

Estrogen Receptor Regulation of Carbonic Anhydrase XII through a Distal Enhancer in Breast Cancer

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Abstract

The expression of carbonic anhydrase XII (CA12), a gene that encodes a zinc metalloenzyme responsible for acidification of the microenvironment of cancer cells, is highly correlated with estrogen receptor α (ER α) in human breast tumors. Here, we show that CA12 is robustly regulated by estrogen via ER α in breast cancer cells, and that this regulation involves a distal estrogen-responsive enhancer region. Upon the addition of estradiol, ER α binds directly to this distal enhancer *in vivo*, resulting in the recruitment of RNA polymerase II and steroid receptor coactivators SRC-2 and SRC-3, and changes in histone acetylation. Mutagenesis of an imperfect estrogen-responsive element within this enhancer region abolishes estrogen-dependent activity, and chromosome conformation capture and chromatin immunoprecipitation assays show that this distal enhancer communicates with the transcriptional start site of the CA12 gene via intrachromosomal looping upon hormone treatment. This distal enhancer element is observed in the homologous mouse genomic sequence, and the expression of the mouse homologue, *Car12*, is rapidly and robustly stimulated by estradiol in the mouse uterus *in vivo*, suggesting that the ER regulation of CA12 is mechanistically and evolutionarily conserved. Our findings highlight the crucial role of ER in the regulation of the CA12 gene, and provide insight into the transcriptional regulatory mechanism that accounts for the strong association of CA12 and ER in human breast cancers. [Cancer Res 2008;68(9):3505–15]

Introduction

Estrogen receptor α (ER α), a hormone-regulated transcription factor and member of a superfamily of nuclear receptors (1, 2), is expressed in ~70% of breast cancers (3). As the major regulator of the phenotypic properties of these breast cancers, ER α markedly influences the pattern of breast cancer gene expression and, perhaps more than any other protein, it defines the distinctly different gene signatures of ER-positive and ER-negative breast cancers (4–7). In our recent breast cancer gene expression profiling studies, we observed the carbonic anhydrase XII gene (CA12) to be robustly stimulated by estradiol (E2) in several ER-containing breast cancer cells (8, 9). Furthermore, from our examination of transcriptional profiling data sets in ER-positive and ER-negative

breast tumors, we found CA12 to show one of the most highly significant positive correlations with ER expression (10–12).

Carbonic anhydrase XII is a transmembrane, extracellular enzyme and member of a family of zinc metalloenzymes that catalyze the reversible hydration of CO₂ to form bicarbonate (H₂O + CO₂ \rightleftharpoons H⁺ + HCO₃[−]), thereby regulating the microenvironment acidity and tumor malignant phenotype (13–16). CA12 was originally identified as a protein overexpressed in renal cancer cells (13), but is now known to be also overexpressed in some other cancers, including breast cancer (17, 18). Although both CA12 and the closely related tumor-associated carbonic anhydrase IX (CA9) are thought to be regulated by hypoxia, only CA12, and not CA9, exhibits a strong positive correlation with ER expression in breast tumors (4, 19), suggesting that CA12 might be under ER regulation. CA12 expression in breast tumors is associated with positive ER α status, lower grade disease, lower relapse rates, and better overall patient survival (20–22).

To understand the mechanistic basis underlying this strong association between ER positivity and CA12 expression, in the work reported here, we have explored the regulation of CA12 by the ER. Our results document that the CA12 gene is under primary transcriptional up-regulation by the estrogen-occupied ER and that this regulation in breast cancer cells is mediated by ER action through a distal enhancer that we have herein characterized. Upon estrogen stimulation, this enhancer binds ER α through an imperfect estrogen response element (ERE) and recruits p160 coactivators. Furthermore, by chromosomal looping, this ER-dependent enhancer communicates with the promoter of the CA12 gene, markedly enhancing the transcription of the CA12 gene. Our findings define a mechanistic basis for the robust coexpression of CA12 and ER in breast cancer.

Materials and Methods

Cell culture and experimental treatments. MCF-7 cells were maintained in minimal essential medium (MEM; Sigma Chemical Co.) supplemented with 5% calf serum (HyClone), 100 μ g/mL of penicillin/streptomycin (Invitrogen), and 25 μ g/mL of gentamicin (Invitrogen). T47D cells were routinely maintained in MEM and antibiotics supplemented with 5% fetal bovine serum (Atlanta Biologicals) and bovine insulin (6 ng/mL; Sigma). All cells were grown in phenol red-free MEM supplemented with 5% charcoal dextran-treated serum for at least 5 days prior to use in experiments.

Animal care and treatments. Eight-week-old ovariectomized C57BL/6 mice were obtained from Harlan Co., and housed under controlled conditions of light and temperature with free access to standard chow and water. All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee. At 16 days after ovariectomy, mice were injected s.c. with E2

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(0.5 µg/animal) dissolved in DMSO then diluted 1:10 in corn oil or with control vehicle DMSO/corn oil alone. At 4 or 24 h after hormone or vehicle injection, uteri were removed, weighed after removal of associated fat, and snap-frozen in liquid nitrogen for RNA isolation.

RNA isolation, reverse transcription, and real-time PCR. Cell and whole uterine total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. RNA samples were reverse-transcribed in a total volume of 20 µL using 200 units of reverse transcriptase, 50 pmol of random hexamer, and 1 mmol/L of deoxynucleotide triphosphates (New England Biolabs). The resulting cDNA was then diluted to a volume of 500 µL with nuclease-free water. Real-time PCR was performed on an ABI Prism 7900HT instrument using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations. Briefly, each PCR contained 1× master mix, 4 µL of the diluted cDNA reaction, and 50 nmol/L of forward and reverse primers designed to yield 80- to 125-bp amplicons. PCR was carried out through 40 cycles (95°C for 15 s, 60°C for 1 min) following an initial 10-min incubation at 95°C. Relative expression levels were calculated as described previously, using acidic ribosomal protein 36B4 mRNA as an internal control (9). Real-time PCR of chromatin immunoprecipitation (ChIP) samples was performed in a similar manner, with appropriate primers.

Small interfering RNA studies. Small interfering RNA (siRNA) duplexes targeting ERα (forward, UCAUCGCAUCCUUGCAAAdTdT; reverse, UUUGCAAGGAAUGCGAUGAdTdT) and control (GL3 luciferase, no. D-001400-01) were obtained from Dharmacon and transfected into cells at a final concentration of 20 nmol/L using DharmaFECT transfection reagent according to the manufacturer's recommendations at 72 h prior to ligand treatment.

Immunoblotting. Whole cell lysates of MCF-7 cells were prepared using 1× cell lysis buffer (Cell Signaling Technology) in the presence of Complete Mini protease inhibitor cocktail (Roche Applied Science). Protein concentration of whole cell lysates was determined by BCA Protein Assay (Pierce). Proteins (20 µg) were boiled in 2× Laemmli buffer and separated by electrophoresis using 10% SDS-PAGE at 150 V for 50 min, and were then transferred to a nitrocellulose membrane (Pall Corp.), using the wet transfer method at 100 V for 90 min. Membranes were blocked with 5% milk in TBS. Rabbit anti-human CA12 primary antibody (13) was incubated with blocked membrane overnight at 4°C. The blot was then washed with TBS containing 0.1% Tween 20 prior to incubation with horseradish peroxidase-conjugated secondary antibody (Zymed Antibodies). The blot was incubated with Super Signal West Femto enhanced chemiluminescence reagents (Pierce) and exposed to film in order to observe protein bands.

