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Rat 17β-Hydroxysteroid Dehydrogenase Type IV Is a Novel Peroxisome Proliferator-Inducible Gene

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SUMMARY

To better understand the molecular mechanisms of the pleiotropic responses induced by exposure to peroxisome proliferator chemicals (PPCs), we conducted a systematic search for genes whose mRNA levels are modulated by the PPC WY-14,643 (WY) in rat liver. The sequence of one up-regulated cDNA (2480 bp) was predicted to encode a protein of 735 aa with 82% identity to the porcine 17β-hydroxysteroid dehydrogenase type IV (HSD IV). Like the porcine enzyme, the rat HSD IV contains a region homologous to yeast hydratase-dehydrogenase-epimerases and to sterol carrier proteins, indicating that the rat HSD IV has broad substrate specificity and contributes to cholesterol metabolism. The rat HSD IV was regulated by diverse PPCs via two distinct mechanisms. Induction of HSD IV and acyl-CoA oxidase (ACO) proteins in rat liver at different treatment times and concentrations of gemfibrozil and di-*n*-butyl phthalate were almost identical, indicating that HSD IV mRNA induction involves the peroxisome proliferator-activated receptor α, a regulator of ACO. In contrast, HSD IV protein levels were only weakly induced by WY, a strong inducer of ACO protein, even though the levels of HSD IV and ACO mRNA were strongly stimulated by WY and gemfibrozil. Thus, HSD IV protein levels were uniquely regulated pretranslationally by WY via a novel mechanism. Increased conversion of estradiol to the less-active estrone by HSD IV induction may explain how phthalate exposure leads to decreases in serum estradiol levels and suppression of ovulation.

A large group of chemicals that induce hepatomegaly and hepatic peroxisome proliferation are hepatocarcinogenic after prolonged administration in rodents. These PPCs have diverse structures, uses, and potentials for human exposure. PPCs include hypolipidemic drugs (clofibrate, GEM), experimental hypolipidemic drugs (WY), plasticizers (DBP), herbicides (chlorophenoxyacetic acids), perfluorinated fluids (perfluorooctanoic acid), and chlorinated solvents (trichloroethylene). The carcinogenicity of some of these chemicals in rodents, coupled with widespread human exposure, suggests that PPCs may contribute to human cancer risk (1). However, the scientific validity of this assumption has not been established.

Exposure of rodents to PPCs leads to a number of well-characterized responses. Changes in the expression of genes involved in metabolism of fatty acids can occur as early as 1 hr (2–4) and precede peroxisome proliferation, which begins ~24 hr after exposure (5). Hepatocyte cell proliferation occurs in two phases: an acute phase at ~1-8 days, followed by a chronic phase for strong PPCs that lasts as long as the animals are fed the PPC (6). Preneoplastic foci occur as early as the 11th week of chronic exposure to WY, and hepatocellular adenomas have been seen at ~52 weeks or later depending on the chemical and dose (7).

Many facets of the molecular mechanism of enzyme induction by PPCs are known. PPCs (8) as well as long-chain unsaturated fatty acids (9) have been shown to activate members of the nuclear receptor superfamily called PPARs. Three mammalian PPAR subtypes have been isolated: termed PPARα, Nucl (also known as PPARδ), and PPARγ (for a review, see Ref. 10). In the presence of these inducers, PPARα, the principal isoform expressed in the liver, activates expression of genes whose products catalyze the me-

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ABBREVIATIONS: PPC, peroxisome proliferator chemical; aa, amino acids; ACO, acyl-CoA oxidase; DBP, di-*n*-butyl phthalate; HSD IV, 17β-hydroxysteroid dehydrogenase type IV; GEM; gemfibrozil; HDE, hydratase/dehydrogenase/epimerase; HSD, 17β-hydroxysteroid dehydrogenase; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SCP2, sterol carrier protein-2; WY, WY-14,643.

tabolism of fatty acids. The genes involved in fatty acid β-oxidation, including ACO (11), and the enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional gene (12, 13), as well as the *CYP4A6* gene encoding a fatty acid ω-hydroxylase (14), have been shown to be activated by PPARα in cell culture. These genes are likely activated by a heterodimer of PPARα and the receptor for 9-*cis* retinoic acid (retinoic X receptor), which recognizes sequences within the 5′ regulatory regions containing two copies of the sequence TG(A/T)CCT in the direct repeat orientation separated by 1 bp (13, 15–19). PPARα was shown to be a global regulator of genes involved in peroxisomal β-oxidation of fatty acids in the liver in that inducibility of these genes by PPCs is abolished in mice that lack a functional PPARα gene (20).

To better understand the molecular mechanisms of the pleiotropic responses induced by exposure to PPCs, we made the first concerted effort to clone and characterize genes that are modulated in the rat liver after exposure to a PPC. Our results demonstrate that PPCs up-regulate HSD IV, originally isolated as a 17β-estradiol dehydrogenase. Our studies imply that PPC-induced expression of HSD IV lowers the levels of estradiol and may account for the toxic effects that some phthalate esters have on ovulation.

Experimental Procedures

Animals. This study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee. For the first three experiments, male F-344 [CDF(F-344)/CrlBR] and Sprague-Dawley (SD-Harlan derived) rats were obtained from Charles River Breeding Laboratories (Raleigh, NC). For the fourth experiment, male Harlan Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Control and treated rats were provided with NIH-07 rodent chow (Ziegler Bros., Gardner, PA) and deionized, filtered water *ad libitum*. Lighting was on a 12-hr light/dark cycle. In the first experiment, F344 rats were administered a single gavage dose of WY (ChemSyn Science Laboratories, Lenexa, KS) at 50 mg/kg of body weight and killed 3 or 24 hr after treatment. In the second experiment, F344 rats were fed a diet containing 1,000 ppm WY or 12,000 ppm GEM (Sigma Chemical, St. Louis, MO) for 3 weeks. In the third experiment, male and female F344 rats and male Harlan Sprague-Dawley rats were fed 500 ppm WY, 8,000 ppm GEM, or 20,000 ppm DBP (Aldrich Chemical, Milwaukee, WI) for 13 weeks. In the fourth experiment (conducted by the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC), male Harlan Sprague-Dawley rats were fed the indicated doses of WY (ChemSyn Science Laboratories, Lenexa, KS), GEM (Sigma Chemical Co., St. Louis, MO), or DBP (ChemCentral, Kansas City, MO) for 1, 5, or 13 weeks. In separate experiments, rats were given 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by gavage for 14 days, which results in a liver tissue concentration of 150 ng of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin/g of liver tissue (21). Chloroform (180 mg/kg of body weight for each of 4 days) and furan (45 mg/kg of body weight for each of 2 days) were administered by gavage in corn oil. Livers from sham-operated and partially hepatectomized rats were isolated at 24 or 36 hr after surgery. At the designated time after treatment, rats were deeply anesthetized with isofluorane anesthesia or pentobarbital injection and killed by exsanguination. The livers were removed, rinsed with isotonic saline, snap-frozen in liquid nitrogen, and stored at −70° until analysis.

