Regulation of Estrogen Receptor-mediated Long Range Transcription via EvolutionarilyConserved Distal Response Elements*

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Nuclear signaling by estrogens rapidly induces the global recruitment of estrogen receptors (ERs) to thousands of highly specific locations in the genome. Here, we have examined whether ER binding sites that are located distal from the transcription start sites of estrogen target genes are functionally relevant. Similar to ER binding sites near the proximal promoter region, ER binding sites located at distal locations are occupied by ERs after estrogen stimulation. And, like proximal binding sites, ERs occupy at distal sites can recruit coactivators and the RNA polymerase transcription machinery and mediate specific structural changes to chromatin. Furthermore, ERs occupied at the distal sites are capable of communicating with ERs bound at the promoter region, possibly via long range chromosome looping. In functional analysis, disruption of the response elements in the distal ER binding sites abrogated ER binding and significantly reduced transcriptional response. Finally, sequence comparison of the response elements at the distal sites suggests a high level of conservation across different species. Together, our data indicate that distal ER binding sites are bona fide transcriptional enhancers that are involved in long range chromosomal interaction, transcription complex formation, and distinct structural modifications of chromatin across large genomic spans.

Estrogens, such as 17β-estradiol (E2),3 are pleiotropic hormones whose effects are responsible for many physiological processes, including normal growth, development, and the precise and coordinated regulation of gene expression in tissues of the reproductive tract, central nervous system, and bone (1, 2).

Estrogens also have important functions in hormone-dependent diseases, such as breast cancer and osteoporosis (1, 2). Selective estrogen receptor modulators, therapeutic agents that act as agonists or antagonists depending on the target tissue, are currently used in the treatment and prevention of these and other hormone-related disorders (1–3). Estrogens and selective estrogen receptor modulators exert their effects through two estrogen receptors (ERs), ER alpha (ERα/ESR1/NR3A1) and ER beta (ERβ/ESR2/NR3A2), which belong to a large superfamily of nuclear hormone receptor proteins (2, 3). ERs share a conserved structural and functional organization with other members of the nuclear hormone receptor superfamily, including domains responsible for ligand binding, dimerization, DNA binding, and transcriptional activation (2, 3).

As their domain structures imply, ERs behave as ligand-inducible, DNA binding transcription factors (2, 3). Their transcriptional activities require the recruitment of a variety of coregulatory proteins by the receptors to estrogen-regulated promoters through either direct or indirect interactions (2, 3). A group of factors, including the p160/steroid receptor co-activator (SRC) family of proteins and the Mediator-like complexes (e.g. TRAP, DRIP, and ARC), have been shown to interact with and stimulate the transcriptional activities of ERs by interacting directly with the ligand binding domain in a ligand and activation function-2-dependent manner (2, 3). Other factors that contain enzymatic activities, such as the histone acetyltransferase p300/CBP and the histone methyltransferase CARM-1, are recruited indirectly by ERs mainly via interactions with the SRC proteins (2, 3). A smaller subset of ER-interacting factors has been shown to bind primarily to the N-terminal A/B region of the receptors, including the RNA-binding protein p68/p72 and SRA (2, 3). Together, these co-regulatory proteins are recruited by ERs in a precise temporal and coordinated manner in response to estrogen to promote local changes in histone modifications, chromatin structure, and the recruitment of RNA polymerase II to the promoters of target genes.

Numerous estrogen target genes have been identified through expression microarray studies (reviewed in Ref. 4); however, it is unclear what fraction of these genes are directly regulated by ERs. Direct regulation by estrogen is largely due to the recruitment of ERs to genomic regions containing sequence specific cis-regulatory motifs (2, 3). These sequences mostly...
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contain a 13-bp palindromic motif (GGTCAnnnTGACC) called estrogen response elements (EREs) (2, 3). Through classic molecular analyses, estrogen-regulated genes were typically found to be associated with a single ER binding site, containing either a full ERE, half ERE, or other binding elements, located near the TSS at the proximal promoter region (5). Well known examples of genes directly regulated by ER whose binding site is at the proximal promoter include CATD, EBAG9, and pS2/TFF1 (5).

