Hormonal status can influence diverse metabolic pathways. Small heterodimer partner (SHP) is an orphan nuclear receptor that can modulate the activity of several transcription factors. Estrogens are here shown to directly induce expression of the SHP in the mouse and rat liver and in human HepG2 cells. SHP is rapidly induced within 2 h following treatment of mice with ethynylestradiol (EE) or the estrogen receptor α (ERα)-selective compound propyl pyrazole triol (PPT). SHP induction by these estrogens is completely absent in ERαKO mice. Mutation of the human SHP promoter defined HNF-3, HNF-4, GATA, and AP-1 sites as important for basal activity, whereas EE induction required two distinct elements located between −309 and −267. One of these elements contains an estrogen response element half-site that bound purified ERα, and ERα with a mutated DNA binding domain was unable to stimulate SHP promoter activity. This ERα binding site overlaps the known farnesoid X receptor (FXR) binding site in the SHP promoter, and the combination of EE plus FXR agonists did not produce an additive induction of SHP expression in mice. Surprisingly, induction of SHP by EE did not inhibit expression of the known SHP target genes cholesterol 7α-hydroxylase (CYP7A1) or sterol 12α-hydroxylase (CYP8B1). However, the direct regulation of SHP expression may provide a basis for some of the numerous biological effects of estrogens.

Estrogens exert biological effects in numerous organs throughout the body. The role of estrogens in reproductive biology, the prevention of postmenopausal hot flushes, and the prevention of postmenopausal osteoporosis are well established (1). Estrogens reduce low density lipoprotein (LDL) cholesterol levels and elevate HDL cholesterol levels (2–4), although these beneficial lipid changes may not translate into favorable clinical results (5). Estrogens may also inhibit the development of colon cancer (5), inhibit the development of Alzheimer’s disease (6), and inhibit development of cataracts (7, 8). The multitude of estrogen responses matches the widespread distribution of estrogen receptors (ERs) throughout numerous organs, with ERs expression the highest in uterus, pituitary, kidney, and adrenal gland and ERβ expression highest in ovary, uterus, bladder, and lung (9).

Although many of these actions of estrogens are due to the classic signaling pathway in which an ER binds to an estrogen response element in the promoter of a gene (10), it is now clear that many actions of estrogens are mediated by interaction of ER with other signaling pathways. For example, estrogens inhibit chronic and acute liver inflammation in the mouse by a mechanism that does not require ER activation of gene expression (11, 12). This in vivo activity of ER correlates with the in vitro ability of ER to inhibit NF-κB signaling (13, 14), likely through a coactivator competition mechanism (15). Similarly, ER can regulate gene expression via interaction with AP-1 response elements (16).

An additional mechanism by which ER could influence diverse signaling pathways is by altering expression levels of other transcription factors. For example, estrogen treatment induces expression of STAT5A in both the kidney (17) and liver (11). Intriguingly, recent microarray studies have demonstrated that chronic treatment with estrogens induces expression of the orphan short heterodimer partner (SHP) receptor in the mouse liver (11). SHP is known to be able to repress the activity of many nuclear hormone receptors in vitro, including ERα and ERβ (18, 19), estrogen receptor-related receptor γ (ERRγ) (20), androgen receptor (21), arylhydrocarbon receptor nuclear translocator (22), hepatocyte nuclear factor 4 (23, 24), constitutive androstane receptor (25), retinoic acid receptor β (25), retinoid X receptor (24), liver X receptor α (26), and liver receptor homologous protein-1 (LRH-1) (27–29). Conversely, SHP stimulates the activity of nuclear factor-κB (30), peroxisome proliferator-activated receptor α (31), and peroxisome proliferator-activated receptor γ (32). Whether all these interactions occur in vivo is less clear, with only the activity of SHP in regulating bile acid synthetic gene expression having been characterized (33, 34). Nevertheless, regulation of SHP expression has the potential to influence a wide array of physiological processes.

Estrogen regulation of SHP expression might thus contribute to many facets of estrogen biology. However, previous studies have not differentiated between a direct induction of SHP expression by estrogens and an indirect induction of SHP as a secondary consequence of prolonged treatment with estrogens. To discern between these two possibilities, both in vivo kinetic studies and in vitro transfection studies were used here to

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The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; AP-1, activating protein-1; CA, cholic acid; CYP, cytochrome P450 enzyme; E2, 17β-estradiol; EE, 17α-ethynyl,17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ERRγ, estrogen-related receptor γ; FXR, farnesoid X receptor; HNF-3, hepatocyte nuclear factor-3; HNF-4, hepatocyte nuclear factor-4; ICAM-1, intercellular adhesion molecule-1; IGF-1, insulin-like growth factor-1; IP3, inositol-1-phosphate synthase; ITF, intestinal trefoil factor; KO, knock-out; PGP dyn, progestaglandin D synthase; PPT, 4-propyl-1,3,5-tris(4-hydroxyphenyl)pyrazole; SHP, small heterodimer partner; SREBP, sterol regulatory element-binding protein; STAT5A, signal transducer and activation of transcription 5A; LRH-1, liver receptor homologous protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LXR, liver X receptor.
characterize estrogen regulation of SHP expression. The results indicate that ERα directly stimulates expression of the SHP gene.

