Estrogen receptor-mediated repression of human hepatic lipase gene transcription

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Abstract **Estrogen replacement therapy in women decreases hepatic lipase (HL) activity, which may account for the associated increase in HDL cholesterol. To investigate whether estrogen decreases HL transcription, transient cotransfection assays with HL promoter and estrogen receptor-α (ERα) expression constructs were performed in HepG2 cells. 17β-estradiol (E_2) decreased transcription driven by the −1557/+41 human HL promoter by up to 50% at 10^{-7} M. Mutation of ERα by deletion of its transactivation domains or ligand-binding domain eliminated E_2-induced repression of the promoter, whereas deletion of the DNA-binding domain of ERα resulted in a 7-fold activation by E_2. The E_2-induced repression was maintained after mutation of a potential estrogen-response element in the promoter. The region of estrogen responsiveness was localized to −1557/−1175 of the HL promoter by deletion analysis. Mutation of an AP-1 site at −1493 resulted in a partial loss of E_2-induced repression, similar to that caused by deletion of nucleotides −1557 to −1366. Gel shift assays with nuclear extracts from E_2-treated HepG2 cells stably expressing ERα demonstrated an increase in binding to an AP-1 consensus oligonucleotide. The AP-1 activator, phorbol 12-myristate 13-acetate, inhibited the HL promoter by greater than 50%. Collectively, the data suggest that estrogen represses the transcription of the HL gene, possibly through an AP-1 pathway.**—Jones, D. R., R. J. Schmidt, R. T. Pickard, P. S. Foxworthy, and P. I. Eacho. **Estrogen receptor-mediated repression of human hepatic lipase gene transcription.** ***J. Lipid Res.*** **2002.** 43: **383–391.**

Supplementary key words HepG2 cells • AP-1 • phorbol ester • very low density apolipoprotein II • promoter regulation • 17β-estradiol

Hepatic lipase (HL) is synthesized in hepatic parenchymal cells and binds to the surface of those cells as well as sinusoidal endothelial cells in the liver (1–3). It is involved in the metabolism of remnant lipoproteins, LDL and HDL. HL functions in a non-catalytic mode to bind lipoproteins, which facilitates cholesterol ester or whole particle uptake by hepatic receptors. It functions in a catalytic mode to hydrolyze triglycerides and phospholipids in lipoproteins, which results in the remodeling of IDL to LDL as well as larger HDL_2 to HDL_3. In the process of remodeling HDL, apolipoprotein A-I (apoA-I) can become dissociated from the particle and eliminated by the kidney. Studies in humans have demonstrated a correlation between HL activity and HDL catabolic rate (4, 5). In healthy, obese, and diabetic populations, high HL activity is associated with low HDL cholesterol (6–13). Allelic variation of the HL gene accounts for 25% of the individual variation in plasma HDL cholesterol levels (14). The inverse relationship between HL activity and HDL cholesterol has been reproduced in rabbits and mice overexpressing the human HL gene and in HL-deficient mice (15–18).

Hepatic lipase is subject to hormonal regulation. Its activity is decreased after the peak of estrogen of the reproductive cycle (19, 20). Women have lower HL activity than men (7), which may account for the higher level of HDL cholesterol in women. Numerous clinical studies in postmenopausal women have demonstrated that estrogen replacement decreases HL activity in association with increased HDL cholesterol (21–24). The mechanism by which estrogen decreases HL activity is not fully understood. The effect can be reproduced in rats accompanied by a reduction of HL mRNA levels, suggesting a transcriptional inhibition (25).

Although estrogen generally regulates gene expression by transcriptional activation (26–28), an increasing number of studies have demonstrated that ligand-bound estrogen receptor-α (ERα) also mediates transcriptional repression (29–32). Estradiol decreases synthesis of type I collagen in murine mesangial cells through a mechanism involving an AP-1 binding motif in the collagen promoter, rather than an estrogen-responsive element (ERE). Suppression appears to occur through enhanced binding of AP-1 (29). In MCF-7 cells, estradiol decreases catachol-*O*-methyltransferase (COMT) mRNA by a mechanism involving two half-palindromic EREs and a region in the dis-

Abbreviations: AF-1, N-terminal ligand independent transactivation domain; AF-2, C-terminal ligand dependent transactivation domain; apoVLDLII, very low density apolipoprotein II; C/EBP, CCAAT/enhancer binding protein; COMT, catachol-*O*-methyltransferase; E_2, 17β-estradiol; ER, estrogen receptor; ERα, estrogen receptor-α; ERE, estrogen-responsive element; HL, heptatic lipase.

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tal promoter containing two CCAAT/enhancer binding protein (C/EBP) sites (30). Estradiol represses phorbolester induced activation of IL-6 transcription in Ishikawa cells by promoting the interaction of ERα with NF-κB and NF-IL6, which inhibits their activation of the IL-6 promoter (31). Estradiol represses apoA-I transcription in HepG2 cells stably transfected with ERα. Binding of ERα to DNA is not required for the effect. Instead, the effect appears to involve preferential partitioning of coactivators to ERα from the transcription factors that bind the apoA-I gene enhancer (32).

The present study was conducted to determine whether estrogen decreases HL expression at the transcriptional level. We demonstrated that estradiol represses HL promoter activity in HepG2 cells transiently expressing ERα. The repression was not observed in the presence of mutant forms of ERα containing a deletion of the AF-1, DNA-binding, AF-2, or ligand-binding domains. The region of estrogen responsiveness within the HL promoter was localized to nucleotides −1557 to −1175. The repression was not mediated by an ERE-like sequence in the proximal 5′ promoter. Instead, an AP-1 site appears to be involved.