To obtain a better understanding of how ER binding directly regulates gene expression under estrogen signaling, we recently mapped the genomic landscape of ERα binding sites in breast cancer cells using a ChIP-and-clone approach called ChIP-PET (Chromatin immunoprecipitation-Paired End diTags) (6). From this study, 1234 ERα binding sites were identified in estradiol-stimulated MCF-7 cells. The majority of these ER binding sites contained full EREs (71%), whereas the rest harbored either half EREs (25%) or had no identifiable ERE motif (4%). Surprisingly, most of the ER binding sites were not concentrated at the proximal promoter region of genes as anticipated but distributed throughout the genome. A large proportion of the binding sites were found at distal regions, 5–100 kb from the 5′- and 3′-ends of the adjacent transcripts. Furthermore, these ER binding sites often occurred as groups of two or more distributed far apart from each other. Whether ERs recruited to these distal binding sites are functional and how they are able to regulate the transcription of target genes from large genomic distances is unclear. Herein, we have examined in detail the functional consequences of ERα recruitment at distal binding sites on coactivator recruitment, histone modification, and ER-dependent transcription. Our results suggest that multiple ERs function cooperatively with each other by communicating through long distance interaction to modify chromatin structure and formation of a stable and active transcription machinery to directly regulate the expression of ER target genes.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—17β-estradiol (E2), 4-hydroxytamoxifen, and raloxifene were purchased from Sigma. TPBM (NSC 95910) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health. Commercially available antibodies raised against the following proteins and histone modifications were purchased from Abcam, Santa Cruz Biotechnology, Upstate, or Labvision following proteins and histone modifications were purchased from Abcam, Santa Cruz Biotechnology, Upstate, or Labvision—17β-estradiol (E2), 4-hydroxytamoxifen, and raloxifene. Commercially available antibodies raised against the following proteins and histone modifications were purchased from Abcam, Santa Cruz Biotechnology, Upstate, or Labvision: H3K4me3 (07-473), H3K9me1 (ab9045), H3K9me2 (07-030), H3K4me3 (07-473), H3K9me1 (ab9045), H3K9me2 (07-441), H3K9me3 (ab8898), acH4 (06-598), acH3 (06-599), acH3K9 (06-942), H3Arg17me2 (07-214), bulk histone H3 (ab1791), normal goat IgG (sc-2028), normal rabbit IgG (sc-2027), and normal mouse IgG (sc-2025).

Cell Culture and Transient Transfection Reporter Assays—Human mammary cancer MCF-7 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, penicillin, streptomycin, and gentamycin in a 37 °C incubator at 5% CO2. Two days prior to transfection, cells were transferred and maintained in phenol red-free Dulbecco’s modified Eagle’s medium/F-12 containing 5% charcoal-stripped fetal bovine serum, penicillin, streptomycin, and gentamycin. A day before transfection cells were plated in 24-well plates at a density of ~60%. Cells were transfected with FuGENETM 6 as recommended by the manufacturer (Roche Diagnostics). Firefly luciferase reporter constructs were transfected together with the pRenilla luciferase-TK plasmid as the internal control at a ratio of 50:1 of firefly to Renilla luciferase. Following an overnight incubation with the transfection mixture, the cells were treated with ethanol or 10 nM E2. After 48 h cells were harvested and firefly and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The relative reporter gene activity was obtained after normalization of the firefly luciferase activity with Renilla luciferase activity. Each experiment was repeated at least three times to ensure reproducibility.

Small Interfering RNA Studies—MCF-7 cells were seeded in Dulbecco’s modified Eagle’s medium/F-12 containing 5% charcoal-stripped fetal bovine serum 1 day prior to transfection. 100 nM siGENOME Non-Targeting siRNA Pool #1 or ERα ON-TARGETplus SMARTpool siRNA (Dharmacon) was then transfected into MCF-7 cells using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol. 48 h following siRNA transfection, the cells were treated with either E2 or vehicle for 45 min (for Western blot analysis, 3C, and ChIP assays) or 8 h (for mRNA analysis). Total cellular RNA was isolated with TRI® reagent (Sigma) and chloroform, ethanol precipitated, and purified using Qiagen RNeasy. The RNA was then reverse transcribed in the presence of oligo(dT)15 primer (Promega), dNTP Mix, and M-MLV RT (Promega). Quantitative PCR of the cDNA was carried out using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7900 real-time PCR machine.

Gel Mobility Shift Assays—Recombinant FLAG-tagged hERα (1–595) was expressed and purified from Sf9 cells as described previously (7). Gel mobility shift assays were performed as described previously (8). Briefly, 32P-radiolabeled probes containing sequences for either TFF1 EREs or the Xenopus vitellogenin ERE were incubated with or without 20 nM of ERα on ice for 15 min in the presence of 100 nM E2. The samples were separated on a 4.8% non-denaturing polyacrylamide gel and exposed on x-ray film.