**FIG. 1.** Estrogens rapidly induce expression of SHP in the mouse liver. Ovariectomized mice were administered a single subcutaneous injection of corn oil/ethanol vehicle (gray circles), 10 μg/kg EE (black circles), or 5 mg/kg PPT (open circles). At 1, 2, 4, 8, or 24 h following treatment, livers (A) and kidneys (B) were removed and mRNA levels for SHP, STAT5A, prostaglandin D synthase (PgD Syn), inositol-1-phosphate synthase (IPS), intestinal trefoil factor (ITF), tissue factor (TF), and CYP3A11 in each individual animal were quantified by real-time PCR. All expression values were normalized for expression of GAPDH and are presented as the mean ± S.E., with six animals per group. The mean expression of each gene in animals receiving a vehicle treatment and assayed at time 0 is defined as 1.0, with all other expression values reported relative to this value.

C, ovariectomized mice were administered a single dose of 10 μg/kg EE or 30 mg/kg GW4064 in methylcellulose Tween vehicle. At 1, 2, 4, 8, or 24 h following treatment, livers were removed and mRNA levels for SHP in each individual animal were quantified by real-time PCR. The mean expression of each gene in animals receiving a vehicle treatment and assayed at time 0 is defined as 1.0, with all other expression values reported relative to this value.

### Table I

| Species | Gene                  | Forward primer | Reverse primer | Probe                  |
|---------|-----------------------|----------------|----------------|-----------------------|
| Human   | ICAM-1                | GCAGACAGTGACCATCTACAGCTT | CTTCTGAGACCTCTGCCTTCGT | CCGGCGCCCAACGTGATTCT |
| Human   | SHP                   | AACTGCCAGACAGACCCCAG | AAGGACTCCAGACAGCATTGAAG | CCTCCCTGGCTGCGGTGCA |
| Mouse   | CYP3A11               | AGGATGAGATCGATGAGGCTCT | TCCAGGTATTCCATCTCCATCAC | CCAACAAGGCACCTCCCACGTATGAT |
| Mouse   | CYP7A1                | AGACCTCCGGGCCTTCCT | ATCACTCGGTAGCAGAAGGCAT | AATCAAAGAGCGCTGTCTGGGTCACG |
| Mouse   | CYP8B1                | TTCGACTTCAAGCTGGTCGA | CAAAGCCCCAGCGCCT | CGACATACCTGTGCCCCCCATTGA |
| Mouse   | Inositol Phosphate Synthase | CCCACGAACCACATGCTATTAG | CCACCTCTCCTGGCTTGATG | TGGGCCTGGACGCTCCATCTTGT |
| Mouse   | Intestinal Trefoil Factor | TCTGTCACATCGGAGCAGTGTAA | AAGCACCAGGGCACATTTG | CCGTGGCTGCTGCTTTGACTCCA |
| Mouse   | Prostaglandin D Synthase | GGTCTCTTGGGATTCCCACA | GCCCCAGGAACTTGTCTTGTT | CCAGGGCCATGACACAGTGCAGC |
| Mouse   | SHP                   | CAGGCACCCTTCTGGTAGATCT | TGTCTTCAAGGAGTTCAGTGATGTC | TCTTCCGCCCTATCATGGGAGACGT |
| Mouse   | STAT5A                | GCTCAGCGCCCACTTCA | GACTCTGCACCACGCCTGT | TGTCACTGAAAAGAATCAAGCGCGCC |
| Mouse   | TF                    | ACCTGGGCCTATGAAGCAAA | GTTGGTCTCCGTCTCCATGAA | TCCTCTCTGTCCCACGGAGGAACTCA |
| Rat     | SHP                   | CCTGGAGGAGCCCTCGT | AACACTGTATGCAAACCGAGGA | TCAGCCCCACCCCCAGCTTG |

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**MATERIALS AND METHODS**

**Animal Studies—** Ovariectomized 129 strain mice (20 g) or Sprague-Dawley rats (200 g) were purchased from Taconic Farms (Lexington, KY). ERα KOCH knockout (obtained from Dr. Ken Korach (35)), ERα KOCH, ERβ KO, and ERα CHER/ERβ KO mice were bred and ovariectomized at Wyeth. All animals were fed a casein-based diet (#8117, Test Diet, Richmond, IN) unless otherwise specified. All compounds tested were synthesized at Wyeth. Compounds given as daily subcutaneous injections were administered in 0.1 ml of 1:1 dimethyl sulfoxide/phosphate-buffered saline or 0.1 ml of 90% corn oil/10% ethanol. Compounds given orally by gavage were administered in 0.1 ml of 0.5% methylcellulose, 2% Tween 80. Compounds given orally in feed were added to the casein diet ground to a powder by mixing. For 5-day treatment studies, cholic acid (CA, Sigma, St. Louis, MO) was added to the ground casein diet. For 5-week cholic acid feeding studies, mice were fed a commercial high fat diet containing 0.5% CA (#21539, Purina, Richmond, IN). Animals were euthanized 2 h following the last treatment, and expression of SHP was quantified by real-time PCR with GAPDH expression used for normalization. The data are presented as the mean ± S.E., with the mean expression value in wild type animals treated with vehicle defined as 1.0. *, p < 0.01 for comparison to vehicle-treated wild type animals.

