Estrogen Induces Estrogen-related Receptor α Gene Expression and Chromatin Structural Changes in Estrogen Receptor (ER)-positive and ER-negative Breast Cancer Cells*

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Estrogen-related receptor α (ERRα), a member of the nuclear receptor superfamily, is closely related to the estrogen receptors (ERα and ERβ). The ERRα gene is estrogen-responsive in several mouse tissues and cell lines, and a multiple hormone-response element (MHRE) in the promoter is an important regulatory region for estrogen-induced ERRα gene expression. ERRα was recently shown to be a negative prognostic factor for breast cancer survival, with its expression being highest in cancer cells lacking functional ERs. The contribution of ERRα in breast cancer progression remains unknown but may have important clinical implications. In this study, we investigated ERRα gene expression and chromatin structural changes under the influence of 17β-estradiol in both ER-positive MCF-7 and ER-negative SKBR3 breast cancer cells. We mapped the nucleosome positions of the ERRα promoter around the MHRE region and found that the MHRE resides within a single nucleosome. Local chromatin structure of the MHRE exhibited increased restriction enzyme hypersensitivity and enhanced histone H3 and H4 acetylation upon estrogen treatment. Interestingly, estrogen-induced chromatin structural changes could be repressed by estrogen antagonist ICI 182 780 in MCF-7 cells yet were enhanced in SKBR3 cells. We demonstrated, using chromatin immunoprecipitation assays, that 17β-estradiol induces ERRα gene expression in MCF-7 cells through active recruitment of co-activators and release of co-repressors when ERRα and AP1 bind and ERs is tethered to the MHRE. We also found that this estrogen effect requires the MAPK signaling pathway in both cell lines.

Estradiol (E2)² is required for the development and function of multiple tissue types and physiological systems in mammals, and it has been implicated in a range of pathological conditions, including the initiation and progression of breast cancer (Refs. 1–3 and references therein). The two genetically distinct nuclear estrogen receptors, ER-α and -β, are thought to mediate most biological effects of E2 (4). Upon binding of a single ligand molecule, the ERs homodimerize and interact directly with estrogen-response elements (ERE) in the regulatory sequences of estrogen target genes (5). In the absence of an ERE, the ERs may still engage via tethering to transcription factors that are bound to a target gene promoter (6–8). ER binding ultimately induces gene expression by modifying the local chromatin structure and facilitating the assembly of a transcription complex in either case (9–11). E2 also elicits various physiological responses that cannot be totally explained by classic ER-mediated actions in the nucleus (see review in 12 and references therein). For example, E2 reportedly elicits rapid effects via cross-talk with intracellular signal transduction and growth factor signaling pathways (13, 14). GPR30, a recently described G-protein-coupled receptor, is reported to mediate ER-independent estrogen signaling under certain conditions (15–17). Therefore, E2 clearly acts through a multitude of complex signaling pathways that may or may not involve the nuclear ER forms.

In contrast to the ERs, the estrogen-related receptors (ERR) -α, -β, and -γ are all orphan nuclear receptors with a high degree of sequence identity to the ERs but do not bind estrogens or any other known natural ligands (18, 19). ERRα is ubiquitous among tissues of the developing embryo and adult (20–22) and regulates an assortment of genes and physiological processes (23–26). Like the ERs, ERRα binds to ERs but is capable of also binding an estrogen-related response element (ERRE) unique to the ERRs (27–29) in monomer or dimer form (30). Once bound to DNA, ERRα recruits co-regulators similar to those recruited by the ERs and thereby influences gene expression, indicating a considerable degree of functional conservation between the ERs and ERRs (31–35). In contrast, however, the ERRs are not ligand-dependent but are constitutively active (36–38) and are able to modulate many ER-regulated physiological pathways and ER target genes (29, 35, 39–41). ERRs are reportedly present in 75% of clinical breast tumor samples and correlates to a more favorable response to endochromatin immunoprecipitation; RT, reverse transcriptase; MNase, micrococcal nuclease; EMSA, electrophoresis mobility shift assay; NPE, nuclear protein extract; oligo, oligonucleotide; pol, polymerase; p-pol, phosphorylated pol; ICI, ICI 182 780.
crine therapy compared with ERα-negative cancers (42). Interestingly, recent clinical studies implicate ERRα as having a potential role in breast cancer progression (43, 44) based largely on its greater prevalence in ERα-negative tumors and correlation with ErbB2, a known marker of aggressive tumors (45). Hence, positive ERRα expression is increasingly associated with an adverse clinical outcome and decreased chance of survival in breast cancer patients (46, 47). ERRα may promote the growth of breast cancers by increasing local estrogen synthesis as it is reported to stimulate the expression of CYP19A1 (P450 aromatase) (48, 49) and SULT2A1 (50), enzymes that contribute to estrogen biosynthesis. Furthermore, we have previously demonstrated that the ERRα gene, ESRRα, is an ER-dependent estrogen target in certain estrogen-responsive tissues and cell lines (21, 51). At this time, however, the contribution of ERRα to hormone resistance and tumor progression in breast cancers remains unknown but may have vital clinical implications.