Differential display of mRNA. To focus on primary PPC-responsive genes, we isolated genes that were altered in expression within 24 hr after a single dose of WY. Genes responsive to WY were identified by the differential display screening technique (22). Total RNA was isolated by modification of the guanidinium isothiocyanate method using RNazol according to the manufacturer's instructions (Tel-Test "B", Friendswood, TX). The cDNA was synthesized from total RNA, and the reverse-transcribed cDNA was amplified by PCR under the conditions described previously (22) with minor modifications. One microgram of total RNA from the livers of control and treated rats was used in each reverse transcription reaction. Because of potential interanimal and assay variability, RNAs from two control and two treated rats were always compared. Only the cDNA bands that showed consistent changes between the pairs of samples were analyzed further. The sequences of the primers used in the display reactions were T_{12}MG and JN-1(5′-GA(G/A)CAGGTGGC(C/T)CAGCTGAAGCA-3), where T_{12} represents 12 Ts and M is an equimolar mixture of A, C, and G. The sequence of the JN-1 primer was originally chosen to selectively amplify members of the *fos/jun* family. This oligonucleotide is predicted to anneal to a region within the cDNA encoding the leucine zipper conserved in this family of proteins. It should be noted that attempts to use this oligonucleotide to isolate members of the *fos/jun* family by differential display failed most likely due to the low stringency of the differential display conditions.

Cloning and sequencing the differentially expressed cDNAs. Differential display gels were soaked in water for 15–20 min to remove urea before drying. cDNA bands were cut from the gels, eluted into water, and amplified by PCR using the same primer pairs used to initially amplify the fragment. The amplified fragments were cloned directly into the vector pCRII using the TA cloning kit according to the instructions of the manufacturer (InVitrogen, San Diego, CA). Plasmids that contained an insert of the correct size were used in subsequent studies. Both strands of individual clones were sequenced according to the dideoxy chain termination method of Sanger *et al.* (23) on double-stranded templates. At least 130 bp of sequence from each cDNA were compared with the entire nonredundant sequence database at the National Library of Medicine using BLAST (24).

Analysis of mRNA expression. A total of 0.5, 5, and 20 μg of total RNA isolated from control rats or rats treated for 3 or 24 hr with a gavage dose of WY was denatured and applied to a nylon membrane using a slot-blot apparatus according to the manufacturer's instructions (Schleicher & Schuell, Hanover, NH). Denatured total RNA was also separated on 1.2% agarose gels and transferred to nylon filters in 20× standard saline citrate (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). The probes for Northern slot-blot were made by PCR using as templates the plasmids containing the cloned inserts and the SP6 and T7 promoter primers in the presence of [α-^{32}P]dCTP (25). The correct sizes and homogeneity of the synthesized fragments were confirmed by denaturing 6% PAGE and native agarose gel electrophoresis. Hybridization and washing conditions were described previously (21). The 1.08-kb *Pst*I cDNA fragment of the ACO cDNA (kindly provided by Dr. Hilde Nebb-Sörensen, Institute of Medical Biochemistry, University of Oslo, Oslo, Norway) was used as a positive control. Albumin was used as a loading control because its level does not change after short term exposure to the PPC WY (26). The probes for Northern analysis were labeled with [α-^{32}P]dCTP using the random-primer DNA labeling kit according to manufacturer's instructions (Stratagene, La Jolla, CA).

Isolation of full-length cDNAs. The pooled cDNAs from the livers of two male F344 rats administered a 24-hr gavage dose of WY (50 mg/kg of body weight) were cloned into the lambda ZAP express vector (Stratagene). Lambda vectors containing the full-length cDNAs for clone 25 were isolated using standard techniques. The cDNA fragments were excised into the pBK-CMV phagemid according to manufacturer's instructions (Stratagene).

Sequencing HSD IV. The full-length HSD IV was sequenced by the chain-termination method (23). The 1.0- and 0.84-kb *Pst*I fragments from the pBK-CMV vector containing the full-length HSD IV cDNA were subcloned into the pBluescript II SK vector (Stratagene).

The 0.58-kb *Pst*I fragment was subcloned into the vector pBK-CMV. Appropriate primers were designed to sequence a small *Pst*I/*Pst*I internal fragment in the original plasmid. Both strands of the two longest isolates (21A and 25A) were sequenced. Using a FastA search, minor differences were noted in the sequence of the two isolates, including a TAGA in the 5′-untranslated region of one of the isolates and the length of the poly(A)$^+$ tail. The predicted protein sequence was compared with the Swiss Prot protein database. Alignments of rat HSD IV to porcine HSD IV, *Candida tropicalis* HDE, and the rat sterol carrier protein-2 were performed with Laser-Gene Navigator.

***In vitro* transcription and translation.** Templates for *in vitro* transcription reactions included the empty vector, pBK-CMV, and the pBK-CMV plasmids encoding the 21A and 25A isolates of rat HSD IV. The plasmids were used to carry out *in vitro* transcription and translation reactions according to the manufacturer's instructions (Promega, Madison, WI). Translation reactions were performed in the presence of ^{35}S-methionine. Labeled proteins were separated by 7.5% SDS-PAGE and visualized by autoradiography. No differences in the size or pattern of bands were observed between templates 21A and 25A (data not shown).

Western blot analysis. Protein extracts (50–120 μg of total protein) made according to Wilcke *et al.* (27) were denatured and size-separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose and visualized with Ponceau Red to confirm transfer. The blotted proteins were probed with polyclonal antibodies followed by anti-rabbit IgG coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (ECL kit, Amersham). The antiporcine HSD IV antibodies and porcine uterine extract were kind gifts from Jerzy Adamski and Peter Jungblut (Max-Planck Institute, Hannover, Germany). The anti-ACO antibody was a kind gift from Stefan Alexson (Huddinge University Hospital, Huddinge, Sweden).

Results

Isolation and sequence of WY-responsive genes. Differential display was used to identify three cDNAs (bands 22, 23, and 25) that were up-regulated in the livers of 24-hr treated rats (Fig. 1A). The cloned cDNA bands were used as probes to confirm WY-induced expression. As shown by Northern slot-blot analysis (Fig. 1B), the positive control ACO was slightly induced at 3 hr and strongly induced at 24 hr, as has been seen in previous studies (2, 3, 28). Clone 22 (Fig. 1B) and clone 23 (data not shown) were induced at 3 and 24 hr but were more abundant after 3 hr. Clone 25 was induced at 24 hr, but there was little if any induction at 3 hr. In addition, clone 25 exhibited higher background expression than ACO in the absence of exposure. The loading control, albumin, exhibited no change in expression after exposure, as previously shown (26).