**FIG. 2. ERα is required for induction of SHP.** A, ovariectomized wild type mice (black bars) or ovariectomized ERα KOCH mice (gray bars) were administered a single subcutaneous injection of corn oil/ethanol vehicle (veh), 10 μg/kg EE (EE), 10 μg/kg EE plus 10 mg/kg ICI182780 (EE + ICI), or 5 mg/kg PPT (PPT). Four hours later the livers were removed and mRNA levels for SHP, STAT5A, PgD syn, IPS, ITF, and CYP3A11 were quantified by real-time PCR. All expression values were normalized for expression of GAPDH and are presented as the mean ± S.E. *, p < 0.01 for comparison to vehicle-treated wild type animals. B, ovariectomized wild type, ERα KOCH, ERβ KO, or ERα CHER/ERβ KO mice were treated by daily subcutaneous injection of vehicle (veh), 10 μg/kg E2 (E2), or 10 μg/kg E2 plus 5 mg/kg ICI182780 (E2 + ICI) for 6 weeks. Livers were removed 2 h following the last treatment, and expression of SHP was quantified by real-time PCR with GAPDH expression used for normalization. The data are presented as the mean ± S.E., with the mean expression value in wild type animals treated with vehicle defined as 1.0. *, p < 0.01 for comparison to vehicle-treated wild type animals.
Sprague-Dawley rats were treated by daily subcutaneous injection of vehicle, 10 μg/kg E2, or 5 mg/kg PPT for 6 weeks. Livers were removed 2 h following the last treatment and expression of SHP was quantified by real-time PCR with GAPDH expression used for normalization. The data are presented as the mean ± S.E., with the mean expression value in wild type animals treated with vehicle defined as 1.0. *, p < 0.01 for comparison to vehicle-treated wild type animals.

during the third cycle, with livers removed for RNA analysis. All treatments were in accord with accepted standards of care as specified by the Wyeth animal care committee.

RNA Analysis—RNA was prepared from frozen organs or from cultured cells by using TRIzol reagent (Invitrogen, Carlsbad, CA) and was treated with DNase to remove contaminating genomic DNA. Following repurification by RNeasy spin columns (Qiagen, Valencia, CA), 100 ng of total RNA was assayed for gene expression by real time reverse transcription-PCR as previously described (12) using the primers and 6-carboxyfluorescein-labeled probes listed in Table I. GAPDH expression was monitored using standard rodent and human assay kits (#4308313 and #402869, respectively, Applied Biosystems, Foster City, CA). Statistical significance was determined by analysis of variance on log-transformed data using Huber weighting.

Cell Culture—HepG2 cells (American Type Culture Collection, Rockville, MD) or Hep9 cells (37) were maintained in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Rockville, MD) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1× glutamax, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% fetal bovine serum. For cycloheximide studies, Hep89 cells plated in deficient growth media (maintenance media without phenol red and supplemented with 10% bovine serum. For cycloheximide studies, Hep89 cells plated in deficient growth media (maintenance media without phenol red and supplemented with 10% bovine serum. For cycloheximide studies, Hep89 cells plated in deficient growth media (maintenance media without phenol red and supplemented with 10% bovine serum. For cycloheximide studies, Hep89 cells plated in deficient growth media (maintenance media without phenol red and supplemented with 10% bovine serum. For cycloheximide studies, Hep89 cells plated in deficient growth media (maintenance media without phenol red and supplemented with 10% bovine serum.

Plasmids—A 1.4-kb region of the human SHP promoter spanning −1383 to +19 was obtained through PCR amplification using 5′-TGAGAAAGATGCCGACACA-3′ forward and 5′-GGGCTCGAGGC-CCCTGTTGCTGTCGCTCA-3′ reverse oligonucleotides with the human BAC clone RP11-285H13 (#MB11201, Invitrogen) as a template. This fragment was then digested with NheI and XhoI and cloned into the luciferase reporter gene pGL3-Basic (Promega, Madison, WI). Each of the human SHP promoter mutations used in this study were created by PCR amplifications using the −1383 SHP promoter as template followed by digestion with appropriate restriction enzymes and ligation into pGL3-Basic.

Transfections—HepG2 cells were seeded into 24-well plates (Falcon, Franklin Lakes, NJ) at a density of 150,000 cells/well in deficient growth media. After 24 h, the cells were transfected with 250 ng of SHP reporter construct, 30 ng of ER expression plasmid, and 100 ng of M19 β-galactosidase control expression plasmid using LipofectAMINE 2000 (Invitrogen) following the manufacturer’s protocol. Six hours later, the cells were treated with MeSO vehicle or 10 μg/ml cycloheximide (Sigma). After 30-min incubation at 37 °C, the medium was further supplemented with MeSO vehicle or 30 nM 17β-estradiol (E2). After incubation for a final 4 h at 37 °C, the cells were harvested for RNA preparation using TRIzol reagent.

Fig. 3. ERα regulates SHP expression in the rat. Ovariectomized Sprague-Dawley rats were treated by daily subcutaneous injection of vehicle, 10 μg/kg E2, or 5 mg/kg PPT for 6 weeks. Livers were removed 2 h following the last treatment and expression of SHP was quantified by real-time PCR with GAPDH expression used for normalization. The data are presented as the mean ± S.E., with the mean expression value in wild type animals treated with vehicle defined as 1.0. *, p < 0.01 for comparison to vehicle-treated wild type animals.

