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Genomic structure of rat 3α-hydroxysteroid/dihydrodiol dehydrogenase (*3α-HSD/DD*, *AKR1C9*)

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Abstract

Rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase (3α-HSD/DD) is a member of the aldo-keto reductase (AKR) superfamily. It is involved in the inactivation of steroid hormones and the metabolic activation of polycyclic aromatic hydrocarbons (PAH) by converting *trans*-dihydrodiols into reactive and redox-active *o*-quinones. The structure of the 5′-flanking region of the gene and factors involved in the constitutive and regulated expression of this gene have been reported [H.-K. Lin, T.M. Penning, Cloning, sequencing, and functional analysis of the 5′-flanking region of the rat 3α-hydroxysteroid/dihydrodiol dehydrogenase gene, Cancer Res. 55 (1995) 4105–4113]. We now describe the complete genomic structure of the rat type 1 3α-*HSD/DD* gene. Charon 4A and P1 genomic clones contained at least three rat genes (type 1, type 2 and type 3 3α-*HSD/DD*) each of which encoded for the same open reading frame (ORF) but differed in their exon–intron organization. 5′-RACE confirmed that the type 1 3α-*HSD/DD* gene encodes for the dominant transcript in rat liver and it was the regulation of this gene that was previously studied. The rat type 1 3α-*HSD/DD* gene is 30 kb in length and consists of nine exons and eight introns. Exon 9 encodes +931 to 966 bp of the ORF and the 1292 bp 3′-UTR implicated in mRNA stability. This genomic structure is nearly identical to the homologous human genes, type 1 3α-*HSD* (chlordecone reductase/DD4, AKR1C4), type 2 3α-*HSD* (AKR1C3) and type 3 3α-*HSD* (bile-acid binding protein, AKR1C2) genes. Three different cDNA's containing identical ORFs for 3α-HSD have been reported suggesting that all three genes may be expressed in rat liver. Using 5′ primers corresponding to the 5′-UTR's of the three different cDNA's only one PCR fragment was obtained and corresponded to the type 1 3α-*HSD/DD* gene. These data suggested that the type 2 and type 3 3α-*HSD/DD* genes are not abundantly expressed in rat liver. It is unknown whether the type 2 and type 3 3α-*HSD/DD* genes represent pseudo-genes or whether they represent genes that are differentially expressed in other rat tissues.

Keywords: 3α-Hydroxysteroid/dihydrodiol dehydrogenase; Aldo-keto reductase; Genomic structure

1. Introduction

Rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase (3α-HSD/DD; AKR1C9) is a member of the aldo-keto reductase (AKR)[3] superfamily. It is a multi-functional oxidoreductase and inactivates circulating steroid hormones, including androgens, progestins and glucocorticoids [1,2], it is involved in bile acid biosynthesis and transport [3,4], and oxidizes polycyclic aromatic hydrocarbon (PAH) *trans*-dihydrodiols (proximate carcinogens) to reactive PAH *o*-quinones with the concomitant production of reactive oxygen species (ROS; $O_2^{-\cdot}$, H_2O_2 and $OH^{\cdot}$) [5–7]. Once formed PAH *o*-quinones enter futile redox cycles to generate *o*-semiquinone radicals and ROS multiple times [8,9]. Amplification of ROS can modify lipids [10], proteins [11] and DNA [12], and may contribute to the cyto- and geno-toxic properties of PAH *o*-quinones [9,13,14].

