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Cloning, Sequencing, and Functional Analysis of the 5′-Flanking Region of the Rat 3α-Hydroxysteroid/Dihydrodiol Dehydrogenase Gene[1]

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ABSTRACT

Rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase (3α-HSD/DD) is a member of the aldo-keto reductase gene superfamily. It displays high constitutive expression and inactivates circulating steroid hormones and suppresses the formation of polycyclic aromatic hydrocarbon *anti*- and *syn*-diol-epoxides (ultimate carcinogens). To elucidate mechanisms responsible for constitutive expression of the *3α-HSD/DD* gene a rat genomic library obtained from adult Sprague-Dawley female liver (*Hae*III partial digest) was screened, using a probe corresponding to the 5′-end of the cDNA (−15 to +250), and a 15.8-kb genomic clone was isolated. Sequencing revealed that 6.3 kb contained exon 1 (+16 to +138 bp) plus additional introns and exons. The transcription start site (+1) was located by primer extension analysis, and the initiation codon, ATG, was located at +55 bp. The remaining 9.5 kb represented the 5′-flanking region of the rat *3α-HSD/DD* gene. A 1.6-kb fragment of this region was sequenced. A TATTTAA sequence (TATA box) was found at 33 bp upstream from the major transcription start site. *cis*-acting elements responsible for the constitutive expression of the rat *3α-HSD/DD* gene were located on the 5′-flanking region by transient transfection of reporter-gene (chloramphenicol acetyl transferase, CAT) constructs into human hepatoma cells (HepG2). CAT assays identified the basal promoter between (−199 and +55 bp), the presence of a proximal enhancer (−498 to −199 bp) which stimulated CAT activity 6-fold, the existence of a powerful silencer (−755 to −498 bp), and a strong distal enhancer (−4.0 to −2.0 kb) which increased CAT activity by 20–40-fold. A computer search of available consensus sequences for *trans*-acting factors revealed that a cluster of *Oct*-sites were uniquely located in the silencer region. Using the negative response element (−797 to −498 bp) as a probe and nuclear extracts from HepG2 cells, three bands were identified by gel mobility shift assay, indicating the presence of protein binding sites in this proposed negative response element. All three bands were supershifted with anti-*Oct-1* mAb, suggesting that *Oct-1* may be the repressor. The 5′-flanking region also contained an AP-1 site, an estrogen response element, and a glucocorticoid response element, which together may comprise a steroid response unit. Although no sequence homology was found to exist between the 5′-flanking region of the rat *3α-HSD/DD* gene and its human orthologue the *DD2* gene, *trans*-acting factor consensus sequences comprising *Oct* sites and steroid response units were conserved. This implies that the expression of the two genes may be regulated by *POU*-domain transcription factors and steroid hormones, respectively.

INTRODUCTION

Rat liver 3α-HSD/DD[3] is a versatile oxidoreductase that: inactivates circulating steroid hormones (androgens, progestins and glucocorticoids) (1–3); oxidizes *trans*-dihydrodiols of PAHs and suppresses formation of the diol-epoxides (ultimate carcinogens; Refs. 4 and 5); and synthesizes and transports bile acids from the sinusoidal to the canicular pole of the hepatocyte (6–8). Its role in steroid and xenobiotic metabolism as well as vectoral transport of bile acids suggests that 3α-HSD/DD is essential for normal hepatic function.

Evidence exists that the oxidation of PAH-*trans*-dihydrodiols catalyzed by 3α-HSD/DD may lead to carcinogen activation. This reaction produces unstable catechols, which autooxidize to yield highly reactive PAH *o*-quinones (9). These *o*-quinones are cytotoxic to hepatoma cells (10), readily form DNA adducts *in vitro* (11), and are direct-acting mutagens in the Ames test (12). During the autooxidation of the intermediate catechol, ROS (superoxide anion, H_2O_2, and hydroxyl radical) and *o*-semiquinone radicals are produced (13). One- or two-electron reduction of the *o*-quinone will regenerate the catechol. By entering this futile redox-cycle, ROS and *o*-semiquinone radicals may be produced multiple times. This mechanism of free radical amplification may contribute to the observed cyto- and genotoxicity of the PAH *o*-quinones (10–12).

The cDNA for rat liver 3α-HSD/DD has been cloned, sequenced and overexpressed in *Escherichia coli* (14–17). The enzyme demonstrates high sequence similarity (>58%) with members of the aldo-keto reductase superfamily, which are monomeric oxidoreductases (M_r 34,000) with overlapping substrate specificity, and catalyze the reduction of carbonyls on a variety of endogenous substrates (steroids and prostaglandins) and xenobiotics. Members of this family include bovine lung prostaglandin F synthase; the rat, bovine, and human aldose reductases; human aldehyde reductase; and human chlordecone reductase. Structural similarity is retained at the three-dimensional level; thus, the X-ray crystal structures of human placental aldose reductase (18) and rat 3α-HSD/DD have been solved (19), and both proteins use the α_8/β_8-barrel or triose-phosphate isomerase barrel scaffold to perform their functions.

In the human liver, at least seven dihydrodiol dehydrogenase isoforms exist (DD1–DD7), of these, DD2 has been assigned as the major bile-acid binding protein and major dihydrodiol dehydrogenase (20). The cDNA for DD2 shows 70% sequence identity at the amino acid level with rat 3α-HSD/DD and is also a member of the aldo-keto reductase superfamily (21, 22). DD2 has since been shown to act as a 3α-HSD on C_{19} and C_{21} 3α-hydroxysteroids and appears to be the human orthologue of the rat liver enzyme (23). Recently, the entire human *DD2* gene has been cloned. It contains 9-exon-intron boundaries, and 851 bp of the 5′-flanking region was sequenced (24). The exon-intron boundaries were completely conserved with other members of the aldo-keto reductase superfamily, suggesting a common genomic organization within members of the family.

Understanding the mechanisms that regulate transcription of the rat *3α-HSD/DD* and human *DD2* genes may provide insight into the control of steroid hormone metabolism and PAH activation and may have relevance to the transcriptional control of other aldo-keto reductases. We and others have shown that in the rat liver, 3α-HSD/DD is hormonally regulated (25–28). The enzyme displays high constitutive

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[3] The abbreviations used are: 3α-HSD/DD, 3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50)/dihydrodiol dehydrogenase, *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase (EC 1.3.1.20); PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; NRE, negative response element; SRU, steroid response unit; CAT, chloramphenicol acetyl transferase; AP-1, activating protein 1; ERE, estrogen response element; GRE, glucocorticoid response element; PRE, progesterone response element; ARE, antioxidant response element; XRE, xenobiotic response element.

expression in male rat liver, comprising 0.5% of the soluble protein. In female rat liver, a 2-fold increase in expression is observed, and this female pattern of expression can be established by administering estrogens to male rats (25–27). Estrogens, dexamethasone, and thyroxine all increase steady-state levels of 3α-HSD/DD mRNA in rat liver (28).