In eukaryotes, genomic DNA is associated with histone proteins to form nucleosomes, the basic unit of chromatin (52). The structure of the nucleosome, and chromatin as a whole, is inherently prohibitive to gene expression because it precludes the access of transcription factors, such as the ERs and ERRs, to specific regulatory regions (53–55). Because ERRα is a constitutively active protein, transcriptional regulation of the ERRα gene may be a primary mechanism for modulating its activity. Hence, the chromatin structure of the ERRα gene warrants investigation. In this study, we used the breast cancer cell lines to characterize the nucleosomal arrangement in an area of the ERRα gene that is known to harbor an MHRE (51). Our results indicate that E2 modulates the local chromatin architecture and histone acetylation levels around the MHRE region in both ER-positive (ER+) and ER-negative (ER−) breast cancer cells. Further probing of mechanistic insights has shown that in ER+ cells ERRα gene expression requires ERRα and AP1 binding, along with ERα being tethered to the MHRE or a nearby AP1 element, favors recruitment of co-activators and release of co-repressors, and ultimately leads to increased ERRα gene expression. Although the mechanisms by which E2 modulates ERRα expression in ER− cells is not yet clear, our studies show that the nucleosome organization of the ERRα gene and E2-induced chromatin modification is similar to that in the ER+ cells. Furthermore, we have shown that the MAPK signaling pathway is required for estrogen action in both cell lines by using a MAPK inhibitor.

**EXPERIMENTAL PROCEDURES**

**Materials**—MCF-7 (HTB-22) and SKBR3 (HTB-30) were obtained from ATCC (Manassas, VA). 17β-Estradiol (E2) was purchased from Sigma. ICI 182 780 (ICI) was from Tocris (Ellisville, MO), and P98059 (PD) was from Calbiochem. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and microccocal nuclease (MNase) from Worthington. The plasmid 0.8-CAT that contains 0.8-kb of the ERRα promoter (51), the plasmid h55 that contains the entire ERRα promoter sequence,3 and AAB-CAT reporter plasmid were all constructed in our laboratory (51, 56). Antibodies for anti-acetylhistone H3 (K9 and K14), anti-acetylhistone H4 (K5, K8, K12, and K16), anti-ERRα (HC-20), anti-c-Jun (sc-45x), anti-RNA polymerase II (N-20), anti-P300 (C-20), anti-CBP (A-22), and anti-RIP140 (H-300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-C terminus of histone H3 (H3-CT-A3S, catalog number 05-928) and rabbit IgG were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-ERB (clone 9.88, IgM) was purchased from Sigma. Anti-ERRα (p2) was produced and verified by our laboratory (57). Anti-phosphorylated RNA polymerase II (Ab5131) was purchased from Abcam (Cambridge, MA).

**Cells Culture and Nuclei Preparation**—MCF-7 cells were cultured in modified Eagle’s medium. SKBR3 cells were cultured in Dulbecco’s modified Eagle’s medium. In both cases, the media were supplemented with 10% fetal bovine serum, and the cells were maintained in a humidified incubator at 37 °C with 5% CO2. At least 2 days prior to any hormonal treatments, the cells were switched to phenol red-free modified Eagle’s medium containing 5% charcoal-stripped fetal bovine serum. Treatments consisted of vehicle (ethanol), 10 nM E2, 1 µM ICI, or 5 µM PD98059 or combinations thereof for multiple time points as indicated by the individual experiment. Nuclei were prepared as described previously (58). Briefly, the cells were rinsed and detached from the dish using a rubber policeman in the presence of cold phosphate-buffered saline and pelleted by centrifugation at 750 × g at 4 °C for 5 min. The cell pellets were resuspended in homogenization buffer containing 0.1% Nonidet P-40 and incubated on ice for 2 min before being lysed by 5 strokes of pestle A in a Dounce homogenizer. Nuclei were sedimented through a 10% sucrose pad by centrifugation at 1,400 g for 2 min, washed with washing buffer to remove traces of Nonidet P-40, and collected by centrifugation at 750 × g for 5 min. The nuclei preparations were then kept on ice until use.

**Micrococcal Nuclease Analysis**—Isolated nuclei were suspended in 200 µl of washing buffer supplemented with 1 mM CaCl2 and then digested with 0–50 units of MNase for 5 min at 30 °C. The reactions were stopped by the addition of 40 µl of 100 mM EDTA, 10 mM EGTA (pH 8.0). Genomic DNA was then purified, digested with the appropriate restriction enzyme (as indicated), and analyzed by Southern blot or reiterative PCR. The first control DNA (C1) was prepared by digestion of purified genomic DNA with 1 unit of MNase/ml for 5 min at 30 °C. The second control DNA (C2) was prepared by double digestion of purified genomic DNA with MNase/ml and the appropriate restriction enzyme.

**Southern Blotting and Reiterative PCR**—For Southern blot analysis, 20 µg of MNase-digested DNA per sample was separated on a 1.5% agarose gel, transferred to Hybond N+ membrane (GE Healthcare), and probed with 32P-labeled DNA fragments. Probe A was radiolabeled by PCR (Fig. 1), and probe B was radiolabeled by random priming (Ready-to-Go beads; GE Healthcare) of the Psf/Sphl genomic fragment (Fig. 1) (51, 56). Hybridization was carried out overnight at 65 °C, and the blots washed and then exposed to x-ray film at −70 °C.