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[3] Current members of the aldo-keto reductase superfamily can be accessed on the AKR web-page at http://pharme26.med.upenn.edu

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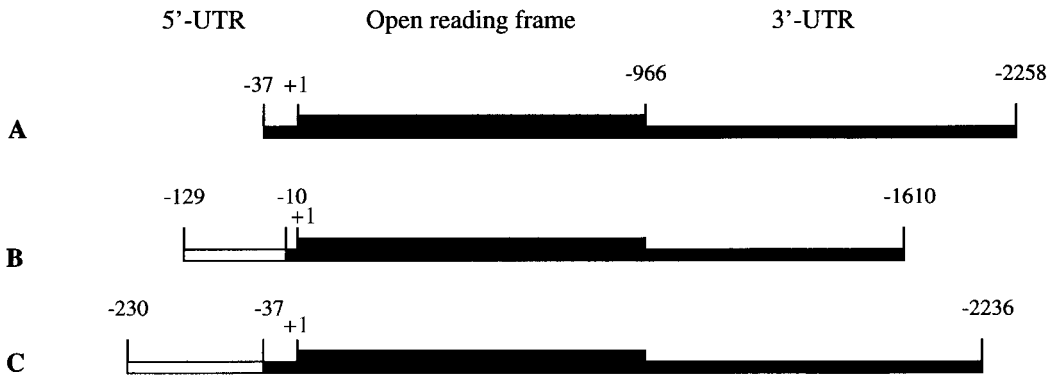

Fig. 1. Structure of rat liver 3α-HSD/DD cDNAs. Schematic comparison of three rat liver 3α-HSD/DD cDNA clones. (A) The clone reported by Stolz et al. [17]; (B), the cDNA cloned by Pawlowski et al. [15] and (C), the cDNA reported by Usui et al. [19]. The clones have identical ORFs (filled boxes) and 3′-UTR (bold lines) but have different 5′-UTRs (bold and open lines).

The cDNA for rat liver 3α-HSD/DD has been cloned [15] and overexpressed in *E. coli* [16]. The recombinant enzyme oxidizes a variety of 3α-hydroxysteroids and PAH *trans*-dihydridiols and is indistinguishable from the native enzyme [16]. Three cDNA clones for rat liver 3α-HSD have been independently reported [15,17–19] (Fig. 1). These clones contain identical sequences for the open reading frame (ORF) and 3′-untranslated region (3′-UTR). The ORFs contain 966 bp and encode a protein of 322 amino acids. However, the cDNA's differ in length and sequence in their 5′-UTRs. The length of the 5′-UTR is −37 bp, −129 bp and −230 bp in the clones reported by Stolz et al. [17]; Pawlowski et al. [15] and Usui et al. [19], respectively. The 5′-UTRs are identical between −10 and −1 bp in cDNA clones reported by Stolz et al. [17] and Pawlowski et al. [15] and are identical between −37 and −1 bp in clones reported by Stolz et al. [17] and Usui et al. [19]. In these comparisons +1 is the translation start site.

Constitutive and regulated expression of the rat 3α-*HSD/DD* gene has been described. The 5′-flanking region of the rat 3α-*HSD/DD* gene has been cloned and functionally characterized [20,21]. A basal promoter, proximal and distal enhancers, and a silencer region have been identified. The silencer region (−797 to −498 bp) binds Oct transcription factors [21]. Analysis of the distal enhancer element identified a 73 bp fragment between −4351 and −4279 bp which contains three putative NF-1 sites. These sites bind NF-1 and in co-transfection paradigms NF-1 can enhance reporter gene activity from these *cis*-elements [22]. In addition, the 5′-flanking region of the gene contains multiple imperfect steroid response elements which may comprise a steroid response unit and contribute to the sexual dimorphic expression of the gene [20]. There are four imperfect half-palindromic glucocorticoid response elements (GRE) in this region. In a previous report we demonstrated that the proximal GRE can function as a *cis*-element for the binding of the occupied glucocorticoid receptor and may be responsible for the induction of 3α-*HSD/DD* gene expression by dexamethasone in rat hepatocytes [23]. In this paradigm high circulating glucocorticoids induce their own metabolism by increasing the level of 3α-*HSD* gene expression.