To delineate the mechanisms involved in the regulation of constitutive expression of the rat *3α-HSD/DD* gene, we now describe the cloning, sequencing, and functional analysis of its 5′-flanking region. Powerful *cis*-acting elements that control constitutive expression of the gene have been identified. We provide evidence that *Oct-1* can bind to a NRE and that *Oct* transcription factors may act as repressors of this gene. Maps of the 5′-flanking regions of the rat *3α-HSD/DD* and human *DD2* genes indicate conservation of transcription factor binding sites, including those for *Oct-1* and those that may comprise a SRU. Our data suggests that both the rat and human genes may be regulated by *POU*-domain transcription factors and steroid hormones, respectively.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical Co. unless otherwise specified. Molecular biology reagents were acquired as indicated. Earle's MEM, fetal bovine serum, and antibiotics were purchased from Life Technologies, Inc. Restriction enzymes were purchased from Boehringer-Mannheim, Promega, or Stratagene. [α-^{32}P]dATP (3,000 Ci/mmol) and [^{14}C]chloramphenicol (50 mCi/mmol) were purchased from NEN-DuPont.

Preparation of Rat Liver Genomic DNA. Rat liver was removed, frozen, crushed, and lysed in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, and 200 μg/ml proteinase K (Boehringer-Mannheim) at 55°C overnight as described previously (29). The cell lysates were subjected to phenol/chloroform extraction and precipitated with 2.5 M ammonium acetate and ethanol. The DNA pellet was washed with 70% ethanol and resuspended in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA.

Genomic Cloning of Rat Liver 3α-HSD/DD. A female Sprague-Dawley rat genomic DNA library, *Hae*III partial digest in a Charon 4A vector (Clontech), was screened as described by the manufacturer. Briefly, approximately 3.0×10^4 plaque-forming units of phage were incubated with *Escherichia coli* host and plated onto each of 150-mm LB agar plates containing 10 mM $MgCl_2$. A total of 1.02×10^6 plaques were screened by transferring the phage DNA to replicate nitrocellulose membranes (Schleicher & Schuell), which were hybridized to a probe corresponding to −15 to +250 bp of the rat liver 3α-HSD/DD cDNA (14) labeled by random priming to a specific activity equal to or greater than 10^9 cpm/μg DNA. The positive clones were subjected to subsequent plaque purification using plate lysate procedures (30). The purified phage DNA was digested with *Eco*RI to release genomic fragments from the Charon 4A vector and subjected to agarose gel electrophoresis, followed by Southern analysis, using the original cDNA probe used in the library screen. Positive fragments were then subcloned into a pBluescript II ks- vector (Stratagene) for sequence analysis using the dideoxy chain termination method (Sequenase version 2.0; United States Biochemical Corp.).

Southern Blot Analysis. Rat liver genomic DNA (10 μg) or genomic DNA clones (2 μg of purified phage DNA) were digested with the appropriate restriction enzymes, and DNA fragments were separated by electrophoresis on a 0.8% agarose gel. Gels were denatured with 50 mM NaOH plus 150 mM NaCl for 30 min, neutralized with 0.1 M Tris-HCl (pH 7.0) plus 150 mM NaCl for another 30 min, and blotted onto a Nytran+ membrane (Schleicher & Schuell). The blots were UV cross-linked (Stratalinker; Stratagene) to immobilize the nucleic acids. The membranes were prehybridized in hybridization buffer (50% formamide, 10% dextran sulfate, 1 M NaCl, and 1% SDS) with 100 μg/ml sheared salmon sperm DNA at 42°C for 2 h, and the probe was then added (10^6 cpm/ml of hybridization buffer). Following hybridization for 16 h at 42°C, the blots were washed twice in 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) at room temperature for 10 min, followed by two high-temperature washes in 0.1× SSC and 1% SDS at 60°C and exposed to X-ray film at −80°C.

Generation of pCAT Constructs. A pCAT plasmid containing the 5′-flanking region of the rat 3α-HSD/DD gene was constructed by performing a double ligation into the multiple cloning site of the pCAT basic vector (pBLCAT3, enhancerless and promoterless; Ref. 50). The ligation reaction contained the pBLCAT3 vector linearized at its *Sal*I and *Bam*HI sites, a 6.2-kb genomic fragment (−6.2 kb to −321 bp) containing a 5′ *Xho*I site (compatible with a *Sal*I overhang) and a 3′ *Eco*RV site (blunt-end), plus a PCR fragment corresponding to −320 bp to +61 bp with respect to the transcription start site containing a 5′ *Eco*RV site and a 3′ *Bam*HI site. The 6.2-kb genomic fragment was derived from the genomic clone by *Pvu*II digestion, subcloning into pBluescript II ks- vector, excision from the vector with *Xho*I and *Eco*RV, followed by purification from agarose gel electrophoresis. The PCR fragment was generated using a 5′ primer corresponding to −797 to −781 with the sequence of 5′-ACAGGACCACACCTTCT-3′ and a 3′ primer complementary to +49 to +73 with the sequences of 5′-GCAGAGATATGGGATC-CATCGCTTG-3′ with an A to G point mutation at +61 (underlined) to generate a *Bam*HI site. The PCR reaction was performed in 100 μl consisting of 50 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 1 mM $MgCl_2$, 2 μg DNA template, 500 nM of PCR primers, 200 mM each deoxynucleotide triphosphate, and 1 unit of Vent DNA polymerase (New England Biolabs) with denaturing at 95°C for 2 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min. The PCR product was directly cloned into pCR-Script sk+ (Stratagene) and sequenced to verify its fidelity. The construct was then digested with *Eco*RV and *Bam*HI to release a 381-bp fragment (−320 bp to +61 bp), which was then purified from an agarose gel. The linearized pBLCAT3 basic vector was dephosphorylated using calf intestine alkaline phosphatase (Boehringer-Mannheim). The two DNA fragments were ligated into the vector using T4 DNA ligase and ATP to incorporate a total of 6.6-kb of the 5′-flanking region of the rat 3α-HSD/DD gene upstream of the CAT reporter gene.

Nested deletion of the pCAT construct was performed by initial digestion with exonuclease III, followed by S1 nuclease digestion and recircularization (Promega protocols). Briefly, the pCAT construct containing 6.6-kb upstream of the rat 3α-HSD/DD gene was first linearized with *Pst*I and *Sph*I and resuspended in 66 mM Tris-HCl (pH 8.0) and 660 mM $MgCl_2$. The deletion reaction was started by the addition of 200 units of exonuclease III (Promega) and incubated at 37°C. Aliquots of the reaction were removed sequentially at 20-s intervals into tubes containing S1 nuclease mix (Promega) and incubated at room temperature for another 30 min. The reaction was terminated by the addition of 300 mM Tris base and 50 mM EDTA, and the S1 nuclease was inactivated by incubating at 70°C for 10 min. The deletion mutants were recircularized by the addition of the Klenow fragment of DNA polymerase I (Promega), a deoxynucleotide triphosphate mix, and T4 DNA ligase. The deletions were sequenced to verify their selection for transient transfection.

Cell Culture, Transient Transfection, and Reporter Gene Assay. The human hepatoma cell line, HepG2 (ATCCHB8065), was maintained in continuous culture in Earle's MEM supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 100 units/ml penicillin, and 10 μg/ml streptomycin (growth medium) at 37°C in a humidified atmosphere with 5% CO_2. Cells were passaged every 7 days with 1:4 dilution.