The WY-responsive cDNA fragments were sequenced and compared with the DNA database at the National Library of Medicine. Clones 22 and 23 were identified as *CYP4A2*, a fatty acid ω-hydroxylase and a gene known to be induced by peroxisome proliferators (29), confirming differential display as a technique appropriate to isolate PPC-responsive genes.

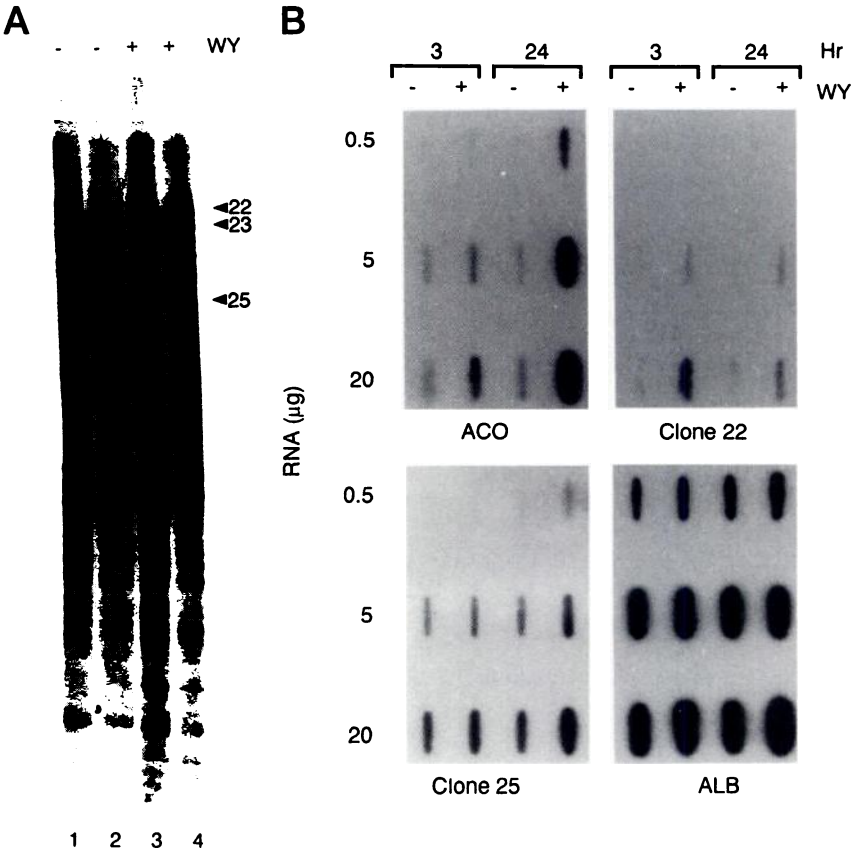

Fig. 1. Identification of WY-responsive genes in rat liver. A, Differential display of liver mRNA from control versus 24-hr WY-treated rats. *Lanes*, gene expression in the liver of either a treated rat (+) administered a gavage dose of WY (50 mg/kg of body weight) or a control rat (−) administered a gavage dose of vehicle alone. *Numbers*, designation of the bands that showed increased expression after exposure and clones derived from them. B, Expression of isolated genes after exposure to WY. Three different amounts of total liver RNA from individual control rats and rats treated for 3 or 24 hr with a gavage dose of WY (50 mg/kg of body weight) were loaded unto nitrocellulose and probed with fragment 22 or 25 and fragments from the positive control ACO or negative control albumin (*ALB*).

A

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GAATTCGGCACGAGTAGACTCATGGCTTCGCCTCTGAGGTTCGACGGGCGTGTGGTCCTGGTCACCGGCGCCGGGGGAGGGTTGGGCAGA   90
                    M  A  S  P  L  R  F  D  G  R  V  V  L  V  T  G  A  G  G  G  L  G  R     23

GCTTATGCCCTGGCTTTTGCAGAAAGAGGAGCATTAGTTGTTGTGAATGACTTAGGAGGGGACTTCAAAGGCGTTGGGAAAGGCTCTTCT   180
 A  Y  A  L  A  F  A  E  R  G  A  L  V  V  V  N  D  L  G  G  D  F  K  G  V  G  K  G  S  S     53

GCCGCAGACAAGGTCGTGGAAGAAATAAGAAGGAGAGGCGGGAAAGCGGTGGCCAATTACGATTCAGTCGAAGCAGGCGAGAAGCTTGTG   270
 A  A  D  K  V  V  E  E  I  R  R  R  G  G  K  A  V  A  N  Y  D  S  V  E  A  G  E  K  L  V     83

AAGACAGCACTGGACACATTCGGCAGAATAGATGTTGTGGTGAACAATGCTGGGATCCTGAGGGACCGTTCCTTCTCTAGGATAAGTGAT   360
 K  T  A  L  D  T  F  G  R  I  D  V  V  V  N  N  A  G  I  L  R  D  R  S  F  S  R  I  S  D    113

GAAGACTGGGATATAATTCAAAGAGTTCATTTGCGGGGCTCCTTCCAAGTGACCCGGGCAGCATGGGATCATATGAAGAAGCAGAATTAT   450
 E  D  W  D  I  I  Q  R  V  H  L  R  G  S  F  Q  V  T  R  A  A  W  D  H  M  K  K  Q  N  Y    143

GGAAGAATCATTATGACGGCCTCAGCTTCTGGAATATACGGCAACTTTGGCCAGGCAAATTATAGTGCTGCAAAGCTGGGCCTTCTGGGT   540
 G  R  I  I  M  T  A  S  A  S  G  I  Y  G  N  F  G  Q  A  N  Y  S  A  A  K  L  G  L  L  G    173

CTCGCCAATACTCTCGTGATTGAAGGCAGGAAGAACAACATTCATTGTAACACCATTGCCCCAAACGCTGGGTCACGGATGACAGAGACG   630
 L  A  N  T  L  V  I  E  G  R  K  N  N  I  H  C  N  T  I  A  P  N  A  G  S  R  M  T  E  T    203

GTGATGCCAGAAGACCTCGTTGAAGCCCTGAAGCCAGAGTATGTGGCACCGCTGGTCCTTTGGCTTTGCCATGAGAGCTGTGAGGAAAAT   720
 V  M  P  E  D  L  V  E  A  L  K  P  E  Y  V  A  P  L  V  L  W  L  C  H  E  S  C  E  E  N    233

GGTGGCTTGTTTGAGGTTGGAGCAGGATGGATTGGAAAATTGCGCTGGGAGAGGACCCTGGGAGCCATTGTCAGGAAGCGGAATCAGCCC   810
 G  G  L  F  E  V  G  A  G  W  I  G  K  L  R  W  E  R  T  L  G  A  I  V  R  K  R  N  Q  P    263

