Interplay between Estrogen Response Element Sequence and Ligands Controls in Vivo Binding of Estrogen Receptor to Regulated Genes*

Received for publication, July 2, 2003, and in revised form, November 13, 2003
Published, JBC Papers in Press, November 14, 2003, DOI 10.1074/jbc.M307076200

Adam J. Krieg‡, Sacha A. Krieg§, Bonnie S. Ahn, and David J. Shapiro¶
From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801–3602

To examine the role of the estrogen response element (ERE) sequence in binding of liganded estrogen receptor (ER) to promoters, we analyzed in vivo interaction of liganded ER with the imperfect ERE in the pS2 gene and the composite estrogen-responsive unit (ERU) in the proteinase inhibitor 9 (PI-9) gene. In transient transfections of ER-positive HepG2-ER7 cells, PI-9 was strongly induced by estrogen, moxestrol (MOX), and 4-hydroxytamoxifen (OHT). PI-9 was not induced by raloxifene or ICI 182,780. Quantitative reverse transcriptase-PCR showed that moxestrol strongly induced cellular PI-9 and pS2 mRNAs, whereas OHT moderately induced PI-9 mRNA and weakly induced pS2 mRNA. Chromatin immunoprecipitation experiments demonstrated strong and similar association of 17β-estradiol-hERα and MOX-hERα with the PI-9 ERU and with the pS2 ER. Binding of MOX-hERα to the PI-9 ERU and the pS2 ER was rapid and continuous. Although MOX-hERα bound strongly to the PI-9 ERU and less well to the pS2 ERE in chromatin immunoprecipitation, gel shift assays showed that estrogen-hERs bind with higher affinity to the deproteinized pS2 ERE than to the PI-9 ERE. Across a broad range of OHT concentrations, OHT-hERα associated strongly with the pS2 ERE and weakly with the PI-9 ERU. ICI-hERs bound poorly to the PI-9 ERU and effectively to the pS2 ERE. Raloxifene-hERα and MOX-hERα exhibited similar binding to the PI-9 ERU and the pS2 ERE. These studies demonstrate that ER ligand and ERE sequence work together to regulate in vivo binding of ER to estrogen-responsive promoters.

The genomic actions of estrogens are mediated by binding of estrogens to intracellular receptor proteins, estrogen receptors (ERs)α and β (1). ERs and other members of the steroid hormone receptor subfamily of nuclear receptors share a common domain structure and overall scheme for transcription activation (2). Binding of potent estrogens, such as 17β-estradiol (E2), or moxestrol (MOX) to the ER may induce dissociation of ER from a heat shock protein-chaperone complex and enable the ER to dimerize and bind to specific DNA sequences termed estrogen response elements (EREs). Transcription activation by ERs is mediated by two interacting activation functions, AF1 and AF2 (3). Whereas AF1 mediates ligand-independent transactivation, AF2 activity depends on estrogen binding to the ER ligand binding domain (LBD). When bound to estrogen, the ER LBD assumes a conformation that enables the recruitment of coactivators, proteins that help assemble a multiprotein complex that facilitates both chromatin remodeling and formation of an active transcription complex (2, 4, 5).

Through both genomic and nongenomic actions, estrogens exert important effects on bone maintenance and remodeling, on neural differentiation, and on the cardiovascular system and regulate the growth and differentiation of cells of the reproductive system. In addition to their normal role in the development of the reproductive system, estrogens can also promote the growth of breast and endometrial cancers. In menopausal women, the loss of estrogen production leads to osteoporosis and hot flashes (6). To prevent estrogen’s detrimental effects while preserving its beneficial actions, a class of pharmacologically active ER ligands known as selective estrogen receptor modulators (SERMs) has been developed. Tamoxifen (TAM), a widely used breast cancer therapeutic, is a prototypical SERM. TAM and its active metabolite, 4-hydroxytamoxifen (OHT), exhibit low estrogenic activity in breast cells and therefore interfere with the estrogen-dependent growth of breast cancers. TAM exhibits slight estrogenic activity in bone cells and can help maintain bone density. However, TAM acts as an estrogen in the uterus, resulting in an increased risk of endometrial cancer. The SERM, raloxifene (RAL), exhibits estrogenic activity in bone cells and has little estrogenic activity in either breast or endometrial tissue (7–9).

Structural studies revealed the molecular basis of the different activities of ER bound to estrogens and SERMs. The ligand binding domain of ER assumes a different conformation when it is bound to potent estrogens or to SERMs and pure antagonists (10–14). These differences in ER conformation influence the ability of coactivators and corepressors to interact with the promoter in a gene- and cell-specific manner. A great deal of research has focused on the ability of ER ligands to influence the binding of coactivators and corepressors (15, 16) and on the effect of cell context on coactivator/corepressor binding (17). However, the interplay between the ERE sequences in specific promoters and the ability of ER bound to different ligands to bind to intracellular promoters containing different EREs has received much less attention.

* This research was supported by NICHD, National Institutes of Health, Grant HD-16720. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: Center for Clinical Sciences Research, Dept. of Radiation Oncology, Stanford University, Stanford, CA 94303-5152.
§ Present address: Stanford University Hospital, Dept. of Gynecology and Obstetrics, Rm. HH-333, Stanford, CA 94305-5152.
¶ To whom correspondence should be addressed: Dept. of Biochemistry, University of Illinois, 600 S. Mathews Ave., Urbana, IL 61801-3602. Tel.: 217-333-1788; Fax: 217-244-5858; E-mail: djsapiro@uiuc.edu.