Genomic cloning, mutagenesis, and luciferase reporter assays. The indicated genomic DNA associated with estrogen receptor-binding and intervening regions was amplified by PCR from human genomic DNA (Roche Molecular Biochemicals) using specific primers and cloned into either pGL3-Promoter or pGL3-Basic luciferase vectors (Promega) using the *Mlu*I and *Bgl*II sites. Site-directed mutagenesis was performed using QuikChange II kit (Stratagene) according to the manufacturer's directions. All constructs were sequenced to verify their correctness. Briefly, 1,000 ng of pGL3 reporter vector and 25 ng of pRL-SV40 were cotransfected into MCF-7 cells in 24-well plates using Lipofectamine 2000 in OptiMEM according to the manufacturer's instructions (Invitrogen). Cells were transfected for 6 h, washed, and treated with the indicated ligands for 16 h prior to cell lysis in 1× passive lysis buffer (Promega) and measurement of luciferase activity in MLX Microtiter Plate Luminometer (Dynex Technologies).

ChIP assays. Whole-genome ERα-binding sites were mapped in MCF-7 cells treated with 10 nmol/L of E2 for 45 min using a ChIP-Paired End diTag (ChIP-PET) cloning and sequencing strategy described previously (23), from which data was obtained on ERα-binding sites near the *CA12* gene. Standard ChIP assays were performed essentially as previously described (24, 25), with a few noted modifications. Following the addition of ethanol vehicle or ligands for the indicated times, MCF-7 cells were cross-linked using 1% formaldehyde at 37°C for 10 min, washed twice with PBS, and harvested in ice-cold PBS plus 1× protease inhibitor cocktail (Roche) and 10 mmol/L of DTT. Cell pellets were first resuspended in nuclei isolation buffer [50 mmol/L Tris (pH 8.0), 60 mmol/L KCl, 0.5% NP40, protease

inhibitor, and 10 mmol/L DTT], centrifuged at $1,000 \times g$ for 3 min, and resuspended in lysis buffer [0.5% SDS, 10 mmol/L EDTA, 0.5 mmol/L EGTA, 50 mmol/L Tris (pH 8.0), protease inhibitor, and 10 mmol/L DTT]. Nuclei were sonicated (Fisher Scientific, Sonic Dismembrator Model 100) thrice at 80% maximum power for 10 s and the sonicate was centrifuged at $14,000 \times g$. The supernatant was diluted 1:4 by dilution buffer [1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris (pH 8), protease inhibitor, and 10 mmol/L DTT] and precleared with 15 µL of preimmune IgG (Santa Cruz Biotechnology, Inc.), 2 µg of salmon sperm DNA, and 50 µL of 25% protein A-agarose slurry (Santa Cruz Biotechnology). Complexes were incubated at 4°C overnight with 2 to 5 µg of antibody, then pulled down at 4°C for 1 h with 60 µL of 25% protein A-agarose slurry and 2 µg of salmon sperm DNA. Antibodies used were for ERα (HC-20, Santa Cruz Biotechnology), RNA Polymerase II (N-20, Santa Cruz Biotechnology), SRC-2 (M-343, Santa Cruz Biotechnology), SRC-3 (H-270, Santa Cruz Biotechnology), and acetylated histone H4 (07-329, Upstate Biotech). Precipitates were sequentially washed with 1 mL of washing buffer [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), and 150 mmol/L NaCl], 1 mL washing buffer II [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.0), 500 mmol/L NaCl], 1 mL washing buffer III [0.25 mmol/L LiCl, 1% NP40, 1% sodium deoxycholate, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 8.0)] and twice with 1 mL of TE [1 mmol/L EDTA and 10 mmol/L Tris-HCl (pH 8.0)]. Chromatin complexes were incubated at room temperature for 20 min with 100 to 300 µL of elution buffer (1% SDS, 0.1 mol/L NaHCO₃). The cross-linking was reversed by incubating at 65°C overnight with 200 mmol/L of NaCl and 200 mg/mL of proteinase K (Invitrogen Corp.). DNA was purified with QIAquick columns (Qiagen) and amplified by real-time PCR.

Chromosome conformation capture assays. MCF-7 cells were grown according to the protocol for ChIP assays above and treated with the indicated ligands for 45 min prior to fixation in 2% formaldehyde at 37°C for 10 min. The formaldehyde was quenched with the addition of 0.125 mol/L of glycine and cells were lysed in lysis buffer [10 mmol/L Tris (pH 8.0), 10 mmol/L NaCl, 0.2% NP40, and 1× Complete Protease Inhibitors (Roche)] at 4°C for 90 min. Nuclei were resuspended in 1× New England Biolabs Buffer 2, 0.3% SDS and incubated at 37°C for 60 min while rotating. Triton X-100 was added to a final concentration of 1.8% to sequester the SDS and incubated at 37°C for 60 min while rotating. The chromatin was then digested overnight using *Mse*I (New England Biolabs) or *Btg*I (New England Biolabs) at 37°C while rotating. SDS was added to a final volume of 1.6% and the samples were heated at 65°C for 20 min. Two-microgram aliquots of the chromatin samples were diluted in ligation buffer containing 1% Triton X-100 and incubated at 37°C for 1 h. The temperature was lowered to 16°C and T4 ligase (New England Biolabs) was added and samples were incubated overnight. The ligated DNA was purified using phenol/chloroform extraction and analyzed using PCR amplification. Resulting PCR products were sequenced and mapped back to the UCSC Genome Browser for verification.

Results

CA12 regulation by estrogen is a primary transcriptional response mediated by the estrogen receptor. In our prior transcriptional profiling microarray analyses of gene expression stimulation by E2 in ER-positive breast cancer and osteosarcoma cells (8, 9, 26), we observed a very marked up-regulation of *CA12* gene expression by E2. To investigate *CA12* regulation by estrogen in breast cancer in greater detail, and to elucidate the mechanism underlying this regulation, we first examined the time course of *CA12* mRNA and protein increases in response to E2 and selective estrogen receptor modulators (SERMs) in ERα-positive MCF-7 and T47D breast cancer cells. *CA12* RNA was significantly stimulated after 2 hours of E2 exposure and continued to increase to maximal stimulation levels by 4 and 8 hours in MCF-7 and T47D cells, respectively, and remained greatly elevated throughout the 48 hours of treatment in both cell lines (Fig. 1A). Increases in

CA12 protein levels were detected in as early as 2 to 4 hours, and continued to increase throughout the time course of treatment (Fig. 1B), consistent with the early and sustained stimulation of CA12 RNA by E2. The SERMs, *trans*-hydroxytamoxifen and raloxifene, induced CA12 RNA by 3-fold to 4-fold, ~40% of that obtained with E2 (Fig. 1C), and in a similar manner, *trans*-hydroxytamoxifen and raloxifene stimulated CA12 protein to approximately one-third that of E2 (Fig. 1D). The ER full antagonist and selective estrogen receptor down-regulator, fulvestrant (ICI 182,780), had no stimulatory effect on CA12 RNA, and it was able to inhibit the E2-, *trans*-hydroxytamoxifen-, and raloxifene-mediated stimulation of CA12 (Fig. 1C). Of the other steroid receptor ligands examined, only dihydrotestosterone was able to mildly stimulate CA12, possibly through androgen receptor or because of its low affinity for ER (27), whereas the glucocorticoid receptor and progesterone receptor agonists, hydrocortisone and medroxyprogesterone acetate, respectively, did not regulate CA12 expression (Fig. 1C).

We next examined the requirement of the estrogen receptor itself for E2-mediated stimulation of CA12 mRNA. MCF-7 cells were pretreated for 60 minutes with vehicle or a log₂ molar excess of fulvestrant prior to treatment with E2; CA12 mRNA and protein levels were assessed after 2 or 8 hours, respectively (Fig. 2A and B). Fulvestrant did not increase CA12 mRNA or protein, but it was able to fully inhibit the E2-mediated up-regulation. We also examined the requirement of ER in CA12 mRNA stimulation by siRNA-mediated depletion of ER α from MCF-7 cells. MCF-7 cells were transfected with siRNA which was shown to deplete cells of >95% of ER α (data not shown). Loss of ER at 72 hours posttransfection

reduced the basal CA12 mRNA level, likely due to possible ligand-independent ER transactivation activity, and abolished the E2- and SERM-mediated stimulation of CA12 (Fig. 2C). These results indicate that ER is required for SERM regulation of the CA12 gene.