ATGACTCCCGAGGCAGTGAGGGACAACTGGGTGAAGATCTGTGACTTCAGCAATGCCAGCAAGCCGAAGAGCATTCAAGAGTCCACAGGT   900
 M  T  P  E  A  V  R  D  N  W  V  K  I  C  D  F  S  N  A  S  K  P  K  S  I  Q  E  S  T  G    293

GGTATAATCGAAGTTTTACATAAAATAGATTCAGAAGGAATCTCACAAAATCACACCGGTCAAGTGGCATCTGCAGATGCATCAGGATTT   990
 G  I  I  E  V  L  H  K  I  D  S  E  G  I  S  Q  N  H  T  G  Q  V  A  S  A  D  A  S  G  F    323

GCTGGCGTCGTTGGCCACAAACTTCCTTCATTTTCTTCTTCATATACGGAACTGCAGTGCATTATGTATGCCCTCGGAGTAGGAGCTTCA  1080
 A  G  V  V  G  H  K  L  P  S  F  S  S  S  Y  T  E  L  Q  C  I  M  Y  A  L  G  V  G  A  S    353

GTCAAAAATCCAAAGGACTTGAAGTTTGTTTATGAAGGGAGTGCTGACTTCTCCTGTTTGCCTACATTTGGAGTCATTGTCGCTCAGAAG  1170
 V  K  N  P  K  D  L  K  F  V  Y  E  G  S  A  D  F  S  C  L  P  T  F  G  V  I  V  A  Q  K    383

TCCTTGATGAGTGGAGGCTTAGCAGAGGTTCCTGGGCTGTCAATCAACTTTGCAAAGGTTCTTCATGGGGAGCAGTACTTGGAGTTGTAT  1260
 S  L  M  S  G  G  L  A  E  V  P  G  L  S  I  N  F  A  K  V  L  H  G  E  Q  Y  L  E  L  Y    413

AAGCCACTTCCCCGATCAGGGGAATTAAAATGTGAAGCAGTTATTGCTGACATCCTGGATAAAGGCTCTGGCATAGTGATTGTTATGGAC  1350
 K  P  L  P  R  S  G  E  L  K  C  E  A  V  I  A  D  I  L  D  K  G  S  G  I  V  I  V  M  D    443

GTCTATTCTTATTCTGGCAAGGAACTTATATGCTATAATCAGTTCTCTGTCTTCGTTGTTGGCTCTGGAGGCTTTGGTGGAAAACGGACA  1440
 V  Y  S  Y  S  G  K  E  L  I  C  Y  N  Q  F  S  V  F  V  V  G  S  G  G  F  G  G  K  R  T    473

TCAGAAAAACTCAAAGCAGCTGTAGCCGTACCAAGTCGGCCTCCAGATGCTGTACTGAGAGATACCACTTCACTGAATCAGGCCGCTCTG  1530
 S  E  K  L  K  A  A  V  A  V  P  S  R  P  P  D  A  V  L  R  D  T  T  S  L  N  Q  A  A  L    503

TACCGCCTCAGTGGAGACTCGAATCCTTTACACATTGACCCGAGCTTTGCGAGCATTGCCGGTTTTGAGAAACCCATATTACACGGATTA  1620
 Y  R  L  S  G  D  S  N  P  L  H  I  D  P  S  F  A  S  I  A  G  F  E  K  P  I  L  H  G  L    533

TGTACTTTTGGGTTTTCTGCAAGGCATGTTTTACAGCAGTTTGCGGATAATGATGTGTCAAGATTCAAGGCCATTAAGGTTCGTTTTGCC  1710
 C  T  F  G  F  S  A  R  H  V  L  Q  Q  F  A  D  N  D  V  S  R  F  K  A  I  K  V  R  F  A    563

AAACCAGTGTATCCAGGACAAACTCTACAAACTGAGATGTGGAAGGAAGGAAACAGAATTCATTTTCAAACCAAGGTCCAAGAGACTGGA  1800
 K  P  V  Y  P  G  Q  T  L  Q  T  E  M  W  K  E  G  N  R  I  H  F  Q  T  K  V  Q  E  T  G    593

GACATTGTCATTTCCAATGCATATGTGGATCTTGTTCCTACATCTGGAGTTTCCGCTCAGACACCTTCTGAGGGTGGAGCACTGCAGAGT  1890
 D  I  V  I  S  N  A  Y  V  D  L  V  P  T  S  G  V  S  A  Q  T  P  S  E  G  G  A  L  Q  S    623

GCTCTTGTATTTGGGGAAATAGGTCGACGCCTCAAGGATGTTGGACGTGAGGTGGTAAAGAAAGTAAATGCTGTATTTGAATGGCATATC  1980
 A  L  V  F  G  E  I  G  R  R  L  K  D  V  G  R  E  V  V  K  K  V  N  A  V  F  E  W  H  I    653

ACGAAAAATGGGAATGTTGCAGCCAAGTGGACCATTGACCTGAAGAACGGCTCTGGAGAGGTTTACCAAGGCCCTGCCAAAGGCTCTGCT  2070
 T  K  N  G  N  V  A  A  K  W  T  I  D  L  K  N  G  S  G  E  V  Y  Q  G  P  A  K  G  S  A    683

GACACGACCATCACAATTTCTGATGAGGATTTCATGGAAGTGGTCCTGGGCAAGCTTAACCCACAGAATGCCTTCTTCAGTGGCAGACTG  2160
 D  T  T  I  T  I  S  D  E  D  F  M  E  V  V  L  G  K  L  N  P  Q  N  A  F  F  S  G  R  L    713

AAGGCCCGAGGAAACATCATGCTGAGCCAGAAGCTACAGATGATTCTGAAAGACTATGCCAAGCTCTGAAGGACCCACTGCGTGCTTTAA  2250
 K  A  R  G  N  I  M  L  S  Q  K  L  Q  M  I  L  K  D  Y  A  K  L  •                          735

TAAAACCAGAATCATTACGTTCTGTCTACGCAGTCATGCTCCAGCCTTCTTTGAAACGATCCACGGTAATGTGCAGCAGAAATTGCTTAA  2340

CATTTTCAGATTCAGATAACTTTCAGATTTTCATTTTCTACTAATTTTTCACATATTATTTTTACAAGGAACTGTAATCTAGCTAGCAAA  2430

TAATTGTTCTGTTCATAGATCTGTATCTTAATAAAAAAAAAAAAAAAAA                                            2480
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Fig. 2. cDNA sequence of full-length clone 25. A, DNA and predicted protein sequence of clone 25. Full-length cDNAs of clone 25 were isolated from a WY-treated rat liver cDNA library. The two clones with the longest inserts were sequenced; they differed only in the length of the poly(A)$^+$ tail and nucleotide positions 15–18 encoding the sequence TAGA found in one clone (shown) but not the other. The sequence begins at the first nucleotide of an *Eco*RI site (GAATTC), 22 nucleotides upstream of the putative initiator methionine. *Double-underlining*, possible polyadenylation signal. B, Confirmation of the open reading frame. The cDNA encoding the clone (*lane 2*) or empty vector (*lane 1*) was transcribed using T3 RNA polymerase and translated using rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The proteins were resolved by 7.5% SDS-PAGE. Almost all of the radiolabeled protein comigrated with the 80-kDa molecular mass standard.