RESULTS

Recently we have demonstrated that treatment of ovariectomized mice with estrogens for 5 days induces expression of SHP in the liver (11). This induction by chronic estrogen treat-
The induction of SHP expression by the ERα is mediated by the aberrantly spliced ERα transcript (ERαKOCH) in several other tissues (40, 41). Chronic treatment of mice with estradiol (E2) in the presence of a FXR agonist PPT and the lack of induction of SHP by estrogens in the ERαKOCH mouse suggest that ERα is responsible for the induction of SHP expression. This induction of SHP by PPT could be mediated either through a direct induction of SHP expression by ERα or through a secondary response subsequent to some other action of estrogen treatment. To delineate between these possibilities, expression of SHP and several other estrogen-regulated genes was determined at various times following treatment of ovariectomized mice with ethynylestradiol (EE) or estrogen receptor agonist PPT (39), at any time point (Fig. 1A). To verify that SHP induction was mediated through ERα, ovariectomized wild type mice or ERαKOCH mice were treated with EE, EE plus the antagonist ICI182780, or PPT (Fig. 2). Induction of SHP expression by EE was inhibited by ICI182780, and neither EE nor PPT treatment induced SHP expression in the ERαKOCH mouse. Similar results were obtained for the STAT5A, PgD synthase, and IPS genes. However, the IIT gene remained responsive to EE and PPT induction in the ERαKOCH mouse. This induction of IIT in the ERαKOCH mouse could be blocked by ICI182780 (not shown) and is likely mediated by the aberrantly spliced ERα transcripts that are found in the ERαKOCH mouse, as has been described for genes in several other tissues (40, 41). Chronic treatment of mice with 17β-estradiol (E2) induced SHP expression in WT and ERβKO mice but not in either ERαKOCH or ERαERβKO mice (Fig. 2B). Together, the induction of SHP expression by the ERα-selective agonist PPT and the lack of induction of SHP by estrogens in the ERαKOCH mouse suggest that ERα is responsible for the direct induction of SHP expression. Finally, the ability of ERα to induce expression of SHP was not limited to the mouse, because both EE and PPT induced expression of SHP in the rat (Fig. 3).

In these studies, the magnitude of gene induction by PPT...
was consistently greater than the magnitude of induction by EE. To determine whether this represented a fundamental difference between PPT, which activates only ERα, and EE, which activates both ERα and ERβ, ovariectomized mice were treated with increasing amounts of either EE or PPT. Both EE and PPT produced very similar dose response curves for induction of SHP (Fig. 4A). The previous differences in the magnitude of SHP induction by PPT as compared with EE reflect simply the dose of compound administered. Approximately 10-fold higher concentrations of PPT than of EE are required to produce the same degree of activation of a synthetic reporter construct in vitro (42), and the dose-response curves of EE and PPT induction of genes such as STAT5a, PgD syn, and IPS show the expected 10-fold shift. In contrast, the ED₅₀ of PPT for SHP induction was ~100-fold higher than the ED₅₀ of EE for SHP induction. This may suggest that the mechanism of ERα induction of SHP expression is different from the mechanism of ERα induction of these other genes.

The high sensitivity of SHP to induction by EE suggested that SHP expression could be regulated by natural fluctuations in estrogen levels. In the mouse, plasma E2 levels are typically about 3-fold higher at proestrus than at estrus (43, 44). In concordance with these levels, mice at proestrus had 4-fold higher levels of liver SHP mRNA than did mice at estrus (Fig. 4B). Thus SHP expression is not only regulated by exogenous administration of estrogens but is also regulated by natural fluctuations of estrogen levels.

To determine whether E2 regulation of SHP gene expression also occurs in human hepatocytes, HepG2 cells, which have lost the ability to express ERα, or Hep89, a derivative of HepG2 cells engineered to constitutively express ERα (37), were treated with E2. No regulation of SHP mRNA levels occurred in the HepG2 cells (Fig. 5A). SHP mRNA was constitutively elevated in the Hep89 cells relative to the HepG2 cells. Treatment of Hep89 cells with E2 stimulated SHP expression, whereas treatment with ICI182780 reduced SHP expression to the level found in HepG2 cells and also blocked E2 stimulation of SHP expression. To verify that the stimulation seen in Hep89 cells was due to ERα, HepG2 cells were transiently cotransfected with a plasmid containing 1383 bp of the human SHP promoter (45) driving expression of a luciferase reporter gene along with either an empty expression vector or an ERα expression vector. Treatment with E2 or PPT induced SHP promoter activity only in the cells cotransfected with ERα (Fig. 5B).

Although the rapid induction of SHP by ERα in vivo suggested a direct activation of the SHP promoter, this was directly confirmed by monitoring the ability of E2 to induce SHP expression in cells treated with cycloheximide. Cycloheximide treatment of Hep89 cells itself increased SHP expression levels, as has been seen in HepG2 cells for at least one other estrogen-regulated gene, the proteinase inhibitor 9 gene (46). In the presence of cycloheximide, E2 treatment still resulted in a further augmentation of SHP expression (Fig. 5C). As a control for inhibition of protein synthesis, treatment of Hep89 cells with cycloheximide induced expression of ICAM-1, a known effect of cycloheximide inhibition of translation of the short-lived IκB protein (47). Finally, ERα induction of SHP promoter activity required an intact DNA binding domain. Cotransfection of an expression plasmid encoding an ERα protein with the DNA binding specificity altered to that of the glucocorticoid receptor failed to activate the SHP promoter following E2 treatment (Fig. 5D), although this chimeric receptor did activate a glucocorticoid response element reporter plasmid following E2 treatment (data not shown). These results suggest a direct activation of the SHP promoter by ERα, potentially through a direct binding mechanism.

