced on either side of the ERE's. Figure 2C shows the positions of those hypersensitive sites.

Estradiol dependent alterations in micrococcal nuclease cutting sites near the ERE

To examine the chromatin structure at this locus in more detail, we digested these same preparations of chromatin with limiting amounts of micrococcal nuclease. In addition to cutting at large windows of exposed DNA like DNAse I, micrococcal nuclease also cuts efficiently at the linker DNA between each nucleosome (20) or at the boundaries of non-nucleosomal proteins (22), and thus can provide supplementary information resulting from changes in the presence and positions of nucleosomes and other chromatin proteins. Digestion of PL3 chromatin with micrococcal nuclease resulted in the release of fragments of genomic DNA whose lengths were integral units of 160 bp (data not shown), typical of yeast nucleosomes (22). The positions of the micrococcal nuclease cutting sites in the vicinity of the ERE's were then mapped after redigestion with *ApaI/BglII* and indirect end labeling analysis with probes A and B as previously described (Figure 3). Although preferential cutting sites for micrococcal nuclease are altered in chromatin relative to the preferred cutting sites in naked DNA, there was no detectable effect of the expression of HEG0 on the pattern of digestion of PL3 chromatin until HEG0 was activated by estradiol, consistent with the results with DNase I digestion. The most dramatic effect of estradiol addition is the sensitivity of a strong naked DNA cutting site