The abbreviations used are: ER, estrogen receptor; E2, 17β-estradiol; MOX, moxestrol; ERE, estrogen response element; CREB, complementery ERE; LBD, ligand binding domain; SERM, selective estrogen receptor modulator; TAM, tamoxifen; OHT, 4-hydroxytamoxifen; RAL, raloxifene; PI-9, proteinase inhibitor 9; ERU, estrogen-responsive unit; ChIP, chromatin immunoprecipitation; AP-1, activator protein-1; FBS, fetal bovine serum; RT, reverse transcriptase; hERα, human estrogen receptor α; CBP, CREB-binding protein.

This paper is available on line at http://www.jbc.org
ER interacts with DNA by direct binding to sequences related to the palindromic 5'-aGGTCAnnnTGACCt-3' (lowercase letters denote nucleotides in the sequence that are not highly conserved), by tethering to other proteins bound at AP-1 and Sp1 sites and through interaction with genes containing isolated GGTCA half-sites or direct repeats (18–22). The most widely studied of these interactions is binding of liganded ER to sequences related to the consensus ERE palindromes. There is now considerable evidence supporting the view that the sequence of palindromic EREs influences ER conformation. Nardulli and co-workers (23, 24) used protease digestion and antibody studies to demonstrate that ER adopts different conformations when bound to different EREs. ER bound to different ligands adopts different conformations when bound to different ERE sequences (25, 26). A more general ability of steroid hormone response elements to influence steroid receptor function is demonstrated by the studies of Yamamoto and co-workers (27) who demonstrated an important role for glucocorticoid response element sequence in glucocorticoid receptor function.

Because previous studies of ER-ERE interaction were primarily in vitro investigations, it was not possible to address the interplay between the effects of ER ligand and ERE sequence on ER binding or to evaluate ER binding to different EREs in the context of cellular genes in their native chromatin context. To address the role of the ERE in binding of ER to promoters of estrogen-regulated genes in intact cells, we used the promoter of the estrogen-inducible gene coding for proteinase inhibitor 9 (PI-9) (22, 28). PI-9 is emerging as an important modulator of apoptotic and inflammatory processes. PI-9 inhibits granulyme B and thereby interferes with granulyme B-mediated apoptosis when tumor cells or cells infected with an intracellular pathogen are targeted by the immune system (29–31). Most researchers also find that PI-9 inhibits caspase-1 and thereby reduces the production of proinflammatory cytokines (32, 33). We previously described PI-9 as an estrogen-inducible gene both in HepG2 cells stably transected with human ERα (HepG2-ER7 cells) and in cultured human liver biopsies (28). Estrogen induction of PI-9 creates an intriguing link between the actions of estrogens, inflammatory processes, and immune function (34).

Estrogen regulates PI-9 gene expression through a sequence located 200 base pairs downstream of the transcription start site (22). This unusual sequence, the PI-9 EREU, consists of an imperfect ERE palindrome followed immediately by a DR13 site (22). We also used chromatin immuno-precipitation (ChIP) with antibodies against ERα to demonstrate direct intracellular binding of ER to the PI-9 EREU (22).

To examine the interplay between different ligands and ERE sequences on the interaction of liganded ER with different estrogen-regulated genes, we carried out studies using intact cells. We analyzed the ability of the potent estrogen MOX, the SERMs raloxifene and 4-hydroxytamoxifen, and the pure anti-estrogen ICI 182,780 to activate expression of a transiently transfected PI-9 promoter (which is probably not fully assembled into a native chromatin structure) and the chromosomal PI-9 promoter. In order to identify other estrogen-inducible genes containing EREs in their promoters, HepG2-ER7 cells were screened for estrogen-dependent gene induction using quantitative RT-PCR. In addition to PI-9, we identified pS2 as a strongly estrogen-inducible gene in cultured liver cells. Estrogen regulates the expression of pS2 through an ERE that differs from the consensus ERE palindrom by 1 nucleotide. pS2 is often used as a prognostic marker in breast cancer cells and has been widely used in studies of ER action (21, 35).

Quantitative RT-PCR was also used to compare induction of PI-9 and pS2 mRNAs in HepG2-ER7 cells treated with MOX, OHT, RAL, and ICI 182,780. In vitro binding studies using deproteinized DNA demonstrated that the estrogen-ER complex exhibited slightly higher affinity binding to the pS2 ERE than to the PI-9 ERE. We used ChIP assays to evaluate binding of ER to EREs in the PI-9 and pS2 promoters. We demonstrated strong sequence-specific association of ER bound to the potent estrogens E2 and MOX with the PI-9 EREU and showed that E2-ER and MOX-ER elicited similar levels of binding to the PI-9 EREU and to the pS2 ERE. We compared the time course of binding of MOX-ER to the PI-9 and pS2 EREs and compared with MOX-ER, OHT-ER binding was much lower for the PI-9 EREU than for the pS2 ERE. The interesting differences in binding of OHT-ER to the PI-9 and pS2 EREs led us to extend our studies to RAL-ER and ICI-ER. RAL and ICI bound to ER exhibited very different patterns of binding to the PI-9 EREU and the pS2 ERE, and their binding was unrelated to their abilities to activate transcription of the EREs. To our knowledge, the differential association of ER with the PI-9 and pS2 EREs represents the first demonstration that differences in estrogen response elements influence the ability of ER bound to different ligands to interact with endogenous promoters in vivo.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HepG2 and HepG2-ER7 cells were maintained in Dulbecco’s minimum essential medium containing 10% dextran/charcoal-treated FBS plus penicillin and streptomycin as described previously (36). MCF-7 cells were maintained in minimal essential medium containing 10% FBS and penicillin/streptomycin. For ChIP assays, MCF-7 cells were switched to Phenol Red-free minimal essential medium containing 10% dextran/charcoal-treated FBS and penicillin/streptomycin for at least 3 days (22).