Reiterative PCR was carried out as follows: 25 µg of purified DNA digested with MNase and Stul was analyzed by linear PCR with a 32P-labeled single strand primer (Table 1) corresponding to the −521/−510 region of the ERRα promoter (51, 56). The

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3 C. T. Teng, unpublished data.
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| TABLE 1 | Primers | Sequence |
|---------|---------|----------|
| For Southern blot | Forward: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Reverse: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Taqman probe: 5’-CAC TCT CGA GCA GCA GCC AGC TCT GC-3’ | |
| For real time RT-PCR | ERα | Forward: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Reverse: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Tagman probe: 5’-CAC TCT CGA GCA GCA GCC AGC TCT GC-3’ | |
| | β2-Microglobulin | Forward: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Reverse: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | c-Myc | Forward: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Reverse: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | β2-Actin | Forward: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |
| | Reverse: 5’-GTC TCT GCC CGA TCA GCC TCG TAC CTC TC-3’ | |

PCR-amplified products and sequencing control (50 ng of ERRα plasmid) were separated on 8% polyacrylamide denaturing gel and analyzed using a PhosphorImager (GE Healthcare). Band sizes were measured by using FragmentNT Analysis software (GE Healthcare).

Nuclear Protein Extraction, Cell Lysate Preparation, and Western Blot Analysis—MCF-7 and SKBR3 cells were treated as described above. Nuclear protein extracts were prepared by using the BD™ TransFactor extraction kit according to the supplier’s instruction (Clontech) and used for ERRα and ERβ detection. Whole cell lysates were prepared for ERRα detection. Protein concentrations were determined (Bradford protein assay, Bio-Rad) and were separated by SDS-10% polyacrylamide gel and 4% stacking gel with Bio-Rad Miniprotein apparatus. After electrophoresis, the proteins were blotted onto polyvinylidene difluoride membrane (Invitrogen) and probed with antibodies specific to ERRα and ERRβ.

RNA Isolation, Reverse Transcription, and Real Time RT-PCR—Total RNA was extracted using the RNeasy mini kit according to the supplier’s protocol (Qiagen). For RT-PCR analyses, cDNA was synthesized as described previously (59). Real time RT-PCR was performed for ERRα and β2-microglobulin gene with Applied Biosystems 7900HT real time PCR System and Taqman Universal PCR Master Mix (Applied Biosystems). Average cycle threshold (Ct) values for ERRα were calculated and normalized to Ct values for β2-microglobulin. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for pS2, c-Myc, and β2-actin genes. Average Ct values for pS2 or c-Myc were calculated and normalized to Ct values for β2-actin. All the primer and probe sequences are presented in Table 1.

Restriction Enzyme Hypersensitivity Analysis—Cell nuclei were prepared after experimental treatment and digested in vitro with MsI, Apol, or Apal at 30 °C for 15 min as described previously (58). The genomic DNA was then purified and digested in vitro with Stul or Sphi. The digested DNA fragments were then amplified by real time primer extension as described above. The PCR-extended products were purified by phenol/chloroform extraction, ethanol precipitation, and analyzed on 8% denaturing polyacrylamide gels. The bands densities were measured by Kodak 1D program, and the percentage of cleavage was calculated as in vivo band density relative to the total band density.

In Vitro Protein Translation, Nuclear Protein Preparation, and Electrophoresis Mobility Shift Assay (EMSA)—ERRα and ERβ were in vitro transcribed and translated using the TNT Coupled Reticulocyte Lysate Systems according to the supplier’s instructions (Promega, Madison, WI). Nuclear proteins of the MCF-7 cells were prepared with the TransFactor extraction kit (Clontech). The MHRE fragment was cut from the AAB-CAT reporter (51) by Nhel and Xhol digestion, gel-purified, and used as the probe in EMSA. The double-stranded MHRE oligos were labeled with [32P]dGTP by fill-in with Klenow large fragment of DNA polymerase I. The antibodies for supershift experiments for ERRα were TE111.5D11 from Neo-Marker (Fremont, CA). ERβ, clone 9.88, IgM, from Sigma, and human lactoferrin antibody prepared in our laboratory (60). The EMSA procedure has been described previously (21, 29).

Chromatin Immunoprecipitation (ChIP) Assay—Native ChIP was used to evaluate histone modifications in cells following treatments (61). Briefly, isolated nuclei were digested with MNase until the chromatin became mono- to trinucleosome in size. ChIP analyses were carried out by using the ChIP assay kit (Upstate Biotechnologies) with minor modifications. Native chromatin was diluted with ChIP dilution buffer and precleared with 80 μl of a salmon sperm DNA-protein A-agarose for 30 min with agitation at 4 °C. Immunoprecipitation was performed overnight (10–14 h) at 4 °C with specific antibodies or with rabbit IgG as a control. After immunoprecipitation, 60 μl of salmon sperm DNA-protein A-agarose were added for 1 more h at 4 °C to capture the immune protein-DNA complexes. The immunoprecipitants were sequentially washed according to instructions, and the immune complexes were eluted with 1% SDS in 0.1 M NaHCO3 and the DNA was purified by phenol/ethanol extraction and precipitation. To study the transcription factor and co-regulator occupancy, standard ChIP was performed in which the chromatin was first cross-linked with 1% formaldehyde at 37 °C for 20 min. The crude cell lysates were sonicated to shear the chromatin to 400–1200-bp size as verified by the agarose gel electrophoresis, and the ChIP assays were performed. After elution, the chromatin was reverse cross-linked with 0.2 M NaCl at 65 °C for 6 h and then the DNA was purified. In Re-ChIP experiments, complexes were eluted by incubation with 10 μM dithiothreitol at 37 °C for 30 min,
diluted 1:50× in ChIP dilution buffer, followed by re-immunoprecipitation with the second antibodies (62) or rabbit IgG as a control. DNA product was detected by PCR with specific forward and reverse primer sequences (Table 1). The PCR conditions were 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s for a total of 30–35 cycles. Real time PCR was performed with Power SYBR Green QPCR master mix (Applied Biosystems) according to the supplier’s instruction.