All reporter gene plasmids were purified prior to transfection by centrifugation through cesium chloride gradients containing ethidium bromide. HepG2 cells were seeded at the concentration of 1.5×10^6 cells/60-mm tissue culture plate for 24 h. Four h prior to transfection, the medium was removed and replaced with a fresh growth medium. For each pCAT construct, DNA-$CaCl_2$ solutions were prepared by mixing 37 μl 2 M $CaCl_2$, 10 μg of pCAT constructs or CAT control plasmids [pBLCAT3 basic (promoter and enhancerless) and pSV-CAT (SV40 promoter); Promega], plus 2 μg of pSV-β-galactosidase containing plasmid (Promega) in a final volume of 300 μl. DNA was precipitated by mixing with an equal volume of 2× HEPES-buffered saline [50 mM HEPES (pH 7.1), 280 mM NaCl, and 1.5 mM Na_2HPO_4; Ref. 31)] with constant agitation. The precipitants were incubated at room temperature for 30 min before their addition to the cell culture medium. After 48 h, the cells were washed twice with PBS (pH 7.1) and harvested using lysis buffer (Promega) for CAT and β-galactosidase enzyme assays.

For each β-galactosidase assay, a cell lysate (100 μl) was incubated in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, and 50 mM β-mercaptoethanol, containing 0.67 mg/ml *o*-nitrophenyl-β-D-galactopyranoside as a substrate in a total volume of 300 μl at 37°C for 2 h. The reaction was terminated by adding

500 μl of 1 M sodium carbonate, and the absorbance of the *o*-nitrophenol anion was read at 420 nm. This end point assay was validated by showing that the absorbance was in the linear range with respect to time and lysate protein. For CAT activity, the volumes of the cell lysates were adjusted to contain the same amount of β-galactosidase activity to normalize for transfection efficiency. Lysates (50–100 μl) were then incubated in a final volume of 125 μl containing 25 mM Tris-HCl (pH 7.5), 40 μM [^{14}C]chloramphenicol (1.0 μCi), and 50 μM acetyl-CoA. The reactions were initiated by the addition of coenzyme and incubated for 8 h at 37°C. The reactions were terminated by extraction with 500 μl ethyl-acetate, the extracts were dried, resuspended in 20 μl ethyl-acetate, and applied to thin layer chromatography plates (Silica Gel IB2; Baker), which were developed in $CHCl_3$/MetOH, 97:3 (v/v). Following autoradiography, the results were quantitated by densitometry of the acetylated and nonacetylated forms of [^{14}C]chloramphenicol using a video densitometer and the UNISCAN software (Analtech).

Preparation of Nuclear Extracts. Nuclear extracts were prepared by the method of Navankasattusas *et al.* (32). Briefly, 6×10^7 HepG2 cells were washed twice with PBS and resuspended in 400 μl of hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM, and 1% Trasylol] containing 0.05% NP40 and disrupted with a Dounce homogenizer. The nuclei were pelleted by centrifugation at 250 × *g* for 10 min at 4°C. The pellet was resuspended in 5 mM HEPES (pH 7.9), 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 300 mM NaCl. Nuclear extracts were clarified by centrifugation at 24,000 × *g* for 20 min at 4°C, aliquoted, and stored at −70°C.

Preparation of Radiolabeled NRE. A DNA fragment corresponding to −797 to −498 bp of the 5′-flanking region of the 3α-HSD/DD gene was PCR amplified using the 5′ primer (−797 bp to −781 bp) and a 3′ primer complementary to −515 to −498 with the sequence of 5′-GCTTGAACTATT-TGCCTT-3′ under the conditions described above. The amplified fragments were subcloned into pCRII vector (Invitrogen), excised from the vector using *Eco*RI, and purified by electrophoresis. The probe was ^{32}P-labeled using the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP and 40 μM dTTP in a final volume of 25 μl. The unincorporated nucleotides were removed by chromatography on Sephadex G-50.

Gel Mobility Shift Assay. Nuclear extracts (10 μg) were incubated with 2 fmol of 300 bp of radiolabeled NRE (50,000 cpm) for 15 min at room temperature in the presence of 0.5 μg sheared salmon sperm DNA and 5 μg poly(dI-dC) (Pharmacia) in a binding reaction of 20 μl containing 40 mM KCl, 25 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, and 5% glycerol. The mixtures were electrophoresed on a 4% polyacrylamide gel in 0.5× TBE buffer, and the gel was dried and subjected to autoradiography. For the competition studies, a 1- to 50-fold molar excess of unlabeled NRE was used. For the supershift assays, 0.5 μl of anti-*Oct-1* mAb (kindly provided by Dr. Winship Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was added to the binding reactions and incubated for another 20 min at room temperature prior to electrophoresis.

Computer Analysis of the 5′-Flanking Region of the Rat *3α-HSD/DD* Gene. The 1.6-kb sequence of the 5′-flanking region of the rat *3α-HSD/DD* gene (positive and negative strands) was used to search the entire GENBANK using FASTA. The same sequence was also used to search the transcription factor database (33, 34) for the presence of transcription factor consensus sequences. In this search, either no mismatches were allowed on either strand, or a default of 15% mismatch was allowed. An identical search was performed against the 850 bp that were sequenced on the 5′-flanking region of the human *DD2* gene (24).

RESULTS

Genomic Southern Analysis and Cloning of the 5′-Flanking Region of the Rat *3α-HSD/DD* Gene. Previously, Southern analysis of rat genomic DNA using either the 5′ region (−129 to +250 bp) or the 3′ region (+854 to +end) of rat liver 3α-HSD/DD cDNA as probes gave simple banding patterns. By contrast, a cDNA probe for the open reading frame (+334 to +511 bp) gave a complex pattern. These results suggested that there was a single copy of the 3α-HSD/DD gene in the genome and that this gene was a member of a multigene family (27). The sequence of the cDNA indicated that this enzyme belongs to the aldo-keto reductase gene superfamily (14–16). When rat genomic DNA was digested with a variety of restriction enzymes and hybridized to a truncated 5′-region of the cDNA (−15 to +250 bp), several bands were detected (Fig. 1*A*). Since this segment of the cDNA does not contain restriction sites for the endonucleases used, it was suspected that the probe detected fragments that contained more than exon 1 of the gene. It was found that *Eco*RI digestion of genomic DNA gave a 4.3- and a 3.0-kb band, which hybridized to the cDNA probe (−15 to +250 bp), and this banding pattern was used to identify positive clones obtained in the screening of the genomic library.