**Transient Transfection Assays**—HepG2 cells were maintained in Dulbecco’s minimum essential medium supplemented with 10% dextran/charcoal-treated FBS. Transient transfections using calcium phosphate coprecipitation were as we recently described (22). HepG2 cells were plated in 12-well plates and transfected with 10 ng of pCMV-SEAP.H9251 and pS2 ERE promoters. We demonstrated an important role for the glucocorticoid response element sequence in glucocorticoid receptor function.

**Experimental Real Time PCR**—HepG2-ER7 cells were grown in medium containing either ethanol vehicle or 10 nM ER ligand (with the exception of raloxifene, which was at 1 µM) for 24 h. Prior to the addition of ligand, the cells were maintained in hormone-free medium. The cells were then harvested using Trizol (Invitrogen) to isolate total RNA. Total RNA was DNase-treated for 20 min and then phenol/chloroform-extracted and precipitated. 1 µg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) with 5 µM random hexamer primers according to the manufacturer’s instructions. 1% of each RT reaction was added to quantitative real time PCRs containing the following in a total volume of 25 µl: 12.5 µl of 2X SYBR Green master mix (ABI, Foster City, CA) and 50 nM forward and reverse primers specific for the genes of interest. Detection and data analysis were carried out with the ABI PRISM 7700 sequence detection system. 18S rRNA expression was used to normalize gene expression for sample-to-sample variation in input and RT efficiency. Primers were designed.

---

**Quantitative Real Time PCR**—HepG2-ER7 cells were grown in medium containing either ethanol vehicle or 10 nM ER ligand (with the exception of raloxifene, which was at 1 µM) for 24 h. Prior to the addition of ligand, the cells were maintained in hormone-free medium. The cells were then harvested using Trizol (Invitrogen) to isolate total RNA following the manufacturer’s protocol. Total RNA was DNase-treated for 20 min and then phenol/chloroform-extracted and precipitated. 1 µg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) with 5 µM random hexamer primers according to the manufacturer’s instructions. 1% of each RT reaction was added to quantitative real time PCRs containing the following in a total volume of 25 µl: 12.5 µl of 2X SYBR Green master mix (ABI, Foster City, CA) and 50 nM forward and reverse primers specific for the genes of interest. Detection and data analysis were carried out with the ABI PRISM 7700 sequence detection system. 18S rRNA expression was used to normalize gene expression for sample-to-sample variation in input and RT efficiency. Primers were designed.
using Primer Express software (Applied Biosystems, Foster City, CA). To ensure specificity, primer sequences were searched against GenBank™ using BLAST.

**Chromatin Immunoprecipitation—**ChIP Assays were performed by a modification of the method we recently described (22) that includes a cell lysis protocol provided by Dr. B. Freeman (University of Illinois). HepG2-ER7 cells were grown to 95% confluence on 150-mm tissue culture dishes and incubated with ligand for the indicated time (in most experiments, 1 h) at 37 °C. The cells were fixed in a final concentration of 1% formaldehyde for 10 min at room temperature and quenched for 5 min by the addition of glycine to a final concentration of 0.125 M. The cells were washed twice with PBS, scraped from dishes in PBS containing 1 mM EDTA, and harvested by centrifugation at 1,000 g at 4 °C. The cell pellets were suspended in 10 mM of nuclear lysis buffer (50 mM HEPE, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% Triton X-100, 50 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 5 μg/ml pepstatin A), and sonicated for 10 min on a Labquake shaker at 4 °C, and the nuclei were pelleted by centrifugation on a clinical centrifuge. Nuclei were resuspended in 10 mM of nuclear wash buffer (10 mM Tris, pH 8.1, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, 50 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 5 μg/ml pepstatin A), and pellets were centrifuged. Nuclear pellets were resuspended in 0.5 ml of SDS lysis buffer (25 mM Tris, pH 8.1, 1% Triton X-100, 1% SDS, 3 mM EDTA, 50 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 5 μg/ml pepstatin A) and sonicated four times for 10 s per pulse, yielding DNA fragments 200–1000 bp in size. Sonicated samples were diluted 10 times in dilution buffer (lysis buffer without SDS) and incubated for 1 h at 4 °C with 50 μl/ml 50% Protein A-Sepharose slurry. 1 ml of precleared sonicate was aliquoted to 1.5 ml of lysis buffer containing 1% formaldehyde for 10 min at room temperature and quenched for 5 min by the addition of glycine to a final concentration of 0.125 M. The nuclei were separated on a 2% agarose gel, stained in ethidium bromide, and visualized by guest on July 24, 2018http://www.jbc.org/Downloaded from

FIG. 1. **OHT induces PI-9 through the ERU.** A, schematic of the PI-9 promoter and sequence of the PI-9 ERU. B and C, the full-length PI-9 promoter luciferase reporter gene construct (22) (A and B), or the PI-9 ERU (A and C) were transfected into HepG2-ER7 cells using calcium phosphate coprecipitation. After 24 h, either ethanol vehicle, 10 nm MOX, 10 μM OHT, 10 μM ICI 182,802, or 780 or 1 μM RAL was added to the medium. Cells were harvested 24 h after the addition of ER ligands and assayed for luciferase activity as described under “Experimental Procedures.” Fold induction represents the increase in luciferase activity for the promoter, with the sample treated with ethanol vehicle set equal to 1. Each bar represents the average of three separate experiments ± S.E.