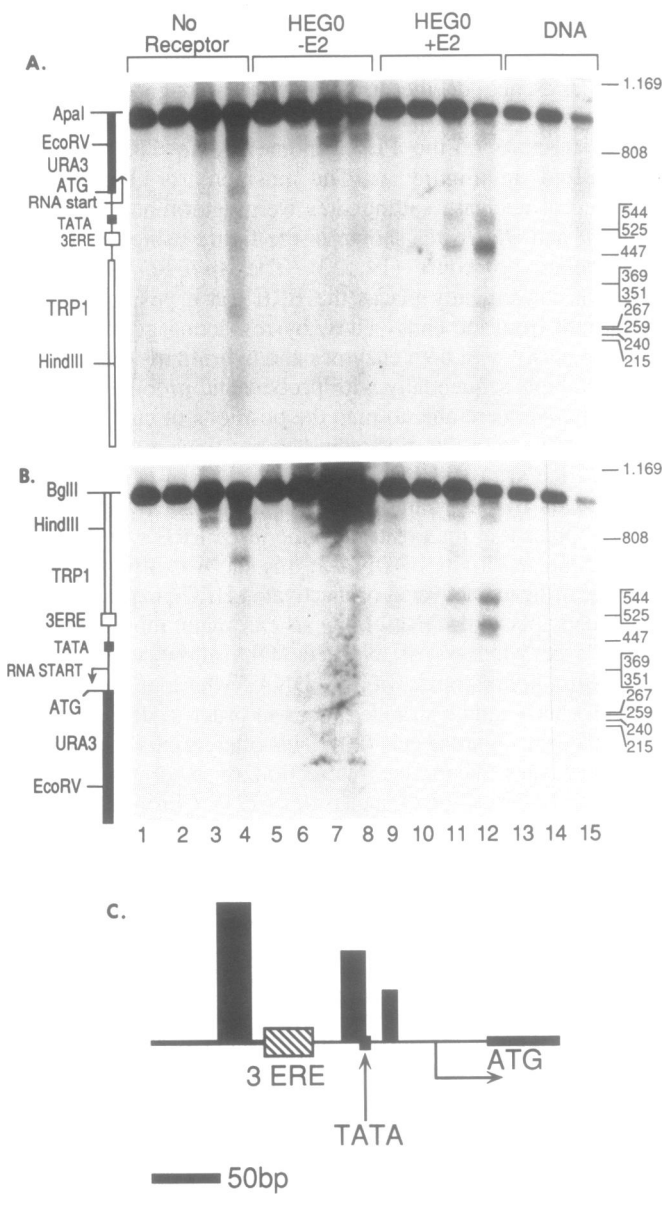


Figure 2. Mapping of the DNaseI hypersensitive sites induced by estradiol. PL3 was transformed with pYE90 (No receptor) or pYE90 expressing HEG0 (HEG0) and was grown in the absence (−E2) or presence (+E2) of estradiol. Chromatin was digested with 2 μg/ml (lanes 1, 5 and 9), 5 μg/ml (lanes 2, 6 and 10), 10 μg/ml (lanes 3, 7 and 11), and 20 μg/ml (lanes 4, 8 and 12) of DNaseI. Since DNaseI displays a limited but not insignificant site specificity (20), it was necessary to similarly digest naked genomic DNA and to compare the preferential cutting sites in chromatin to that of naked DNA. Naked DNA (DNA) was digested with 0.2 μg/ml (lane 13), 0.5 μg/ml (lane 14) and 1.0 μg/ml (lane 15) of DNaseI. After digestion, DNA was purified and re-digested with ApaI and BglII, and Southern blot analysis was performed, hybridizing with A.) probe A or B.) probe B. The positions of HindIII digested SV40 molecular weight markers as they migrated in the right most DNA lane are shown to the right (individual lanes show slight variations in migration, this was controlled for by including markers in each lane as described in Materials and Methods). A diagram of the ApaI/BglII fragment is shown to the left of each autoradiogram to serve as a reference. C. Positions of DNaseI cutting sites in the vicinity of the ERE's were mapped based on the migration of molecular weight markers that were included in each of the DNA lanes shown in A.) and B.) (see Materials and Methods). These positions were calculated relative to the BglII and ApaI sites using probes A and B, and the width of the bars shown represents the difference between the calculated cutting site positions using these two probes. The lengths of the vertical lines are intended to reflect the relative frequencies of digestion but are not necessarily in calculated proportion.