MATERIALS AND METHODS

Plasmid constructs

The human hepatic lipase gene promoter was PCR-amplified from human genomic DNA (Clontech) using Amplitaq polymerase (Gibco BRL). The resulting product was cloned into pGem-T (Promega) and digested with *Kpn*I and *Hind*III to release the promoter segment encompassing 1,557 base pairs upstream to 41 base pairs downstream of the transcription start site. This segment was ligated to *Kpn*I/*Hind*III-digested pGL2BasicLuc (Promega) to create the −1557/+41HLLuc plasmid. The −1557/+153HLLuc construct was prepared by *Xma*I/*Hind*III digestion of the −1557/+41HLLuc plasmid followed by ligation of a *Xma*I/*Hind*III double-stranded synthetic linker, extending the promoter sequence to nucleotide +153. Progressive 5′ promoter deletions were prepared by PCR-amplification (Pfu polymerase, Stratagene) of regions of the −1557/+41 promoter with the following endpoints: −1366/+41, −1175/+41, −775/+41, −375/+41, and −202/+41. PCR primers were designed to allow for ligation of each of the resulting deletions into the *Kpn*I (5′) and *Hind*III (3′) sites of *Kpn*I/*Hind*III-digested pGL2BasicLuc.

The Wisconsin Package software (version 9.1 by the Genetics Computer Group, Madison, WI) was used to locate potential transcription factor binding sites within the 5′ promoter. Mutations of the ERE-like, ERE half-sites, and AP-1 sites within the −1557/+41HLLuc reporter were made using Promega's Altered Sites II in vitro mutagenesis system. ERE and AP-1 site mutations are indicated in Figs. 4A and 6A, respectively.

The pCMVERα and the ERα mutant expression vectors were provided by Dr. Benita Katzenellenbogen (University of Illinois at Urbana Champaign). The ligand-binding domain, the N-terminal ligand independent transactivation domain (AF-1), the DNA-binding domain, and the C-terminal ligand dependent transactivation domain (AF-2) deletion mutants refer to deletions of ERα regions E/F, A/B, B/C/D, and amino acids 531–595, respectively (28). The pRSVERα vector was constructed by digesting pCMVERα with *Sal*I. The resulting *Sal*I fragment, containing the full-length ERα cDNA, was ligated into *Xho*I-digested pREP4 (Invitrogen). The pβ-actinLuc plasmid was prepared by cloning the *Hind*III/ *Xba*I fragment of pSP-luc+ (Promega) into *Hind*III/*Xba*I-prepared pSP72 (Promega) to create pSP72luc+. *Hind*III/*Bam*HI double-digest of pSP72luc+ yielded the luciferase cDNA for cloning into the human β-actin promoter-containing pBAP (provided by Dr. Na Yang, Lilly Research Laboratories, Indianapolis, IN). The very low density apolipoprotein II (apoVLDLII) Luc construct was prepared by PCR amplification of the chicken apoVLDLII promoter from chicken genomic DNA (Clontech) using primers incorporating 5′ *Kpn*I and 3′ *Hind*III restriction sites. The resulting product was double digested and cloned into *Kpn*I/*Hind*III-digested pGL2EnhLuc (Promega). Vector pRSVLacZ was purchased from ATCC. All plasmids were purified on Qiagen Maxi Purification columns according to the manufacturer's instructions and subjected to sequencing analysis to verify insert orientation and accuracy.

Transient cotransfections and reporter activity assays

HepG2 cells (ATCC HB8065) were grown at 37°C, 5% CO_2 in DMEM-F-12 (3:1, v/v; Gibco BRL), 10% FBS (Gibco BRL), 20 mM HEPES, 50 μg/ml Tobramycin, and 1 μg/ml Nucellin Zn. Cells between passage numbers 4 and 12 were used for cotransfection assays because these were most responsive to estrogen. Cotransfections were performed as per Henry et al. (33) with modifications. Briefly each 100 mm plate of confluent cells was trypsinized (0.05% trypsin, 0.53 mM Na EDTA) and seeded into three 100 mm plates. Twenty-four hours later, the cells were trypsinized and seeded at 2.5×10^6 cells per 100 mm plate. After an additional 24 h, cells were cotransfected using Lipofectamine Plus according to the manufacturer's instructions (Gibco BRL). Each cotransfection reaction contained a mixture of luciferase reporter (6.7 μg of −1557/+41HLLuc or equimolar concentrations of the mutated HL promoter constructs), pRSVLacZ (1.7 μg), pRSVERα (1.3 μg or an equimolar amount of ERα deletion mutants), and pSp72 carrier DNA to bring total transfected DNA to 10 μg/plate. Six hours after incubation with the DNA-Lipofectamine Plus mixture, transfected cells were washed two times in PBS. Individual plates were trypsinized and seeded into 96-well plates at approximately 15,000 cells/well. Wells contained the appropriate reagents in media containing 5% charcoal-activated/dextran-treated FBS (Hyclone). E_2 was from Sigma. Thirty-six to forty-eight hours later, luciferase and β-galactosidase assays were performed on cell extracts as described by Henry et al. (33). Luciferase activity was normalized to β-galactosidase activity. The β-galactosidase activities were similar for vehicle- and E_2-treated cells, suggesting that decreases in transcriptional activities did not reflect cellular toxicity. Differences between dose groups for a given construct were assessed by one-way ANOVA with pairwise contrasts examined using *t*-tests on mean differences.

Stable ERα expression in HepG2 cells

Double digest of pCMVERα with *Eco*RI and *Bam*HI released full-length ERα cDNA which was cloned into *Eco*RI/*Bam*HI-digested pcDNA3.1(−) from Invitrogen. The correct orientation of the insert was confirmed by restriction digest and DNA sequencing. HepG2 cells were transfected as described above with 10 μg of *Sca*I-linearized plasmid/100 mm plate. Forty-eight hours following transfection, cells were trypsinized and seeded 1:4, v/v in DMEM/F-12 (3:1, v/v), 10% FBS, 20 mM HEPES, 50 μg/ml Tobramycin, and 1 μg/ml Nucellin Zn with selection in 800 μg/ml G418 (Gibco). After 2 weeks of selection, individual colonies were expanded in media containing 400 μg/ml G418. Individual colonies were characterized for ERα expression by Western blot as described below.