**RESULTS**

**OHT Strongly Activates a Transfected PI-9 Promoter**—Since OHT reportedly exhibits some agonist activity in HepG2 cells and in liver, we compared the ability of OHT and the potent estrogen mestosterone to activate expression of transiently transfected PI-9 promoter-luciferase reporter gene constructs. This plasmid contains the entire functional PI-9 promoter region (~1482 to +314; Fig. IA) (22, 28). Transfected cells were treated with MOX, OHT, ICI 182,780, or RAL. Consistent with our earlier work (22, 28), MOX strongly activated the PI-9 promoter (Fig. IB). The antagonist ICI 182,780 and the SERM RAL exhibited little or no ability to activate the PI-9 promoter. Surprisingly, OHT activated the PI-9 promoter ~5-fold. We previously showed that the ERU was both necessary and sufficient for strong estrogen induction of PI-9 and that the consensus AP-1 site in the PI-9 5'-flanking region plays no role in estrogen induction (22). Since OHT-ER is more effective in tethering to AP-1 sites than E2-ER (38), it was important to determine whether the ERU was sufficient for OHT induction.
A construct containing the PI-9 ERU (Fig. 1A) cloned upstream of a luciferase reporter construct was effectively activated by OHT (Fig. 1C). These data demonstrate that OHT induction of PI-9 is mediated by the ERU, a site we previously demonstrated binds MOX-ER in vivo.

The Breast Cancer Prognostic Marker pS2 Is Strongly Estrogen-inducible in Liver Cells—PI-9 is induced by estrogen and OHT through an unusual element containing an imperfect ERE palindrome followed by a direct repeat of ERE half-sites separated by 13 nucleotides (Fig. 1A). To evaluate the interaction of liganded ER with different classes of ERE, we wanted to compare the ability of ER bound to different ligands to interact with the nontraditional PI-9 ERU and with a gene containing a classical imperfect ERE. Although numerous estrogen-inducible genes have been identified and studied in MCF-7 breast cancer cells (15–17, 21), PI-9 is constitutively overexpressed in MCF-7 cells and is not further inducible by estrogen or other agents.

We therefore attempted to identify additional estrogen-inducible genes in hepatoma cells. Although early passages of HepG2 human hepatoblastoma cells contained ER (39, 40), this cell line lost ER over time, and the currently used strains of HepG2 cells are ER-negative. To restore ER expression, HepG2 cells were stably transfected to express hER (28), the ER subtype normally expressed in liver (41).

Previous work in several laboratories suggested several candidate genes that were potentially estrogen-inducible in hepatocytes (Table I). Since our objective was to find genes exhibiting strong estrogen induction using the conditions that produced a strong PI-9 induction, we used the same conditions employed for PI-9 induction without attempting to optimize induction of individual mRNAs. HepG2-ER7 cells were treated with 10 nM MOX, RNA was isolated, and mRNA levels were determined using quantitative real time PCR with SYBR Green detection. Of the genes screened, the breast cancer marker gene pS2 (42), complement C3 (53), cathepsin D, cortisol-binding globulin (CBG) (54), retinol-binding protein 4 (RBP4), thyroid hormone-binding globulin (TBG) (6), sex hormone-binding globulin (SHBG) (42), and insulin-like growth factor-binding protein 1 (IGFBP1) (54).

HepG2-ER7 cells, maintained in Dulbecco’s Modified Eagle’s Medium containing 10% dextran/charcoal-FBS were treated with 10 nM MOX for 24 h. RNA was harvested and reverse transcribed, and the resulting cDNA was quantitated by real time PCR as described under “Experimental Procedures” using primers specific for the indicated genes. Induction of each mRNA was normalized to the ethanol control for that mRNA, which was set equal to 1. Genes were selected based on reports of estrogen induction in mammalian liver or in ER-positive HepG2 cells. If known, the sequence of the gene’s functional estrogen response elements is listed in the second column of the table. For comparison, the sequence of the consensus ERE from the Xenopus laevis vitellogenin A2 gene is shown at the top of the second column. The genes tested were PI-9 (22), clotting factor XII (Factor XII) (6, 51), 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA Red) (52), Breast Cancer Marker gene pS2 (trefoil factor 1) (pS2) (42), complement C3 (53), cathepsin D, cortisol-binding globulin (CBG) (54), retinol-binding protein 4 (RBP4), thyroid hormone-binding globulin (TBG) (6), sex hormone-binding globulin (SHBG) (42), and insulin-like growth factor-binding protein 1 (IGFBP1) (54).

**Table I**

| Gene       | ERE sequence (cERE: AGGTCAAGTCGGACCT) | MOX induction |
|------------|---------------------------------------|---------------|
| PI-9       | AGGGGAGGCTTCGACTGCGACTGGGCTGTCCTGACCT | ~15×          |
| Factor XII | AGGGGAGGCTTCGACTGCGACTGGGCTGTCCTGACCT | ~2×           |
| HMG-CoA Red| AGGGGAGGCTTCGACTGCGACTGGGCTGTCCTGACCT | ~1×           |
| pS2 (TFF1) | AGGGGAGGCTTCGACTGCGACTGGGCTGTCCTGACCT | ~35×          |
| Complement C3 | AGGGGAGGCTTCGACTGCGACTGGGCTGTCCTGACCT | ~1×           |
| Cathepsin D | AGGGGAGGCTTCGACTGCGACTGGGCTGTCCTGACCT | ~1×           |
| CBG        | Unknown                               | ~1×           |
| RBP4       | Unknown                               | ~1×           |
| TBG        | Unknown                               | ~1×           |
| SHBG       | Unknown                               | ~1×           |
| IGFBP1     | Unknown                               | ~2×           |

MOX is a weak inducer of cellular PI-9 and pS2 mRNAs—To compare the regulation of PI-9 and pS2 mRNAs, HepG2-ER7 cells were treated with either 10 nM moxestrol, 10 nM ICI 182,780, 10 nM OHT, or 100 nM OHT for 24 h, and the levels of PI-9 and pS2 mRNAs were determined by quantitative real time PCR. As expected, MOX induced PI-9 mRNA ~15-fold and pS2 mRNA ~35-fold, whereas ICI 182,780 did not induce PI-9 or pS2 mRNA. OHT at 10 or 100 nM induced PI-9 mRNA 3–4-fold and pS2 mRNA 2.5–3.5-fold (Fig. 2). OHT induced cellular PI-9 mRNA ~25% as well as MOX but was a weaker inducer of pS2 mRNA (7% of the level seen with MOX). OHT was a less effective activator of the cellular PI-9 gene (Fig. 2) than of the transfectable PI-9 promoter (see Fig. 1B).