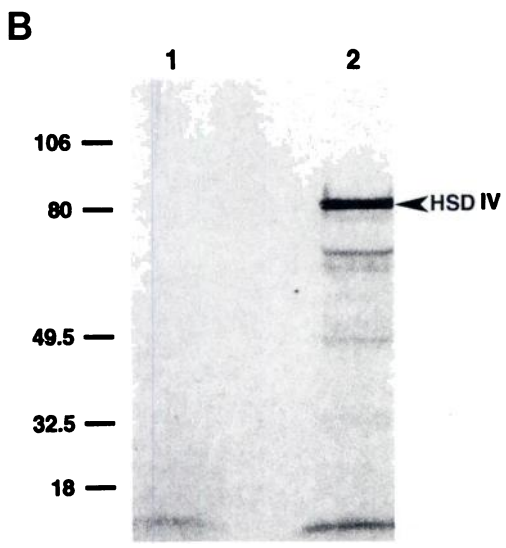

Fig. 2B.

The clone 25 sequence had no exact match to anything in the gene database, indicating that it was a novel cDNA.

Sequence analysis of full-length clone 25. Using the clone 25 fragment as a probe, a number of cDNAs (2.3–2.6 kbp) were isolated from a cDNA library made from rat liver mRNA 24 hr after WY exposure. Preliminary Northern blot hybridization studies using the clone 25 as a probe demonstrated hybridization to a single 2.4-kbp mRNA (data not shown), indicating that in our library screen full-length cDNA clones were isolated. The two largest cDNA inserts were sequenced, and the putative open reading frame was translated. The 2,480-bp cDNA was predicted to encode a 79,438-Da protein of 735 aa (Fig. 2A) and a pI of 8.58. The nucleotide sequence flanking the putative ATG start codon (AGACTCATGGG) was similar to the consensus sequence for eukaryotic translation initiation sites [GCC(G/A)CCATGGG] proposed by Kozak (30). Two potential polyadenylation signals exist in the 3′ end of the clone 25 mRNA. These include the sequence AAUAAU consistent with the consensus signal (AAUAAA) found 29 nucleotides upstream of the poly(A)$^+$ tail and the sequence UCUGUUCA consistent with the proposed GU-rich element consensus YGUGUUYY (for a review, see Ref. 31). The size of the encoded protein was determined by *in vitro* transcription and translation reactions using plasmids encoding the full-length clone 25. SDS-PAGE of the proteins (Fig. 2B) revealed a predominant 80-kDa protein identical in size to that predicted.

Comparison of the putative open reading frame of the full-length cDNA with the protein data base showed 82% identity and 88% similarity to the porcine HSD IV (32), also known as 17β-estradiol dehydrogenase. The rat protein is predicted to be 2 aa shorter than the porcine enzyme. Like the porcine enzyme (33), the rat HSD IV protein exhibits homology to two types of proteins: mammalian sterol carrier proteins and yeast dehydrogenase-hydratase multifunctional proteins, including the *Candida tropicalis* HDE (34) and the *Saccharomyces cerevisiae* Fox2 (35). In addition, the rat HSD IV encodes a putative carboxyl-terminal peroxisomal localization signal, AKL (for a review, see Ref. 36). This is the same carboxyl-terminal sequence as that found in the rat SCP2, which has been immunolocalized to peroxisomes (37).

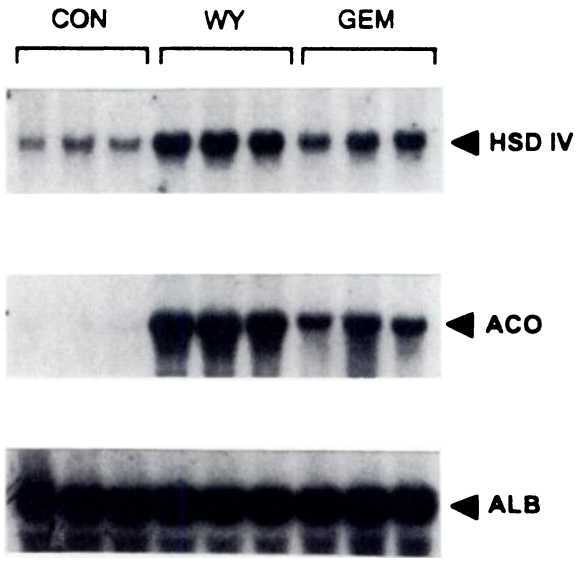

Fig. 3. Increased expression of HSD IV mRNA after PPC exposure. Total RNA was isolated from livers of control rats (*CON*) and from rats fed 1,000 ppm WY or 12,000 ppm GEM in the diet for 3 weeks. Fifteen micrograms of total RNA was size-separated, transferred to a nylon membrane, and probed with either the full-length rat HSD IV cDNA, a fragment of the ACO cDNA, or a fragment of the albumin (*ALB*) cDNA, which was used as a loading control.

HSD IV mRNA expression after exposure to PPC. Northern blot analysis was performed to determine the extent of the increase in expression after exposure to PPC. When rats were fed WY (1,000 ppm) or GEM (12,000 ppm) for 3 weeks, there was an increase in the levels of HSD IV mRNA in the liver (Fig. 3). ACO was also induced, although the level of induction was higher, partly due to the higher level of expression of HSD IV in the absence of exposure. At these concentrations, WY was a better inducer of both HSD IV and ACO. HSD IV could also be induced *in vitro* by WY and ciprofibrate in the rat hepatoma cell line H4IIEC3 (data not shown). This cell line seems to be an appropriate cell model for understanding the molecular mechanism of the induction of HSD IV. The HSD IV induction by PPCs was shown to not be linked to a general cell proliferation response because chloroform, furan, and partial hepatectomy protocols that induce liver cell proliferation by different mechanisms do not alter the level of HSD IV mRNA (data not shown). Other PPC-responsive genes isolated by differential display have been shown to be induced by diverse protocols that induce cell proliferation.[4]

HSD IV protein levels after exposure to PPCs. The level of responsiveness of HSD IV protein to diverse PPC exposure conditions was determined by Western analysis of liver whole-cell extracts. Male and female F344 rats were fed 500 ppm WY, 8,000 ppm GEM, or 20,000 ppm DBP in the diet for 13 weeks. These doses of WY (38) and GEM (39) are close to those shown to elicit a maximal response in ACO activity. An 80-kDa immunoreactive protein observed in the control animals was shown to be induced in all treated groups (Fig. 4A). This 80-kDa protein is the full-length rat HSD IV because it comigrates with the full-length porcine HSD IV (data not shown). The livers from rats treated with GEM and, to a lesser extent, WY and DBP, also contained a 62-kDa immu-

[4] J. C. Corton, R. C. Cattley, and E. S. Moreno, unpublished observations.