Western analysis

An aliquot of cells from each of the transient cotransfections was used for confirmation of expression of ERα from pRSVERα

and pCMVERα. The cells were rinsed in Ca^{2+}- and Mg^{2+}-free PBS, 100 μl of lysis buffer (1% Triton X-100, 1 mM EDTA, 50 mM Tris, pH 7.5, 150 mM NaCl, plus Boehringer Mannheim Complete EDTA-free protease inhibitor cocktail) was added, the cells were vortexed, and incubation was carried out at room temperature for 30 min. Following centrifugation of the cells, the extract supernatant was analyzed for protein concentration using the Pierce Bradford assay. Samples of extract were separated on 4–20% polyacrylamide Tris/Glycine gels (Novex), transferred to nitrocellulose membranes, and analyzed using the Amersham Life Sciences ECL detection kit. The primary antibody was mouse anti-human ERα (Cat. No. 05-394, Upstate Biotechnology) and the secondary antibody was HRP-conjugated goat anti-mouse IgG (Bio-Rad).

RNA isolation and RNase protection analysis

HepG2 cells stably expressing functional ERα were treated with either 0.01% ethanol or E_2 for 48 h with replacement of fresh media at 24 h and at 30 min prior to harvest. Parental HepG2 cells were treated in parallel. Total RNA was then isolated according to Promega's RNAgents Total RNA Isolation System. RNA was quantitated and purity was assessed spectrophotometrically. For each sample 10 μg of RNA was subjected to RNase protection analysis utilizing Ambion's RPA III kit. Human β-actin probe and the RNA size standard were labeled with [α^{32}P]-CTP using Ambion's Maxiscript system. The HL probe template was prepared by reverse transcribing HL message from total human liver RNA (Clontech) and cloning it into pGem-T. After accuracy of the insert sequence was confirmed through DNA sequencing, the plasmid was linearized with *Nco*I and used as an SP6 polymerase template in the presence of [α^{32}P]CTP to generate HL probe. Hybridizations were carried out at 42°C overnight. RNase A/T1 (1:100, v/v dilution) digests were performed at 37°C for 45 min. Samples were then resolved on a Novex 6% polyacrylamide TBE/Urea gel. The gel was fixed in 10% acetic acid, dried, and exposed to a phosphoimager screen. A Molecular Dynamics phosphoimager was used to quantitate protected HL and β-actin signal. For each sample HL message intensity was normalized to β-actin message intensity.

Nuclear extract preparation and electrophoretic mobility shift assay

HepG2 cells stably expressing functional ERα were treated with either 0.01% ethanol (vehicle) or 10^{-6} M E_2 dissolved in ethanol for 48 h with replacement of fresh media at 24 h and at 30 min prior to harvest. Nuclear extract preparation and AP-1 consensus gel shift assays were performed as per Silbiger et al. (29). Protein concentration was measured by colorimetric assay (Pierce). Four micrograms of nuclear extract were mixed with 5 μg of poly (dI:dC) in the presence of ^{32}P end-labeled AP-1 consensus oligonucleotide (Promega). The sequence of the oligo-