In addition to the ER itself, we also determined the requirement of ongoing transcription and translation for E2 stimulation of CA12. MCF-7 cells were pretreated for 60 minutes with the RNA polymerase inhibitor actinomycin D or the translational inhibitor cycloheximide prior to treatment with E2 (Fig. 2A). The E2-mediated stimulation of CA12 mRNA (or *trans*-hydroxytamoxifen- or raloxifene-mediated increase in CA12 mRNA; data not shown) was inhibited by actinomycin D, but not cycloheximide, suggesting that ongoing transcription, but not synthesis of new protein factors, is necessary for CA12 mRNA stimulation by the ER. Taken together, these results suggest that CA12 regulation by estrogen is a primary transcriptional response mediated by the ER.

Because of our observations of the requirement of ER for CA12 regulation, we examined CA12 expression in primary ER-positive breast tumors by analysis of several gene expression data sets from ER-positive breast tumors (Fig. 2D). These analyses reveal a very positive correlation of CA12 expression with ER α expression in primary breast tumors, shown in the scatter plots in Fig. 2D. Our findings highlighting the crucial role of ER in CA12 up-regulation may account for the robust coexpression of CA12 and ER α observed in human breast cancers.

E2-bound ER is recruited to a distal region upstream of the CA12 transcription start site *in vivo*. The ER primarily functions as a signal-activated transcriptional transactivator through direct

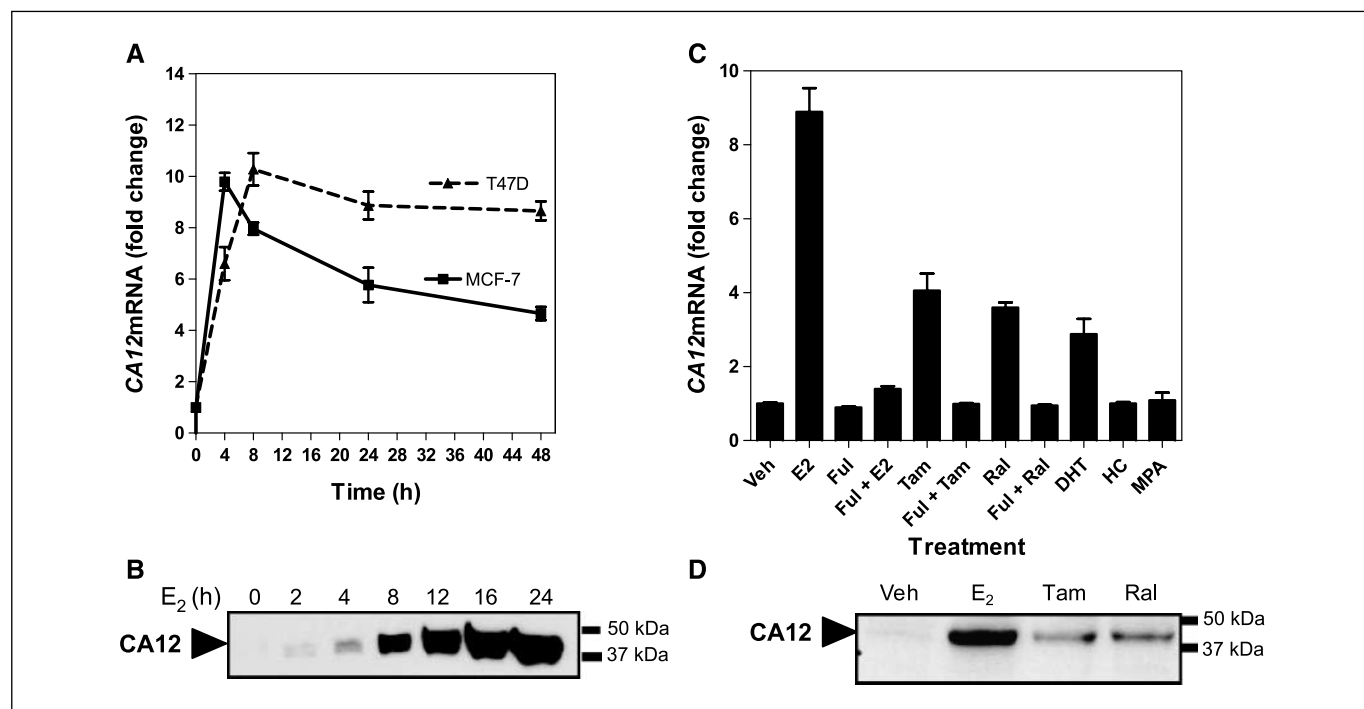


Figure 1. Various estrogen receptor ligands increase CA12 levels in breast cancer cells. **A**, CA12 mRNA is induced in a time-dependent manner by E2 in ER α -expressing MCF-7 and T47D breast cancer cells. Cells were treated with 10 nmol/L of E2 for 0 to 48 h. RNA was isolated, reverse-transcribed, and cDNA measured by quantitative PCR using primers for CA12 and internal control 36B4 mRNA. **B**, CA12 protein levels are induced by E2 in a time-dependent manner. MCF-7 cells were treated with 10 nmol/L of E2 for 0 to 24 h and total cellular lysates were used for CA12 immunoblotting. **C**, CA12 mRNA is induced by ER agonists. MCF-7 cells were treated for 8 h with vehicle (0.1% ethanol) or with intracellular receptor ligands E2 (10 nmol/L), fulvestrant (ICI 182,780, *Ful*; 1 μ mol/L), fulvestrant + E2, *trans*-hydroxytamoxifen (*Tam*; 100 nmol/L), fulvestrant + *trans*-hydroxytamoxifen, raloxifene (*Ral*; 100 nmol/L), fulvestrant + raloxifene, dihydrotestosterone (*DHT*; 10 nmol/L), hydrocortisone (*HC*; 10 nmol/L), or medroxyprogesterone acetate (*MPA*; 10 nmol/L). Cells were then harvested and qRT-PCR performed as above. **D**, E2 and the SERMs induce CA12. MCF-7 cells were treated for 8 h with 10 nmol/L E2, 100 nmol/L *Tam*, or 100 nmol/L *Ral* and CA12 protein levels assessed by immunoblotting as above.