ChIP, 3C, and ChIP-3C/ChIP-Loop Assays—ChIP experiments were performed as essentially described (6). Briefly, after 45 min of drug treatment cells were cross-linked with 1% (v/v) formaldehyde (Sigma) for 10 min at room temperature and stopped with 125 mM glycine for 5 min. Cross-linked cells were washed with phosphate-buffered saline, resuspended in lysis buffer, and sonicated for 8–10 min in a Biorupter (Diagenode) to generate DNA fragments with an average size of 500
Chromatin extracts were diluted 5-fold with dilution buffer, pre-cleared with Protein-A- and/or -G-Sepharose beads, and immunoprecipitated with specific antibody on Protein-A- and/or -G-Sepharose beads. After washing, elution and de-cross-linking, the ChIP DNA was detected by either traditional PCR (25–35 cycles) or by quantitative real-time PCR analyses with SYBR green master mix on the ABI Prism 7900.

3C was performed as described previously (9) with modifications. Briefly, MCF-7 cells were treated as mentioned in the ChIP protocol up to the cross-linking step with 1% formaldehyde. Nuclei were resuspended in 500 μl of 1.2X restriction enzyme buffer at 37 °C for 1 h. 7.5 μl of 20% SDS was added, the mixture was incubated for 1 h, followed by addition of 50 μl of 20% Triton X-100, and then incubation for an additional 1 h. Samples were then incubated with 400 units of selected restriction enzyme at 37 °C overnight. After digestion, 40 μl of 20% SDS was added to the digested nuclei, and the mixture was incubated at 65 °C for 10 min. 6.125 ml of 1.15X ligation buffer
and 375 μl of 20% Triton X-100 was added, the mixture was incubated at 37 °C for 1 h, and then 2000 units of T4 DNA ligase was added at 16 °C for a 4-h incubation.

Samples were then de-cross-linked at 65 °C overnight followed by phenol-chloroform extraction and ethanol precipitation. All primers had to be within a region of ±150 bp from the restriction enzyme digestion site. PCR products were amplified with AccuPrime Tag High Fidelity DNA Polymerase (Invitrogen) for 40 cycles. PCR products were run on a 2% agarose gel. Each validation experiment was repeated at least twice.

ChIP-3C/ChIP-Loop assays were performed as essentially described previously with slight modifications (10, 11). Briefly, antibody-specific immunoprecipitated chromatin was obtained as described above for ChIP assays. Chromatin still bound to the antibody-Protein-A-Sepharose beads was digested with restriction enzyme, ligated with T4 DNA ligase, eluted, and de-cross-linked. After purification, the ChIP-3C material was detected for long range interaction with primers from the distal and promoter ER binding regions. Primer sequences used for ChIP, 3C, and ChIP-3C assays are available upon request.

Evolutionary Conservation Analysis—The proximal promoter sequence of TFF1 (from 1 kb upstream to 200 bp downstream of the TSS) in human (hg17), chimp (panTro1), rhesus (rheMac2), mouse (mm7), rat (rn4), dog (canFam2), and cow (bosTau2) were retrieved and aligned using ClustalW. A similar procedure was used for the region upstream of TFF1 and intronic to TMPRSS3 in which the distal EREs are located. Here, a 2-kb window (chr21:42,668,352–42,670,351) was used to include the full intron and the two flanking exons.

RESULTS

Estrogen Up-regulated Genes Are Often Associated with Multiple ER Binding Sites—Our previous genome-wide ERα binding site study in MCF-7 breast cancer cells showed that estrogen up-regulated genes in general were more significantly associated with ER binding compared with down-regulated genes (6). When we examined the location of ER binding with respect to the up-regulated genes, we observed that many genes, including previously known estrogen direct targets such as TFF1, CYP1B1, SIAH2, CTSD, and GREB1, were associated with multiple ER binding sites that are distributed across large genomic distances of up to 100 kb apart (Fig. 1). From these observations, it appears that transcriptional regulation of a large subset of estrogen up-regulated genes may require the actions of not one but multiple ERs functioning collectively through a mechanism involving long distance chromatin interaction.