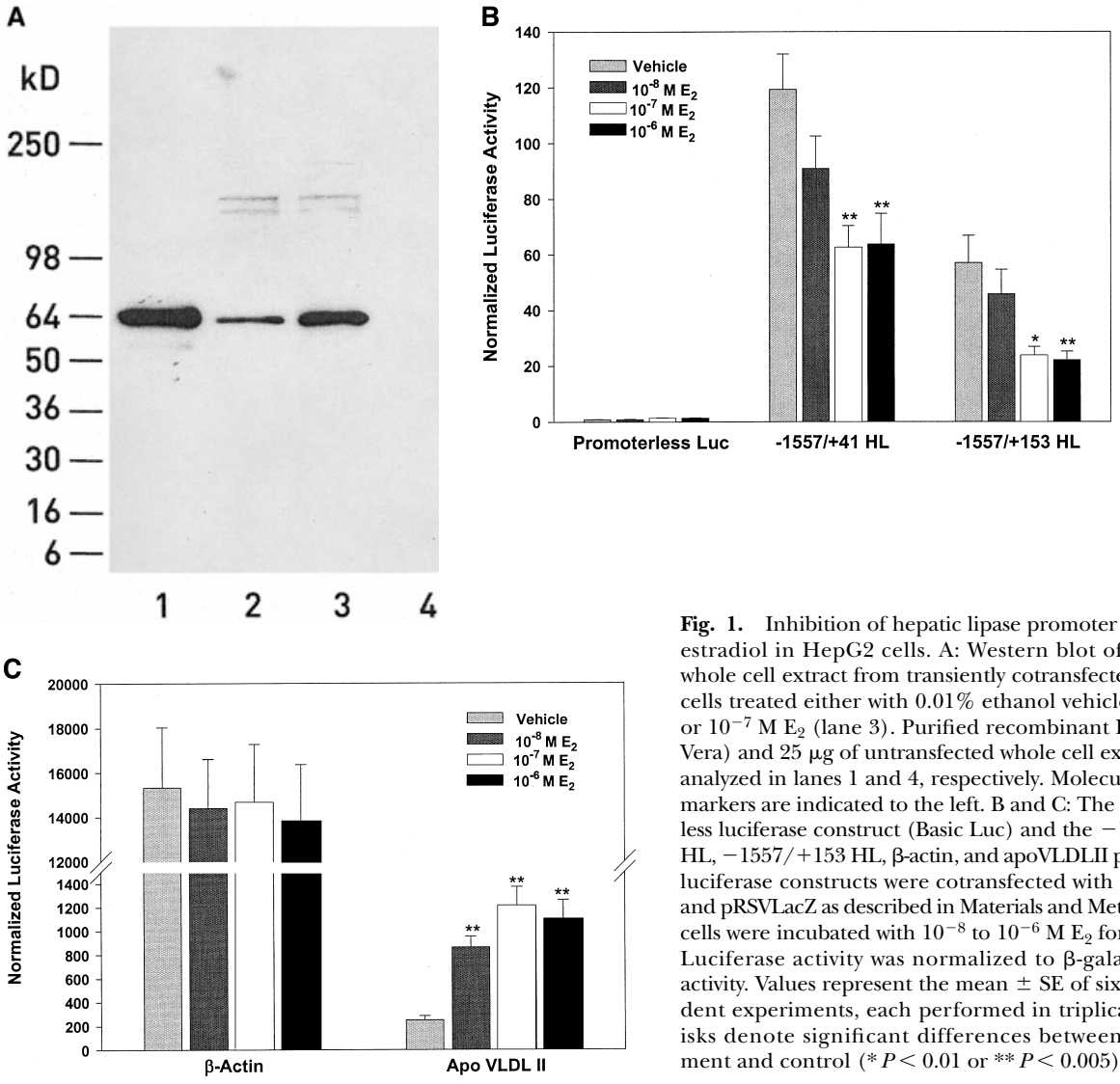


Fig. 1. Inhibition of hepatic lipase promoter activity by estradiol in HepG2 cells. A: Western blot of 25 μg of whole cell extract from transiently cotransfected HepG2 cells treated either with 0.01% ethanol vehicle (lane 2) or 10^{-7} M E_2 (lane 3). Purified recombinant ERα (PanVera) and 25 μg of untransfected whole cell extract were analyzed in lanes 1 and 4, respectively. Molecular weight markers are indicated to the left. B and C: The promoterless luciferase construct (Basic Luc) and the −1557/+41 HL, −1557/+153 HL, β-actin, and apoVLDLII promoter/luciferase constructs were cotransfected with pRSVERα and pRSVLacZ as described in Materials and Methods. The cells were incubated with 10^{-8} to 10^{-6} M E_2 for 36–40 h. Luciferase activity was normalized to β-galactosidase activity. Values represent the mean ± SE of six independent experiments, each performed in triplicate. Asterisks denote significant differences between E_2 treatment and control (* $P < 0.01$ or ** $P < 0.005$).

nucleotide was as follows with the consensus AP-1 site indicated in bold: 5′-CGCTTGA**TGAGTCAG**CCGGAA-3′. The sequence of the unrelated oligonucleotide used to test for specificity of binding was the following: 5′CCTGCCGGTAGAACGTGGGCC TCTCCTGAGACATGTGATGGTGATGGTGATGCATGGCAGA TCTGG-3′. Electrophoresis was performed on a 5% polyacrylamide gel in Tris-borate buffer. A Molecular Dynamics phosphoimager was used to quantitate bands.

RESULTS

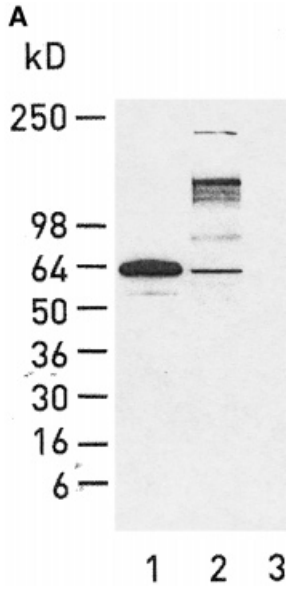
Estrogen represses HL gene transcription in transient cotransfection assays of HepG2 cells

To determine if estrogen regulates HL promoter activity, transient transfection experiments were performed in HepG2 cells using an HL promoter-luciferase reporter construct. The expression of ERα in untransfected HepG2 cells was undetectable in Western blots, and incubation with E_2 (10^{-8} to 10^{-6} M) had no effect on the HL promoter (data not shown). In subsequent experiments, an ERα expression vector (pRSVERα) was cotransfected with the HL promoter construct. ERα expression in HepG2 cells cotransfected with the pRSVERα construct was confirmed by Western blots (**Fig. 1A**). A control vector lacking promoter and enhancer sequences failed to drive transcription of the luciferase gene in the absence or presence of E_2 (Fig. 1B). Two different HL promoters were used to evaluate the regulation by E_2. The first contained sequence extending from −1557 to +41 base pairs relative to the transcription start site. E_2 in the range of 10^{-8} to 10^{-6} M decreased transcription driven by this promoter by up to 50%. Basal transcription driven by the second promoter, −1557/+153 HL, was lower than that of −1557/+41 HL. This is most likely due to the presence of a transcriptional silencer located between nucleotides +28 and +129 (34). E_2 repressed the activity of the −1557/+153 promoter by a maximum of 61%. The relatively weak transcriptional activity of both HL promoters in HepG2 cells is consistent with previous observations (34–36).

The β-actin promoter was evaluated to determine if transcriptional repression was a general phenomenon induced by E_2 in HepG2 cells. β-Actin mRNA expression is unresponsive to estrogen treatment in vitro and in vivo in rats (37, 38). The transcriptional activity of the human β-actin promoter, which was over 120-fold stronger than that of the HL promoter, was not affected by E_2 treatment (Fig. 1C). The chicken apoVLDLII promoter was also included as a control because the gene is highly inducible by estrogen in vivo (39). Basal activity of the apoVLDLII promoter was similar to that of the HL promoter, but it was increased approximately 5-fold in the presence of E_2 (Fig. 1C).

Estrogen decreases HL mRNA in a stable HepG2 cell line expressing ERα

E_2 did not repress HL mRNA in the parental HepG2 cell line (**Fig. 2B**). Because ERα levels are undetectable in the parental cells, a HepG2 line was established that stably expresses human ERα under the control of the CMV pro-