**Statistical Analysis**—Statistical analyses were carried out using the Student t test. p values of <0.05 were considered to be statistically significant.

**RESULTS**

The ERRα Gene MHRE Is Assembled Over a Single Nucleosome—We demonstrated previously that estrogen stimulation of the ERRα expression is mediated through the MHRE located between bp −709 to −649 of the ERRα promoter (51, 63). To study the influence of estrogen on the chromatin dynamics in this portion of the ERRα promoter, we had to first determine the nucleosome positions around the MHRE region. Two probes (A and B) were selected to map nucleosome positions between bp −1205 and −41 relative to the transcription start site (Fig. 1A). To map nucleosome positions from the 5′ end of the 1-kb target region, nuclei from MCF-7 cells were digested with increasing concentrations of MNase; the DNA was purified, cut with StuI, blotted, and hybridized with probe A (Fig. 1B). A similar process was used to map the nucleosome position from the 3′ end but instead cutting with SphI and hybridizing the resulting blot with probe B (Fig. 1C). Two control DNA samples were included, one with MNase digestion only (C1) and the other digested again with the restriction enzyme (C2).

Nuclei digested in vivo produced a ladder when stained with ethidium bromide, each band indicating the presence of a nucleosome (data not shown). As expected,
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A top panel, detection of ERα and ERβ GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNAs in different cell lines. Total RNA was prepared from different cell lines, and RT-PCR was performed. Lower panel, detection of ERα and ERβ GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNAs in different cell lines. Total RNA was prepared from different cell lines, and RT-PCR was performed.

B, E2-induced pS2 (top panel) and c-Myc (lower panel) expression. The mRNA levels were determined by SYBR Green real time PCR. C, E2-induced ERRα expression. Top panel, Taqman real time RT-PCR. Cells were treated with either vehicle or 10 nM E2 for 0.5–24 h. Total RNA was prepared and used for mRNA determination. Lower panel, Western blotting. Whole cell lysate was prepared from cells treated with E2 for 24 h and used in Western blotting for ERRα. β-Actin was used as loading control. D, dose response of ERRα induction by E2 in SKBR3. Various concentrations of E2 were added to the cell culture for 8 h. The ERRα mRNA level was measured by Taqman real time RT-PCR. Data are presented as means ± S.E. for at least three independent experiments at each datum point. * indicates p < 0.05 for vehicle-treated versus E2-treated cells.

FIGURE 2. Estrogen induction of ERRα expression in ER-positive MCF-7 and ER-negative SKBR3 cells. A, top panel, detection of ERα and ERβ GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNAs in different cell lines. Total RNA was prepared from different cell lines, and RT-PCR was performed. Lower panel, detection of ERα and ERβ GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNAs in different cell lines. Total RNA was prepared from different cell lines, and RT-PCR was performed. B, E2-induced pS2 (top panel) and c-Myc (lower panel) expression. The mRNA levels were determined by SYBR Green real time PCR. C, E2-induced ERRα expression. Top panel, Taqman real time RT-PCR. Cells were treated with either vehicle or 10 nM E2 for 0.5–24 h. Total RNA was prepared and used for mRNA determination. Lower panel, Western blotting. Whole cell lysate was prepared from cells treated with E2 for 24 h and used in Western blotting for ERRα. β-Actin was used as loading control. D, dose response of ERRα induction by E2 in SKBR3. Various concentrations of E2 were added to the cell culture for 8 h. The ERRα mRNA level was measured by Taqman real time RT-PCR. Data are presented as means ± S.E. for at least three independent experiments at each datum point. * indicates p < 0.05 for vehicle-treated versus E2-treated cells.

Southern blots with probe A or probe B detected at least three nucleosome boundaries with an overlapping region around bp −480 to −778 (Fig. 1, B and C), indicating that the MHRE region of the ERRα promoter is organized into regular nucleosome arrays. The ERRα promoter produced comparable results using the same mapping technique in SKBR3, HK2, and HepG2 cell lines (data not shown).