To understand the molecular basis of how ERs function together across large genomic distances, we examined the recruitment of ERα to the regulatory region of a well characterized E2-responsive gene, TFF1. From our ERα ChIP-PET analysis, ERα appears to be recruited at two main locations, 1) at the proximal promoter (~−400 bp) which has been previously characterized and 2) a novel region ~10 kb upstream of the TSS (Fig. 1). The ERα binding site at the proximal promoter has been shown to harbor a functional ERE motif in previous studies (12, 13). Sequence analysis of the distal ER binding site showed there are two potential ERE motifs (Fig. 2). As expected, ERα bound efficiently to radiolabeled double stranded probes containing sequences of the promoter ERE (ERE I) and the positive control, vitellogenin ERE. ERα also bound to probes containing sequences of the two EREs (ERE II and III) found in the distal ER binding site (Fig. 2B). Mutating the ERE sites in the gel mobility shift probes completely abrogated the binding of ERα to all 3 ERE sites (data...
not shown). In addition to gel shift assays, ChIP analyses were performed in MCF-7 cells to assess the *in vivo* binding of ERα at the corresponding ERE locations. ERα was recruited in a ligand-dependent manner to the promoter region, which encompasses ERE I, and to the distal ER binding site, which contains ERE II and III (Fig. 2, C and D). Taken together, these results indicate that ERα occupies multiple EREs at the proximal and distal regulatory regions of TFF1.

**Estrogen Stimulates the Accumulation of ERα, Coactivators, and RNA pol II to the Proximal and Distal ER Binding Sites of TFF1**—Estrogen-dependent transcription involves a large number of coactivators that function with the liganded ER to modify histones, alter chromatin structure and recruit RNA polymerase II (1–3). To examine the repertoire of coactivators recruited to ER binding sites of TFF1, we performed ChIP assays targeting the proximal and distal binding sites with antibodies against various proteins that are known coactivators of ERα. As expected, stimulation of MCF-7 cells with E2 resulted in enhanced recruitment of activation function-2-dependent coactivators, including SRCs (SRC-1 and AIB1), CBP, GCN5, CARM1, and the AF-1-dependent coactivators, p68 and SRA, to ERE I in the ER binding site near the proximal promoter (Fig. 3). These same coactivators also bound to both ERE II and ERE III in the distal ER binding site in a similar manner. Interestingly, in addition to coactivators, RNA pol II (both the phosphorylated and unphosphorylated forms) was also recruited to all three ERE sites, indicating that the distal EREs may have similar functional properties as the proximal ERE.

The recruitment of ER, coactivators, and the RNA polymerase machinery to the distal ER binding site of TFF1 suggests that regulatory elements located far away may play an important role in estrogen-dependent transcription. Because our binding experiments were limited to only the ERE sites, it is possible that we may have overlooked important information regarding the recruitment of ER, coactivators, and RNA polymerase to other regulatory regions of TFF1. We therefore expanded our analysis by scanning across a 19-kb region of the TFF1 locus by ChIP quantitative PCR, which included not only the proximal and distal ERE binding sites, but also surrounding upstream and downstream regions such as the coding and non-coding region of the TFF1 gene. As shown in Fig. 4, estrogen induced the recruitment of ERα at three major locations along the 19-kb TFF1 locus with one sharp distinct peak at the pro-

**FIGURE 3.** The distal ER binding site of TFF1 functions by recruiting coactivators and the basal transcription machinery. ChIP assays using antibodies against ERα, coactivators, and RNA pol II were performed with MCF-7 cells treated with or without E2 as described in Fig. 2.

**FIGURE 4.** Estrogen induces the recruitment of ERα and coactivators to specific sites while inducing RNA pol II spreading across large genomic regions. ChIP scanning of the TFF1 locus. ChIP assay was performed with MCF-7 cells treated with or without E2 as described in Fig. 2 using antibodies against ERα, SRC3, CBP, phosphorylated (pol II Ser 5), and un-phosphorylated (pol II) RNA pol II and IgG. ChIP material was quantified by quantitative PCR with multiple primers spanning from −12 kb to +7 kb from the TSS of TFF1. Each graph represents the average of three independent experiments ± S.E.
moter corresponding to ERE I, and another two (one small and one large) peaks at 9 and 10 kb corresponding to ERE II and ERE III, respectively. The recruitment of coactivators AIB1 and CBP also generated similar binding profiles as ERα/H9251. In contrast, estrogen induced a very different pattern for RNA pol II recruitment compared with ERα/H9251 and the coactivators. Both the phosphorylated and un-phosphorylated RNA pol II bound to three main regions with very broad occupancy spanning 4–5 kb in width. Two of these regions co-localized with the proximal and distal ER binding sites, whereas a third RNA pol II peak appeared after the TFF1 gene. Collectively, our ChIP analyses suggest that the distal EREs can recruit the same set of factors as the ERE at the proximal promoter of TFF1.