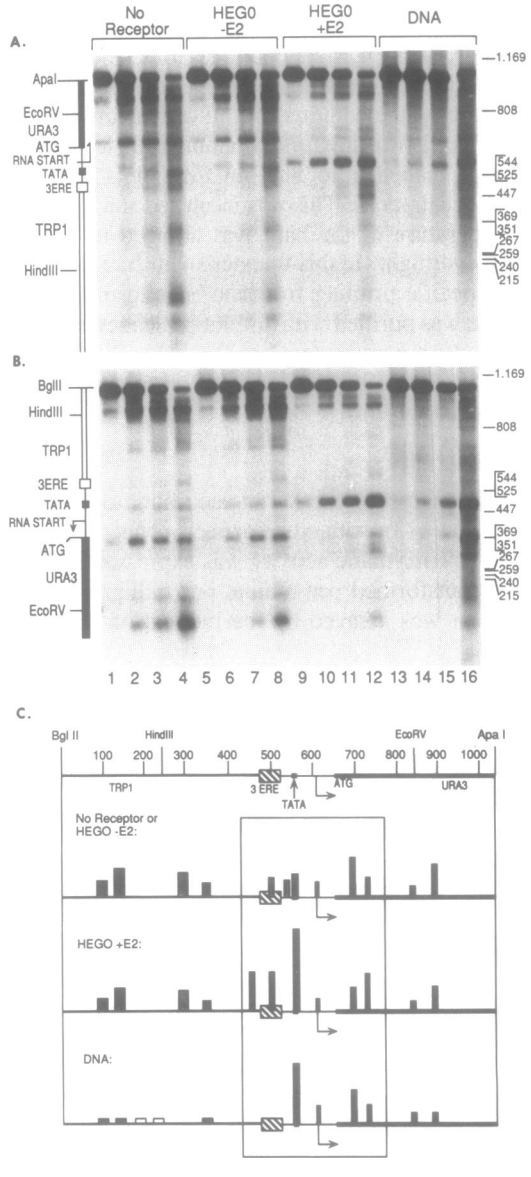


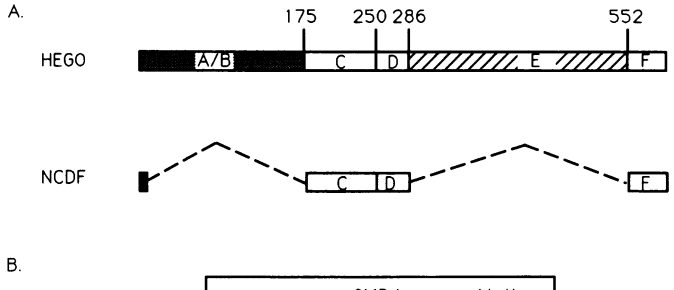
Figure 3. Mapping the micrococcal nuclease cutting sites in PL3 chromatin. Chromatin prepared from PL3 was digested with 100 units/ml (lanes 1, 5 and 9), 200 units/ml (lanes 2, 6 and 10), 400 units/ml (lanes 3, 7 and 11) or 800 units/ml (lanes 4, 7 and 12) of micrococcal nuclease. Naked DNA was digested with 0.5 (lane 13), 1.0 (lane 14), 2.0 (lane 15), and 5.0 (lane 16) units/ml of micrococcal nuclease. DNA was subsequently purified and re-digested with ApaI and BglII, and Southern blot analysis was performed, hybridizing with A.) probe A or B.) probe B. A reference diagram of the ApaI/BglII fragment is shown to the left of each autoradiogram, as in Figure 2. Due to the extreme sensitivity of the cutting site near the TATA box, especially in induced chromatin, the first cut within the fragment usually occurs at this site making sites distal to the TATA box difficult to detect without prolonged exposure of the autoradiogram. Thus, sites downstream of the TATA box are more readily visualized with probe B and sites upstream of the TATA box are more readily visualized with probe A. C.) Positions of the micrococcal nuclease cutting sites were calculated as for the DNaseI cutting sites in Figure 2. Micrococcal nuclease exhibits considerable site specificity (20), therefore, the the naked DNA (DNA) cutting sites are included for comparison. Sites that were detectable under all conditions are indicated by the black bars, sites found only in naked DNA are in white, and sites found only in chromatin are stippled. Sites whose sensitivity is altered by estradiol are contained within the boxed region. Some of the cleavage sites not seen in the autoradiograms were detected in longer exposures and only the positions of the bands that were repeatedly seen in multiple experiments are indicated. As in Figure 2, the lengths of the vertical lines are intended to reflect the relative frequencies of digestion but are not necessarily in calculated proportion.

located very close to the TATA box of the URA3 promotor. In the absence of estradiol (or in the absence of HEG0) this site is partially protected relative to naked DNA and an additional cutting site upstream of the TATA box, not found in naked DNA, can be observed (Figure 3A and C). Upon the addition of estradiol the site near the TATA box becomes the dominant cutting site and the upstream cutting site is no longer observed. In addition, a site upstream of the ERE's was found only in induced chromatin, and a site mapping within the ERE's that was not detected in naked DNA was enhanced by the addition of estradiol. Finally, reciprocal changes were seen in the sensitivities of two sites downstream of the transcription start site. The positions of micrococcal nuclease cutting sites are shown in Figure 3C, with the sites affected by estradiol contained within the boxed area.

Chromatin structure alterations are not the result of induced levels of transcription

The changes in chromatin structure could be the direct result of receptor binding, however, an alternative explanation is that they are the result of transcription *per se* and/or the binding of basic transcription factors. This was of particular concern since one of the most prominent changes in both DNaseI and micrococcal nuclease sensitivity occured very near to the TATA box. To distinguish between these possibilities we removed all known transcriptional activating domains of HEG0 and tested whether DNA binding alone can alter chromatin structure. Figure 4A shows a schematic diagram of HEG0 and the truncated receptor derivative, NCDF. NCDF contains deletions of the A/B activating domain and the hormone binding domain (HBD or E domain); both of these domains contain transcriptional activating regions that function in yeast (Pierrat,B., Heery,D., Lemoine,Y. and R.L., submitted) and mammalian cells (23). The binding of this deletion derivative to the ERE-URA3 promotor resulted in less than 0.5% the level of induction of URA3 achieved by HEG0 (Figure 4B). Since the hormone binding domain of the ER has been removed in NCDF, the binding of this protein to DNA is independent of the addition of hormone (24), thus a comparison can be made between the effect of NCDF expression, independent of the presence of estradiol, and the effect of the ligand bound HEGO.