**17β-Estradiol and Moxestrol Induce Similar Binding of ER to the PI-9 ERU and to the pS2 ERU**—The prototypical estrogen, 17β-estradiol, is rapidly metabolized in HepG2 cells and other p450-containing liver cells (36, 42). Studies of hepatic gene regulation lasting more than a few hours therefore require the use of unacceptably high micromolar concentrations of E₂ (42). Although structurally very similar to E₂, MOX contains modifications that make it similar to ethynylestradiol and greatly reduce the ability of p450s to degrade MOX. Most studies of hepatic gene regulation therefore use MOX (22, 28, 36, 42). Although our previous studies demonstrated that both E₂ and MOX induced PI-9 mRNA (28), since our studies involved the role of ER ligands in controlling interaction of ER with EREs, it was important to compare the ability of E₂ and MOX to recruit ER to the PI-9 ERU and to the pS2 ERU. We therefore used chromatin immunoprecipitation to compare association of E₂-ER and MOX-ER to the PI-9 ERU and the pS2 ERU (Fig. 3A). In cells exposed for 60 min to 10 nM MOX or to 10 or 100 nM E₂, there was virtually identical association of ER with the PI-9 ERU or the pS2 ERU (Fig. 3B). Since MOX-ER and E₂-ER were not recruited to an upstream region of the PI-9 gene used as a control (Fig. 3A), recruitment was specific for the PI-9 ERU (Fig. 3B, PI-9 upstream). A control antibody (M2 anti-FLAG antibody) did not elicit specific immunoprecipitation of ER bound to the PI-9 ERU or the pS2 ERU (Fig. 3B, M2). Since MOX and E₂ elicited similar binding of the ER to the test genes and use of MOX allowed direct comparison of ER binding to our studies of mRNA levels (Figs. 1 and 2 and Table I), we used MOX in our ChIP experiments that employed HepG2 cells.

* A. Krieg and D. Shapiro, unpublished observations.
Endogenous PI-9 and pS2 mRNAs are strongly induced by moxestrol and weakly induced by OHT. HepG2-ER7 cells were treated with 10 nM MOX (black bar), 10 nM ICI 182,780 (ICI, vertically striped bar), 10 nM OHT (gray bar), 100 nM OHT (cross-hatched bar), or ethanol vehicle (white bar). RNA was harvested and reverse transcribed, and the resulting cDNA was quantitated by real time PCR with SYBR Green detection as described under “Experimental Procedures” using primers specific for PI-9 or pS2, respectively. Fold induction represents the increase in SYBR Green signal for each of the ligands with the signal generated by the ethanol vehicle set equal to 1. The data represent the mean ± S.E. for three separate experiments.

**Fig. 2.** Endogenous PI-9 and pS2 mRNAs are strongly induced by moxestrol and weakly induced by OHT. HepG2-ER7 cells were treated with 10 nM moxestrol and fixed at intervals of 0, 20, 40, 60, 80, 100, and 120 min. Chromatin was prepared and immunoprecipitated as described under “Experimental Procedures” using the indicated antibodies. Immunoprecipitated chromatin was amplified using primers encompassing the promoter regions containing the PI-9 ERU or the pS2 ERE.

MOX-ER exhibited nearly maximal interaction with the PI-9 ERU and with the pS2 ERE 20 min after administration of MOX to the medium and exhibited little change in association with these elements over the 120-min time course of the experiment (Fig. 4). Similar results were observed with the general coactivator CBP, except that CBP binding showed a moderate increase between 20 and 40 min. Control experiments demonstrated that the nonspecific M2 monoclonal antibody did not select ERU or ERE-containing complexes in immunoprecipitations (Fig. 4, M2). On the PI-9 ERU and the pS2 ERE, histone H4 acetylation is substantial at time 0 and only increases modestly following MOX treatment. Histone H3 acetylation on the PI-9 ERU showed a moderate increase with increasing time of exposure to MOX. Although both PI-9 and pS2 mRNAs show strong estrogen induction, they are each expressed at a low basal level in HepG2-ER7 cells. Since the increases in acetylation over time are modest, both promoters may reside in active domains of chromatin in the absence of hormone.

**Fig. 3.** MOX and E2 induce strong and equivalent binding of ER to the PI-9 ERU and the pS2 ERE. A shows the regions containing the PI-9 ERU and the pS2 ERE that were amplified by PCR for the ChIP assays and the location of an upstream region in the PI-9 gene used as a control for non-sequence-specific binding. In B, HepG2-ER7 cells were incubated for 60 min in medium containing ethanol vehicle, 10 nM MOX, 10 nM E2, or 100 nM E2. The cells were fixed, lysed, and sonicated for ChIP assays as described under “Experimental Procedures.” PCR of immunoprecipitated DNA generated the appropriately sized ~300-nucleotide product for the +48/+334 primer set flanking the PI-9 ERU and for the −448/−146 primer set flanking the pS2 ERE.

hERα Rapidly Associates with the PI-9 ERU and pS2 ERE in Vivo—Most studies of the kinetics of in vivo binding of ER to EREs employed chromatin immunoprecipitation assays in MCF-7 breast cancer cells (15, 16). Since there was no information on the kinetics of ER association with EREs in HepG2-ER7 cells, we determined the time course of recruitment of MOX-ER to the PI-9 ERU and to the pS2 ERE (Fig. 4). HepG2-ER7 cells were treated with MOX and fixed with formaldehyde every 20 min over 2 h. ChIP was then performed with antibodies against ERα and the general transcription coactivator CBP.