In this study, we demonstrate the presence of three rat 3α-*HSD/DD* genes which contain exons that code for identical ORF's. The three genes differ in their exon–intron organization. 5′-RACE and RT-PCR confirmed that the type 1 3α-*HSD/DD* gene is abundantly expressed in rat liver. The structure of this gene is given and its dominant transcript corresponds to the gene on which promoter analysis has been previously reported. The cDNA corresponding to this gene is that previously reported by Stolz et al. [17]. It is unknown whether the type 2 or type 3 genes are expressed or whether they are pseudo-genes. The possibility remains that tissue specific expression of 3α-HSD/DD may be governed by different genes that encode for the same protein but differ in their promoter regions.

2. Materials and methods

2.1. Chemicals and reagents

Adult male and female Sprague-Dawley rats (200 ± 20 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). The animals were allowed free access to food and water for 7 days before use. A rat liver genomic library in a Charon 4A vector was purchased from Clontech Laboratories (Palo Alto, CA). Bacteriophage P1 clones were obtained from Genome Systems (St. Louis, MO). *Vent* DNA polymerase was acquired from New England Biolabs (Beverly, MA). Avian myeloblastosis virus (AMV) reverse transcriptase and Expand™ long

Table 1
Oligonucleotide primers used for identification of exon–intron junctions and estimation of intron sizes

Exon number	Forward primer	Reverse primer
1	43–59 bp[a], 5′-TTCATTCCTGTACTGGG-3′	
2	148–165 bp, 5′-GACTCTGCTTATTTGTAC-3′	114–94 bp, 5′-AGTAGCCTTGATAACTTCAT-3′
3	253–273 bp, 5′-CTTTGGAGCACTTTCCATAGA-3′	278–257 bp, 5′-TCTATGGAAAGTGCTCCAAAG-3′
4	424–440 bp, 5′-GTGGATATCTGTGACAC-3′	408–391 bp, 5′-TTTTCCATGCTCATCTGC-3′
5	547–567 bp, 5′-AAATACAAGCCTGTGTGCAAC-3′	567–546 bp, 5′-GTTGCACACAGGCTTGTATTT-3′
6	637–654 bp, 5′-CTGGTTTCCTACTGCACG-3′	593–577 bp, 5′-TTGAGATAAAGGTGACA-3′
7	760–777 bp, 5′-CTAGTTGCCCTTCGCTAC-3′	722–706 bp, 5′-AAGAACTGGATCATCTA-3′
8	849–870 bp, 5′-TTTTGAATTCCAGTTGGCTTCA-3′	870–849 bp, 5′-TGAAGCCAACTGGAATTCAAAA-3′
9		969–949 bp, 5′-CTATTCATCAGTAAATGGATG-3′
9		2258-2241 bp, 5′-TCTGATCAAGGAGACAAT-3′[b]

[a] Numbers refer to cDNA sequence.
[b] 18 bp located after the polyadenylation signal in cDNA cloned by Stolz et al. [17].

template PCR system was obtained from Boehringer Mannheim (Indianapolis, IN). [α–^{32}P]dATP (3000 Ci/mmol) and [γ–^{32}P]-dATP (3000 Ci/mmol) were purchased from NEN-DuPont (Boston, MA). The pCRII cloning vector was obtained from Invitrogen (San Diego, CA).

2.2. Cloning and purification of rat 3α-HSD/DD genomic clones

Rat 3α-HSD/DD genomic DNA was cloned from a female Sprague-Dawley rat liver genomic library, with *Hae*III partial digest in Charon 4A vector. Briefly, a total of 2×10^6 plaques were screened by hybridization to a random primed probe corresponding to −15–+250 bp and +850–+1610 bp of the rat liver 3α-HSD/DD cDNA [15]. The positive clones were subsequently purified using plate purification procedures as previously described [21]. The purified phage DNA was digested with *Eco*RI to release genomic fragments from the Charon 4A vector and electrophoresized on an agarose gel followed by Southern analysis using the same probes used for genomic screening to confirm the identity of the positive clones.