The TFF1 Locus Is Marked by Distinct Histone Modifications before and after E2 Stimulation—Modification of specific residues on histone tails have been correlated with certain transcriptional outcome (14, 15). For example, acetylation of histone H3 and acetylated histone H4, mono-, di-, and tri-methylated histone H3K4, mono-, di-, and tri-methylated histone H3K9, acetylated histone H3K9, and di-methylated histone H3R17. Anti-bulk histone H3 was used as a control. Each graph represents the average of three independent experiments ± S.E.

FIGURE 5. Distinct histone modifications across the TFF1 locus. ChIP scanning assays were performed as described in Fig. 4 with antibodies against ERα, acetylated histone H3, acetylated histone H4, mono-, di-, and tri-methylated histone H3K4, mono-, di-, and tri-methylated histone H3K9, acetylated histone H3K9, and di-methylated histone H3R17. Anti-bulk histone H3 was used as a control. Each graph represents the average of three independent experiments ± S.E.

little is known about the effects of estrogen signaling on specific histone modifications. This is particularly most evident across large genomic regulatory regions.

To examine the changes of histone modification across the entire TFF1 locus under estrogen signaling, we performed similar ChIP scanning experiments as described above. As shown in Fig. 5, bulk acetylated histones H3 and H4, which are histone marks associated with gene activation, were enhanced across the TFF1 locus with two broad peaks surrounding the distal and proximal ER binding sites. These modifications also extended throughout the gene. Mono- and dimethylation at H3K4 produced a similar profile as bulk histone acetylation, but these modifications were already present at high levels and were not further enhanced by estrogen stimulation. In contrast, tri-methylation of H3K4 was found broadly distributed at the promoter and gene regions under un-induced state but was further enhanced after estrogen stimulation. The profile for acetylated H3K9 was similar to H3K4me3 with extensive modifications at the promoter region but minor at the distal ER binding site. We
same set of coactivators and marked by similar histone modifications. A surprising observation was that RNA polymerase was recruited to both binding regions. These findings suggest that under estrogen stimulation the distal ER binding site may exist in close proximity with the ER binding site at the promoter region, possibly via long distance chromatin interaction. To explore whether this occurs, we used chromosome conformation capture (3C), an assay developed for detecting long range chromosome interactions (17). As shown in Fig. 6B, a ligation product was detected after estrogen stimulation between the promoter and enhancer (P-E) region of TFF1 compared with the promoter and a control (P-C) region, suggesting the promoter and distal ER binding sites interact in close proximity with each other.

Although the long range chromosome interaction at TFF1 is dependent on estrogen stimulation and occurs around the distal and promoter ER binding sites, it is possible that the interaction could be mediated through other sequence elements or indirectly via a non-estrogen signaling pathway rather than direct ERα binding to EREs. First, to determine if the observed long range interaction is dependent on ERα, we knocked down the level of ERα protein in MCF-7. siRNA targeted to ERα efficiently reduced the amount of ERα protein compared with control siRNA (Fig. 6C). The lower level of ERα resulted in a dramatic decrease in TFF1 expression (Fig. 6D), and ERα recruitment to the ER binding sites (Fig. 6E). Importantly, the depletion of ERα abolished the long range chromosomal interaction between the distal

also examined di-methylation of H3R17, which has been shown to be associated with activation of estrogen-regulated genes (16). ChIP scanning shows that this mark superimposed the profile of ERα. Taken together, our results show that ERα via recruitment of coactivators at the distal and proximal ER binding sites can induce extensive changes in chromatin structure across the entire span of the TFF1 locus.

**ERα Directly Mediates Long Distance Chromatin Interactions at the TFF1 Locus**—Our ChIP experiments showed that both the distal and promoter ER binding sites were occupied by the