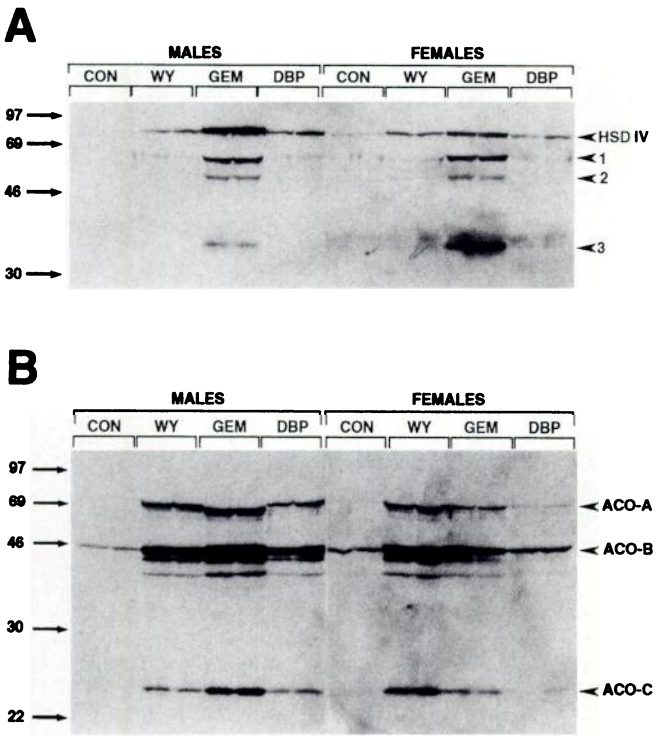

Fig. 4. Increased expression of HSD IV protein after PPC exposure. Whole-cell liver extracts from male and female F344 rats fed a control diet (*CON*) or a diet of WY (500 ppm), GEM (8,000 ppm), or DBP (20,000 ppm) for 13 weeks were separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against (A) HSD IV or (B) ACO. A, *Arrowhead HSD IV*, position of the full-length 80-kDa rat HSD IV protein; *arrowheads 1–3*, position of immunoreactive fragments of HSD IV with molecular masses of 62, 56, and 35 kDa, respectively. B, *Arrowheads*, position of ACO forms A–C, which have molecular masses of 72, 52, and 22 kDa, respectively; *numbers on the left*, position of molecular mass markers (kDa).

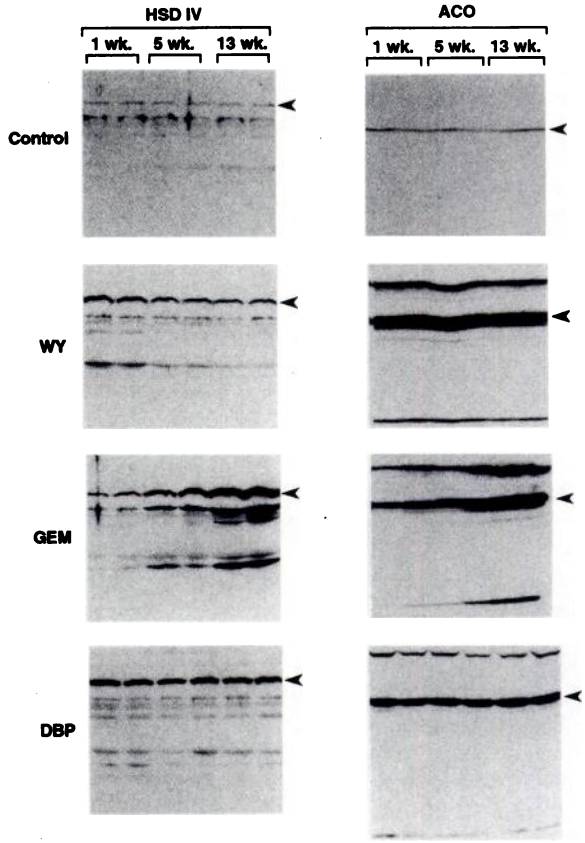

Fig. 5. Time course of induction of HSD IV and ACO proteins by diverse PPCs. Liver whole-cell extracts from male Sprague-Dawley rats fed a control diet (*CON*) or a diet containing WY (500 ppm), GEM (8,000 ppm), or DBP (20,000 ppm) for 1, 5, or 13 weeks were separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to HSD IV or ACO. *Left*, *arrows*, position of the full-length 80-kDa HSD IV protein. *Right*, *arrows*, position of the 52-kDa ACO-B protein.

noreactive protein. Additional immunoreactive proteins of 56 and 35 kDa were seen in the livers of GEM-treated rats only. In the GEM-treated samples, the 80- and 62-kDa forms of HSD IV were distorted in their migration because of the massive amount of the bifunctional protein (74 kDa) found in these induced samples. In male rats, GEM elicited by far the greatest increase in HSD IV protein; WY and DBP elicited approximately the same level of induction. Female rats had a somewhat different pattern of HSD IV induction to that seen in males (i.e., WY was a better inducer of HSD IV than DBP, although GEM was again the best inducer).

ACO is a useful protein to compare with HSD IV because much is known about its regulation by PPC. The purified, induced enzyme from rat liver consists of three polypeptide components: ACO-A (72 kDa), ACO-B (52 kDa), and ACO-C (22 kDa), respectively (40). Available evidence indicates that ACO-B and ACO-C are formed *in vivo* from ACO-A by post-translational proteolytic cleavage (40). As with HSD IV, ACO was induced to a greater extent by GEM compared with DBP in both males and females (Fig. 4B). In contrast to HSD IV, WY is a potent inducer of ACO, being almost equal to GEM in males and much stronger than GEM in females.

Both HSD IV and ACO levels were induced by GEM and DBP to higher levels in males than in females. This observation is consistent with a number of studies showing that PPCs are often better inducers of enzymes and peroxisome proliferation in males than in females (41 and references therein). In contrast, WY was a better inducer of HSD IV and ACO in females than in males, indicating that differences in responses between males and females can be variable.

To further explore differences in the ability of these PPCs to increase expression of HSD IV and ACO, we examined their pattern of expression at 1, 5, and 13 weeks after initiation of the feeding study using the same dietary concentrations of WY, GEM, and DBP as above. In this and the dose-response study discussed below, we used male Sprague-Dawley rats. No strain-specific differences between the Fisher and Sprague-Dawley rats were evident in response to the three PPCs (data not shown). Fig. 5 (*top*) demonstrates the lack of differences in the expressions of HSD IV and ACO over the time of the experiment in the control animals. The kinetics of the induction of HSD IV and ACO by GEM and DBP were very similar. GEM-induced levels of the two proteins were greatest at 13 weeks, even though significant induction was observed at 1 and 5 weeks. Differences in protein levels between 1 and 5 weeks but not between 5 and 13 weeks could be in part explained by differences in feed consumption.[5] DBP, on the other hand, elicited the greatest increase for both proteins at 1 week, with levels at 5 and 13

[5] P. J. Sausen and R. C. Cattley, unpublished observations.