MNase digestion provides only an estimation of nucleosome positions. Therefore, high resolution PCR-based mapping was carried out to better demarcate the MHRE nucleosome position (Fig. 1D). The ERRα gene promoter was sequenced to provide a landmark (Fig. 1D, lanes 1–4). Reiterative PCR products identified two clusters of MNase sensitivity (Fig. 1D, lanes 6–9, arrows) centering on bp −628 and −774 (147 bp), suggesting the expected nucleosomal size of ~146 bp. These two sites correspond to the nucleosome containing the MHRE and were consistent with the sites identified by Southern blot (Fig. 1, B and C). Based on probe locations and fragment sizes, we concluded that the ERRα MHRE is organized into a single nucleosome.

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induction of ERRα expression in SKBR3 cells occurred at 10 nM E2 (Fig. 2D), similar to that described previously in MCF-7 cells (51).

E2 Induces MHRE Nucleosome Modification in Both MCF-7 and SKBR3 Cells—We hypothesize that E2 induction of ERRα involves chromatin remodeling around the MHRE, making it more accessible to transcription factors. Such remodeling of the region can be revealed via increased sensitivity to restriction endonucleases digestion, such as those for MslI and BstI which are within the MHRE, and Apal which is located close to the 3'-nucleosome boundary (Fig. 3A).

MCF-7 cells were treated with E2 for 15–120 min, and hypersensitivity at the MslI sites was examined using the oligo D primer (Fig. 3A). In untreated cells, oligo D primer produced a single DNA fragment extending to the StuI site at bp −1215, indicating that both MslI sites in the MHRE were not accessible. Within 15 min after E2 exposure, however, both MslI sites exhibited increased sensitivity, as indicated by the production of two small PCR fragments by oligo D (MslI at −666 and MslI at −689) (Fig. 3B). As expected, the Apal site located outside the nucleosome was not affected by E2 treatment (Fig. 3C). The percent cleavage at each of the MslI sites was comparable after E2 treatment and exhibited no change from 15 to 120 min of E2 exposure (Fig. 3, B and C).

Estrogen-induced nucleosome hypersensitivity was also detected at the Apal site when using oligo A in reiterative PCR. The Apal site lies 3' boundary of the nucleosome containing the MHRE (Fig. 3A). Interestingly, this E2-induced hypersensitivity could be blunted by the pure anti-estrogen ICI (Fig. 3D). These results demonstrate that estrogen-induced chromatin modifications within the ERRα promoter in MCF-7 cells is mediated by ERRα.

Because ERRα induction by E2 in ER-negative SKBR3 cells exhibited a markedly delayed peak relative to MCF-7, our evaluation of structural changes in the chromatin in these cells was equally delayed until 2–6 h after E2 exposure. Nonetheless, E2-induced hypersensitivity around the MslI site was obvious within 2 h after E2 exposure (Fig. 4A), whereas the Apal site outside the MHRE nucleosome showed no change (Fig. 4B). Surprisingly, E2-induced hypersensitivity around the Apal site was not blocked by ICI as was observed in the MCF-7 cells but enhanced (Fig. 4C). The ICI-induced increase of hypersensitivity at the Apal site in SKBR3 cells was consistent with the increased expression of ERRα gene after ICI or ICI and E2 treatment (Fig. 4D).

E2 Induces Histone Acetylation and Co-regulator Exchange in the MHRE Nucleosome—Acetylation of lysine residues in histone tails reduces their affinity for DNA, relaxing the arrangement of chromatin structure and thereby facilitating access of transcription factors and RNA polymerase II (53, 55). We therefore chose to evaluate the acetylation of histones H3 and H4 of the MHRE nucleosome using native ChIP assay at various time points after E2 exposure (Fig. 5A). In MCF-7 cells, histone H3 acetylation occurred within 5 min of E2 exposure and
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The restriction enzyme ApaI hypersensitivity was analyzed as described in E2, ICI, or E2 and ICI for 24 h, and the whole cell lysate was prepared. The gradual decline to basal levels within 1 h (Fig. 5B). E2-induced acetylation of histone H4 followed a similar trend but was less robust (Fig. 5B). Loss of histone acetylation but not total histone at the MHRE was verified using an antibody against the C terminus of the histone H3 (Fig. 5C). These data demonstrate that rapid but transient acetylation of histone H3 and H4 of the MHRE nucleosome precedes active transcription in MCF-7 cells.

We examined whether events occur in the ER− cells after E2 treatment consistent with the delayed expression observed earlier in SKBR3 cells (Fig. 5D). Although the ER-positive MCF-7 cells exhibited maximum histone acetylation within 15 min of E2 exposure (Fig. 5B), histone acetylation at MHRE vicinity in SKBR3 cells was on by 15 min but persisted up to 120 min (Fig. 5D). We also found that RNA polymerase II (pol II) and phosphorylated pol II (p-pol II) were recruited to the MHRE nucleosome in E2-treated SKBR3 cells in a temporal pattern consistent with the 120-min peak of histone acetylation (Fig. 5E) which was subsequently followed by an increase in ERRα expression.

We also examined co-regulator occupancy at the MHRE nucleosome in MCF-7 cells using a standard ChIP assay, including a formaldehyde fixation step to ensure the detection of loosely bound proteins (Fig. 6A). Our results indicate that p300 is recruited to the MHRE nucleosome within 5 min of E2 treatment, followed by CBP, the latter exhibiting two distinct periods of peak binding at 15 and 60 min. Interestingly, the co-repressor RIP140 was present on the MHRE nucleosome prior to E2 treatment but was quickly released within 15 min after E2 exposure. The recruitment of p-pol II to the MHRE was trailing behind CBP; it peaked at 30 and 120 min (Fig. 6B). These results are consistent with the current model of cyclical exchange between co-regulators at the chromatin level during estrogen regulation of target genes (62, 65).