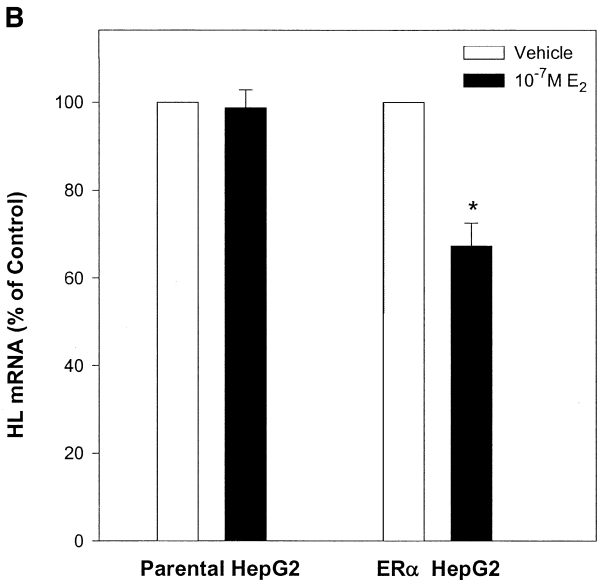


Fig. 2. Estrogen represses HL mRNA in HepG2 cells expressing ERα. A: Stable expression of ERα in the HepG2 cell line was confirmed by Western blot. Lane 1 contains 50 ng of recombinant ERα (PanVera), lane 2 contains 50 μg of whole cell extract from HepG2 cells stably expressing ERα, and lane 3 contains 50 μg of whole cell extract from untransfected HepG2 cells. B: HL mRNA was measured by RNase protection analysis in parental HepG2 cells or those stably expressing ERα after treatment with 10^{-7} M E_2 for 48 h. HL mRNA values were normalized to β-actin mRNA. The HL/β-actin mRNA ratios in replicate experiments were as follows: parental HepG2 cells treated with the vehicle (0.025, 0.071, 0.033) or 10^{-7} M E_2 (0.027, 0.069, and 0.042); and ERα-expressing HepG2 cells treated with vehicle (0.14, 0.09, 0.018, and 0.055) or 10^{-7} M E_2 (0.08, 0.07, 0.014, and 0.031). In the ERα-expressing HepG2 cells, treatment with estrogen reproducibly decreased the HL/β-actin mRNA ratio compared with the vehicle-treated control cells in each experiment. The results of each experiment were expressed as the percentage of the vehicle-treated cells and the mean and SE of those values are presented. Asterisks denote a statistically significant difference when percentage reductions were compared by paired *t*-test (* $P = 0.013$).

moter. The cells expressed a high level of ERα protein as determined by Western analysis (Fig. 2A). The functionality of the stably expressed ERα was demonstrated by its repression of the −1557/+41 HL promoter and a 3- to 4-fold activation of the apoVLDLII promoter (data not shown). Treatment of the ERα expressing HepG2 cells with 10^{-7} M E_2 decreased HL mRNA by 33% (Fig. 2B).

Intact ERα is required for repression of HL transcription

To evaluate the requirement of the intact estrogen receptor in the repression of the HL promoter by E_2, cotransfection experiments were conducted in the absence or presence of wild-type or mutated forms of ERα. In the absence of a receptor, treatment of HepG2 cells with E_2 caused no change in the activity of the −1557/+41 HL promoter (**Fig. 3**). In the presence of wild-type ERα, E_2 repressed HL promoter activity by up to 48%, similar to that shown previously. The E_2-induced repression of the HL promoter was lost in cells cotransfected with ERα containing mutated ligand-binding. Likewise, E_2 responsiveness was lost upon mutation of either of the transactivation domains, AF-1 or AF-2. E_2 caused a 7-fold stimulation of HL promoter activity in cells transiently expressing ERα with a deleted DNA-binding domain. These data indicate that an intact ERα is required to repress HL transcription in the presence of E_2.

Repression of transcription by estrogen is not mediated by the ERE-like element in the HL promoter

A putative ERE within the rat HL promoter was presumed to mediate the estrogen-induced decrease of HL activity observed in rat (40). The human −1557/+41 HL promoter contains an imperfect palindromic ERE sequence at nucleotide −978 (**Fig. 4A**). To determine if this element mediates the effect of E_2 on the HL promoter, a scramble mutation was introduced in this sequence. Mutation of this ERE-like sequence did not affect E_2 repression of HL promoter activity (Fig. 4B). Conversion of the ERE-like site to a consensus ERE by changing five nucleotides resulted in an E_2-dependent stimulation of promoter activity of up to 2-fold. The results demonstrate that estrogen-dependent down-regulation of HL transcription does not directly involve the ERE-like sequence identified within the human HL promoter.

Localization of the HL 5′ promoter region involved in estrogen repression

In an attempt to identify transcriptional elements responsible for estrogen-mediated down regulation in HepG2 cells, progressive 5′ promoter deletion constructs

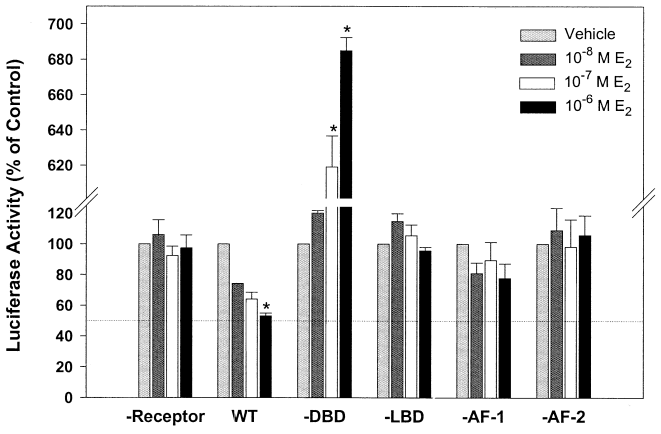


Fig. 3. Intact estrogen receptor-α is required for repression of HL transcription. The −1557/+41HLLuc promoter construct was cotransfected with the wild-type ERα expressed from pCMVERα (WT), or ERα with deletions of the DNA-binding domain (−DBD), ligand-binding domain (−LBD), N-terminal transactivation domain (−AF-1), or C-terminal transactivation domain (−AF-2). The expression of the ER mutants was confirmed by Western analysis (data not shown). The cells were incubated with 10^{-8} to 10^{-6} M E_2 for 36–40 h. The luciferase activity is expressed as a percentage of the vehicle-treated control for each ERα construct. The values represent the mean ± SE from three independent experiments, each performed in triplicate. Asterisks denote significant differences from the vehicle-treated cells (* $P < 0.005$).