Bacteriophage P1 clones containing rat 3α-*HSD/DD* genes were screened using a pair of primers corresponding to intron 2 sequences: 5′ primer (5′-GTATTTTGTATATGGGATGCTTG-3′) and a 3′ primer (5′-ACCCTAACTAAGACAGTGCAT-3′), which generate a 138-bp fragment from rat genomic DNA. PCR screening was conducted by Genome Systems (St. Louis, MO) to yield positive clones. The P1 clones were purified through centrifugation using cesium chloride gradients containing ethidium bromide.

2.3. PCR amplification of 3α-HSD/DD genomic fragments from λ and P1 genomic clones

To identify nucleotide sequences for exon–intron junctions and to determine the sizes of introns for the type 1 3α-*HSD/DD* gene, pairs of PCR primers corresponding to each exon were synthesized (Table 1). For example, to amplify intron 1, the forward primers from exon 1 and the reverse primer from exon 2 were used in a PCR reaction. PCR were first heated at 94°C for 2 min followed by 35 cycles of PCR with 1 min denaturation at 94°C, 2 min of annealing at 55°C and 2–10 min polymerization at 68°C, depending on the sizes of introns, using the Expand™ long template PCR system. The amplified DNA fragments were subjected to purification by agarose gel electrophoresis and subcloned into a pCRII vector for subsequent restriction endonuclease digestion and dideoxysequencing.

2.4. Total and poly(A)$^+$ RNA extraction

Total RNA and poly(A)$^+$ RNA were extracted from rat liver to identify 3α-HSD/DD transcripts. Total RNA extraction was performed using TRIzol reagent (Life Technology). Briefly, 100 mg of rat liver was excised and homogenized in 1 ml TRIzol reagent and incubated at 30°C for 30 min. RNA was isolated by chloroform extraction followed by isopropanol precipitation. For poly(A)$^+$ RNA extraction, rat liver was excised and 100 mg of the tissue was homogenized in a Dounce homogenizer with Micro-FastTrack™ lysis buffer (Invitrogen). Poly(A)$^+$ RNA was isolated through binding to oligo(dT)-cellulose and ethanol precipitation according to the manufacturers protocol.

2.5. Rapid amplification of 5′ cDNA end (RACE) PCR

To identify 5′-UTR sequences that correspond to the dominant 3α-HSD/DD transcripts in rat liver, 5′-RACE was performed using rat liver total RNA as template and the Marathon cDNA amplification kit (Clontech Laboratories). Rat 3α-HSD/DD mRNA was