Estrogen Induces ERRα Expression in MCF-7 Cells via an ERRα-mediated Mechanism—The ERs do not bind the ESRRα23-response element located within the MHRE region (33). We confirmed these findings by EMSA using in vitro transcribed/translated ERα and ERβ (Fig. 7A, lanes 2 and 3). However, nuclear protein extract (NPE) from ER-positive MCF-7 cells clearly contains a component(s) capable of binding the MHRE (Fig. 7A, lanes 4 – 9, arrows). When combined with ERα or ERβ, the NPE of MCF-7 cells continues to produce a complex with the MHRE; and furthermore, antibodies to ERRα supershift this complex (Fig. 7A, compare lanes 7 – 9). Collectively, these data indicate the following: (a) MCF-7 nuclei contain protein(s) capable of binding the MHRE, and (b) this complex recruits ERα but not ERβ.

ChIP analyses were performed to further confirm the tethering of ERα to the ERRα MHRE in MCF-7 cells after E2 treatment. Repeated experiments indicated that endogenous ERRα is recruited to the MHRE within 15 min, peaks at 30 min, recedes to base line within 60 min but returns again to maximum levels at 120 min, suggestive of a cyclical binding (Fig. 7B).

ERRα and ERRγ are known MHRE-binding proteins (63, 66) and therefore candidate components of the MHRE binding complex detected in MCF-7 NPEs. The ERRα could potentially be tethered to the MHRE and co-occupy the region with DNA-bound ERRs (29) or, alternatively, may be tethered to AP1 (67–69) or SP1 (7, 70) complexes bound to their consensus binding sites located upstream as well as within the MHRE nucleosome. To discern among these possibilities, we first used ChIP assays to demonstrate that ERRα and AP1 interact with their corresponding binding elements in the nucleosome (Fig. 7C). ERRα
binding is largely constitutive but clearly increased by E2, peaking 45 min after treatment and then gradually decreasing to below base-line levels by 90 min. This suggests a dynamic relationship between ERRα and the MHRE in response to E2 treatment. Interestingly, AP1 is not constitutively bound to the MHRE nucleosome but becomes so within 15 min after E2 treatment, and cycles in 45-min intervals thereafter for at least 120 min. Furthermore, maximum AP1 recruitment to the MHRE precedes that of ERα, the latter peaking 30 min after E2 treatment. Additionally, we have shown by using ChIP and re-ChIP experiments that ERα is a co-occupant with both AP1 and ERRα on the MHRE nucleosome (Fig. 7, D and E), although its occupation with the latter appears more robust. These data confirm that the ERRα is an estrogen-responsive transcription factor in MCF-7 cells, as well as indicates that ERα may serve as a transducer for this estrogen action via co-occupancy with DNA-bound ERRα and AP1 proteins.

E2-induced ERRα Expression Can Be Blocked by ERK Inhibitor—Estrogen triggers a variety of second messenger signaling events independent of the ERs in addition to the classical ER-mediated gene activation (12). Among the many potential pathways, we first tested an inhibitor of ERK pathway, PD98059, in ER-negative SKBR3 cells. The result showed that the E2-induced expression was completely blocked by the ERK inhibitor (Fig. 8A). Similar experiments were performed on the ER-positive MCF7 cells with a different treatment regime that matches the ERRα expression. Like with the SKBR3 cells, the ERK inhibitor also blocked the E2-induced expression (Fig. 8B). Because ERα is functionally regulated via phosphorylation by several protein kinases (71), it is expected that the suppression of the MAPK signaling pathway in ER-positive cells blunts ERα-mediated estrogen action. These results indicate that estrogen regulation of the ERRα gene expression in ER-positive and ER-negative breast cancer cells is measurable and depends on MAPK signaling pathways.

DISCUSSION

There is mounting clinical evidence linking ERRα expression to tumor progression and less favorable prognoses in breast cancer patients (43, 44, 46, 47). We have previously demonstrated that estrogens induce ERRα expression in MCF-7 cells, an ERα-positive breast cancer cell line. We have been able to replicate these findings as well as demonstrate estrogen induction of ERRα expression in SKBR3 cells, an ER-negative breast cancer cell line. Furthermore, we have demonstrated that estrogen causes structural remodeling of the nucleosome containing the ChIP products. B, time-dependent histone acetylation (Ace) in MCF-7 cells. Cells were treated with vehicle or 10 nm E2 for various times. Acetylation status of histone H3 and H4 was determined by native ChIP assay. DNA purified from the immunoprecipitated chromatin was amplified by real-time PCR. C, total histone H3 was determined by native ChIP assay. D, time-dependent histone acetylation in SKBR3 cells. Cells were treated with E2 for 15 and 120 min. The status of histone H3 and H4 acetylation was determined by native ChIP assay. con, control. E, recruitment of RNA pol II and phosphorylated RNA polymerase II (p-pol II) to the MHRE nucleosome in SKBR3 cells. Cells were treated with 10 nm E2 for various time points, and the standard ChIP assays were performed to detect the presence of pol II and p-pol II on the MHRE nucleosome. The PCR products of the ChIP assays from repeated experiments are presented as means ± S.E.
an MHRE in the ERRα gene promoter in both cell lines, as evident by increased hypersensitivity to restriction enzymes and increased histone acetylation. We also demonstrated that estrogen induces ERRα, AP1, and ERα occupancy at the MHRE nucleosome and recruits co-activator and RNA polymerase II to this region in MCF-7 cells, whereas in SKBR3 cells, we showed the recruitment of RNA polymerase II. Although ERRα induction requires the MAPK pathway in both cell types, the rapid response to estrogen in the ERα-positive MCF-7 cells may contribute by the tethering of ERα to ERRα and AP-1, each bound to their respective response elements.