**Fig. 4.** MOX-ER rapidly associates with the PI-9 ERU and with the pS2 ERE in HepG2-ER7 cells. HepG2-ER7 cells were treated with 10 nM moxestrol and fixed at intervals of 0, 20, 40, 60, 80, 100, and 120 min. Chromatin was prepared and immunoprecipitated as described under “Experimental Procedures” using the indicated antibodies. Immunoprecipitated chromatin was amplified using primers encompassing the promoter regions containing the PI-9 ERU or the pS2 ERE.

Estrogen activation of gene expression is often accompanied by increased histone acetylation mediated by the histone acetyltransferase activity of CBP and other histone acetyltransferases (15, 16). To determine the level of acetylation of histones H3 and H4, we used antibodies against acetylated core histones to carry out ChIP assays of the regions of the genes containing the PI-9 ERU and pS2 ERE. In both cell lines, association of MOX-ER with the PI-9 ERU and with the pS2 ERE at different times, there was no indication of the differences in the association of MOX-ER with the PI-9 ERU and the pS2 ERE 20 min after administration of MOX to the medium and exhibited little change in association with these elements over the 120-min time course of the experiment (Fig. 4). Similar results were observed with the general coactivator CBP, except that CBP binding showed a moderate increase between 20 and 40 min. Control experiments demonstrated that the nonspecific M2 monoclonal antibody did not select ERU or ERE-containing complexes in immunoprecipitations (Fig. 4, M2). On the PI-9 ERU and the pS2 ERE, histone H4 acetylation is substantial at time 0 and only increases modestly following MOX treatment. Histone H3 acetylation on the PI-9 ERU showed a moderate increase with increasing time of exposure to MOX. Although both PI-9 and pS2 mRNAs show strong estrogen induction, they are each expressed at a low basal level in HepG2-ER7 cells. Since the increases in acetylation over time are modest, both promoters may reside in active domains of chromatin in the absence of hormone.

**Fig. 5.** MCF-7 cells exhibited several interesting outcomes not predicted by prior work in MCF-7 cells. Elegant studies in MCF-7 cells described a periodic oscillation in the association of estrogen-ER with the pS2 ERE and with the EREs of other estrogen-regulated genes (16). Although there were small differences in the association of MOX-ER with the PI-9 ERU and the pS2 ERE at different times, there was no indication of the periodic oscillation seen in MCF-7 cells. Second, whereas the pS2 and PI-9 genes are strongly induced by MOX in the HepG2-ER7 cells (Fig. 2), the changes in histone acetylation we observed were modest. To determine whether these effects were due to differences in cell culture conditions or ChIP protocols or reflected differences between HepG2-ER7 and MCF-7 cells, we determined the time course of association of ER with the pS2 ERE in MCF-7 cells.

The time course of association of estrogen-ER with the pS2 ERE was similar in MCF-7 cells and in HepG2-ER7 cells (Fig. 5). In both cell lines, association of ER and CBP with the ERE was nearly maximal 20 min after the addition of estrogen to the culture medium and did not show much change over the time course of the experiment (Figs. 4 and 5). Consistent with earlier studies in MCF-7 cells (15, 16), there was a strong time-dependent increase in histone H4 acetylation and a moderate...
increase in histone H3 acetylation (Fig. 5). Since pS2 gene expression is strongly induced by estrogen in both the HepG2-ER7 cells and the MCF-7 cells, the differences in histone acetylation we observed may reflect the different intracellular environments of the two cell lines.

In Vitro, ER Exhibits Higher Affinity Binding to the Deproteinized pS2 ERE than to the PI-9 ERE—In ChIP assays, MOX-ER demonstrated strong binding to the PI-9 ERU and the pS2 ERE. However, the -fold induction of pS2 mRNA is actually higher than the -fold induction of PI-9 mRNA (Fig. 2). We therefore used electrophoretic mobility shift assays to compare binding of purified ER to deproteinized DNA probes of similar length containing the consensus cERE, the pS2 ERE, or the PI-9 ERU. As expected, estrogen-ER complex bound to the cERE with higher affinity than to the pS2 ERE or the PI-9 ERU. The affinity of ER for the pS2 ERE was about 2-fold higher than its affinity for the PI-9 ERU (Fig. 6). Neither -fold induction of pS2 mRNA than its affinity for the PI-9 ERU (Fig. 6). Neither -fold induction of pS2 mRNA than its affinity for the PI-9 ERU (Fig. 6). Neither -fold induction of pS2 mRNA than its affinity for the PI-9 ERU (Fig. 6). Neither -fold induction of pS2 mRNA than its affinity for the PI-9 ERU (Fig. 6).
ability of an ER ligand to activate transcription of one of the genes and the ability of that ligand bound to ER to interact with the cellular gene. Interestingly, ICI-ER clearly bound to the pS2 ERE and the PI-9 ERU, indicating that binding of ER to a pure antagonist, such as ICI 182,780, is sufficient to recruit ER to intracellular EREs.