Analysis of the SHP promoter region up to −1383 failed to identify any good matches to a consensus estrogen receptor response element (ERE), although several ERE half-sites were identified. Analysis of promoter elements required for basal and ERα-induced SHP expression in hepatocytes was performed by cotransfection of a series of SHP promoter mutations (delineated in Fig. 6) into HepG2 cells (Fig. 7 and Supplemental Table SI). Four major elements (HNF-4, HNF-3, GATA, and AP-1 as shown in Fig. 6) driving basal expression were identified. Thus, removal of an HNF-4 consensus element at −550 by a 5′ deletion (−560 compared with −529, Fig. 7A), by an internal deletion (del −580/−529, Fig. 7C) or by destruction of the HNF-4 site with an SpeI restriction site (LS-554/−548, Fig. 7D) all reduced basal activity by about 2-fold. Second, removal of an HNF-3 consensus element at −465 by a 5′ deletion (−461 compared with −429, Fig. 7A), by a 3′ deletion (del −462/−126 compared with del −468/−126, Fig 7B), by an
SHP Induction by ERα

Fig. 7. Elements mediating basal SHP promoter activity. HepG2 cells were cotransfected with 250 ng of SHP luciferase reporter plasmid containing various regions of human SHP promoter sequence, 30 ng of pcDNA3 ERα expression plasmid, and 100 ng of M19 β-galactosidase control plasmid. After treatment with vehicle for 16 h, cell extracts were prepared and assayed for luciferase and β-galactosidase activities. All luciferase values were normalized for β-galactosidase expression. The luciferase activity in cells transfected with the promoterless pGL3-Basic luciferase plasmid and treated with vehicle was defined as 1.0 for each independent transfection study. All plasmids were assayed in at least three independent transfections. A, results from a series of 5' progressive truncations of the SHP promoter. B, results from a series of 3' progressive truncations of the upstream SHP promoter joined to the proximal SHP promoter at position −126. C, results from a series of small internal deletions in the SHP promoter. D, results from a series of clustered point mutations at the indicated positions produced by introduction of an SpeI restriction site into the SHP promoter.

internal deletion (del −480/−429, Fig. 7C), or by destruction of the HNF-3 site with an SpeI restriction site (LS −467/−462, Fig. 7D) reduced basal promoter activity by 3- to 10-fold. Third, removal of a GATA site at −450 by a 3' deletion (del −430/−126 compared with del −456/−126, Fig. 7B) or by destruction of the GATA site with an SpeI site (LS −455/−450, Fig. 7D) also reduced basal promoter activity by about 2- to 4-fold. Introduction of an SpeI site between the HNF-3 and GATA elements (LS −461/−455) did not alter basal promoter activity. Fourth, removal of an AP-1 site at −265 by a 5' deletion (−279 compared with −229, Fig. 7A), by a 3' deletion (−260/−126 compared with −267/−126, Fig. 7B), by an internal deletion (del −267/−179, Fig. 7C), or by destruction of the AP-1 site with an SpeI site (LS −266/−261, Fig. 7D) all lowered basal promoter activity by 2- to 4-fold. Thus the major elements regulating basal SHP expression in HepG2 cells appear to be HNF-3, GATA, AP-1, and HNF-4 sites, in approximately that order of importance.

None of these elements was required for E2 induction of the SHP promoter. Thus promoter constructs in which the HNF-4 site (LS −554/−549), the HNF-3 site (LS −467/−462), the GATA site (LS −455/−450), or the AP-1 site (LS −266/−261) were disrupted all were still induced by E2 (Fig. 8C). Removal of either the HNF-3 or GATA site actually increased induction of the E2-responsive element to the remaining SHP promoter activity in the absence of these constitutive elements. Analysis of the series of 5' deletions suggested that the region between −316 and −229 was involved in E2 induction (Fig. 8A). Similarly, the 3' series of deletions suggested that the E2 response element was located between −307 and −273 (Fig. 8B). However, in both series of mutations, the loss of E2 induction occurred over a series of progressive deletions, and a complete sudden loss of E2 induction did not occur. To further characterize this region in detail, a series of clustered point mutations covering the SHP promoter between −309 and −261 were assayed for E2 induction (Fig. 8C). No single mutation could be identified that completely eliminated E2 induction. However, a set of mutations at the 5' end of this region (LS −285/−280, LS −307/−302E, and LS −303/−298) and another set of mutations at the 3' end of this region (LS −278/−273, LS −272/−267) partially reduced E2 inductions. When a mutation from the 5' set was combined with a mutation from the 3' set (LS −307/−302E and −278/−273), E2 induction of the SHP promoter was completely abolished. A combination of two mutations that individually did not alter E2 induction (LS −297/−292 and −266/−261E) also did not reduce E2 induction. Thus E2 induction of the SHP promoter relied upon two distinct but closely linked sites.