Figure 5A and B shows the DNaseI and micrococcal nuclease digestion patterns of chromatin prepared from PL3 expressing NCDF. Representative samples from PL3 expressing HEG0 and grown in the presence and absence of estradiol are included for comparison (The digestion pattern of chromatin from PL3 in the absence of a receptor is identical to HEG0−E2; see Figure 3A and B). Expression of NCDF induced DNaseI sensitive sites at the same locations as HEG0. In addition, the dramatic increase in sensitivity of a micrococcal nuclease cutting site near the TATA



Receptor	OMPdecase activity	
	-E2	+E2
HEGO	<0.01	25.00
NCDF	0.12	0.08

Figure 4. Enzyme induction by HEG0 and NCDF. A. Schematic diagram of HEG0 and NCDF. The A/B domain of HEG0 was replaced with a small N-terminal leader sequence and the E domain (HBD) was deleted to make NCDF (see Materials and Methods). Numbers above the domains refer to the amino acids of the estrogen receptor. B. Enzyme activity in PL3 expressing HEG0 or NCDF. Receptors were expressed from a multicopy plasmid, pYE90. Receptor protein levels were verified to be similar by Western blotting (not shown).

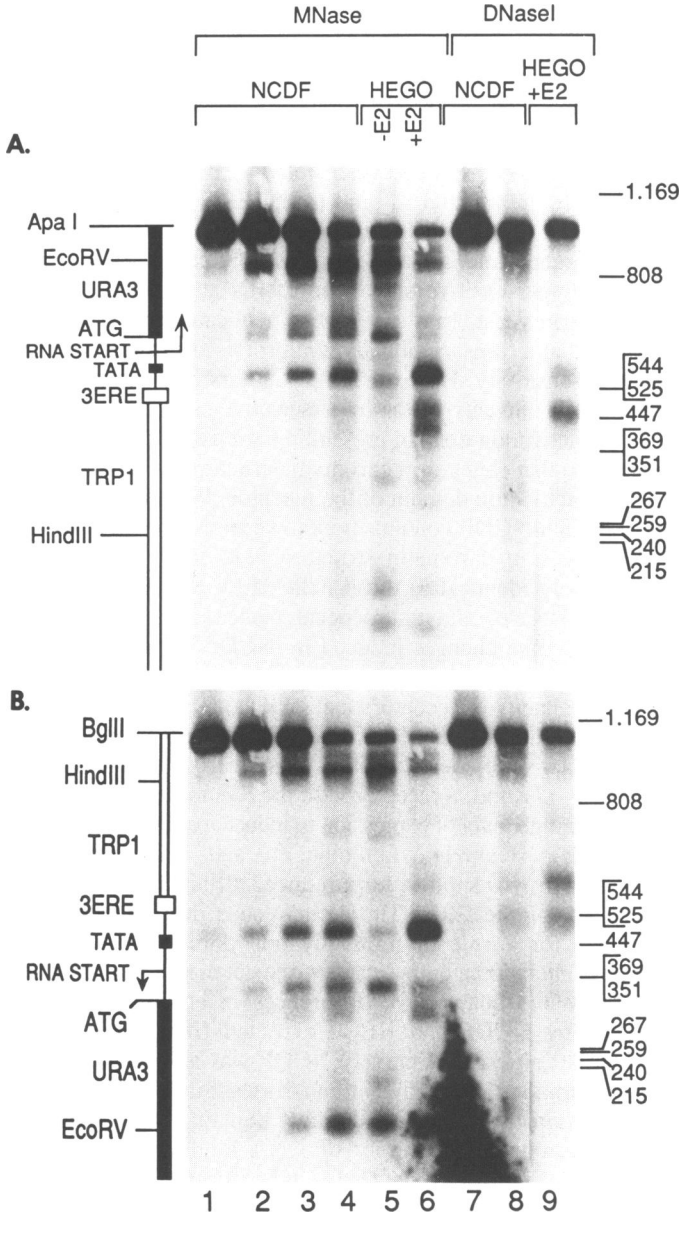


Figure 5. Chromatin structure changes induced by NCDF in PL3. PL3 was transformed with pYE90 expressing NCDF and chromatin was prepared and analyzed as in previous figures. Chromatin was digested with 100, 200, 400, or 800 units/ml of micrococcal nuclease (MNase — lanes 1−4, respectively) or with 10 and 20 μg/ml DNaseI (lanes 7 and 8, respectively). Southern analysis was performed as before, hybridizing with A.) probe A or B.) probe B. Lanes 5, 6 and 9 are samples of the same DNA shown in lanes 8 and 12 of Figure 4 and lane 9 of figure 2, respectively, run in parallel for comparison.

box, loss of an adjacent upstream cutting site, the appearance of a site upstream of the ERE, an increase in sensitivity of a site within the ERE's, as well as reciprocal changes in sensitivity of sites downstream of the RNA start site, are all induced by NCDF. Thus, although the extent to which nuclease sensitivity of chromatin was altered in the presence of NCDF is quantitatively less than in the presence of HEG0 (as is expected since NCDF cannot bind DNA as tightly as HEG0 — see discussion), qualitatively, NCDF induced precisely the same alterations in chromatin structure as were induced by HEG0.