To explore in further detail whether direct binding of ERα to the EREs in the distal and proximal ER binding sites is necessary for chromosome interaction, we specifically blocked ERα from binding to DNA with TPBM, a newly identified small molecular inhibitor that binds to the DNA binding domain of Eras (18). As shown in Fig. 6G, TPBM significantly reduced the estrogen-stimulated recruitment of ERα to ERE I, II, and III and the Erα-mediated long range chromosomal interaction between the distal and proximal ER binding sites of TFF1 (Fig. 6H).
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FIGURE 7. ERα, coactivators, and RNA polymerase II form a long distance chromosomal structure between the enhancer and proximal promoter of TFF1. A, schematic diagram showing the primers and restriction enzyme used in the ChIP-3C/ChIP-Loop assay at the TFF1 locus. B, ChIP-3C/ChIP-loop assay was performed on ERα-immunoprecipitated ChIP material obtained from MCF-7 cells treated with and without E2 for 45 min as described in Fig. 2. Ligation product was detected by PCR using one primer near the distal ER binding site and another primer near the proximal ER binding site as shown in A. ChIP-3C assays were performed as described in B using antibodies against ERα, CBP, AIB1, and RNA pol II (C), acetylated histone H3, acetylated histone H4, and acetylated histone H3K9 (D). ChIP-3C assays were performed at short term (0, 15, 30, 45, and 60 min) (E) and long term (45 min versus 180 min) (F) exposure to E2.

In addition to the DNA binding domain, we also examined the role of the ligand binding domain of ERα in mediating chromosomal interaction. For this, we treated MCF-7 cells with estrogen, or the selective estrogen receptor modulators, tamoxifen and raloxifene, which bind to the ligand binding domain and inhibit ERα transcriptional activity by switching it from recruiting coactivators to corepressors (2, 3). As shown in Fig. 6, long range chromosomal interactions was observed between the distal and promoter ER binding sites of TFF1 whether MCF-7 cells were treated with estrogen, tamoxifen, or raloxifene (although at a lower level for tamoxifen and raloxifene), suggesting that the ligand binding domain of ERα is important in mediating both long range chromatin interactions in transcriptional activation and repression. Overall, our results show that the long range chromosome interaction between the distal and proximal ER binding sites at the TFF1 is mediated by the direct binding of ERα to the ERs.

Estrogen Stimulates the Formation of a Transcriptionally Active Complex at the TFF1 Promoter via ERα-mediated Long Distance Chromosomal Interactions—ERα recruits coactivators such as SRCs and CBP/p300 to activate transcription (1–3). As shown in Fig. 3, coactivators are recruited to both the distal and proximal ER binding sites of TFF1. To determine if these factors are involved in or form part of the long range chromosome interaction at TFF1 we used a technique called ChIP-coupled 3C/ChIP-loop assay (10). This assay is a variation of the 3C assay in which an immunoprecipitation step is included to enrich for factor-bound chromatin.

As shown in Fig. 7B, a ligation product was detected in the ChIP-3C assay when we used an antibody against ERα, suggesting that ERα is part of the long range chromosomal interaction complex between the distal and proximal ER binding site of TFF1. Long range interaction between two ER binding sites was also detected when antibodies against AIB1, CBP, and RNA pol II were used in the ChIP-3C/ChIP-loop assay (Fig. 7C). Because similar histone modification marks appear at both the distal and proximal ER binding sites (Fig. 5), we examined whether long range interaction can be detected using specific histone modification antibodies. Similar to ERα, coactivators, and RNA pol II, acetylated histones H3, H4, H3K9, and dimethylated H3R17 were also found to be part of the long range interaction complex (Fig. 7D).

Next, we examined the kinetics of the long range chromosome interaction at various times after estrogen stimulation. Interaction was detected within 15 min of estrogen treatment, continued until 45 min, and then disappeared by 60 min (Fig. 7E). The disappearance was not permanent as long range interaction formed again after 3 h of estrogen stimulation (Fig. 7F). The observed appearing and disappearing of the chromatin interaction correlates well with and is likely due to the cyclical binding of ERα as shown previously by several groups (19, 20). Taken together, our 3C and ChIP-3C/ChIP-loop results suggest that, under a transcriptionally active condition, the TFF1 regulatory region is folded into a looped structure consisting of the distal and proximal ER binding sites, ERα, coactivators, RNA pol II, and specific histone modifications. Furthermore, this interaction appears to be highly dynamic.

The Distal ERE Is Required for Maximal Transcriptional Activation of TFF1—To determine whether the ERs in the ER distal binding site can function as transcriptional enhancers, we cloned the ER binding site into a luciferase reporter construct for transient transfection assays. The distal ER binding site was obtained by PCR amplification of genomic DNA, inserted into pGL4-TATA (pGL4.10 containing a minimal TATA box), and transiently transfected into hormone-deprived MCF-7 cells. As
activation of the TFF1 promoter. Taken together, our transient transfection results suggest that ERE III in the distal ER binding site of TFF1 plays a crucial role in the long distance transcriptional regulation of the gene.