The previous finding that an intact DNA binding domain was required for ERα activation of the SHP promoter suggested that ERα might bind to one of these two regions. Purified human ERα bound to an oligonucleotide containing the −309 to −267 region of the SHP promoter (Fig. 9A). This binding could be competed by an authentic ERE, but not by a mutated ERE, and inclusion of an antibody directed against ERα produced a supershifted complex. Binding analysis of the series of mutations tested in HepG2 cells revealed that the LS −285/−280 mutation alone was able to disrupt binding of ERα to this fragment of the SHP promoter (Fig. 9B). This mutation destroys an ERE half-site (GACTG, Fig. 6) present within this region. ERα direct recognition of this ERE half-site likely contributes to E2 induction of the SHP promoter, although other interactions must also contribute to this induction based upon the transfection results.

The identified ERα binding site comprises one arm of the
inverted repeat IR-1 FXR binding site within the SHP promoter, suggesting that simultaneous occupancy of this site by both ER<sub>H9251</sub> and FXR should not occur. To determine whether ER<sub>H9251</sub> and FXR agonists could produce an additive induction of SHP expression, ovariectomized mice were fed a diet containing an ER agonist (EE), an FXR agonist (GW4064), or both together. Both EE and GW4064 individually stimulated expression of SHP (Fig. 10). However, the addition of EE to GW4064 treatment did not produce any further stimulation of SHP expression. EE induction of STAT5A, PgD syn, IPS, and ITF was not altered by treatment with GW4064. To verify that these results were not unique to GW4064, ovariectomized mice were fed either a control diet or a diet containing cholic acid (to activate FXR) along with subcutaneous EE treatment. EE activated SHP expression in the mice consuming the control diet but not in mice consuming the cholic acid-containing diet (Fig. 11A). The same result was obtained if the study period was extended to 5 weeks to ensure equilibrium had been obtained (Fig. 11A). The inability of ER<sub>H9251</sub> to activate SHP expression in animals in which FXR was activated is consistent with the overlapping ER<sub>H9251</sub> and FXR response elements identified by the transfection analysis and binding studies.

One of the known in vivo effects of SHP induction is to repress expression of the bile acid synthetic pathway genes CYP7A1 and CYP8B1. However, treatment with EE for either 5 days or 5 weeks induced expression of SHP, but no corresponding reduction in CYP7A1 or CYP8B1 mRNA levels occurred (Fig. 11A). Similar results were obtained using E2 (data not shown). Induction of SHP mRNA levels by either 5-day or 5-week treatment with CA significantly more than did EE treatment (Fig. 11B). Addition of 0.1% CA was sufficient to repress expression of CYP7A1 and CYP8B1 by 80–90%, with this amount of CA inducing SHP expression to a lesser extent than did EE treat-
ment. Thus, although the magnitude of SHP induction by EE would appear to be sufficient to produce a significant reduction in CYP7A1 and CYP8B1 expression, no such reduction occurred.

**DISCUSSION**

Previously we have demonstrated that chronic dosing of mice with 17β-estradiol increased expression of SHP (11). Here, we demonstrate that this is due to a direct induction of SHP promoter activity mediated by ERα. In cultured HepG2-expressing ERα, E2 induced expression of SHP in the presence of cycloheximide, and cotransfection of an ERα expression plasmid conferred estrogen responsiveness upon a reporter plasmid driven by the human SHP promoter. Estrogen regulation of the SHP promoter was localized to between −309 and −267 and was distinct from four major sites (HNF-4, HNF-3, GATA, and AP-1) controlling basal SHP promoter activity in HepG2 cells. This region contained two elements important for maximal induction by estrogens, with one element located between −309 and −298 and a second element located between −285 and −267. Both elements are highly conserved among the human, mouse, and rat promoters (Fig. 6B). The second element contained an ERE half-site that bound to purified ERα and contributed to the induction of the SHP promoter by EE treatment of HepG2 cells. The related nuclear hormone transcription factor ERRγ can also bind to ERE half sites (48) and activates the SHP promoter (20). However, ERRγ activation does not utilize this ERE half-site but, rather, utilizes an SP1 site located at −80 in the SHP promoter (49).