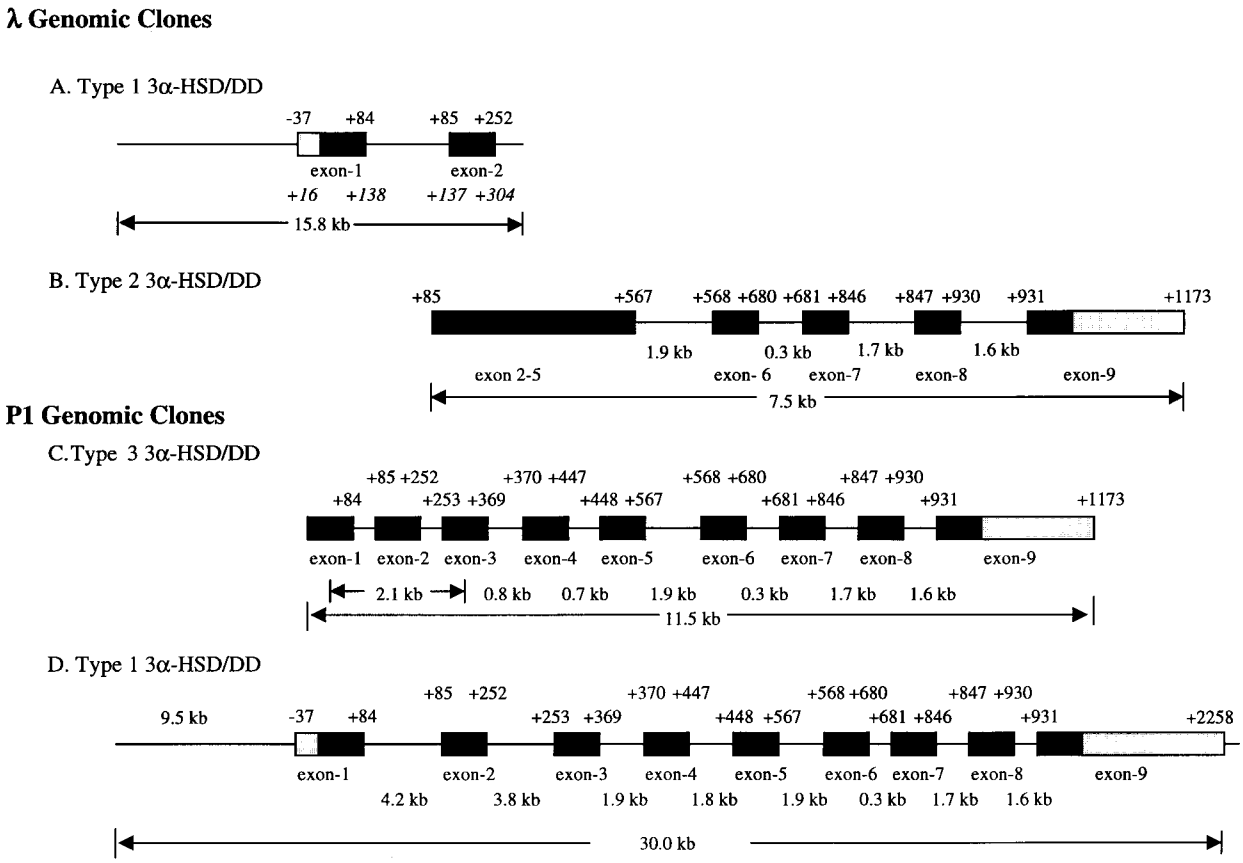

Fig. 2. Organization of the rat type 1, type 2 and type 3 3α-*HSD/DD* genes. Two 3α-HSD/DD genomic clones were isolated from rat λ genomic library and purified. The phage DNA was subjected to restriction enzyme digestion and PCR analysis. (A) Structure of the original 15.8-kb genomic clone containing the 5′-flanking region of type 1 3α-*HSD/DD* gene; (B) partial structure of the second genomic clone (7.5 kb of an 11.5 kb clone is shown) containing the type 2 3α-*HSD/DD* gene. The P1 clone was also subjected to PCR analysis and sequencing. (C) Structure of the type 3 3α-*HSD/DD* gene; (D) entire structure of the type 1 3α-*HSD/DD* gene. Filled boxes indicate exons, the numbering above a gene corresponds to the cDNA sequence where +1 is the translation start site, the numbering in italics beneath a gene are with respect to the transcription start site as determined by the size of a primer extension fragment, and the sizes of introns are shown.

reverse transcribed using oligonucleotides complementary to +126 to +104 bp (5′-TATAGCTATTTTA-GTAGCCTTGA-3′) and AMV reverse transcriptase. Second strand cDNA was synthesized using RNaseH and DNA polymerase. The double-stranded cDNA was ligated to adaptors provided in the amplification kit followed by PCR amplification using adaptor primer 1 as a forward primer and a primer complementary to +32 bp to +11 bp (5′-TTTAGTGCTACA-CGCAGAGATA-3′) of the 3α-HSD/DD cDNA as a reverse primer with 1 min denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C using *Vent* DNA polymerase. The amplified product was subcloned into the pCRII vector and subjected to dideoxysequencing.