Chromatin structure is an inherent barrier that must be overcome to allow transcription and gene expression. Hormone-response elements in the promoters of steroid hormone target genes are often distinctively positioned in the nucleosome in such a way that they are not easily accessible by transcription factors and transcription initiation complex (72, 73). In the presence of hormone, however, activated receptors are bound to the nucleosome, which in turn recruits co-regulators that act to further modify the core histone tails and produce chromatin structure that is more permissible. These events allow the recruitment of additional transcription factors and the generation of a transcription initiation complex.

The promoter of the ubiquitously expressed ERRγ gene is no exception. It is organized such that a functional MHRE lies within a single nucleosome, which also harbors consensus AP1 (6) and SP1 (74, 75) sites just downstream. This arrangement suggests the two regulatory elements may cooperate to bring about estrogen stimulation of the ERRγ gene. Indeed, we have demonstrated that ERRγ and AP1 are integrally involved in ERRα-mediated estrogen induction of the ERRγ expression in MCF-7 cells. We believe that SP1 also participates in estrogen-stimulated activity of the ERRγ gene. By using EMSA, ChIP, and re-ChIP approaches, we have shown that ERα is present in the protein complex assembled on the MHRE nucleosome region of the ERRγ gene in MCF-7 cells in response to estrogen. Recruitment of ERα to the MHRE nucleosome after E2 treatment was cyclical but exhibited a temporal pattern such that peak binding occurred at 120 min, coinciding with maximum AP1 occupancy, which then led to increased acetylation of histones H3 and H4, recruitment of p300, and the release of the constitutively bound co-repressor RIP140. Therefore, ERRγ binding to the MHRE in its own promoter is a decisive step toward inducing the overall dynamic exchanges necessary to allow for its expression in ER-positive breast cancer cells.

The organization of the ERRα MHRE nucleosome may largely contribute to estrogen responsiveness because it contains three distinct but potentially estrogen-influenced regulatory elements, an MHRE, AP1, and SP1. Modification of any one of these elements through ERRα or even alternative signaling pathways could initiate a chain reaction of chromatin modification and co-regulator recruitment, thus beginning activation of transcriptional activity. We have produced evidence that estrogen clearly induces a more “open” chromatin configuration around the MHRE, as illustrated by increased restriction enzyme hypersensitivity assay in both MCF-7 and SKBR3 cells. Interestingly, the two MspI sites within the MHRE appear less accessible than the ApaI site located just outside the MHRE but still within the nucleosome. This difference could be an artifact of differential activities between the two restriction enzymes, or

**FIGURE 6.** Estrogen induces co-regulator dynamics and recruitment of RNA polymerase II in the MHRE nucleosome in MCF7 cells. Cells were treated with vehicle or 10 nM E2 for 5–60 min, and the standard ChIP assay was performed. A, co-regulator dynamics. Specific antibodies to co-activators p300 and CBP and co-repressor RIP140 were used in the study. B, RNA polymerase II recruitment. Specific antibody to phosphorylated polymerase II was used. DNA was amplified by PCR, and the relative band intensity of repeated experiments was determined by using the Kodak 1D software. Data are presented as means ± S.E.
Possibly because of \textit{ERR} masking the MslI sites when bound to the MHRE.

Although estrogen induces measurable chromatin remodeling, independent of the ER status of the cell type, the mechanisms involved may differ. In MCF-7 cells, estrogen-induced \textit{ERR} expression occurred in two distinct waves. Our results indicate that the first wave of estrogen induction follows a current model of ER-mediated transcriptional activation where ER indirectly interacts with a complex bound to the MHRE. Whether this or another mechanism is responsible for the second wave of induction is unclear. Estrogen induction of \textit{ERR} possibly because of \textit{ERR} masking the MslI sites when bound to the MHRE.