A total of 1.02×10^6 plaques from a rat genomic library were screened using the 5′-end of the cDNA for rat liver 3α-HSD/DD (−15 to +250 bp). Following a secondary screen, 34 positive clones were identified, and these gave 6 different banding patterns following *Eco*RI digestion. One 15.8-kb genomic clone gave four bands of 6.5, 4.3, 3.0, and 2.0 kb in size after *Eco*RI digestion (Fig. 1*C*). Southern analysis showed that two of these bands, corresponding to 4.3 and 3.0 kb, hybridized to the same probe used in the genomic Southern analysis (Fig. 1*B*). Both of these fragments were subcloned into the *Eco*RI site of pBluescriptII ks- (Stratagene) and subjected to dideoxy sequencing. The fidelity of the 15.8-kb genomic clone was established since the 4.3-kb *Eco*RI fragment contained the first 84 bp of the open reading frame of the cDNA for rat liver 3α-HSD/DD. To obtain sequence further 5′-upstream of the gene, the 15.8-kb genomic clone was digested with *Pvu*II, and two fragments of 8.0 and 2.0 kb in length were found to hybridize to the 5′-end of the cDNA (−15 to +250 bp). Additionally, the 8.0-kb fragment was found to hybridize to a probe prepared by PCR amplification of a portion of the gene corresponding to −704 to −196 bp. These data confirmed that the 8.0-kb fragment contained the 5′-flanking region of the rat *3α-HSD/DD* gene. The 8.0-kb fragment was subcloned into the pBluescriptII ks- vector and then subjected to *Xho*I and *Eco*RV digestion to release a fragment of 6.2 kb, which resided 5′-upstream of the *Eco*RV site found at −321 bp relative to the transcription start site. This 6.2-kb fragment was used to generate pCAT reporter gene constructs (see "Materials and Methods").

Organization of the 5′-Flanking Region of the Rat *3α-HSD/DD* Gene. The four *Eco*RI fragments of the 15.8-kb genomic clone and the *Pvu*II fragments were subcloned into pBluescriptII ks- for sequencing. By using oligomers that contained sequence from the *Eco*RI fragments to prime DNA sequencing from the *Pvu*II fragments, it was found that the *Eco*RI fragments were arranged in the following order (5′ to 3′): 6.5, 2.0, 4.3, and 3.0 kb in the rat *3α-HSD/DD* gene (Fig. 2). The 4.3-kb fragment contained 1060 bp upstream of the ATG translation start codon, and primer extension analysis revealed a major transcription start site at 55 bp (+1) upstream of the start codon (27). A sequence corresponding to TATTTAA (TATA box) was located at −33 bp (88 bp upstream from the translation start site) and presumably binds RNA polymerase II. No CAAT box was evident. Based upon alignment with a full-length cDNA clone, this 4.3-kb fragment also contained the entire first exon +16 bp to +138 bp, followed by at least the first intron. Since the sequence at the 3′-end of this fragment gave no open reading frame, it ends on an intron. The 3.0-kb *Eco*RI fragment of the genomic clone, which hybridized to the 5′-end of the cDNA (−15 to +250 bp) also gave no open reading frame on either its 5′- or 3′-ends, indicating that it contained at least one exon sandwiched by intron sequences. Lou *et al.* (24) has shown that intron-exon junctions are conserved in the three members of the aldo-keto reductase gene superfamily whose gene structure is known, *i.e.*, human hepatic *DD2*, human placental aldose reductase, and mouse major vas deferens protein genes. This conservation predicts

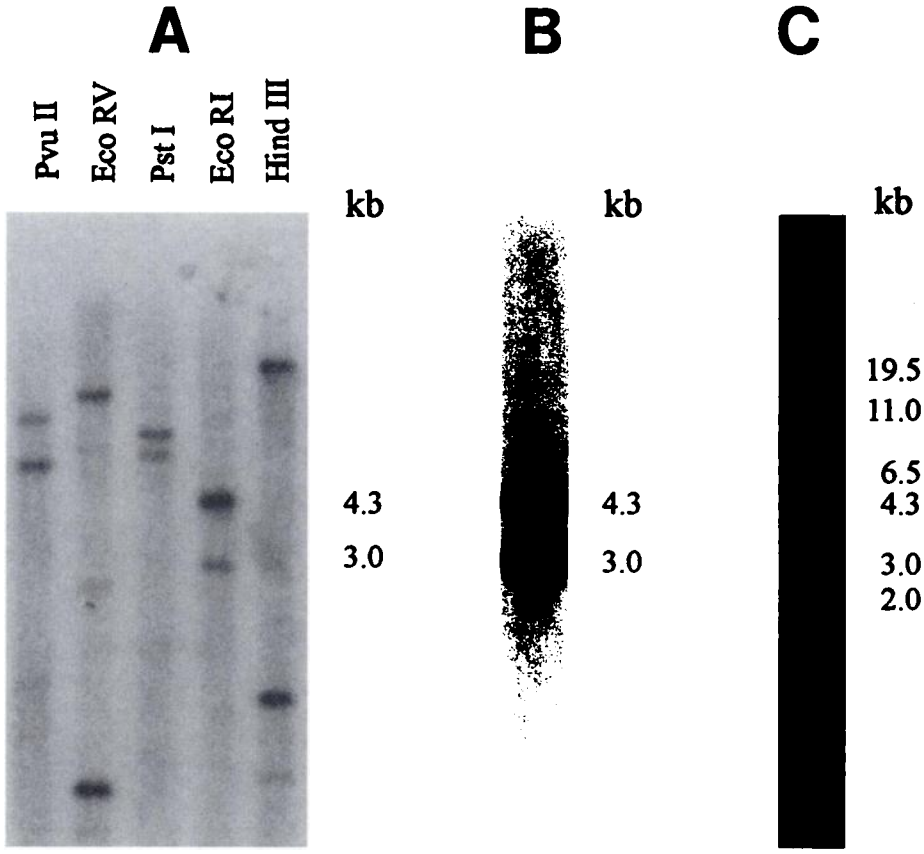

Fig. 1. Southern blot analysis of rat liver genomic DNA and the 15.8-kb genomic clone. *A*, a total of 10 μg genomic DNA purified from female rat liver was digested with the restriction enzymes indicated; fragments were separated by agarose gel-electrophoresis and subsequently hybridized to a ^{32}P-labeled probe derived from the 5′-end of the 3α-HSD/DD cDNA (−15 to +250 bp). *B*, the phage DNA containing the genomic sequence of 3α-HSD/DD was digested with *Eco*RI to separate the genomic insert from the Charon 4A vector, and fragments were separated by gel electrophoresis and hybridized as before. *C*, ethidium bromide staining of fragments released by *Eco*RI digestion of the genomic clone giving four bands of 6.5, 4.3, 3.0, and 2.0 kb in size. Two bands of the same size (4.3 and 3.0 kb) appeared in the Southern analyses of both the genomic DNA (*A*) and the phage DNA clone (*B*) following *Eco*RI digestion.

that exon 1 resides between +16 to +138 bp and that exon 2 resides between +139 and +306 bp.

Sequence Analysis of the 5′-Flanking Region of the Rat *3α-HSD/DD* Gene. A total of 1647 bp upstream of the ATG translation start site has been sequenced (Fig. 3). To obtain insight into the identity of transcription factors that may control constitutive and regulated expression of the rat *3α-HSD/DD* gene, a computer search of the transcription factor data base (33, 34) was performed in which either no mismatches were allowed or 15% mismatches were allowed on either strand. With no mismatches, the search revealed the presence of intact consensus sequences for AP-1, AP-2, C/EBP, and multiple *Oct* transcription factors (including *Oct-1, Oct-2, Oct-3, Oct-5, Oct-6, Oct-7, Oct-8, Oct-9,* and *Oct-10*). Incomplete steroid-hormone response elements, such as an ERE and a GRE were also present (Fig. 3). If a 15% mismatch was allowed in the search for consensus sequences, there were two partial EREs, three partial GREs, six partial PREs, and two imperfect AP-1 sites scattered in the 1.6-kb upstream sequence. The presence of a cluster of putative steroid response elements and AP-1 sites suggests that these may comprise a SRU (35). Because 3α-HSD/DD plays an important role in the metabolism of PAH, the lack of a XRE was notable. This *cis*-element is activated upon binding the liganded aryl hydrocarbon receptor and is located on the 5′-flanking region of the rat *CYP1A1* gene, which is involved in PAH activation (36, 37).