2.6. Reverse transcription (RT) PCR

To detect the presence of multiple 3α-HSD/DD transcripts in rat liver, total RNA (1.5 μg) from rat liver was first annealed to oligo(dT)$_{12–18}$ primers and reverse transcription was performed at 42°C for 1 hr using AMV reverse transcriptase in a total volume of 20 μl. Aliquots of the reaction (5 μl) were PCR amplified using 200 nM 5′ primers corresponding to 5′-CTCAGGTTTCTCACACTCCTGG-3′ (−129−−107 bp, in the 5′-UTR reported by Pawlowski et al., [15]), to 5′-TTCCTATGGCGTCTTGGAAGTACC-3′ (−124−−101 bp, in the 5′-UTR reported by Usui et al., [19]) or to 5′-AGACCCAAGACCTGCTTGGTT-3′ (−37−−17 bp, in the 5′-UTR reported by Stolz et al., [17]) plus 200 nM of a 3′ primer complementary to +423–+404 bp (5′-ACTGTTTCAAACAATAGT-TTT-3′) in a total volume of 50 μl, heated at 94°C for 2 min and subjected to 30 cycles of PCR in a Perkin-Elmer thermal cycler with 1 min denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C using *Vent* DNA polymerase. Aliquots (5 μl) of

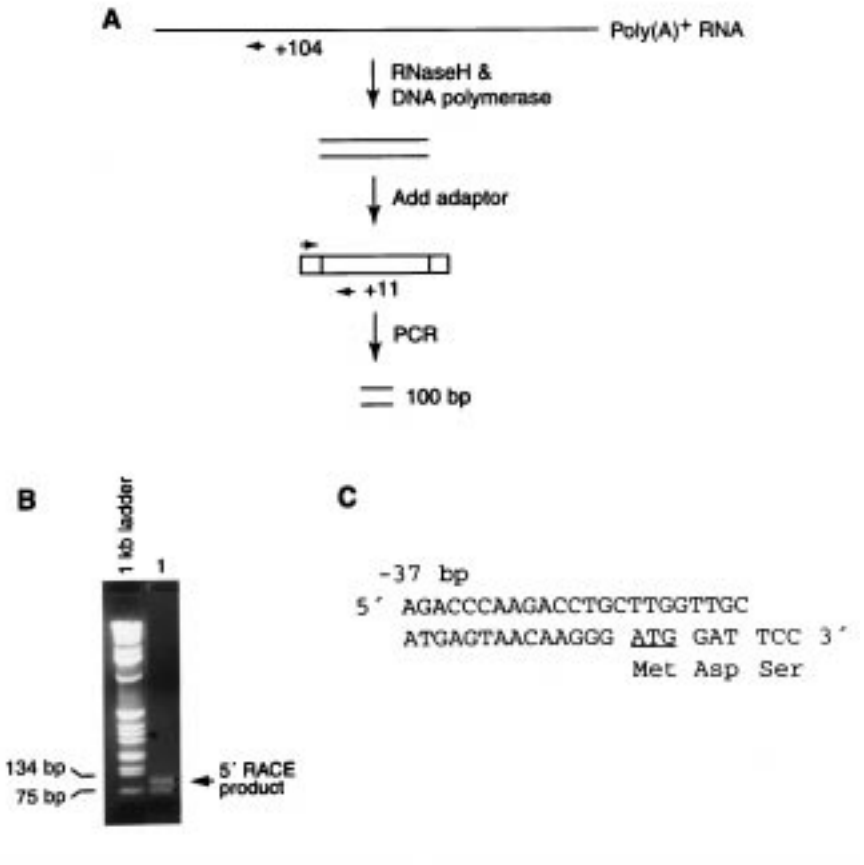

Fig. 3. Determination of the sequence of the 5′-UTR of the 3α-HSD/DD mRNA predominantly expressed in rat liver using 5′ RACE. (A) Strategy for 5′-RACE; (B) gel electrophoresis of 5′-RACE product (100 bp) separated on 1.5% agarose gel and (C) nucleotide sequence of the amplified DNA product where the ATG start codon is underlined. The lower band on the gel is from the 5′-adaptor primer.

the reaction were electrophoresed on an 1% agarose gel. In this nomenclature +1 is the translation start site.