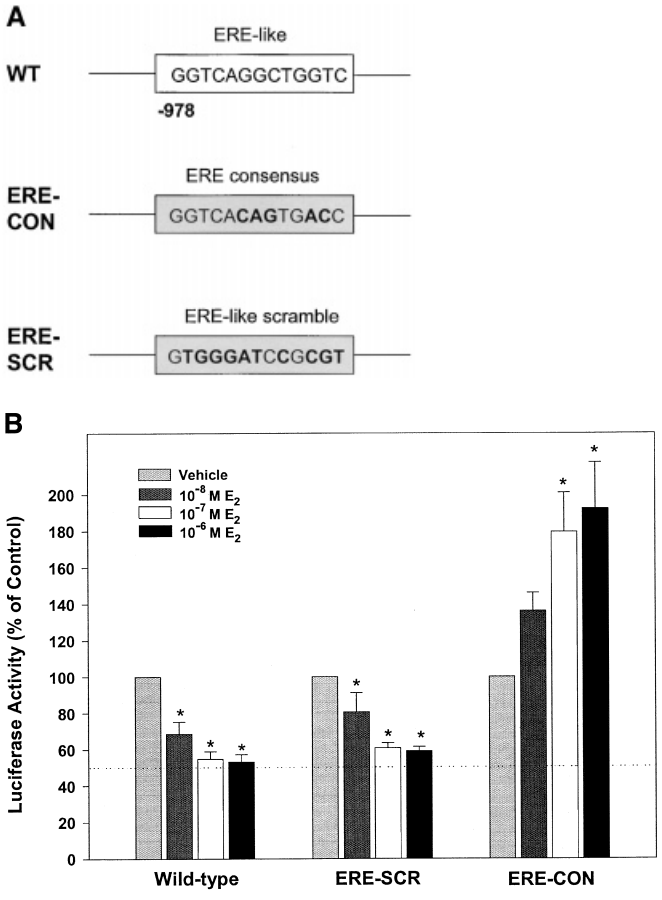


Fig. 4. Repression of HL transcription by estrogen is not mediated through the ERE-like sequences in the 5′ promoter. A: Schematic diagram of the native HL promoter sequence (wild-type) containing an ERE-like site. The ERE-CON variant of the HL promoter was made by mutating the ERE-like sequence to an ERE consensus sequence. ERE-SCR denotes a scramble mutation of the ERE-like sequence. Mutated nucleotides are depicted in bold type. B: Cotransfections and incubation with E_2 were performed as described in Fig. 1. The luciferase activity is expressed as a percentage of the vehicle-treated control for each promoter construct. Values represent the mean ± SE of three independent experiments, each performed in triplicate. Asterisks denote significant differences from the vehicle control (* $P < 0.005$).

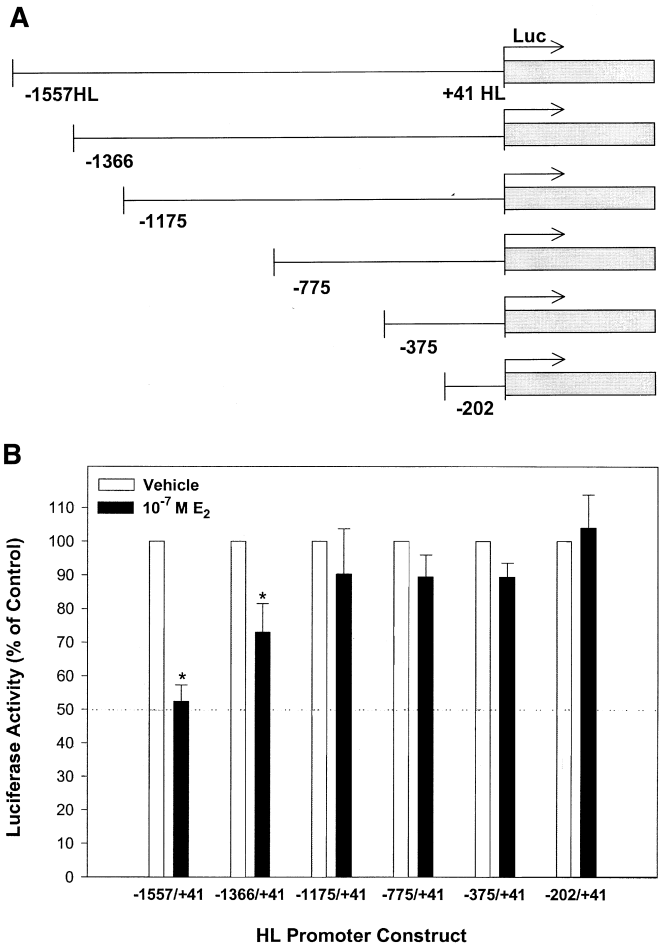


Fig. 5. HL 5′ promoter deletion analysis to identify the region of estrogen responsiveness. A: Schematic diagram depicting the native HL promoter-luciferase construct, extending from nucleotide −1557 to +41 of the HL gene, and the deletion mutants. B: Cotransfections and incubations with 10^{-7} M E_2 were performed as described in Fig. 1. The luciferase activity is expressed as a percentage of the vehicle-treated control for each promoter construct. Values represent the mean ± SE of six independent experiments, each performed in triplicate. Asterisks denote significant differences from the vehicle control (* $P < 0.01$).

were cotransfected with pRSVERα (**Fig. 5A**). Transcription driven by the −1557/+41HL promoter was decreased by nearly 50% at 10^{-7} E_2 (Fig. 5B). Deletion of nucleotides from −1557 to −1366 muted the repression by E_2 (27% decrease). E_2 did not significantly repress the activity of the HL promoters containing additional progressive 5′ deletions (−1175/+41, −775/+41, −375/+41, and −202/+41). The deletional analysis indicates that the estrogen responsive region of the HL promoter is located between nucleotides −1557 and −1175.

The role of AP-1 sites in HL promoter repression by estrogen

The −1557/+41HL promoter contains several AP-1 sites that were shown previously to regulate basal transcriptional activity (35). The AP-1 site at −1493 is within the region of estrogen responsiveness. To determine the role of the −1493 AP-1 site in the E_2-dependent repression, the site was mutated in the −1557/+41HL promoter and cotransfected with pRSVERα in HepG2 cells. The maximum inhibition of this promoter construct (32%) was muted compared with the effect on the native −1557/+41HL promoter (**Fig. 6B**). The HL promoter containing a mutation of the AP-1 site at −1059, which is outside the region of estrogen responsiveness, was repressed by E_2 comparably to the native promoter.

To further explore a role of AP-1 in the estrogen repression of the HL promoter, HepG2 cells were cotransfected with pRSVERα and the −1557/+41 HL promoter in the presence of phorbol 12-myristate 13-acetate (PMA), an AP-1 activator. PMA (10^{-7} M) inhibited HL promoter activity by 56% (**Fig. 7**).