The 5′-flanking region of the rat *3α-HSD/DD* gene was compared with the 5′-flanking region of the human *DD2* gene, its human orthologue (24). Although there was no homology between the two sequences, there was a remarkable conservation of *cis* elements (Fig. 4). The 5′-flanking regions of both genes contained AP-1 sites, AP-2 sites, EREs, GREs, PREs, and *Oct* clusters. The presence of these common transcription factor consensus sequences suggests that the expression of these two genes may be regulated in a similar manner

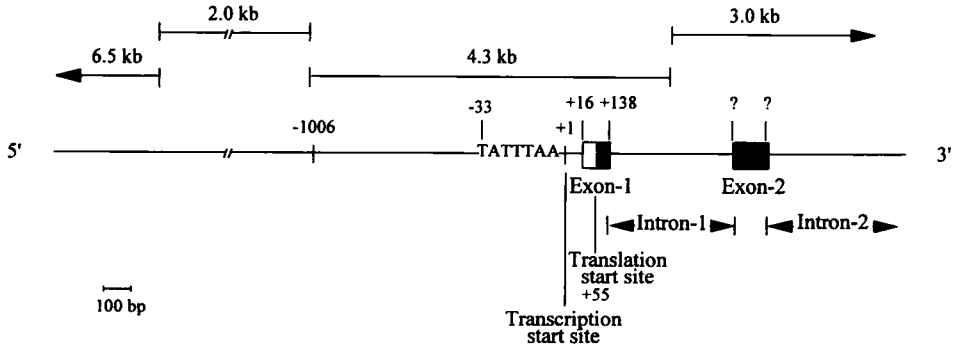

Fig. 2. Organization of the *Eco*RI fragments obtained from the 15.8-kb genomic clone within the rat *3α-HSD/DD* gene. The four *Eco*RI fragments present in the 15.8 kb genomic clone are arranged in the following order (5′ to 3′): 6.5, 2.0, 4.3, and 3.0 kb in the rat *3α-HSD/DD* gene. Restriction mapping and sequence analysis revealed that the 4.3-kb fragment contains 1060 bp upstream of the ATG initiation start codon at +55 bp, exon 1 (+16 to +138 bp), and at least intron 1. The 3.0 kb codes for additional exons and introns downstream of the 4.3-kb fragment. The 2.0-kb fragment is adjacent to the 5′-end of the 4.3-kb fragment; and the largest fragment, 6.5 kb, corresponds to DNA sequence further upstream of the 5′-flanking region of the rat *3α-HSD/DD* gene. A putative TATA box with the sequence of TATTTAA is located at −33. The position of the major transcription start site was located by primer extension analysis and is indicated.

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5'TATATGGAAT CATTACCAGT TGTGGGTGGG AAGCATAAAT ATCTAGAAAA TTNAAGTCTG -1534
                    AP-1                      C/EBP
NAAAATCTAT AATTGAAGTA ATAATTCAGC CAACCATGAA TGGAAAAGTA AACAGCTATA -1474

GAATGTCCAA AAGAAATATA AAGTTATTCA CTGTTTAAAG CAATCAAGAA AATGCTAATC -1414
                                            C/EBP
AAAGCTGTCC TATACCAATC TGATGTCTGA CATTAAGAAA AATATGAACC AATAAATGCT -1354
GRE

GATGAGNAAG GGAGATGGTG GGGGCCTTCT ACATGGCTGG TAAAAAGGTA ATTATTTTAG -1294

CCATTATGGA CATCAGCATA AATTTAGCTT CAGAAGCTAA AAACAGAATT TCCTTTTGGT -1234

GCAGATATTC TGCTCCTTCA TATAAATGTG AAATAATCAA AGTCAGTATA ATAAAAGTAT -1174
                                  ERE
CCATGTGTCT TAGGGTTTCA TGGCTATGTA TAGACACCAT GACCAAGGCA AGCTTTATAA -1114
                AP-2
AAACAACATT TAATTGGGGC TGGCTTACAG GTTCAGAGGT TCAGTCTAGT ATCAACAAGG -1054

CAGGAACATG GTAGCATCCA GGCAGGCATG GTTCAGGAGG AGCTGAGCTA CATCTTCATC -994

TGAAGGCTGC TAGCAGAATA CTGACTTCCA GGCAGCTAGG TTGAGGGTCT TTTTTTTTTT -934

TTTTTTTTTT GGTTCTTTTT TTCGGAGCTG GGGACCGAAC CCAGGGCCTT GCGCTTCCTA -874

GGTAAGCGCT CTACCACTGA GCTAAATCCT CAGCCCCGGT TGAGGGTCTT AAAGCCACAG -814

TGACACACTT ACTCAAACAG GACCACACCT TCTAATAGTG CCATTCCTTG GAGCAAGCAT -754
Oct-1
ATGCAAATCA TCACAGTATA CATCCACTCT CTTTAAAGTG CTTTTTTATT TTTTATTTTT -694
Oct-1
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ACAATGTCCA GGATGTGGAA TCAGCCCAGG TGCCAATTGA TGGATACATG GATAAAGCCA -634

AGTGTGGTAC CTATTTAAGA TAGAGTTTTA TTTGGGAGAG ACAAATAATA TCATTTGCAC -574

CAAAATGGAT GGAGCTTTGT GAAAATCATC TTGTTAAACA TAAAACCTCC AACTCAGAAA -514

GGCAAATAGT TCAAGCATCC CTACAGATGG TGTCTAGTCT TTGAAAAAGA CATGAAAGGA -454

GAAGGTAGAC AATTTAGGGG GGCAGAGAGG GAACAGAAGG AGCAGTGGGA GAGGTAAGGG -394

TGATCAAGAA ACATTGTGTC TGTATGGAAA TGTTACAATG AGACTCTTAC TGTTTAGAAA -334
          C/EBP
GTAAAGAAGA GATATCCTTT TCAAATGAGA AACCAGAGTC CAGTAGCTGA AGTTACGTTT -274
                                  ERE
TAGGTAACGT ATAATTGCTT ACCATCGGTC AGTCTCCCCT TAACAATTTA CAGAAGTAAA -214

TCTGGATTGT GAACAGAACC TTTCCTTCTA CAAGAAATTC CTGGTTTCAT TTTCATGTTT -154
C/EBP
CATTAATTTA TTGGACACTC GTGCACACAC ACATGCAAGC AAGCCTCTAG TTTTCAGTCT -94
                                                    C/EBP
CTGTGGACTA TTGCTTTAAG CTGACTCCTC CCACATGCCA TTGTTTAACT TTCCAGGAGA -34
                                  +1
TATTTAAGGA GCTGTAACAA TTTCGCTGGC TTTTATCCTG TGCAAAAAGA AGACCCAAGA +27