DISCUSSION

The results presented in this report show that ER induced alterations in chromatin structure in the vicinity of an ERE in yeast are dependent on the presence of ligand. These changes can be induced by a protein consisting of little more than the DBD of the hER and do not require induced levels of transcription. The simplest interpretation of these results is that the ER requires ligand to bind to the ERE *in vivo*.

In support of our conclusions, Pham *et al.* (5) have shown that both estradiol and an anti-estrogen (nafoxidine) can induce DNaseI hypersensitive sites near ERE's at an ER responsive promotor in yeast. In a subsequent publication (11), they also showed that a transcriptionally compromised receptor can alter DNaseI sensitivity. However, these authors found that the exact pattern and intensity of the hypersensitive sites depended upon the transactivation domain present in various ER derivatives and concluded that changes in chromatin structure are influenced by the transactivation domain of the receptor. We have found that, even though NCDF contains no known transactivation domain, the changes in chromatin structure induced by NCDF were qualitatively identical to those induced by HEG0 as measured by both DNaseI and micrococcal nuclease sensitivities. The intensity of the changes induced by NCDF is reduced relative to HEG0, however, this is expected due to the reduced DNA binding capacity of receptors that lack the HBD (3). In fact, we have found that, while HEG0 can activate transcription in PL3 with similar efficiencies when expressed from a low copy or a multicopy plasmid, a receptor with the E domain (HBD) deleted requires a high level of expression to induce maximal transcription (Pierrat,B., Heery,D., Lemoine,Y., and R.L., submitted), providing *in vivo* support for a reduced affinity of HBD deleted receptors for the ERE. Thus, our results do not provide any evidence for a role of transactivation domains in chromatin structure alterations, but we cannot rule out that such a role exists.