Although estrogen regulation of gene expression has been typically considered as being mediated by a canonical inverted repeat ERE, a growing number of genes are known to require additional elements for estrogen regulation. For example, ERE half-sites in combination with SP1 sites can confer estrogen regulation to multiple genes (50), whereas an AP-1 site is required for function of an ERE within the pS2 gene promoter (51). Neither of these mechanisms are likely for the SHP promoter, because the upstream region has poor homology to either SP1 or AP-1 binding sites. The one functional AP-1 site in the SHP promoter as identified here and by others (52) is clearly dispensable for estrogen regulation of SHP promoter activity. Interestingly, SREBP-1 is required for estrogen induction of LDL receptor expression (53), and our analysis of the human LDL receptor promoter suggests the presence of an ERE half-site −15 nucleotides upstream from the SREBP-1 binding site (data not shown). The corresponding position relative to the ERE half-site is within the −309/−289 element, and this element has partial homology to the SREBP-1 consensus binding site (54). However, this element also contains a base change known to disrupt SREBP-1 binding in the LDL receptor promoter (55). Furthermore, cycloheximide treatment rapidly diminishes SREBP-1 protein levels (56), suggesting that cycloheximide treatment likely would have blocked E2 induction of the SHP promoter if SREBP-1 were required. Finally, many genes that utilize half-ERE sites to confer regulation have multiple functional half-sites, ranging from two active half-sites in the prothymosin α promoter (57) to numerous active sites in the NHE-RF gene promoter (58). The −309/−298 element contains a near match (AGGgCA) to an ERE binding site (54). However, this element also contains a base change known to disrupt SREBP-1 binding in the LDL receptor promoter (55). Furthermore, cycloheximide treatment rapidly diminishes SREBP-1 protein levels (56), suggesting that cycloheximide treatment likely would have blocked E2 induction of the SHP promoter if SREBP-1 were required. Finally, many genes that utilize half-ERE sites to confer regulation have multiple functional half-sites, ranging from two active half-sites in the prothymosin α promoter (57) to numerous active sites in the NHE-RF gene promoter (58). The −309/−298 element contains a near match (AGGgCA) to an ERE half-site. Although this site did not demonstrate binding by ERα in gel shift assays, the ability of ERα to interact with this site may be greater in the context of the full promoter with associated transcription factors bound.

The direct activation of the SHP promoter by ERα in cultured cells was reflected by activation of the SHP promoter in vivo. Thus, either subcutaneous or oral administration of EE or the ERα-specific agonist PPT rapidly induced SHP expression, with peak SHP expression occurring at 1–2 h following treatment. Interestingly, a set of transcription factors exhibited these rapid inductions. For example, STAT5A was also rapidly induced by estrogen treatment. These kinetics were similar to those seen for gene induction in the kidney (17) and for immediate response genes in the uterus (59). The induction of SHP was blocked by ICI182780 treatment and was completely absent in ERαKOCH mice. Although the plasma concentrations of

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**Fig. 10. EE induction of gene expression in the presence of GW4064.** Ovariectomized mice were fed a casein-based powdered diet supplemented to deliver no treatment (−), 10 μg/kg/day EE (EE), 10 mg/kg/day GW4064 (GW), or 10 μg/kg/day EE plus 10 mg/kg/day GW4064 (EE + GW). After 5-day treatment, livers were removed and mRNA levels for SHP, STAT5A, PgD syn, IPS, and ITF in each individual animal were quantified by real-time PCR. All expression values were normalized for expression of GAPDH and are presented as the mean ± S.E., with six animals per group. The mean expression of each gene in animals receiving a vehicle treatment is defined as 1.0, with all other expression values reported relative to this value. *, p < 0.01 for comparison to untreated animals.
EE and PPT were not measured in these studies, the induc-
tions of SHP and STAT5A in the liver as well as STAT5A and
tissue factor in the kidney give a pattern very similar to the
plasma concentration profile following administration of a sin-
gle dose of EE to women, suggesting that expression levels of
these genes all rapidly reflect circulating estrogen levels.
Whereas all genes in the kidney were rapidly induced, in the
liver several genes such as PgD syn, IPS, ITF, and others had
much slower kinetics of induction. Because SHP is demon-
strated here to be a direct target of ER
\(^{36427}\)/H9251
, and the presence of a
perfect ERE in the mouse STAT5A promoter (not shown) sug-
gests it may also be a direct target of ER
\(^{36427}\)/H9251
, it may be that
estrogen induction of a small number of transcription regula-
tors such as SHP and STAT5A mediate the subsequent slower
induction of many additional genes.

The ER binding site in the SHP promoter utilizes one of the
inverted repeats of the FXR binding site (29), suggesting that
FXR and ER\(^{36427}\) should not be able to simultaneously occupy this
site. In concordance with this, EE treatment failed to further
induce expression of SHP in mice in which FXR was activated
either by treatment with GW4064 or by consumption of a diet
containing cholic acid. Interestingly, the human SHP promoter
has also been shown to bind LXR at a DR-4 site, which also
overlaps the FXR IR-1 element (60), in a manner analogous to
the ER\(^{36427}\) binding site. Thus at least three nuclear hormone
receptors, FXR, LXR, and ER\(^{36427}\), all bind to the same region of
the human SHP promoter. In the mouse and rat, the LXR DR-4
binding site is disrupted by a single nucleotide change that
disrupts LXR activation. Thus no activation of SHP expression
occurs after treatment of rodent hepatocytes with an LXR
agonist (60). Whether ER\(^{36427}\) interaction with the human SHP
promoter influences LXR activation of SHP expression remains
to be determined.