CCTGCTTGGT TGCATGAGTA ACAAGCG ATG GAT TCC ATA TCT CTG CGT GTA GCA +81
                                  Met Asp Ser Ile Ser Leu Arg Val Ala
CTA AAT GAT GGT AAC TTC ATT CCT GTA CTG GGG TTT GGA ACC ACT GTG +129
Leu Asn Asp Gly Asn Phe Ile Pro Val Leu Gly Phe Gly Thr Thr Val

CCT GAG AAG 3'
Pro Glu Lys
```

Fig. 3. Nucleotide sequence and identification of putative *cis*-acting elements in the 5′-flanking region of the rat *3α-HSD/DD* gene. A total of 1647 bp upstream of the translation start site of the rat *3α-HSD/DD* gene was sequenced. The major transcription start site (+1) is located 55 bp upstream of the ATG initiation codon. A putative TATA box, with the sequence of TATTTAA at −33 bp, and exon 1 (+16 to +138 bp) are *underlined*. *cis*-acting elements containing consensus sequences for AP-1, AP-2, *Oct* transcription factors, and steroid hormone response elements (ERE and GRE) are represented by *arrows*, indicating whether the sequence is found on the positive or complementary strand.

and, in particular, both may be regulated by steroid hormones via a SRU.

When the 1.6 kb of the 5′-flanking region of the rat *3α-HSD/DD* gene was used in a homology search against the genomic database, two stretches of sequence were identified that had high sequence identity with noncoding regions of seven rodent genes. The first, stretch which resides between −1161 and −942 bp showed 85% identity with a 244-nucleotide overlap from intron 3 of the rat lysozyme gene, 82% identity with a 233-nucleotide overlap from intron 1 of the mouse IgE-binding lectin gene, and 78% sequence identity with a 238-nucleotide overlap from the 5′-flanking region of the rat metallothionein-1 pseudogene b. The second stretch, which resides

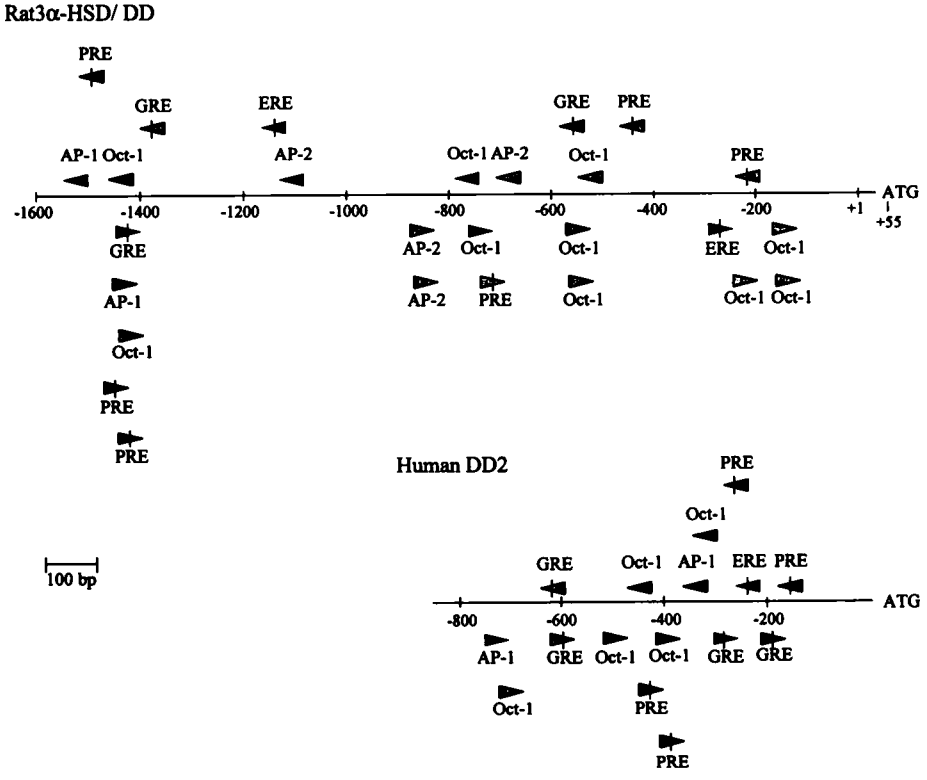

Fig. 4. Comparison of *cis*-acting elements on the 5′-flanking regions of the rat *3α-HSD/DD* and the human *DD2* genes. The 5′-flanking regions of both genes share several common *cis*-acting elements including AP-1, C/EBP, ERE, and GRE. If 15% mismatches were allowed for comparison of transcription factor consensus sequences, *Oct* transcription elements, a PRE, and SP-1 were also present in the 5′ upstream of the two genes. The *direction* of the *arrow* indicates whether the consensus sequence is on the positive or complementary strand. *Filled arrows*, a perfect match with a consensus sequence; *shaded arrows*, a 15% mismatch with a consensus sequence; ⇥, a perfect match with a half-palindromic sequence; ⇥, a 15% mismatch with a half-palindromic sequence.

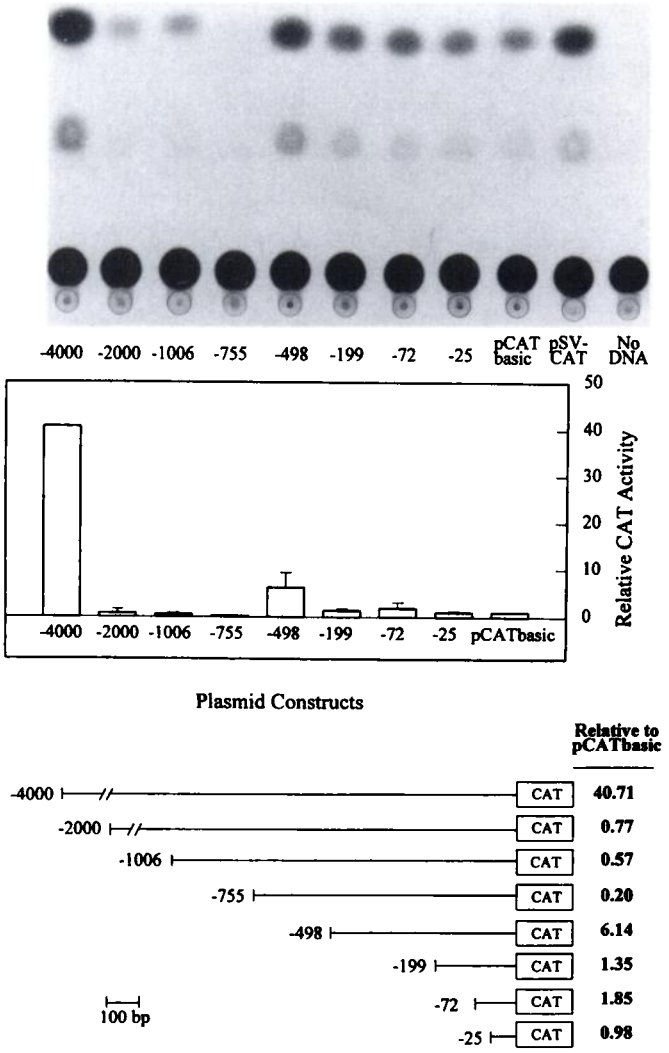

Fig. 5. pCAT reporter gene constructs containing the 5′-flanking region of the rat *3α-HSD/DD* gene and analysis of CAT expression following transient transfection into HepG2 cells. A stretch of 6.6 kb immediately upstream of the rat *3α-HSD/DD* gene and a series of 5′ nested deletions of this region were fused to a CAT reporter gene (*bottom panel*) as described in "Materials and Methods." The CAT constructs were transfected into a human hepablastoma cell line, HepG2, and CAT activity was determined by the incorporation of the 1,3-acetyl groups into [^{14}C]chloramphenicol. Mono- and diacetylated chloramphenicol were separated by thin layer chromatography followed by autoradiography (*top panel*). The CAT activity for different constructs is reported relative to that observed with the pCAT-basic vector (1.0) from three separate experiments; bars, *SE* (middle panel). All values were normalized for the size of the CAT constructs and for β-galactosidase activity cotransfected as pSV-β-galactosidase.

between −968 and −835 bp shared 85% sequence identity with a 134-nucleotide overlap from the 3′-flanking region of the rat *CYPIID4* gene, 98% sequence identity with a 101-nucleotide overlap from intron 1 of the rat troponin T cardiac isoform gene, 99% sequence identity with a 109-nucleotide overlap from intron 6 of the rat ATP-citrate lyase gene, and a 100% sequence identity with a 44-nucleotide overlap with a intergenic sequence from the rat γ-crystallin gene cluster. To date, no function has been assigned to these highly conserved noncoding sequences.

Functional Analysis and Identification of *cis*-Acting Elements That Control Constitutive Expression of the Rat *3α-HSD/DD* Gene Using CAT Reporter Gene Constructs. To locate functional *cis*-acting elements that regulate constitutive expression of the rat *3α-HSD/DD* gene, 6.6 kb of the 5′-flanking region (−6.6 kb to +61 bp, where +55 is the initiation codon) was inserted into a pCAT basic vector (minus promoter and enhancer, pBLCAT3), and a series of nested deletions on the 5′-flanking region were transiently transfected into the human hepatoma cell line (HepG2). As a control, HepG2 cells were also transfected with the pCAT basic vector (promoter and enhancerless) and the pSV-CAT vector (SV40 promoter). Heterologous transfection into HepG2 cells was chosen since pCAT constructs have been used successfully in these cells to locate *cis*-acting elements in the Ya subunit of the rat glutathione-*S*-transferase gene (38) and the rat *CYP1A1* gene (39). Measurement of CAT activity in transfected cells indicated that the basal promoter was located between −199 and +55 bp since enzyme activity was elevated 1.35 to 1.85 times over the pCAT basic vector (Fig. 5). CAT activity was elevated 6-fold above basal levels when the pCAT construct contained −498 to +55 bp of the upstream sequence, and the CAT activity was similar to that observed with the positive control, the pSV-CAT vector. By contrast,