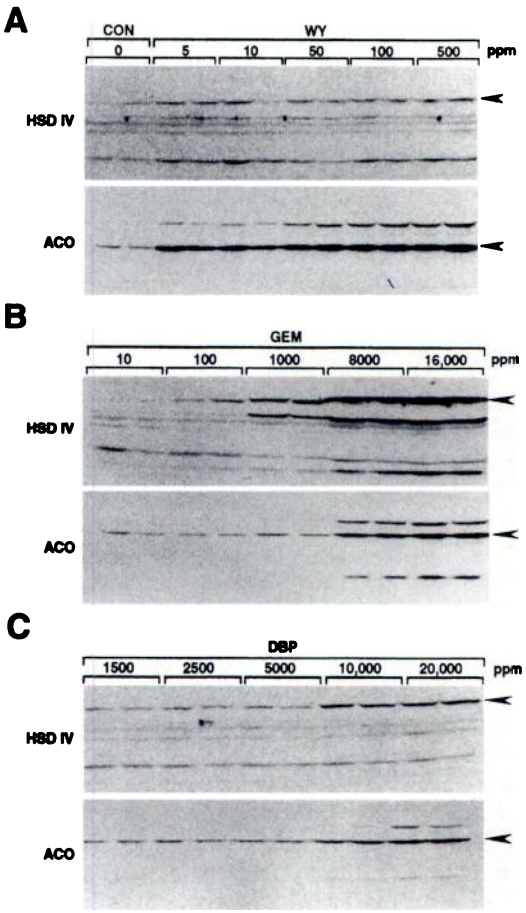

Fig. 6. Response of HSD IV protein levels to different dietary concentrations of PPCs. Liver whole-cell extracts from male Sprague-Dawley rats fed (A) a control diet or a diet containing 5 different concentrations of (A) WY, (B) GEM, or (C) DBP for 13 weeks were separated by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to HSD IV (*top*) or ACO (*bottom*). *Arrows*, positions of the 80-kDa full-length HSD IV (*top*) or the 52-kDa ACO-B protein (*bottom*).

weeks remaining essentially unchanged. Induction of both proteins by WY was also shown to be maximal at 1 week. Surprisingly, the levels of the full-length HSD IV and the 35-kDa fragment steadily decreased at 5 and 13 weeks of WY treatment, whereas ACO levels remained unchanged.

The responses of HSD IV and ACO to different dietary concentrations of WY, GEM, and DBP after 13 weeks of exposure were also assessed. Induction of both proteins was observed at 5 ppm WY, the lowest dose tested, but the levels of induction differed dramatically (Fig. 6A). The induction of ACO continued to increase with increasing dose of WY, becoming maximal at 100 or 500 ppm WY. In contrast, there was little if any increase in induction of HSD IV at doses of >5 ppm WY. GEM induction of HSD IV and ACO followed approximately the same dose response except that HSD IV induction was detected at a 10-fold-lower concentration (100 ppm) than the minimal concentration needed to induce ACO (Fig. 6B). Both proteins were induced to the greatest extent at the highest dose used in this study. Compared with WY and GEM, the rats were resistant to even high doses of DBP in the diet. HSD IV and ACO were minimally induced by 10,000 ppm DBP and induced to the greatest extent by 20,000 ppm (Fig. 6C). There is a good correlation between the levels of ACO protein induction observed in this study and induction of ACO enzyme activity by different doses of WY (38) and GEM (39) observed earlier.

Porcine HSD IV enzymatic activity has been purified as both an 80-kDa protein and a predominant 32-kDa, amino-terminal fragment, which may be the result of cleavage by a protease present in peroxisomes (42). This protease has been proposed to recognize and cleave near the sequence A(A/V)P (43), which is found in the porcine enzyme but is not conserved in the rat sequence. A putative protease site, AVP (aa 482–484) is found in the rat but not the porcine enzyme. Cleavage at this site in the rat protein would generate two fragments of ~52 and ~27 kDa encoding the dehydrogenase and SCP2 functions, respectively. Comparison of the proteolytic fragments of HSD IV produced in rat liver and porcine endometrium demonstrated the increased size of the rat liver 35-kDa fragment compared with the porcine 32-kDa fragment (data not shown). This indicates that the site of proteolysis in the porcine enzyme is not conserved in the rat protein and that the predicted protease site in the rat protein is not functional.

Discussion

In a screen for WY-responsive genes, we identified a member of the HSD family as a PPC-inducible gene. The putative sequence of the protein based on the cDNA sequence is 82% identical and 88% similar to the type IV originally isolated as a 17β-estradiol dehydrogenase from porcine uteri (42). Homology between the porcine and rat proteins is preserved in regions that have defined functions in the short-chain dehydrogenase family (44) to which all the HSD family members belong. The HSD family includes four isotypes that catalyze the interconversion of the active 17β-hydroxysteroids and the less active or inactive ketosteroids (for a review, see Ref. 45). The HSD types I and III are found primarily in the steroid-producing tissues, ovary and testis, respectively. They mainly catalyze the reduction reaction resulting in the production of active estrogens or androgens. The HSD types II and IV are found in peripheral tissues and act to oxidize and decrease the activity of androgens and estrogens. The importance of the HSD family of enzymes in maintaining proper levels of these hormones is highlighted by the discovery of inactivating mutations in the HSD type III gene that result in genetic males with female external genitalia (46).

The PPC-induced rat HSD IV mRNA and protein levels were shown to be regulated by two distinct mechanisms. The first mechanism is very similar to that which increases the levels of ACO. The HSD IV and ACO mRNAs accumulated in parallel in rat liver after 24-hr or 3-week treatments with GEM or WY and in a rat hepatoma cell line after treatment with WY or ciprofibrate. Western analysis of HSD IV and ACO protein levels demonstrated increased expression by GEM and DBP exposure that was very similar over time and at different doses. Because ACO is positively regulated by PPARα (11, 20), the predominant PPAR subtype in the liver, it is likely that HSD IV is activated by PPARα in a fashion similar to that of ACO and the other β-oxidation genes. Preliminary analysis of HSD IV expression in mice that lack a functional PPARα (20) demonstrates that inducibility but

not constitutive expression of HSD IV is dependent on PPARα in the kidney.[6]