ERE III of TFF1 Is Evolutionarily Conserved—DNA sequences that are functionally important are more likely to be retained and unchanged through evolution. To examine whether the EREs of TFF1 are evolutionarily conserved, we compared the sequences of the EREs to different species to determine how it might function together with the proximal ER binding site, we cloned a 10.5-kb region of the TFF1 promoter, which covers both the proximal and distal ER binding sites into pGL4.10. The 10.5-kb TFF1 promoter construct was highly responsive to E2 (Fig. 8C). In deletion analysis, removal of ERE III from the 10.5-kb TFF1 promoter (d1) drastically reduced (but did not completely abrogate) the transcriptional activity and estrogen response of the promoter. Continued deletion of the TFF1 promoter had little effect on the activity and response of the promoter until the proximal promoter ERE I was removed (d5).

In addition to deletional analysis, we also performed mutational analysis of the ERE sites individually and in various combinations in the context of the full-length promoter (Fig. 8D). Our results show that mutating either ERE I (mt1) or ERE II (mt2) did not reduce the transcriptional activity of the promoter. In contrast, mutating ERE III (mt3) alone was enough to significantly lower the transcriptional activity and estrogen response of the TFF1 promoter. Further mutational analysis consisting of ERE III with either ERE I (mt5) or ERE II (mt6) completely abrogated the activity of the promoter, indicating ERE III can function together with either ERE I or ERE II for maximal transcriptional activation of the TFF1 promoter. The results were also obtained for the double ERE mutant construct (mt3). These results indicate that the distal ER binding site is a bona fide transcriptional enhancer acting mainly through ERE III.

To examine the activity of the distal ER binding site in the context of the entire TFF1 promoter and also to determine how it might function together with the proximal ER binding site, we cloned a 10.5-kb region of the TFF1 promoter, which covers both the proximal and distal ER binding sites into pGL4.10. The 10.5-kb TFF1 promoter construct was highly responsive to E2 (Fig. 8C). In deletion analysis, removal of ERE III from the 10.5-kb TFF1 promoter (d1) drastically reduced (but did not completely abrogate) the transcriptional activity and estrogen response of the promoter. Continued deletion of the TFF1 promoter had little effect on the activity and response of the promoter until the proximal promoter ERE I was removed (d5).

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ERE III of TFF1 Is Evolutionarily Conserved—DNA sequences that are functionally important are more likely to be retained and unchanged through evolution. To examine whether the EREs of TFF1 are evolutionarily conserved, we compared the sequences of the EREs to different species whose genomes have been sequenced (Fig. 9). Sequences
homologous to the promoter and to the distant region in various primates, rodents, and carnivores were retrieved and
aligned to assess the level of conservation of the three EREs. We observed that both ERE I and ERE II were only conserved within
primates, while ERE III appeared to trace back deeper into the
mammalian phylogeny. The highly conserved nature of the ERE
III together with the above functional results suggests that the
ERE III is the primary functional binding site for regulating
TFF1.

**DISCUSSION**

We recently reported the whole genome cartography of ERα
binding sites in MCF-7 cells (6). A significant portion of the
binding sites identified were located at large genomic distances
from the TSS of E2-regulated transcripts (Fig. 1). It is unclear
whether these binding sites are simply platforms or docking
stations for ERα binding or functional sites that have a role in
estrogen-mediated transcription. Herein, using the TFF1 locus
as a model to investigate transcriptional regulation by ERα,
we provide evidence that distal ER binding sites are
bona fide transcriptional enhancers that are critical for achieving maximum
transcriptional activity. Our data reveal the distal ER binding
site of TFF1 is occupied by the same suite of transcription fac-
tors as the proximal ER binding site (Figs. 2–4). This complex
consists of ERα, coactivators, and the RNA polymerase
machinery. Furthermore, we show that the distal ER binding
site can communicate with the proximal ER binding site via
long range chromosomal interaction (Fig. 6). This interaction
appears to involve multiple factors, including the receptor,
coactivators, and RNA polymerase II (Fig. 7). Functional anal-
ysis of the distal ER binding site reveals that the ERE motifs
located in these regions play a major role in determining the
overall transcriptional strength of the promoter (Fig. 8). Finally,
a comparison of the TFF1 ER binding sites across different spe-
cies indicate there is a high level of evolutionarily conservation
in particular with the distal ER binding site (Fig. 9). Taken
together, our results reveal an additional level of transcriptional
regulation by ERs in the estrogen signaling pathway, namely
long range chromosome interactions.