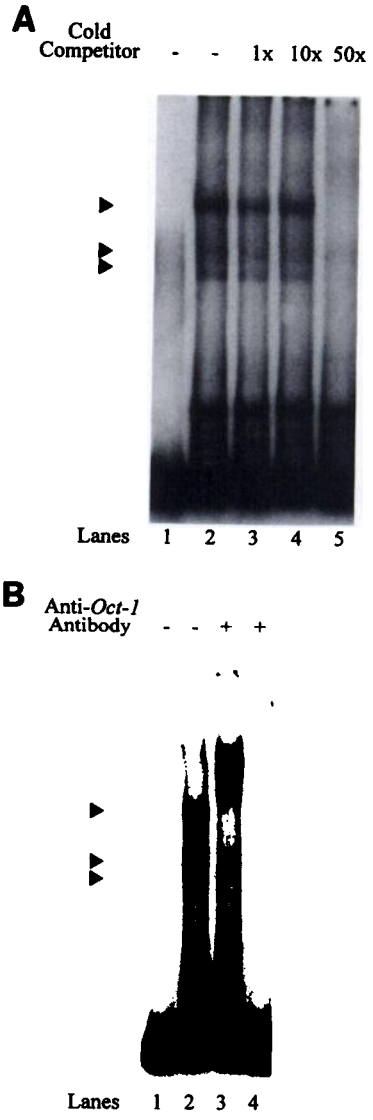

Fig. 6. Gel mobility shift assay to measure the binding of HepG2 cell nuclear extract to the NRE. A double-stranded DNA probe corresponding to −797 to −498 bp of the 5′-flanking region of the *3α-HSD/DD* gene was PCR amplified and end-labeled using Klenow fragment. The binding reaction was performed at room temperature in the presence of 2 fmol radiolabeled probe and 10 μg HepG2 nuclear extract. Some reactions contained a molar excess of unlabeled probe as competitor. All reactions were analyzed by PAGE. *A: Lane 1*, radiolabeled DNA probe in the absence of nuclear extract; *Lanes 2–5*, radiolabeled DNA probe in the presence of nuclear extract plus the molar excess (0–50-fold) of competitor DNA as indicated. *B*, supershift was achieved by adding anti-*Oct-1* mAb to the binding reaction. *Lane 1*, radiolabeled probe; *Lane 2*, radiolabeled probe plus HepG2 nuclear extract; *Lane 3*, radiolabeled probe plus HepG2 nuclear extract plus anti-*Oct-1* mAb; *Lane 4*, radiolabeled probe plus anti-*Oct-1* mAb alone.

when sequence further 5′ upstream (−755 to −498) was included in the construct, CAT activity decreased to 20% of the level observed with the basal promoter, indicating the presence of a powerful silencer or NRE. CAT activities increased gradually with the inclusion of sequence further 5′-upstream (−755 bp to −2 kb). A very strong enhancer element was located between −2.0 to −4.0 kb since inclusion of this sequence in the CAT reporter gene constructs resulted in a 20–40-fold increase in CAT activity over that observed with the basal promoter.

Characterization of Nuclear Factors That Bind to the NRE of the Rat *3α-HSD/DD* Gene. To characterize nuclear factors that bind to the NRE and as a result repress CAT reporter gene activity, gel mobility shift assays were performed. HepG2 nuclear extracts were used to bind to the radiolabeled double-strand NRE (−797 to −498 bp). Three DNA-protein complexes with retarded electrophoretic mobility were identified. Formation of these complexes was effectively competed out by the addition of a 50-fold molar excess of the unlabeled NRE (Fig. 6*A*, *Lane 5*). Analysis of the NRE for consensus sequences for *trans*-acting factors indicated that two *Oct* sites were uniquely located in this region of the gene. To determine whether *Oct* transcription factors were responsible for the formation of the NRE-protein complexes, anti-*Oct-1* mAb was included in the binding reactions, and gel mobility shift assays were repeated. In the presence of anti-*Oct-1* mAb, the three bands were supershifted, indicating that the *Oct-1* antibody associates with the NRE-protein complexes (Fig. 6*B*, *Lane 3*). By contrast, anti-*Oct*-1 mAb was unable to form a complex with the labeled NRE alone. These results suggest that *Oct-1* can bind to the NRE region of the rat *3α-HSD/DD* gene and may participate in repressing constitutive expression.

DISCUSSION

We describe the isolation of a 15.8-kb genomic clone for rat liver 3α-HSD/DD. Over 9.5 kb of this clone corresponds to the 5′-flanking region of the gene. *cis*-Regulatory elements responsible for constitutive expression were identified by heterologous transfection of pCAT reporter gene constructs into HepG2 cells. Reporter gene constructs located a basal promoter (−199 to + 55 bp), a proximal enhancer (−498 to −199 bp), a strong silencer (−755 to −498 bp), and a powerful distal enhancer (−4.0 to −2.0 kb) on the 5′-flanking region of the rat *3α-HSD/DD* gene.

Although the strength of the proximal enhancer presumably contributes to the high level of constitutive expression of the *3α-HSD/DD* gene observed (0.5% of the soluble protein in male rat liver), by far the greatest effect on pCAT expression is obtained with a reporter gene construct containing −2.0 kb to −4.0 kb of the 5′-flanking region of the *3α-HSD/DD* gene. In this construct, CAT activity is elevated 20–40-fold over that observed in the pCAT-basic vector. This distal enhancer may play a greater role than the proximal enhancer in causing high level constitutive expression of the *3α-HSD/DD* gene. Recently, a powerful distal enhancer (−6069 to −5900) was located on the 5′-flanking region of the rat α-fetoprotein gene. DNase I footprinting revealed five nuclear protein binding domains within this enhancer. Band shift assays and oligonucleotide competition studies indicated the existence of binding sites for the liver-specific transcription factors HNF-3 and C/EBP in this region (40). Work is in progress to identify the sequence of the powerful distal *cis* element(s) on the rat *3α-HSD/DD* gene and the *trans*-acting factors that bind to these elements.