**DISCUSSION**

**Regulation of PI-9 Gene Expression by OHT**—Proteinase Inhibitor 9 is an intracellular inhibitor of both the proapoptotic serine protease, granzyme B, and the proinflammatory protease, caspase-1 (29–31, 33, 48). The primary role of caspase-1 is to mediate immune and inflammatory actions by catalyzing the maturation of the proinflammatory cytokines, interleukin-1β...
and interleukin-18. We find that estrogen induction of PI-9 blocks production of interleukin-1β in a liver cell culture model for inflammatory disease. The potentially important role of PI-9 in inflammatory and immune processes makes the study of its regulation by estrogens and SERMs particularly relevant. When we compared the ability of ER ligands to activate the PI-9 promoter in transiently transfected HepG2 cells, the SERMs OHT and RAL exhibited dramatically different effects. Whereas RAL elicited little or no activation of the PI-9 promoter, 10 nM OHT activated the transfected PI-9 promoter about half as well as the potent estrogen, moxestrol. Induction of PI-9 by OHT was mediated primarily, and perhaps exclusively, through the ERU. A construct containing a single copy of the ERU was activated ~7-fold by MOX and 4-fold by OHT (Fig. 1C). These data were surprising, since our earlier studies using Northern blots to analyze PI-9 mRNA levels indicated that OHT and TAM were weak inducers of cellular PI-9 mRNA (28). We used quantitative RT-PCR to analyze the effect of OHT on cellular PI-9 mRNA levels in a more precise way. Similar concentrations of OHT were clearly more effective in activating expression of the transiently transfected PI-9 promoter than the cellular PI-9 gene (Figs. 1, 2, and 8A). The chromatin structure on transiently transfected DNA is probably incompletely organized and is thought to be more open and accessible to transcription factors than cellular chromatin (43, 44). A likely explanation for the weaker OHT induction of cellular PI-9 expression is that the native chromatin structure on the endogenous PI-9 gene interferes with the ability of OHT-ligated ER to function as well as it does on a transiently introduced promoter.

We previously showed that MOX-ER associates with the PI-9 ERU in HepG2-ER7 cells (22). Recent reports demonstrated binding of OHT-ER to several estrogen-inducible promoters in both breast and endometrial cell lines (15–17). To evaluate the ability of OHT-ER to activate transcription and interact with genes other than PI-9, we undertook a small scale screen to identify additional estrogen-inducible genes in HepG2-ER7 cells. Although the test genes were chosen because previous studies in whole animals or cultured cells suggested that they might be estrogen-inducible in liver cells, only PI-9 and pS2 exhibited strong induction by moxestrol. It is possible that more detailed studies using induction conditions optimized for individual genes might have enabled us to demonstrate estrogen induction of additional mRNAs. The modest number of strongly estrogen-inducible mRNAs we observed is consistent with our earlier conclusion (28) that HepG2-ER7 cells do not contain extremely high levels of hERs that would result in widespread and potentially inappropriate activation of candidate estrogen-regulated genes. Across a wide range of concentrations, OHT had much less ability to activate expression of the pS2 gene than the PI-9 gene (Fig. 8A).

Interaction of MOX-ER with the PI-9 ERU and pS2 ERE—Since there had been little prior work analyzing the in vivo interaction of ER with ERE-containing genes in ER-positive HepG2 cells, it was important to determine the basic parameters of ER-ERE interaction in these cells. Several features of these studies stood out. ChIP assays of pS2 in MCF-7 cells usually yielded stronger signals than the same assays in HepG2-ER7 cells. This may be due to the presence of higher levels of ER in MCF-7 cells than in HepG2-ER7 cells (50,000–100,000 molecules of ER/cell in most lines of MCF-7 cells and ~30,000 molecules of ER/cell in HepG2ER7 cells) (28, 37). Since our ER immunoprecipitations use a mixture of three monoclonal antibodies that recognize epitopes in different regions of the ER, it is unlikely that masking of the epitopes recognized by the ER antibody is responsible for these cell-specific differences in recruitment of ER to the pS2 gene. Analysis of histone acetylation also suggested that there were only modest changes in histone H3 and H4 acetylation around the pS2 gene following estrogen treatment of HepG2-ER7 cells. The MCF-7 cells exhibited the expected large increase in acetylation around the pS2 gene. Since MOX elicits a robust 30-fold induction of pS2 mRNA in HepG2-ER7 cells, the weaker ER recruitment and less dramatic changes in histone acetylation are unlikely to result from poor activation of the pS2 gene. Differential accessibility of the histone antibodies in the two cell lines also seems improbable. The differences in histone acetylation seen on the pS2 promoter in these two cell lines may represent cell-specific influences on chromatin organization and the ability of the genes to be activated or reflect different basal levels of pS2 gene expression in MCF-7 cells and HepG2-ER7 cells. Although MOX-ER produces strong signals from both the PI-9 ERU and the pS2 ERE in ChIP assays that reflect intracellular binding, in gel shift assays using deproteinized DNAs, the pS2 ERE exhibits a higher affinity for the pS2 ERE than the PI-9 ERU.

ChIP of HepG2-ER7 cells treated with 10 nM MOX over a time course of 2 h demonstrates that the ER rapidly associates with the PI-9 ERU and with the pS2 ERE and remains associated with both promoters over the course of the experiment. These data were surprising, since an important study emphasized that estrogen-ligated ER and coregulators interact with pS2 and other ERE-containing genes in an oscillatory fashion, with time-dependent cycles of protein binding and release (16). To determine whether these differences reflected different modes of interaction in HepG2-ER7 cells and MCF-7 cells, we determined the time course of ER binding to the pS2 ERE in MCF-7 cells. Binding of estrogen-ER and of CBP to the pS2 gene in MCF-7 cells was also rapid and showed little or no fluctuation over the time course of the experiment. Our studies are consistent with a recent study in MCF-7 cells (15). The presence or absence of a pattern of oscillations in binding of ER to the pS2 ERE may reflect differences in cell culture methodology in different laboratories. In some cases, cell culture conditions may produce a partially synchronized cell population whose growth is coordinately stimulated by estrogen.