Although estrogen induces measurable chromatin remodeling, independent of the ER status of the cell type, the mechanisms involved may differ. In MCF-7 cells, estrogen-induced \textit{ERR} expression occurred in two distinct waves. Our results indicate that the first wave of estrogen induction follows a current model of ER-mediated transcriptional activation where ER indirectly interacts with a complex bound to the MHRE. Whether this or another mechanism is responsible for the second wave of induction is unclear. Estrogen induction of \textit{ERR} individually or in combination. The protein complexes were detected by EMSA on a nondenaturing gel. Antibodies (Ab) used in supershift experiments were ER\textsubscript{\alpha}, ER\textsubscript{\beta} (commercial), and human lactoferrin (83). Retarded protein complexes are indicated by arrows, and the supershifted band is marked (SS). B, E\textsubscript{2},-induced ER\textsubscript{\alpha} occupancy on the MHRE nucleosome. Cells were treated with 10 nM E\textsubscript{2} for 15–120 min. The presence of ER\textsubscript{\alpha} on the chromatin was immunoprecipitated with the ER\textsubscript{\alpha} antibody in ChIP assay. DNA products were determined by real time PCR, and a representative PCR gel picture is shown on top. C, E\textsubscript{2} induces AP1 and ERR\textsubscript{\alpha} recruitment to the MHRE nucleosome. Same experiment as in D, but an ERR\textsubscript{\alpha} antibody was used in the first ChIP assay.

\textbf{FIGURE 7.} \textit{ERR} tethered to MHRE nucleosome in MCF-7 cells. A, EMSA. The \textit{in vitro} transcribed and translated ER\textsubscript{\alpha} and ER\textsubscript{\beta} and nuclear protein extract (NPE) from MCF-7 cells were incubated with labeled MHRE oligos at room temperature individually or in combination. The protein complexes were detected by EMSA on a nondenaturing gel. Antibodies (Ab) used in supershift experiments were ER\textsubscript{\alpha}, ER\textsubscript{\beta} (commercial), and human lactoferrin (83). Retarded protein complexes are indicated by arrows, and the supershifted band is marked (SS). B, E\textsubscript{2},-induced ER\textsubscript{\alpha} occupancy on the MHRE nucleosome. Cells were treated with 10 nM E\textsubscript{2} for 15–120 min. The presence of ER\textsubscript{\alpha} on the chromatin was immunoprecipitated with the ER\textsubscript{\alpha} antibody in ChIP assay. DNA products were determined by real time PCR, and a representative PCR gel picture is shown on top. C, E\textsubscript{2} induces AP1 and ERR\textsubscript{\alpha} recruitment to the MHRE nucleosome. Loading of ER\textsubscript{\alpha}, AP1, and ERR\textsubscript{\alpha} on the MHRE nucleosome were determined by standard ChIP assay. The PCR product was scanned and presented as the relative density from two experiments. The data are presented as means \pm S.E. * indicates \( p < 0.05 \) for vehicle versus E\textsubscript{2}-treated cells.

\textbf{FIGURE 8.} MAPK inhibitor blocked the E\textsubscript{2}-induced ERR\textsubscript{\alpha} expression in SKBR3 and MCF-7 cells. Cells were treated as indicated for 8 and 0.5 h for SKBR3 cells and MCF-7 cells, respectively. The RNA was prepared from repeated experiments, and the ERR\textsubscript{\alpha} levels were determined by real time PCR. Data are presented as means \pm S.E. * indicates \( p < 0.05 \) for vehicle versus E\textsubscript{2}-treated cells.
in ERα-negative SKBR3 cells was much delayed, as were the expected changes in chromatin structure, histone acetylation, and protein recruitment. Interestingly, ICI did not block E2-induced transcriptional activity in the ER-negative breast cancer cells but rather enhanced it (Fig. 4D). This result was supported by the restriction enzyme hypersensitivity assay of the MHRE nucleosome treated with both E2 and ICI (Fig. 4C).

These findings establish the estrogen responsiveness of ERRα gene in an ER-negative environment.

The G-protein-coupled receptor, GPR30, has been recently implicated in mediating E2 actions in certain cell-specific contexts (76). For example, phytoestrogens such as genistein and quercetin stimulate the estrogen-responsive c-FOS gene in ERα-negative SKBR3 cells via GPR30 (77). Furthermore, the selective estrogen receptor modulators tamoxifen and ICI are reported to function as agonists for GPR30 in SKBR3 cells (76). Although the potential role of GPR30 in the E2-induced ERRα expression in SKBR3 cells is not known, we certainly recognize it as a candidate. We have demonstrated that E2 induction of ERRα relies on the MAPK signaling pathway as it is susceptible to a MAPK inhibitor. Stimulation of the MAPK pathway by estrogen can lead to the activation of ERRα via phosphorylation (22, 78). Phosphorylated ERRα binds DNA and interacts with co-activators more efficiently. Therefore, it is possible that ERRα enhances its own expression through autoregulation (30, 33).

We are only beginning to understand the clinical importance of ERRα expression in breast cancer. The association of ERRα expression in breast cancer with a poor clinical outcome suggests it plays a role in tumor progression and aggressiveness (43, 44, 46). Evidence of similar correlates that appear in cancers of hormonally responsive cancers in an estrogen-independent manner. In addition, ERRα and ERα may antagonize each other’s function on certain promoters (40, 57). The correlation between overexpression of ERRα and CYP19A1 (49), a downstream target of ERRα, in breast cancer cells suggests that ERRα may contribute to increased local production of estrogens in mammary glands. Likewise, ERRα reportedly increases SULT2A1 expression and therefore may increase the pool of aromatase substrates (50). These findings suggest a potential feed forward loop in which local production of estrogen increases ERRα expression in aggressive breast tumors.

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