SHP has been suggested to regulate the activity of numerous
transcription factors in transfection assays. However, in ani-
mals the only clearly defined regulatory effect for SHP is in the
bile acid feedback pathway in which FXR induction of SHP
inhibits expression of CYP7A1 and CYP8B1 (28, 29). This has
been most clearly demonstrated in the SHP KO mouse, in
which expression of CYP7A1 is constitutively elevated (33, 34).
SHP repression of CYP7A1 expression is mediated by inhibi-
tion of a\(_{1}\)-fetoprotein transcription factor (LRH-1) activation of
the SHP promoter (28, 29), whereas SHP repression of CYP8B1
expression appears to differ between species, with SHP inhibi-
tion of HNF-4\(^{374}\)/H9251
1-fetoprotein transcription factor responsible for repression of human
CYP8B1 promoter activity (23) and SHP inhibition of a\(_{1}\)-feto-
protein transcription factor responsible for repression of rat
CYP8B1 promoter activity (61). Surprisingly, although the

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**Fig. 11.** EE induction of SHP does not repress CYP7A1 or CYP8B1 expression. A, ovariectomized mice were fed either a casein-based diet
\((-\text{)}\) for 5 days, a casein-based diet supplemented with 0.3% cholic acid (CA, \(\text{+}\)) for 5 days, a control diet \((-\text{)}\) for 5 weeks, or a high fat diet containing
0.5% CA \(\text{(+)}\) for 5 weeks. All animals received daily subcutaneous injections of vehicle \((-\text{)}\) or 10 \(\mu\)g/kg EE \(\text{(+)}\). After 5 days of treatment, livers
were removed 1 h after the last subcutaneous treatment. After 5 weeks of treatment, livers were removed 24 h after the last subcutaneous
treatment. Levels of SHP, CYP7A1, and CYP8B1 mRNA were quantified by real-time PCR with normalization for GAPDH expression. Values
shown are the mean ± S.E. determined from six animals per group, with gene expression in animals consuming the control diet and treated with
vehicle for either 5 days or 5 weeks defined as 1.0. *, \(p < 0.01\) for comparison to control diet vehicle animals. B, ovariectomized mice were fed a
casein diet supplemented with various concentrations of CA. After 5 days of treatment, liver mRNA levels for SHP, CYP7A1, and CYP8B1 were
determined by real-time PCR with normalization for GAPDH expression. Values shown are the mean ± S.E. of determinations, with 12–18 animals
total per group. The expression level of each gene in the absence of CA supplementation was defined as 1.0. *, \(p < 0.01\) for regulation by CA.
magnitude of induction of SHP by EE was similar to that produced by 0.1% cholic acid (which resulted in 90% repression of expression of CYP7A1 and CYP8B1), EE treatment did not produce the expected repression of either CYP7A1 or CYP8B1 expression. Similar results were found for E2 treatment (data not shown), and there was no correlation between SHP expression and CYP7A1 and CYP8B1 repression in cycling animals (data not shown). Some FXR ligands such as GW4064 both induce SHP expression and repress CYP7A1 and CYP8B1 expression (29, 33), whereas others such as guggulsterone induce SHP expression but fail to repress either CYP7A1 or CYP8B1 expression (62), as seen here for EE. It is not yet clear whether a similar pattern will emerge for ERα, with some ERα ligands both inducing SHP and repressing CYP7A1 and CYP8B1.

The basis for the lack of repression of CYP7A1 and CYP8B1 repression despite the induction of SHP in EE-treated mice remains to be determined. In SHP KO mice, cholic acid still represses CYP7A1 expression via alternative mechanisms (33, 34). SHP induction by itself might not be sufficient to mediate repression of CYP7A1 and CYP8B1 without these additional signaling pathways. FXR activation could provide additional signals such as production of a SHP ligand with the ability to confer SHP repression upon CYP7A1 and CYP8B1 promoters. A second possibility is that ERα sends a stimulatory signal to the CYP7A1 and CYP8B1 promoters to counteract the SHP repression signal. However, ERα does not stimulate activity of either the human CYP7A1 or CYP8B1 promoters in cotransfection assays, and EE treatment of mice did not increase mRNA levels for either LRH-1 or HNF-4α (data not shown). A third possible explanation relies on the ability of liganded ERα to bind to SHP, which then represses ERα activity (18, 19, 63). If the affinity of SHP for liganded ERα were significantly greater than for either LRH-1 or HNF-4α, then the SHP repression by ERα activation might be sequestered by the active ERα and thereby be unable to mediate repression of LRH-1 or HNF-4α activity. In this model, it would be expected that EE treatment could reverse bile acid-mediated repression of CYP7A1 and CYP8B1 expression, but only a very small effect in this direction was seen in the studies performed here. Whatever the basis, it appears that in vivo mechanisms have been provided to prevent cross-talk between SHP utilization in bile acid signaling pathways and in estrogen signaling pathways.

SHP is expressed in many tissues of the body aside from liver (25, 45), suggesting that regulation of bile acid synthesis is unlikely to be its sole role. Estrogen treatment can regulate SHP expression in organs other than the liver and coexpression of ERβ in HepG2 cells can induce SHP promoter activity (data not shown), suggesting that all in vivo estrogenic regulations of SHP need not necessarily be mediated through ERα. The conservation of estrogen regulation between rodents and human suggests estrogen regulation of SHP in these organs may play a role in the biology of estrogen. For example, postmenopausal women have increased amounts of white adipose tissue, and hormone replacement therapy decreases the level of white adipose tissue (64–66). Similarly, white adipose tissue is increased by ovariectomy (67) or in ERαKO mice (68). Inactivating mutations in the SHP gene have been associated with mild obesity in the Japanese population (69). Given the direct regulation of SHP by ERα demonstrated here, these findings suggest the hypothesis that regulation of SHP levels are an important part of the mechanism by which estrogens regulate adipose tissue levels. Further analysis of the role of estrogen induction of SHP in specific organs other than the liver will be necessary to delineate the contributions of SHP to estrogen physiology.

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