Reporter gene constructs also identified the presence of a NRE between −755 and −498 bp. The NRE contains two *Oct*-binding sites, which exist between −755 and −744 and −586 and −574 bp, and were referred to as *Oct* site I and *Oct* site II, respectively. Gel retardation assays with HepG2 nuclear extracts indicated the formation of three NRE-protein complexes. All of these complexes were supershifted by the anti-*Oct-1* mAb. These data would support the hypothesis that *Oct-1* binds to both sites in the NRE. The binding of monomeric *Oct* (M_r 90,000) to sites I and II in the NRE is anticipated to yield two different complexes because their neighboring DNA sequence is different, and these complexes should be resolved by band shift assay. When *Oct-1* binds to both sites, a third complex of lower mobility would form. The super shift data suggest that each complex contains *Oct-1*. *Oct* transcription factors are members of the *POU*-domain transcription family; they bind to the conserved octamer nucleotide sequence (*5′-ATGCAAAT-3′*) (41). Interestingly, the 5′-flanking region of the rat *CYP1A1* gene contains a NRE that binds *Oct-1* as a transcription factor (39). Cotransfection of pCAT constructs containing the 5′-flanking region of the rat *CYP1A1* gene with an expression vector for *Oct-1* in HepG2 cells verified that *Oct-1* was responsible for the repression of *CYP1A1* gene transcription (42). Similar cotransfection studies with pCAT constructs containing the 5′-flanking region of the 3α-HSD are not possible since the NRE is able to silence all CAT activity in the absence of cotransfected *Oct-1*. It is of interest that two important genes involved in PAH metabolism (*CYP1A1* and *3α-HSD/DD*) may be silenced by the same transcription factor family.

Previous studies have shown that 3α-HSD/DD displays a sexual dimorphic expression in rat liver (25–27). Enzyme activity, enzyme protein, and mRNA are all twice as high in females relative to males (27). Furthermore, the female pattern of expression can be achieved by administering estrogens to male rats (27). Studies in rat hepatocytes have also revealed that steady-state levels of mRNA for 3α-HSD/DD were up-regulated by dexamethasone and thyroxine (28). Of these two hormones, thyroxine was found to increase *3α-HSD/DD* gene transcription in nuclear run-off assays, whereas dexamethasone increased mRNA stability.

The possibility that hormones regulate the transcription of the rat *3α-HSD/DD* gene is supported by the presence of multiple steroid hormone response elements on the 5′-flanking region of the gene. Clusters of steroid response elements are often found on the 5′-flanking region of steroid responsive genes interrupted with intervening sequences for additional transcription factors, *e.g.*, AP-1 (35), and together they comprise a SRU. The putative SRU on the 5′-flanking region of the *3α-HSD/DD* gene consists of two perfect half-palindromic EREs, three imperfect GREs, six imperfect PREs, and two imperfect AP-1 sites. The two EREs differ from a complete palindrome by only 2 bp on the right arm. Imperfect palindromic EREs can increase the transcription of estrogen-responsive genes in a synergistic manner (43). Furthermore, the distal GRE on the (+) strand at −1408 and on the (−) strand at −1378 are almost perfect GREs and are within 70 bp of a proximal AP-1 site (at −1451). Stimulation of gene transcription by steroid hormone receptors can be mediated at half-palindromic sequences, provided there is cooperativity with adjacent promoter elements. For example, glucocorticoid receptors interact with AP-1 transcription factors at the AP-1 binding site to either block or stimulate transcription of the proliferin gene (44). The presence of the EREs would be consistent with the regulation of 3α-HSD/DD expression by estrogens *in vivo* (25–27). By contrast, the presence of the GREs would be inconsistent with the failure of glucocorticoids to increase 3α-HSD/DD transcription in rat hepatocytes (28).

The rat *3α-HSD/DD* gene and the human *DD2* gene contain a number of perfect and imperfect AP-1 sites. On the murine glutathione-*S*-transferase Ya subunit gene (45) and the human quinone reductase gene (46), these sites correspond to AREs that are activated by the monofunctional inducers described by Talalay and coworkers (47,

48). These inducers are characterized by an electrophilic Michael acceptor, which can activate binding of *fos/jun* heterodimers to AP-1 sites. Ciaccio *et al.* (49) demonstrated marked up-regulation of *DD2* mRNA in human colon cells upon treatment with ethacrynic acid and dimethyl maleate (both Michael acceptors), suggesting that the human *DD2* gene may be up-regulated by monofunctional inducers. Furthermore, ethacrynic acid-induced CAT activity in Hepa-1 cells stably transfected with a pARE-tk-CAT construct and increased AP-1 binding activity in colon cell nuclear extracts (49). These indirect findings suggest that one or more of the AP-1 sites on the rat and human genes may function as an ARE. Interestingly, the rat ARE is responsive to ROS (39, 46). Hydrogen peroxide is generated during the oxidation of PAH *trans*-dihydrodiols catalyzed by 3α-HSD/DD, and the resultant PAH *o*-quinones generate additional oxidative stress. We have obtained preliminary data that ROS up-regulate 3α-HSD/DD mRNA in rat hepatoma cells (H4IIe) and up-regulate human *DD2* mRNA in human hepatoma cells (HepG2) maintained in continuous culture.[4] Whether ROS stimulate AP-1 binding activity on the 5′-flanking regions of the rat *3α-HSD/DD* and human *DD2* genes is under investigation.

In summary, the analysis of the 5′-flanking region of the rat *3α-HSD/DD* gene and its similarity with the human *DD2* gene suggests that both genes may be under the same transcriptional regulation. *POU*-domain family transcription factors (*Oct*) may act as negative regulators of constitutive expression. *Trans*-acting factors involved in increasing gene expression may include steroid hormone receptors and members of the AP-1 transcription factor family.

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