Despite the quantitative differences in chromatin structure induced by NCDF and HEG0 expected from their different binding affinities, qualitatively NCDF was able to produce the same alterations in sensitivity to nucleases. Since NCDF consists of little more than the DNA binding domain (DBD) of HEG0, this implies that the activity of DNA binding is induced by estradiol. We do not know why the expression of the 3ERE-URA3 promotor is slightly elevated by the binding of NCDF; it is possible that the changes in chromatin structure brought about by NCDF binding allow a certain amount of basal level expression that would otherwise be repressed. Regardless of the mechanism, it is unlikely that this barely detectable level of expression can account for the changes in chromatin structure that are readily detectable with NCDF. In addition, we have examined the chromatin structure at this same promotor containing only 1ERE, and have found that HEG0 can readily induce the expression of this reporter gene but that the changes in chromatin structure described above are barely detectable (D.M.G., unpublished observations). Thus, strong changes in chromatin structure require 3 binding sites for the receptor, but, there is no correlation between the level of transcription induced at the URA3 promotor and the alterations in chromatin structure. Taken together, these results strongly suggest that DNA binding *per se* causes the observed changes in chromatin structure at the ERE-URA3 promotor.

Our results also suggest that the receptor:DNA interaction is relatively weak, since a micrococcal nuclease cutting site located within the ERE's is not protected but actually enhanced by the addition of estradiol. Thus, the gap between DNaseI hypersensitive sites at the ERE's observed in Figure 2 is unlikely to be a 'footprint' due to protection by the receptor itself, as has been suggested by others using similar systems (5, 11). In fact, we were not successful in detecting the protection by HEG0 of guanine residues in the ERE using *in vivo* DMS footprinting (D.M.G., unpublished observations), raising the possibility that the ER binds weakly or transiently *in vivo*. Other laboratories have come to similar conclusions for the glucocorticoid receptor (7, 9). Hormone dependent DMS protection at a GRE has been reported (6), however, careful studies done by Raymond Pictet and co-workers (9) have shown this same footprint to be due to another protein with an overlapping binding site, leading to the 'hit and run' hypothesis that, in response to ligand, the glucocorticoid receptor interacts only briefly with the glucocorticoid response element (GRE) to recruit the binding of other transcription factors. Our results would be consistent with such a theory for the ER.

We have not attempted to map the positions of nucleosomes in this report, however, the positions of micrococcal nuclease cutting sites at the ERE-URA3 promotor shown in Figure 3 are too close together to accommodate a positioned nucleosome, even in the absence of an ER, while cutting sites within the URA3 and TRP1 structural genes are consistent with the presence of stable nucleosomes. Since in chromatin there is a cutting site within the ERE's that is not detected in naked DNA, it is unlikely that the ERE is embedded within a positioned nucleosome. Thus, if the binding of the receptor displaces a positioned nucleosome as has been shown for the glucocorticoid receptor (7, 8), it must be a nucleosome positioned upstream of the ERE's and not including the promotor elements or the ERE's. We do not know if this is a general feature of chromatin structure near an ERE or whether it is a result of the specific position of the ERE in our constructs; we have not placed the ERE in different positions to address this question. There is some evidence, however, that this particular structure is not due to the presence of the ERE's. It is apparent that the DNA within the adjacent ERE-URA3 promotor is not completely naked, since there are significant differences between the preferred micrococcal nuclease cutting sites in chromatin as compared to naked DNA (Figure 3). These differences could be due to the presence of an unstable nucleosome that is not capable of completely protecting the TATA region, or they could be due to the binding of other yeast proteins such as GA−BF, which has been shown to bind sites located upstream and downstream of the TATA box of the URA3 promotor (25). The binding of GA−BF could preclude the binding of a nucleosome at this particular promotor, independent of the presence of the ERE.

Our results demonstrate conclusively that activation of the ER by ligand (or by the removal of the HBD) is necessary to induce

the chromatin structure changes detected in this report. While it is still formerly possible that HEG0 contacts the ERE in the absence of hormone but remains 'invisible' to detection by DNase I or micrococcal nuclease, the simplest interpretation for our results is that the ER requires ligand to bind DNA *in vivo*. Thus, the conflicting results obtained *in vitro* (see Introduction) can be explained if the receptor has the potential to bind DNA in the absence of ligand, but is prevented from doing so *in vivo* by its association with other nuclear proteins in a 'docking complex', as has been proposed (26). As a result, the method of receptor preparation may be critical to its DNA binding activity *in vitro*.

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