To determine if estrogen treatment alters the binding of nuclear factors to an AP-1 site, an electrophoretic mobil-

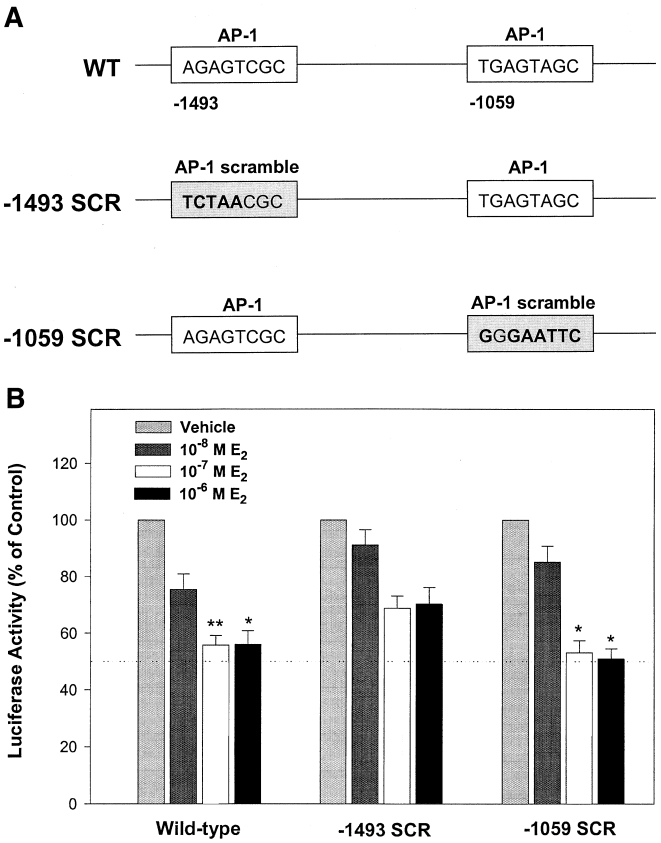


Fig. 6. The effect of mutations to AP-1 sites in the HL promoter on the repression by estrogen. A: Schematic diagram of the native HL promoter sequence (wild-type) containing AP-1 sites. The HL promoter variants contained scramble mutations to the AP-1 sites located at −1493 (−1493 SCR) and −1059 (−1059 SCR). Mutated nucleotides are indicated in bold. B: The wild-type −1557/+41 HL promoter or the variants containing AP-1 site mutations were cotransfected with pRSVERα in HepG2 cells as described in Fig. 1. The cells were incubated with E_2 for 36–40 h prior to quantitation of luciferase activity. The luciferase activity is expressed as a percentage of the vehicle-treated control for each promoter construct. Values represent the mean ± SE of three independent experiments, each performed in triplicate. Asterisks denote significant differences from the vehicle control (* $P < 0.05$ and ** $P < 0.01$).

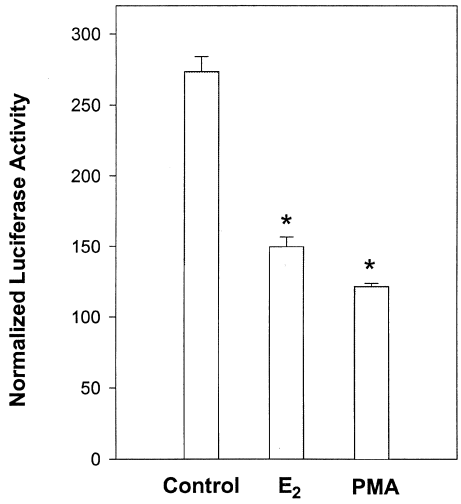


Fig. 7. Inhibition of hepatic lipase promoter activity by phorbol ester. The wild-type −1557/+41 HL promoter was cotransfected with pCMVERα in HepG2 cells as described in Fig. 1. The cells were incubated with 10^{-7} M E_2 or 10^{-7} M phorbol 12-myristate 13-acetate (PMA) for 36–40 h prior to quantitation of luciferase activity. Values represent the mean ± SE of three replicate assays from the same experiment. Asterisks denote significant differences from the vehicle control (* $P < 0.0005$).

ity shift assay was performed with a radiolabeled oligonucleotide containing a consensus AP-1 site (TGAGTCAG). A single DNA-protein complex was formed using nuclear extracts from untreated HepG2 cells that stably express ERα (**Fig. 8**). The specificity of the complex was demonstrated by competition with a 100-fold excess of unlabeled oligonucleotide probe. A 100-fold excess of unrelated oligonucleotide failed to compete for the complex. Treatment of the HepG2 cells with 10^{-6} M E_2 resulted in a 1.6-fold increase in the complex.

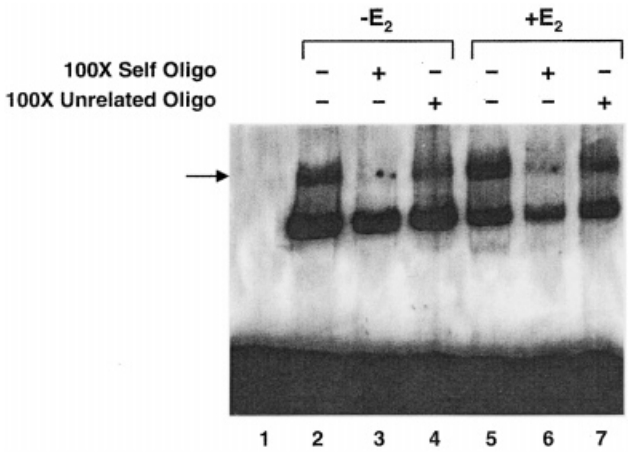


Fig. 8. Estrogen increases binding of nuclear factors to a consensus AP-1 sequence. Nuclear extracts were prepared from HepG2 cells stably expressing ERα that were treated with vehicle or 10^{-6} M E_2 for 48 h. Binding of the extracts to a consensus AP-1 site contained within a 21-base pair probe (Promega) was evaluated in an electrophoretic mobility shift assay, as described in Materials and Methods. The arrow indicates the specific AP-1 complex. The intensity of the bands was determined by phosphoimaging.