Interplay between ER Ligands and ERE Sequence Controls ER Binding—OHT-ER and TAM-ER bound much less well to the PI-9 ERU than MOX-ER. Poor binding of OHT-ER to the PI-9 ERU was observed across a broad range of OHT conen-
trations. In contrast, OHT-ER bound nearly as well as MOX-ER to the pS2 ERE. Since OHT has little or no ability to induce pS2 mRNA (~5% of the level seen with MOX) and is a moderately effective inducer of PI-9 mRNA (at 10 nM OHT, ~20% of the level seen with MOX), binding of OHT-ER to the PI-9 ERE and the pS2 ERE is clearly not related to the ability of OHT to activate transcription of these genes. Important studies of the effect of cell context on transactivation by SERMs indicate that in breast cancer cells, OHT-ER recruits corepressors to EREs and AP-1 sites, whereas in endometrial cells it can recruit coactivators to ER binding sites (17). Since OHT-ER and MOX-ER exhibit nearly equal interaction with the pS2 ERE in HepG2-ER7 cells, OHT-ER clearly does not recruit coactivators to the pS2 gene. 10 nM OHT induces PI-9 mRNA to levels ~20% of those seen with MOX, and OHT-ER binds to the PI-9 ERE roughly 20% as well as MOX-ER. These data raise the possibility that OHT-ER recruits coactivators to the PI-9 ERE. Testing this possibility directly was not feasible. The CBD and GRIP-1 antibodies available to us produced signals in the ChIP assays ~4-fold lower than those seen with our ER antibodies. Since MOX-ER also binds to the PI-9 ERE ~5-fold better than OHT-ER, the expected signals for GRIP-1 and CBD binding are expected to be very much lower than those seen with MOX, and in our studies these signals proved too faint for reproducible analysis. An unidentified liver-specific coactivator might also mediate the weak OHT activation of PI-9.

The inference that ERE binding might be related to transactivation potential is derived in part from a recent report that the pure agonist ICI 184,684 did not recruit ER to the pS2 ERE in MCF-7 cells. In contrast, partial agonists such as OHT did recruit ER (47). We find that whereas the similar compound ICI 182,780 exhibits negligible ability to induce PI-9 and pS2 mRNAs, ER liganded to ICI is recruited to both genes. The failure to observe ICI-ER recruitment to the pS2 ERE may be due to the use of MCF-7 cells in those studies or to differences in the ChIP assay protocol used by those researchers.

The effect of ER ligand on association with the pS2 ERE and with the PI-9 ERE is highlighted by the differential recruiting of the ER to the respective promoters in cells treated with ICI 182,780, OHT, and RAL. Whereas OHT is a partial agonist of PI-9, ICI 182,780 and RAL have very little if any ability to induce PI-9 mRNA (Fig. 1B and data not shown). OHT, ICI 182,780, and RAL do not effectively activate the pS2 gene in HepG2-ER7 cells. ER bound to ICI 182,780 associates with the PI-9 promoter slightly more effectively than OHT-ER, whereas RAL-ER robustly binds the PI-9 ERE with an efficiency that approaches that of MOX-ER. Since RAL is a poor activator of both PI-9 and pS2 gene transcription, it is unlikely that the enhanced binding observed on the PI-9 promoter is due to coactivator-mediated stabilization of the binding of RAL-ER to the ERE. Conversely, ER bound to OHT or to ICI 182,780 strongly associates with the pS2 ERE, whereas ER bound with RAL binds approximately as well as ER-MOX. Since ICI 182,780 and RAL do not induce either PI-9 mRNA or pS2 mRNA, their different abilities to induce ER binding to the two EREs in these genes reflect intrinsic properties of these EREs.

The most straightforward explanation for the differential association of ER with the pS2 ERE and PI-9 ERE is that the ligand-induced conformation is more "receptive" to binding to specific ERE sequences. Structural studies of ER LBDS bound to several different ligands reveal structural differences between ER LBDS bound to strong agonists, SERMs, and pure antagonists (10–14). Our data suggest that in intact cells, the nature of the hormone ligand influences ER interactions with DNA. The pS2 gene contains a "classical" imperfect ERE palindrome that differs from the consensus sequence by 2 nucleotides followed by a direct repeat of ERE half-sites separated by 13 nucleotides (22). Investigations in several laboratories on both ER and glucocorticoid receptor suggest that hormone response elements can function as allosteric effectors modulating the conformation of steroid receptors (23, 24, 27). The conformation of the ER when bound to DNA in vitro is also sensitive to different ligands (25, 26, 49, 50). We propose that when the ER binds an ERE in the cell, the protein must strike a compromise between the conformations induced by ligand and DNA. How well the ER associates with a given promoter would therefore depend on the influences of ligand and ERE on receptor conformation. In the case of the PI-9 ERE, the ER conformation required to bind the ERU may be more compatible with the conformation imposed by MOX and RAL than with those imposed by OHT and ICI 182,780. Alternatively, DNA binding may be differentially stabilized by conformation-sensitive interactions with chromatin remodeling complexes or unidentified factors. Our in vitro demonstration that both ligand and ERE sequence influence the ability of the ER to interact with promoters of estrogen-responsive genes provides one mechanism by which SERMs elicit their promoter-specific actions.

Acknowledgments—We are grateful to Drs. B. Freeman, M. Bagchi, and A. Nardulli and their co-workers for helpful conversations about CHIP assays and to J. Clemons for help in preparing the graphics and manuscript.

REFERENCES
Interplay between Estrogen Response Element Sequence and Ligands Controls in Vivo Binding of Estrogen Receptor to Regulated Genes
Adam J. Krieg, Sacha A. Krieg, Bonnie S. Ahn and David J. Shapiro

J. Biol. Chem. 2004, 279:5025-5034.
doi: 10.1074/jbc.M307076200 originally published online November 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307076200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 53 references, 19 of which can be accessed free at
http://www.jbc.org/content/279/6/5025.full.html#ref-list-1