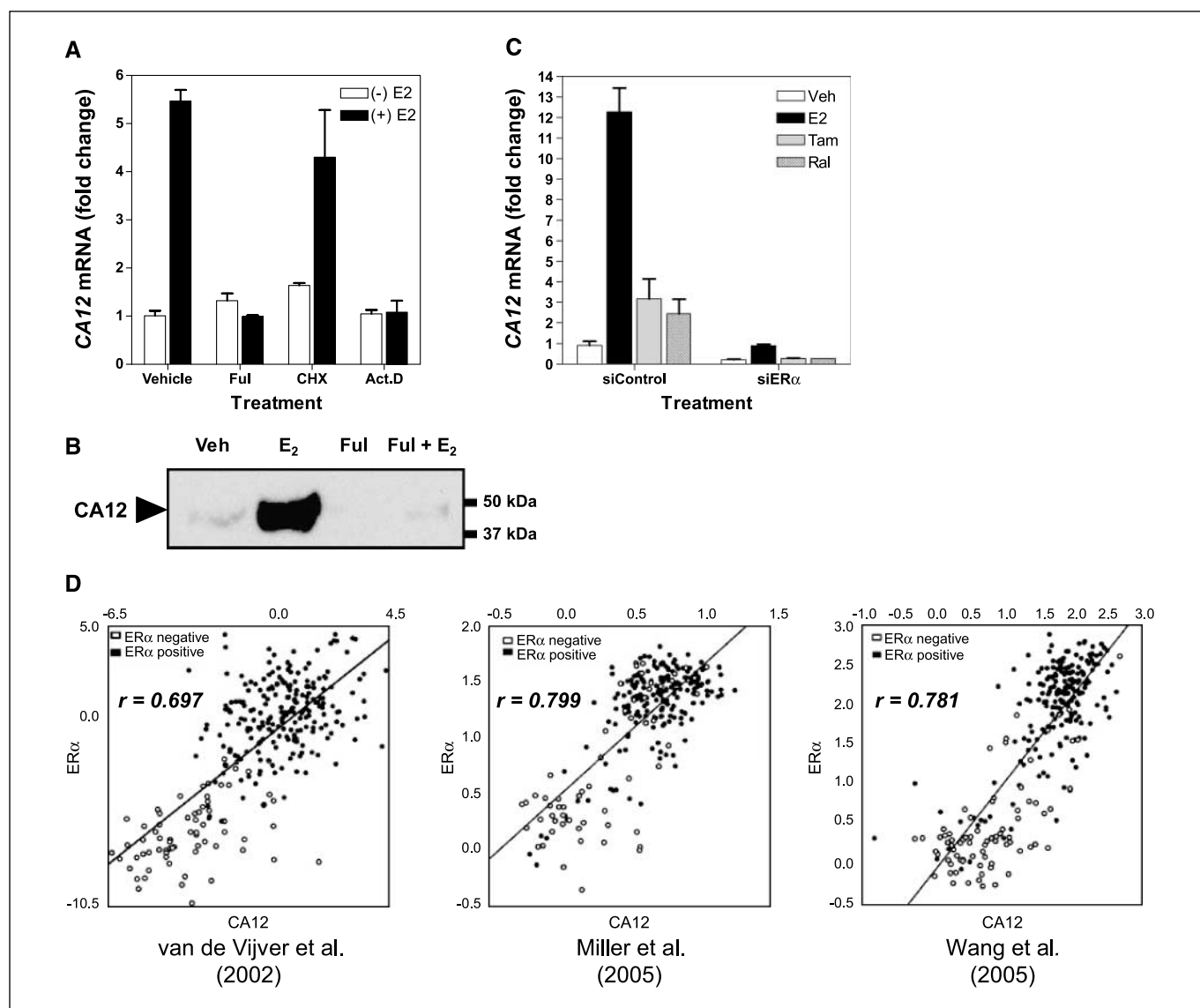


Figure 2. E2 stimulation of *CA12* gene expression is sensitive to actinomycin D and fulvestrant (ICI 182,780), but not cycloheximide, and requires ER α . Strong association of *CA12* and ER α in breast tumor data sets. **A**, *CA12* mRNA induction by E2 is blocked by pretreatment with the transcriptional inhibitor actinomycin D, or the pure ER antagonist fulvestrant, but not the translational inhibitor cycloheximide. MCF-7 cells were pretreated for 60 min with 0.1% DMSO, 1 μ M of fulvestrant, 10 μ g/mL of cycloheximide (CHX), or 5 μ M of actinomycin D (Act.D) and then 0.1% ethanol or 10 nmol/L of E2 was added for 2 h. qRT-PCR for *CA12* mRNA was performed. **B**, ER antagonist fulvestrant blocks E2 stimulation of *CA12*. MCF-7 cells were treated for 8 h with 0.1% ethanol, 10 nmol/L of E2, 1 μ M of fulvestrant, or both E2 and fulvestrant prior to cell lysis and immunoblotting for *CA12*. **C**, *CA12* mRNA induction is ER-dependent. MCF-7 cells were transfected with 5 nmol/L of siControl or 5 nmol/L of siRNA against ER α for 72 h. Cells were then treated for 4 h with 0.1% ethanol, 10 nmol/L of E2, 100 nmol/L of *trans*-hydroxytamoxifen, or 100 nmol/L of raloxifene prior to RNA isolation and qRT-PCR analysis. **D**, scatter plots and correlation between *CA12* and ER α RNA expression in breast tumors from the indicated studies. The plots were generated from the Oncomine Database. x and y axes, fold changes in expression for *CA12* and ER α (ESR1), respectively.

binding to DNA response elements or other protein transcription factors (24, 28). To examine the role of ER in regulating *CA12* mRNA, we used a series of ChIP experiments to investigate the recruitment and binding of ER to chromatin. Genome-wide ChIP-PET experiments using an antibody against ER α to capture DNA loci bound by ER after 45 minutes of E2 exposure in MCF-7 cells showed a cluster of ER-binding DNA fragments ~6 kb upstream from the transcriptional start site (TSS) of the *CA12* gene (Fig. 3A and B). Further examination of this ChIP-PET cluster of bound DNA fragments at ~6 kb revealed a cluster of five overlapping fragments, and two single upstream ChIP-PET DNA fragments considered to be experimental noise (Fig. 3B; ref. 23).

To validate and further examine the extent of ER binding within the *CA12* genomic region, we performed ChIP scanning for ER α in MCF-7 cells treated for 45 minutes with vehicle or 10 nmol/L of E2 and amplified recovered ChIP DNA fragments using 100 bp primer sets tiled approximately every 500 bp from -6.5 kb to the transcriptional start site of the *CA12* gene (Fig. 3C). Of note, within this region, there are four predicted imperfect EREs and multiple response elements for factors to which ER α is known to tether (e.g., activator protein-1), suggesting multiple putative ER-binding sites. ChIP scanning for ER α binding revealed robust E2-induced binding approximately at the -6 kb binding region previously shown to bind ER α through the genomewide ChIP-PET experiments, and

low-level binding of ER α at the proximal promoter region, but near-background level binding at intervening positions (Fig. 3C). These ChIP assays further define the one robust ER α -binding region at a distal region ~6 kb upstream from *CA12*.

E2- and SERM-induced transcription factor recruitment and chromatin modifications to *CA12* genomic regions *in vivo*. To better understand the regulation of the *CA12* gene, we further examined the recruitment of ER, coactivators, RNAPII, and permissive histone modifications at the enhancer, proximal promoter, and additional loci in MCF-7 cells treated with vehicle, 10 nmol/L of E2, or 100 nmol/L of *trans*-hydroxytamoxifen. ChIP experiments coupled with quantitative reverse transcription (qRT)-PCR showed specific and robust recruitment of both E2- and *trans*-hydroxytamoxifen-bound ER α at the enhancer region after 45 minutes, with minimal binding to a region upstream of the enhancer or to an intermediate position (Fig. 4, *middle*) at approximately -4 kb. The binding of ER to the enhancer region in E2- or *trans*-hydroxytamoxifen-treated cells remained elevated

over the vehicle at 4 and 24 hours of exposure. At the proximal promoter (TSS), the largest subunit of RNA polymerase II was bound to a certain degree in the absence of hormone, but increased 7-fold after 45 minutes of E2, consistent with a well-established role of E2-mediated formation of productive transcriptional complexes. Consistent with the lower potency of *trans*-hydroxytamoxifen in the stimulation of *CA12* gene expression, *trans*-hydroxytamoxifen-treated cells showed less RNAPII recruitment at the TSS. RNAPII was not appreciably recruited to areas upstream of the TSS with either E2 or *trans*-hydroxytamoxifen treatments.