TFF1 was one of the first estrogen-responsive genes identi-
fied (21, 22). Previous studies mapped a single ERE-like motif at
−405 to −393 bp upstream of the TSS (12, 13). Since then, this
regulatory region of TFF1 has been studied extensively and now
is viewed as the model promoter for examining transcriptional
regulation by ERs. However, our study clearly demonstrates
that transcription of TFF1 is controlled not only by a single ER
at the proximal promoter but also EREs located far away from
the gene. We present several pieces of supporting evidence to
show this, including the detection of long range chromosomal
interaction between the distal site with the proximal site (Fig.
6). This observation is similar to the interaction reported
recently by another group while this study was in progress (10).
In addition to interaction studies, we provide functional anal-
ysis showing that maximal transcriptional activity of the TFF1
promoter requires multiple EREs working together (Fig. 8).
Finally, we also show that the distal ER binding site is highly
conserved across many species of vertebrates (Fig. 9). Together,
these results clearly indicate that future experiments using the
TFF1 promoter as a model for examining the mechanism
of transcriptional regulation by ERs should consider the contri-
butions of all the EREs in the analysis.

What are the critical factors that are necessary in the forma-
tion of long range chromosomal interaction under estrogen sig-
naling? We detected long range chromosome interaction only
in the presence of estrogen (Fig. 6), suggesting that ERα is
required and most likely act as anchor points at the two ends
on the interaction. However, ERα almost certainly is not the only
factor that is required in mediating the interaction. The RNA
polymerase machinery and coactivators, including p68, AIB1,
and CBP/p300, are recruited by ERα (Figs. 3 and 4) to the
ER binding sites and are also found to be part of the looped tran-
scription complex (Fig. 7). It is conceivable they could also potentially have roles that are important in forming and maintaining of the chromosome interaction besides their regular functions. Recently, coregulatory factors such as FOXA1 and GATA3 have been reported to play an important role in regulating ER-dependent transcription (10, 23). These factors are highly enriched near ERα binding sites, thus they may have a role in mediating long range chromosomal interaction. We have also observed long range interaction at the TFF1 locus under anti-estrogen conditions such as tamoxifen and raloxifene (Fig. 6I). This result raises the question whether there are similar or distinct factors that are required to mediate long range chromosomal interaction under transcriptional activation and repression. In future studies, it will be interesting to explore these possibilities further.

To date, most studies examining the transcriptional regulation of estrogen target genes have created a simple model whereby ERα is recruited to a single ERE located at the proximal promoter of the gene to activate transcription (5). However, emerging evidence from our laboratory and others suggests that transcriptional regulation by estrogens may be much more complicated involving long range chromosomal interactions that are mediated by multiple ERs (and therefore multiple ERs) (6, 10). Although the current study focused only on the TFF1 locus, we have similar results indicating the transcription of many estrogen target genes, including GREB1, P2RY2, and SIAH2, are also regulated by multiple EREs/ERs and long range chromosomal interactions.4 Long range transcriptional regulation may not only be limited to ERs but may also be used by other nuclear receptors, because recent studies suggests that transcription may not only be limited to ERs but may also be used by other nuclear receptors, because recent studies suggests that hormone signaling, regulation of transcription involving interactions between enhancers and promoters of genes has been reported in several other systems, including immune response (27, 28), development (29–31), and more recently maintenance of pluripotency in stem cells (32). Currently, the best characterized system is the β-globin locus, in which the coordinated formation of DNA loops and the specific expression of transcripts at this region are mediated by the transcription factors, GATA1 and EKLF (29, 30).

From this study and previously published results by others, it is now well accepted that long range chromosomal interaction is a basic mechanism of transcriptional regulation. However, one major question currently eluding the field is, how common is this mechanism used for regulating transcription inside the cell? Our genome-wide analysis of ERα binding sites in MCF-7 cells revealed that distal binding sites represent the majority of the binding sites in the genome, suggesting there is a strong possibility that long range chromosomal interaction may be a general mode of transcriptional regulation by the estrogen receptor in breast cancer cells. However, to identify all these interactions using 3C would be tedious, time consuming, and probably impossible. Newer techniques such as 4C (33, 34) and 5C (35) are improvements on 3C, but these techniques are not genome-wide. Thus, to determine whether long range chromosomal interaction is indeed widely utilized by ERs and other transcription factors, the future challenge is to develop methods that will be able to map all the long range interactions in the genome.

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