HSD IV expression is also regulated by a second mechanism unique to WY exposure. Although HSD IV and ACO mRNA levels were coordinately induced by WY and GEM, HSD IV protein levels were induced only weakly by WY. Under the same conditions, ACO protein levels were induced to high levels. In contrast, GEM was a strong inducer of both HSD IV and ACO mRNA and protein levels. This unexpected minimal inducibility of the full-length HSD IV by WY was probably not due to increased proteolysis because a corresponding increase in the 35-kDa immunoreactive fragment or other fragments was not observed. Thus, in WY-treated animals, the HSD IV mRNA, although synthesized to high levels, was not efficiently translated. The WY-induced inhibition of translatability of the HSD IV mRNA was sensitive to low WY levels in the diet. The HSD IV protein was minimally induced at 5 ppm, and little induction of HSD IV levels was seen at higher doses. In contrast, the ACO protein levels were significantly induced at 5 ppm, and they continued to rise, becoming maximal at 100–500 ppm. In addition, the negative regulation of HSD IV expression increased over time; the WY-induced HSD IV and 35-kDa fragment levels were maximal at 1 week but steadily decreased over time. The phenomenon of depressed protein levels could be controlled at the level of mRNA translocation to the cytoplasm or subsequent translation of the mRNA. Many recent studies demonstrate that negative regulation of protein levels often occurs at the level of translation initiation (for a review, see Ref. 47). Determination of the molecular mechanism of this putative translational repression by WY may lead to the identification of additional WY-induced genes that are regulated by a similar mechanism.

HSD IV has a subcellular location that is unique among the HSD family members. The porcine HSD IV has been recently localized by immunogold electron microscopy to peroxisomes in pig endometrium and kidney (48). The rat HSD IV was also localized to liver peroxisomes, as shown by gradient centrifugation cosedimentation of HSD IV with ACO and catalase, known peroxisomal proteins (data not shown). A peroxisomal location of HSD IV was predicted by the peroxisomal signal sequence found at the carboxyl termini of both the pig and rat proteins. In contrast to HSD IV, HSD type I is found in the cytosol, and types II and III are found in microsomes (45). The unique location of HSD IV indicates that the protein performs functions distinct from those of the other members of the HSD family.

The rat HSD IV, like that of the porcine (32), human (49), and mouse (50) proteins, possesses a multidomain structure that is unique within the HSD family. Based on amino acid sequence homologies, HSD IV was predicted to encode at least two functions (33): a dehydrogenase activity within the amino-terminal 290 aa and a sterol carrier protein activity within the carboxyl-terminal 123 aa. The amino-terminal 290 aa of HSD IV exhibit significant homology to the dehydrogenase domains in *C. tropicalis* HDE (34) and the *S. cerevisiae* Fox2 (35) proteins, which are repeated twice in each protein. The first 289 aa of the rat HSD IV exhibit 51% identity and 65% similarity to the first dehydrogenase repeat (aa 1–287) and 44% identity and 58% similarity to the second repeat (aa 314–590) of the *C. tropicalis* HDE protein. The homologous regions include defined or putative functions in short-chain dehydrogenase (44). These include the cofactor binding site at rat HSD IV aa 15–22, encoding the sequence TGAGGGLG [compared with the consensus sequence TG(A/G)(A/G/S)XG(I/L)G] and the active site at aa 164–168 encoding the sequence YSAAK [compared with the consensus sequence YXA(S/A)K]. Homology between the rat HSD IV and the three other isoforms of HSD is almost wholly restricted to these regions (data not shown). This region of homology in the Fox2 protein has been shown to encode a D-3-hydroxyacyl-CoA dehydrogenase activity (35). The carboxyl-terminal aa 600–735 of HSD IV exhibit 39% identity and 55% similarity with the rat sterol carrier protein-2. SCP2 (also called nonspecific lipid transfer protein) is a highly conserved protein known for its stimulatory effects on various aspects of cholesterol metabolism; these include conversion of lanosterol into cholesterol, cholesterol esterification, bile acid formation, and steroid hormone synthesis (for a review, see Ref. 51). All amino acids found to be critical in human SCP2 activity (52) have been conserved in HSD IV, indicating that there is functional conservation between the proteins. The structure/function relationships within HSD IV have recently been unequivocally determined by expressing each domain of the porcine protein and assaying its function (53). The amino-terminal 323 aa are able to carry out the dehydrogenase reaction not only with steroids at the C17 position but also with 3-hydroxyacyl-CoA. The carboxyl-terminal 141 aa like SCP facilitate the transfer *in vitro* of 7-dehydrocholesterol and phosphatidylcholine between membranes. In addition, the central part of the protein catalyzes the 2-enoyl-acyl-CoA hydratase reaction. The HSD IV described here is identical to a recently purified dehydrogenase (54) that preferentially uses a bile acid precursor as a substrate (53). A role in bile acid synthesis for the rat HSD IV might explain why two apparently unrelated functions have been coupled in the same reading frame. The SCP2-like sequence found in the HSD IV carboxyl terminus may be required for movement of the bile acid precursor from the membrane to the HSD IV active site. In fact, SCP2 has been shown to have stimulatory effects on bile acid formation (55). The coordinate increase in the two functions by PPC may partially explain why there is an increase in bile acid formation *in vivo* after treatment with a PPC (56).

What would be the physiological consequences of overexpression of HSD IV? Porcine (42) and human (49) HSD IV principally catalyze the oxidation of the most-active estrogen estradiol to the less-active estrogen estrone. One prediction of continued exposure to PPCs in responsive rodent species, therefore, would be a decrease in circulating estradiol levels. Decreases in serum estradiol levels have not been seen in male rats; rather, under some but not all conditions of PPC exposure, the serum estradiol levels are increased and have been attributed to an increase in liver aromatase activity (57). In contrast, treatment of female rats with di(2-ethylhexyl) phthalate, a PPC similar in structure and activity to DBP, results in a dramatic drop of serum estradiol levels to 25–30% of normal levels (58). Suppression of estradiol levels secondarily results in increased serum follicle-stimulating hormone levels and an absence of luteinizing hormone surges necessary for ovulation. As a result, exposure to di(2-ethylhexyl) phthalate suppresses ovulation and results in polycys-

[6] J. C. Corton, unpublished observations.

tic ovaries in female rats (58). The decrease in serum estradiol levels could be due to lowered secretion of estradiol from preovulatory granulosa cells (59). Mouse granulosa cells have been shown to express the HSD IV protein (50), although the effect of PPC exposure on increased levels of HSD IV protein is not known in these cells. Alternatively, increased metabolism by induced levels of HSD IV in the liver or in other tissues could play an important role in modulating serum estradiol levels.

In summary, we identified the rat HSD IV as a peroxisomally located, PPC-inducible gene unique to the family of HSD. The overexpression of HSD IV by PPCs in liver and extrahepatic tissues could lead to altered intracellular and extracellular estrogen levels and consequently disrupt normal homeostasis of estrogen-dependent functions, a concept we are currently exploring.

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