We also examined the recruitment of both of the coregulators SRC-2 and SRC-3 to the enhancer region in the presence of E2 (Fig. 4), and found a large change in the recruitment of both at 45 minutes, and also at the 4- and 24-hour time periods monitored. In the presence of *trans*-hydroxytamoxifen, coactivator recruitment at the enhancer was comparable with that of vehicle, suggesting that the *trans*-hydroxytamoxifen-bound ER does not efficiently recruit p160 coregulators, as others have observed (29). To

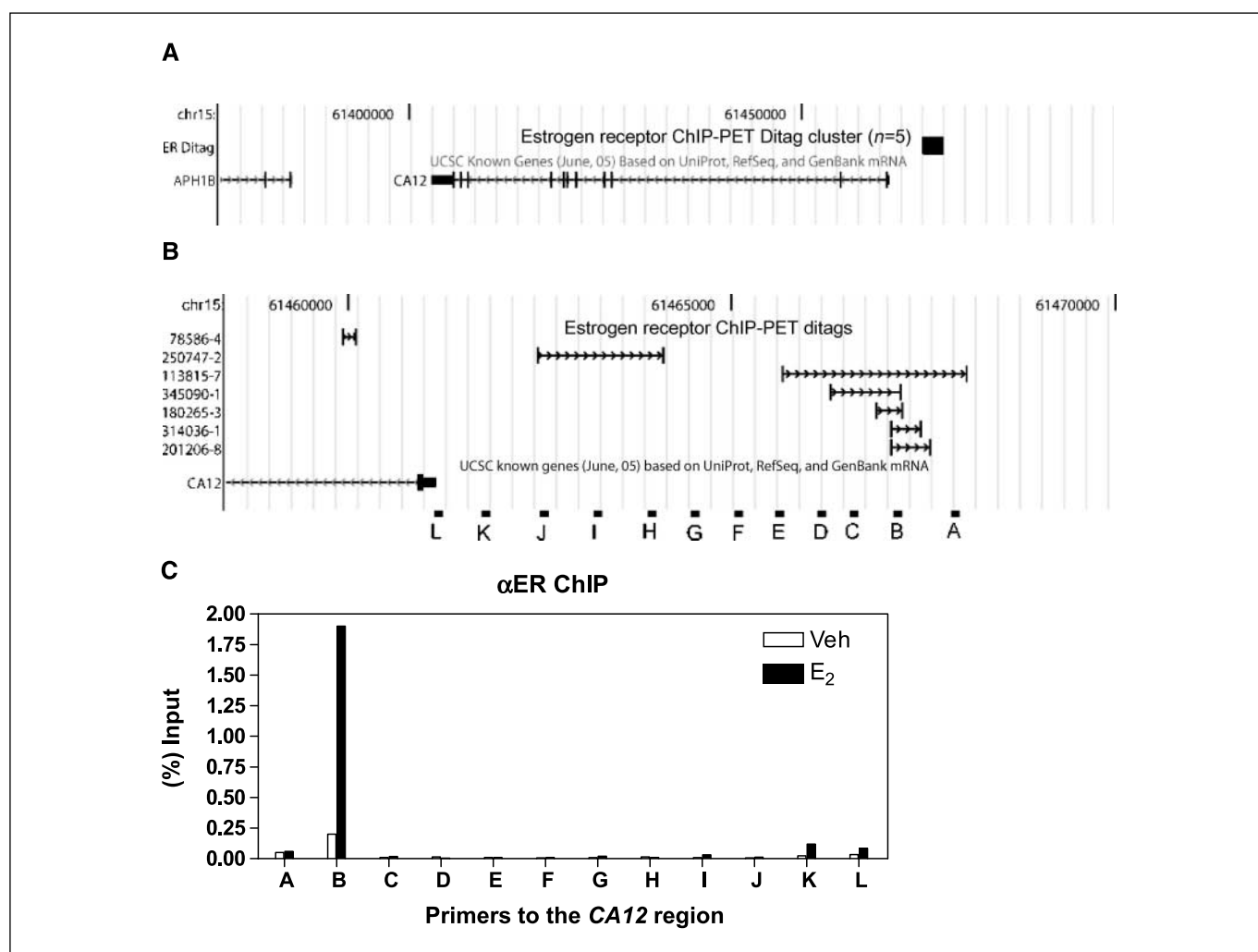


Figure 3. E2-occupied ER is recruited to a distal region 6.5 kb upstream of the *CA12* transcription start site *in vivo*. **A**, whole-genome ER α -binding sites were mapped in MCF-7 cells treated with 10 nmol/L of E2 for 45 min using the ChIP-PET strategy (23) and mapped to the *CA12* genomic region in the UCSC Genome Browser (Hg17). A singular ChIP-PET cluster ~6 kb 5' to the *CA12* transcriptional start site (*top*), with a higher resolution map with individual fragments indicated (*bottom*). **B** and **C**, ChIP scanning of the *CA12* genomic region *in vivo* validates ChIP-PET identification of putative *CA12* enhancer. **B**, a schematic representation of chromosome 15 and primer set locations (A–L) immediately 5' to the *CA12* transcriptional start site. **C**, E2-occupied ER α is recruited to an upstream region ~6 kb 5' to the *CA12* gene. MCF-7 cells were treated for 45 min with control 0.1% ethanol or 10 nmol/L of E2, subjected to ER α ChIP, and immunoprecipitated DNA amplified using PCR primers as denoted in **B** and recovered DNA represented as a percentage of the input.