DISCUSSION

Estrogen has been found to decrease post-heparin plasma HL activity in humans and rats (20–25). The effect is associated with decreased HL mRNA in rats (25). This observation suggested that E_2 represses transcription of the HL gene. We have now confirmed that E_2 causes a transcriptional down-regulation of the HL gene using cotransfection assays in HepG2 cells. The repression of HL promoter activity was dependent on the concentration of E_2 and an intact ERα. The repression was not observed after deletion of ligand-binding, AF-1, AF-2, or DNA-binding domains of ERα. Although these studies were focused on ERα, we also observed repression in the presence of ERβ (unpublished results). The results of the cotransfection experiments were supported by the finding that HL mRNA was decreased by E_2 in a HepG2 cell line expressing ERα. The human β-actin and chicken apoVLDLII promoters were evaluated to rule out non-specific or toxic effects of E_2 on the HepG2 cells. The β-actin promoter was not affected by E_2 in the cotransfection assays. The apoVLDLII promoter was strongly induced, which is consistent with in vivo observations (39). The data generated in the cotransfection model were reproducible in a large number of experiments, demonstrating that the decrease in HL expression caused by E_2 is due to transcriptional repression.

We considered the possibility that the imperfect palindromic ERE-like sequence at nucleotide −978 of the human HL promoter mediated the estrogen-dependent repression. To evaluate this hypothesis, a scramble mutation was introduced into the sequence. The repression by estrogen was maintained in this mutant form of the promoter. The ERE-like sequence was also converted to a consensus ERE, resulting in activation of HL promoter activity in the presence of E_2. Thus, the native ERE-like sequence is not required for the estrogen-dependent repression. Furthermore, a consensus ERE in the context of the HL promoter functions as a classical ERE, in that it mediates the activation of gene transcription. This suggests that the estrogen repression of the native HL promoter does not involve a classical ERE-mediated mechanism.

The region of E_2 responsiveness in the HL promoter was localized to nucleotides −1557 to −1175 by deletion analysis. Within this region there is an AP-1 site at −1493 that was shown previously to regulate basal HL transcription (35). We found that mutation of the −1493 AP-1 site in the HL promoter resulted in a partial loss of the E_2 mediated repression, comparable to the loss of repression observed after deletion of a broad region surrounding the site (nucleotides −1577 to −1366). Mutation of an AP-1 site at −1059, which is outside of the region of estrogen-responsiveness, had no effect on the E_2-dependent repression. Therefore, the −1493 AP-1 site appears to have a role in the E_2-dependent repression of the HL promoter. We found that E_2 increased HepG2 cell nuclear factor binding to a consensus AP-1 oligonucleotide probe. Estrogen was found to increase murine mesangial cell nuclear factor binding to an AP-1 consensus oligonucleotide by

increasing *c*-fos expression (29). Thus estrogen may increase AP-1 activity in HepG2 cells, although we have not determined this. Consistent with this hypothesis was our finding that the AP-1 activator, PMA, decreased HL promoter activity by more than 50%.

The basal expression of the HL gene is under the control of multiple transcription factors acting at multiple *cis*-acting elements (34, 35, 41). Each of the regulatory elements appears to have a minor role in transcriptional regulation rather than any single element having a major role (35). This is consistent with our observation that mutation of the AP-1 site at −1493 resulted in a partial loss of the repression, but the response was completely lost only after a broad region of the promoter was deleted. There are several genes whose transcription is repressed by estrogen through an AP-1 site (29, 42, 43). The list includes lipoprotein lipase, which is in the same gene family as HL (42). The inhibition of the lipoprotein lipase promoter is entirely mediated by an AP-1 site, unlike the repression of HL by estrogen. Our data suggest that E_2 represses the HL promoter via multiple weakly active sites, including the AP-1 site at −1493.

The observation that an AP-1 site rather than the ERE-like site is involved in the repression suggests a non-classical pathway of estrogen signaling is involved. This is not unexpected, in that repression of gene expression by estrogen is generally considered to occur by a non-classical mechanism. Estrogen signaling by the classical pathway requires the interaction of estrogen receptor (ER) via its DNA-binding domain with an ER-binding element in responsive genes. Repression, in contrast, often involves the interaction of ER with co-activators or co-repressors of the estrogen-responsive gene. Tumor necrosis factor-α gene transcription is repressed by an AP-1-dependent mechanism involving the interaction of the estrogen receptor AF-2 domain with transcriptional coactivators (43). ERα inhibits gene transcription in erythroid precursor cells by the interaction of its AF-2 domain with the transcription factor GATA-1 (44, 45). Estrogen inhibits apo(a) expression by a mechanism that involves the interaction of the ERα transactivation domains with coactivators of the apo[a] gene (46). ERα prevents the binding of the coactivators to the apo[a] gene independent of its own binding to DNA. Similarly, ERα represses IL-6 gene transcription by interacting with the transactivators, NF-KB and C/EBP (31, 47). Since ERα does not bind with high affinity to the IL-6 promoter, it regulates IL-6 transcription through protein/protein interactions.

There are parallels with ERα-mediated repression of IL-6 that may help explain the mechanism of HL repression. In both cases, deletion of the AF-2 transactivation domain of ERα led to a loss of the estrogen repression. Interestingly, the repression was lost and the promoters were actually activated by estrogen in the presence of the DNA-binding domain mutant of ERα. The intact DNA-binding domain is required for the interaction of ERα with transactivators of the IL-6 gene (31, 47), as well as for the interaction of ER with the transcription factor *stat*5, which regulates the expression of milk protein genes (48). A mutation to ERα that alters its interaction with other proteins can transform the receptor from a transcriptional activator to a repressor (49). Thus, the mutation to the DNA binding domain may have transformed ERα from a repressor to an activator of the IL-6 promoter by altering its interaction with coactivators and/or corepressors. ERα may also repress HL transcription by such a mechanism, perhaps involving interactions with AP-1 proteins, based on our observation that the repression is mediated by an AP-1 site. Indeed, the AP-1 protein, *c*-jun, binds to ER, possibly through its DNA binding domain (50).

In summary, we have demonstrated that estrogen represses human HL promoter activity. The effect is mediated by a broad region of the promoter from nucleotides −1557 to −1175, including an AP-1 site at −1493. The findings may explain the effects of estrogen on hepatic lipase in humans.

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