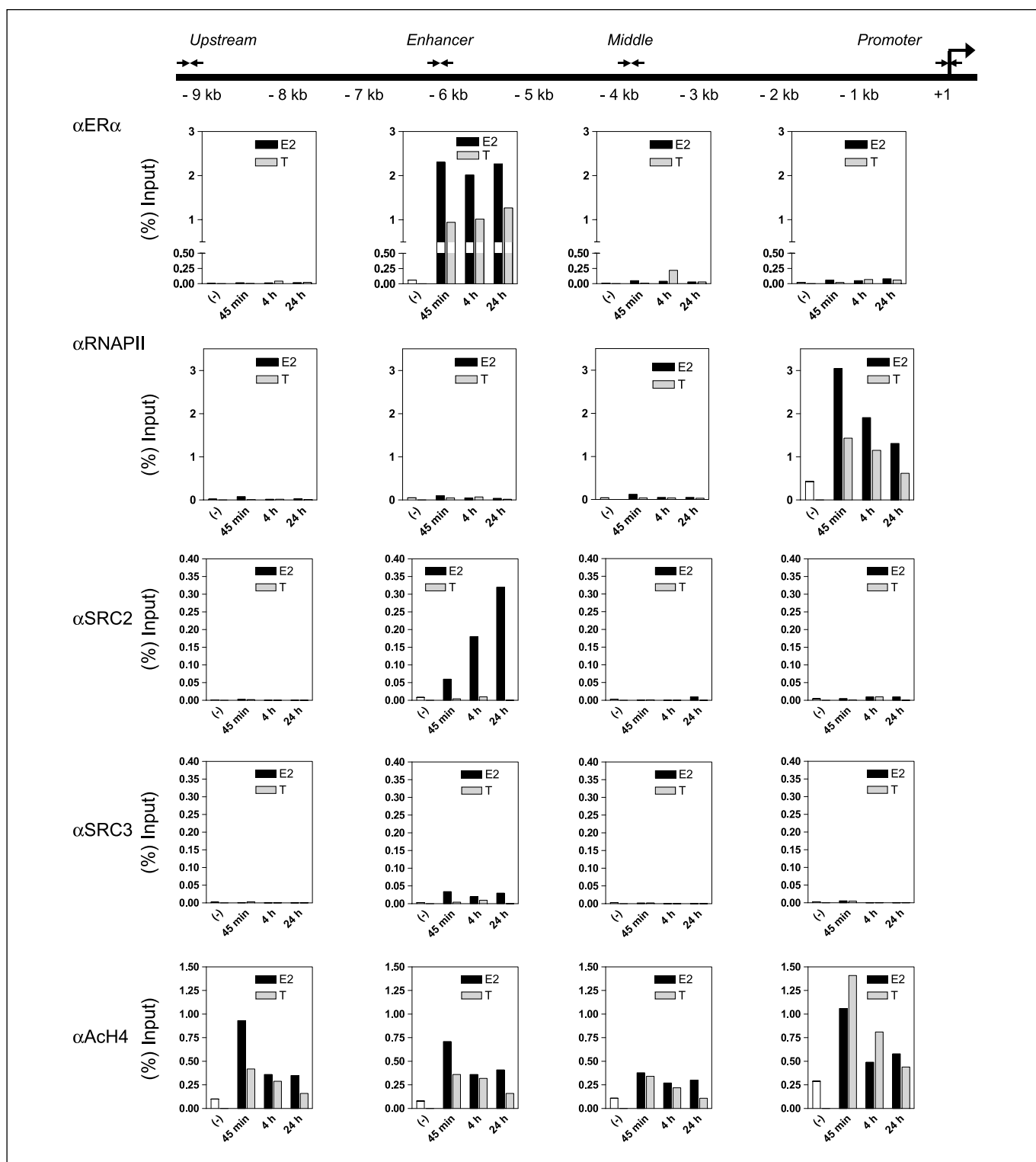


Figure 4. E2- and SERM-induced transcription factor recruitment and chromatin modifications to the *CA12* genomic region *in vivo*. E2- and *trans*-hydroxytamoxifen-treated MCF-7 cells were examined for recruitment of ER α , RNA polymerase II, SRC-2, and SRC-3 binding and acetylated H4 modifications within the *CA12* genomic region using various primer sets at denoted positions (top). Immunoprecipitated DNA, expressed as a percentage of the input, from experiments using specific indicated antibodies (left) and amplified using primer sets as denoted above.

determine the degree of chromatin modifications consistent with gene activation, we also examined the level of histone H4 tail acetylation (AcH4), considered to be a general marker of acetylated, "relaxed" histones permissive of transcriptional regula-

tion. We observed a marked increase of AcH4 at the enhancer region in both E2 and *trans*-hydroxytamoxifen-treated cells (E2 > *trans*-hydroxytamoxifen), which was greatest at 45 minutes and decreased at later times. This E2- and SERM-mediated increase

in AcH4 was noted at the TSS and also, interestingly, at the middle and particularly at the upstream regions shown not to bind ER. These are supportive of findings by others showing a high level of histone lysine acetylation, consistent with chromatin remodeling, at the active enhancer and promoter of stimulated genes (30), as well as more broadly in nearby regions.

Estrogen regulation of the cloned CA12 enhancer is mediated by an imperfect ERE. The *in vivo* recruitment of agonist-bound ER α and coactivator proteins, and histone modifications associated with transactivation, suggest that the identified region ~6 kb upstream of the TSS is an enhancer for the CA12 gene. Sequence analysis of putative transcription factor-binding sites revealed one imperfect ERE with a 1 bp mismatch at approximately -6047 (relative to TSS; Fig. 5A). To understand the *cis* elements involved in recruiting ER α and facilitating transactivation of CA12, we cloned the ~6.9 kb fragment of genomic DNA containing the putative upstream enhancer spanning a region approximating the captured DNA fragments binding ER α (ChIP-PET experiments; Fig. 3B) to just upstream of the CA12 TSS (-6832 to +46, Chr15:61461083-61467960). In addition, we also cloned a truncated 1.8 kb fragment approximating the overlapping ChIP-PET cluster of ER α -binding fragments (-6832 to -4999), and both full-length (-6832 to +46) and truncated (-6832 to -4999) genomic fragments were then subcloned into luciferase reporter

vectors to assay putative estrogen responsiveness (Fig. 5A). After transfection into MCF-7 cells and exposure to 10 nmol/L of E2 for 16 hours, the full-length reporter was stimulated approximately eight times over vehicle-treated transfectants or empty vector (Fig. 5B). In addition, the truncated reporter approximating the greatest overlap of ChIP-PET fragments (-6832 to -4999) was able to stimulate reporter activity upon treatment with E2 comparable to that of the full-length reporter (-6832 to +46; Fig. 5B), implying that the E2-responsive region is located in the far-upstream genomic region.

As noted above, the ER-binding region at approximately -6 kb has one imperfect ERE (Fig. 5A), which seemed a likely putative *cis*-regulatory element to direct ER α -dependent transactivation. To examine this, we mutated both the single consensus half-ERE (Mut. 1.0), or both the consensus half-ERE and imperfect arm of the ERE (Mut. 1.1). As seen in Fig. 5A and B, mutation of either one or both arms of the ERE abolished the ability of E2 to stimulate the -6832 to -4999 reporter, strongly suggesting that ER regulation of the cloned CA12 enhancer is dependent on an imperfect (1 mismatch) ERE at -6047 relative to the TSS.

The ER-binding distal enhancer communicates with the transcriptional start site of the CA12 gene via intrachromosomal looping upon estrogen treatment *in vivo*. The observed *in vivo* recruitment of E2-bound ER to a putative distal enhancer

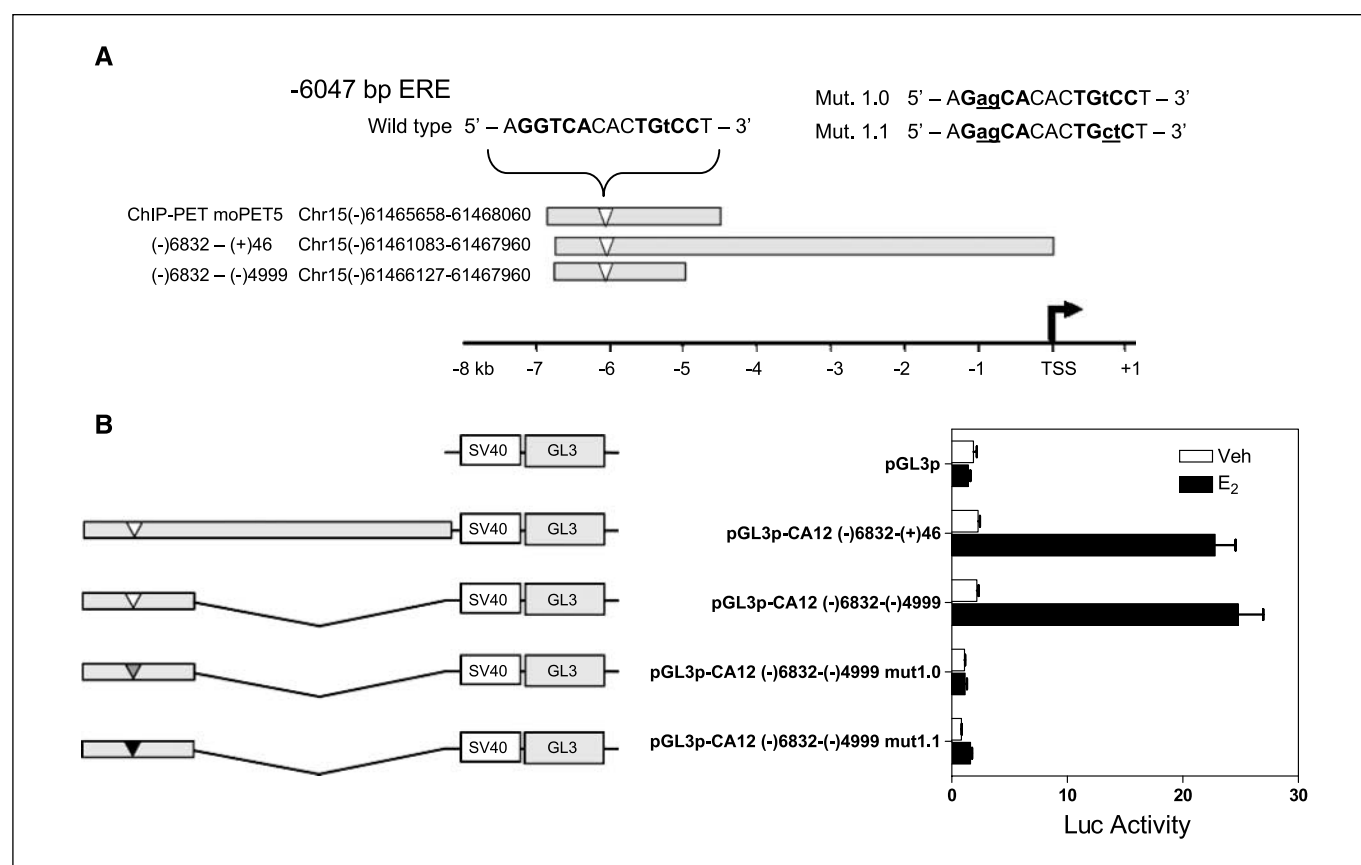


Figure 5. Estrogen regulation of the cloned CA12 enhancer is mediated by an imperfect ERE. *A*, a schematic representation indicates genomic regions identified as binding ER α by ChIP scanning and ChIP-PET analysis (ChIP-PET moPET5) and cloned genomic regions used for *cis*-element reporter assays. At the open triangle position is the wild-type imperfect ERE sequence (top left), as well as the mutated sequences used (top right; mutated nucleotides underlined and bases differing from wild-type in lowercase). *B*, cloned CA12 enhancer reporter activity is dependent on an imperfect ERE. Full-length, truncated, mutated, and empty reporter plasmids (left, 1 μ g each) were cotransfected with pRL-SV40 (25 ng) into MCF-7 cells in 24-well plates. At 24 h after transfection, cells were treated for 16 h with 0.1% ethanol or 10 nmol/L of E2, lysed, and assayed for Firefly and Renilla luciferase activities (right). Open triangles, wild-type imperfect ERE ~6 kb upstream of CA12 TSS; gray triangle, mutant 1.0; and black triangle, mutant 1.1.

region, and the E2- and ERE-dependent activation of the cloned ER-binding enhancer in transiently transfected MCF-7 cells together suggest that the E2-mediated stimulation of *CA12* is via an ER-dependent upstream enhancer ~6 kb upstream from the *CA12* gene. To test the *in vivo* utilization of this enhancer, we used chromosome conformation capture (3C) assays to examine the putative communication of the ~6 kb enhancer and the *CA12* proximal promoter. Briefly, after 45 minutes of E2 treatment at 10 nmol/L, MCF-7 cells were fixed with formaldehyde and chromatin was isolated, digested with *MseI*, and subjected to dilute intramolecular ligation before de-cross-linking, DNA isolation, and PCR amplification of DNA fragments of interest. Chromatin regions which were in close proximity with each other at the time of fixation were examined using PCR primers complementary to genomic DNA from the enhancer or proximal promoter regions. Specifically, primers were used in variable combinations to examine the presence of enhancer DNA only, proximal promoter DNA only, or all possible DNA species only produced by intramolecular ligation of DNA derived from the respective enhancer and promoter regions after fixation and digestion (Fig. 6, top).

As shown in Fig. 6A (middle), we observed a ligase- and E2-dependent 780 PCR product amplified using the "A" forward primer from the enhancer region and "F" reverse primer from the proximal promoter region of *CA12* (Fig. 6A and B). Normally separated by ~6 kb, the DNA amplified in the resulting PCR fragment was purified, sequenced, and mapped via BLAT to the UCSC Human Genome Browser, and resulting sequence analysis indicated that the E2- and ligase-dependent product was derived from both *CA12* enhancer and promoter regions (Fig. 6A, bottom). The estrogen-enhanced communication of the distal enhancer and proximal promoter in the 3C assay strongly suggests that this ER-binding enhancer is functionally active *in vivo*. Taken together with the ChIP assays in which we did not observe appreciable *in vivo* recruitment of ER α or RNAPII in the intervening regions between the ER-binding enhancer and RNAPII-binding promoter, these 3C data suggest that the ER-binding enhancer transactivates the *CA12* gene via intrachromosomal looping.

Conservation of the *CA12* enhancer in mammalian genomes and of *CA12* regulation by estrogen receptor. Analysis of the upstream region of *CA12* by Vertebrate Multiz Alignment reveals a high degree of multispecies conservation within the newly described ERE-containing enhancer region (Fig. 6B). Within the enhancer region (Fig. 6B), there is only a 1-bp mismatch between the human and mouse 15mer ERE. To determine whether there is estrogen regulation of *Car12*, the mouse orthologue of the *CA12* gene, ovariectomized female mice were treated with E2 or vehicle, and uteri were collected at 4 or 24 hours postinjection (Fig. 6C). At both time points examined, *Car12* gene expression was robustly stimulated by E2. These results provide strong evidence that the ER regulation of *CA12* is both mechanistically and evolutionarily conserved.

Discussion

Our studies reveal that the ER robustly up-regulates *CA12* gene expression in breast cancer cells and that this transcriptional regulation is mediated by a hormone-responsive enhancer located ~6 kb upstream of the start site of transcription of the *CA12* gene. This marked regulation of *CA12* by the ER may account for the

strong coexpression of ER and *CA12* that is observed in breast tumors.

Dynamic signal-specific assembly of transcription factors at enhancers is an increasingly recognized aspect of biological control of genes essential for developmental and hormonal response programs. Recent studies have suggested that the majority of estrogen-responsive genes may be under the control of ER-binding sites at a considerable distance (>5 kb) from the target RNA-coding loci (23, 31, 32), but there has been only limited evidence that they function as genomic regulatory elements for these relatively distant hormone-regulated genes. Here, we describe the regulation of the carbonic anhydrase XII (*CA12*) gene by agonist-bound ER through a long-range distal enhancer that we have characterized through *in vivo* ChIP-scanning across the *CA12* genomic region and ChIP-PET analysis, and by the ability of this element to strongly activate hormone-dependent expression of a reporter. This enhancer contains an imperfect ERE which we show to be essential for its ER regulation through mutagenesis and transfection studies. 3C and ChIP assays show a physical interaction between this distal enhancer and the *CA12* promoter in breast cancer cells upon E2 treatment, indicating a direct role for the enhancer in *CA12* expression.

Associated with the recruitment of ligand-occupied ER to hormone-dependent enhancers is the recruitment of coregulators (2, 33, 34), some with histone acetyl transferase activity, resulting in distinct changes in histone acetylation status and chromatin conformational changes. In the case of *CA12* gene regulation, we observed markedly increased recruitment to the enhancer of the p160 coregulators, SRC2 and SRC3 that have histone acetyltransferase (HAT) activity. Of note, increased histone H4 acetylation status was observed not only at the enhancer and promoter regions, but also broadly throughout the upstream 5'-flanking region from the promoter to the enhancer, and even at a more upstream region, suggesting that chromatin changes are affected over a broad region after receptor occupancy by ligand. As expected, RNA polymerase recruitment was only observed at the promoter.

SERMs such as tamoxifen and raloxifene, shown to be effective in both the prevention and treatment of breast cancer (35, 36), often have mixed agonist-antagonist activities on estrogen-regulated genes in breast cancer (26, 37, 38). Tamoxifen was a weak stimulator of *CA12* RNA and protein expression compared with E2. In keeping with this, tamoxifen was less effective in increasing the recruitment of ER to the enhancer, in recruiting RNA polymerase II to the *CA12* promoter, and in augmenting the acetylation of histone H4. As observed previously for estrogen-regulated gene expression by SERMs (29), tamoxifen did not recruit the SRC coregulators, suggesting that other coregulators are likely involved in eliciting the weak agonistic activity of tamoxifen on this gene.

Interestingly, the distal enhancer element displays synteny with the homologous mouse genomic sequence, and its robust stimulation by E2 in the mouse uterus highlights that ER regulation of *CA12* is mechanistically and evolutionarily conserved. Other approaches, including bioinformatic coupled with genome-wide nuclear receptor-binding site analyses, have suggested the likely conservation of gene-regulatory mechanisms at other estrogen-responsive genes across mammalian species (23, 32).

Gene regulation by long distance enhancers has recently been observed for other nuclear receptors, such as the androgen receptor in its control of the prostate-specific antigen gene (39). Also, recent reports have documented long distance enhancer

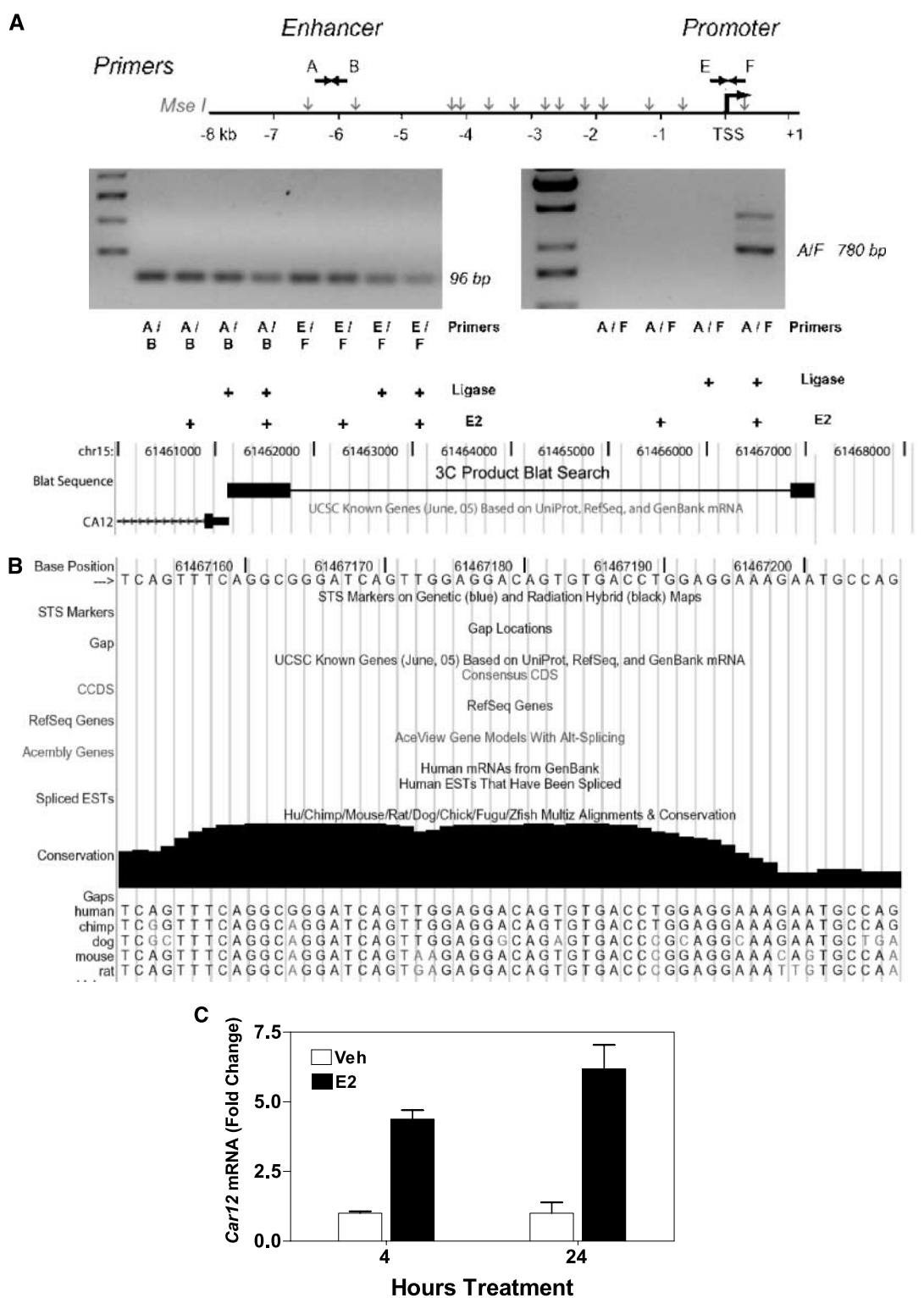


Figure 6. ER-binding distal enhancer communicates with the transcriptional start site of the *CA12* gene via intrachromosomal looping upon estrogen treatment *in vivo*; enhancer shows conservation between humans and mice. **A**, schematic of the *CA12* genomic region, *MseI* restriction enzyme cut sites, and primer positions for 3C assays (top). *CA12* enhancer and promoter show enhanced communication upon E2 treatment in MCF-7 cells *in vivo* (middle). Cells were treated for 45 min with 0.1% ethanol or 10 nmol/L of E2, cross-linked, and subjected to 3C analysis using the indicated primer pairs. E2- and ligase-dependent 3C PCR product was purified, sequenced, and mapped to the human genome by UCSC BLAT indicating proximal and distal DNA communication (bottom). **B**, Vertebrate Multiz Alignment shows conservation of the -6 kb *CA12* ERE-containing enhancer region. **C**, *Car12* mRNA is induced by E2 in the mouse uterus. Eight-week-old female mice were ovariectomized and at 16 days post-ovariectomy, they were injected with vehicle or E2. Uteri were collected 4 or 24 h later and total RNA was isolated and subjected to qRT-PCR for *Car12* mRNA.

regulation of *GREB1* (gene regulated in breast cancer-1), encoding a protein with an unknown function but suggested to contribute to the enhancement of proliferation of MCF-7 cells by E2 (40, 41). In the case of *GREB1*, its stimulation by E2 is mediated by the binding of ER to three consensus EREs spread over ~20 kb of upstream flanking sequences (41, 42).

CA12 is a membrane zinc metalloenzyme that is present in a variety of normal tissues but is overexpressed in some cancers (13, 20, 21, 43–47). In MCF-7 cells, we find that CA12 and CA9 are the only carbonic anhydrases that are expressed (D.H. Barnett and B.S. Katzenellenbogen, data not shown). CA12 and CA9 mRNA and protein levels are stimulated by hypoxia in a variety of cancer cell lines, and their expression is down-regulated by a return to normoxia (13, 17, 47, 48). Of note, however, we find that only CA12, and not CA9, is regulated by estrogen, and likewise, only CA12, and not CA9, exhibits a strong positive correlation with ER expression in breast tumors (4, 19). The activity of CA12 as a metalloenzyme, catalyzing the reversible hydration of carbon dioxide to form bicarbonate, is likely involved in modulating a variety of physiologic processes including transport of carbon dioxide and other solutes, as well as acidification of microenvironments that can modulate the tumor malignant phenotype (16, 49, 50). That CA12 expression in breast tumors is associated with lower grade disease, positive ER α status, and lower relapse rates and better overall patient survival (20–22) suggests that the estrogen receptor regulation of CA12 expression may be an important variable in this more optimal breast tumor phenotype.

Gene expression microarray profiling has documented ER α as a master transcriptional regulator of the phenotype and behavior of

~70% of human breast cancers, and that the gene expression signatures in ER α -positive and ER α -negative breast tumors are profoundly different (5–7, 19). CA12 is one of the genes whose expression is most highly correlated with ER α in breast cancer. In fact, for comparison with the correlations shown in Fig. 2D for CA12 and ER α , we examined the association of ER α with progesterone receptor, a well-characterized ER target gene and useful clinical marker, in the same three studies of primary breast cancer gene expression. Interestingly, the correlation of progesterone receptor with ER in the studies by van de Vijver (0.296), Miller (0.287), and Wang (0.374) was considerably less than the correlation of CA12 with ER in the same data sets (Fig. 2D), highlighting the robust association of CA12 and ER expression. Our findings reveal a transcriptional regulatory mechanism that likely underlies this robust coexpression of CA12 and ER α in human breast cancers. In addition, our findings imply that involvement of long distance enhancers in the regulation of estrogen-responsive genes in breast cancer may be more frequent than previously appreciated.

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Estrogen Receptor Regulation of Carbonic Anhydrase XII through a Distal Enhancer in Breast Cancer

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