3. Results

3.1. Identification of multiple 3α-HSD/DD genes from a rat genomic library

We have previously reported the partial cloning and characterization of the rat 3α-*HSD/DD* gene from a rat genomic DNA library, *Hae*III partial digest in a Charon 4A vector [21]. A genomic clone (=15.8 kb) contained 9.5 kb of the 5′-flanking region of the gene followed by exon 1 (+16–+138 bp; 122 bp), intron 1 (=5.0 kb), exon 2 (167 bp) and a portion of intron 2 (Fig. 2A). Using primer extension analysis, a major transcription start site was located at 55 bp upstream from the ATG translation start codon [20] and numbering was relative to the transcription start site. The 5′-upstream region of the genomic clone encoded the −37 bp that were found in the 5′-UTR of the 3α-*HSD/DD* cDNA reported by Stolz et al. [17]; thus exon 1 covers −37–+84 bp of the cDNA (122 bp) where +1 is the translation start site. Although the two exon sequences were identical to the corresponding region of ORF in the three cDNA's reported [15,17,19], the 37 bp sequence reported by Stolz et al. [17] did not match the 5′-UTR in the other two cDNA clones. The presence of three cDNA species with different 5′-UTRs may result from cloning artifacts, alternative splicing at exon 1, or they may represent different gene products. The presence of the −37 bp fragment plus +84 bp of the ORF in the original clone verified that the 5′-flanking region that we previously reported belonged to a 3α-*HSD/DD* gene. Functional analysis of the 5′-flanking region of this gene has been reported [21].

To obtain the entire 3α-*HSD/DD* gene, probes corresponding to the 5′-end of the cDNA (−15–+250 bp) and the 3′-end of the cDNA (+850–1610 bp) were used to identify a second genomic clone of 11.5 kb in length (Fig. 2B). The identity of the positive clone was confirmed by restriction digestion and Southern analysis. After plaque purification, the phage DNA was

used as a template using the primer sets described in Table 1 for PCR reactions. These primer sets were based on the presumed location of the exons based on the structures of related *AKR* genes. Intron sizes were determined from the amplified products; and exon–intron junctions were determined by subsequent subcloning of these PCR products into the pCRII vector followed by dideoxysequencing. This genomic clone did not contain the 5′-flanking region of the first gene and exon 1 was absent. Unlike the first genomic clone which encoded exon 2 from +85 to +252 bp, this clone contained an exon which spanned exon 2 through exon 5 (+85–+567 bp, assuming that exons are conserved relative to the human 3α-HSD genes) and was followed by 4 additional exons (+568–+680 bp, +681–+846 bp, +847–+930 bp). The ninth exon (+930–+1173 bp) extended into the 3′-UTR of the cDNA's reported by Stolz et al. [17]. and Pawlowski et al. [15].

The intron sizes in this gene were 1.9, 0.3, 1.7, and 1.6 kb, respectively (Fig. 2B). The ORF predicted by the exon sequences in this genomic clone were identical to those predicted by all three cDNAs. The difference in the structure of exon-2 in both clones suggested the presence of at least two rat 3α-*HSD*/*DD* genes. Based on their chronology the first genomic clone contained the type 1 3α-*HSD*/*DD* gene and the second genomic clone contained the type 2 3α-*HSD*/*DD* gene. If both of these genes were transcribed this would lead to at least two 3α-HSD/DD mRNA species and would provide an explanation for the presence of more than one cDNA. Thus it became important to determine which gene encoded for the dominant transcript in rat liver, so that the remainder of its structure could be determined.

3.2. 5′-RACE identified the rat type 1 3α-HSD/DD gene as the gene that encodes the predominant transcript

Northern analysis using a probe for the ORF showed one major transcript (2.7 kb) for rat 3α-HSD/DD in male and female rat liver [20]. Since the three cDNAs contain identical ORFs, it is unlikely that this method would distinguish between multiple 3α-HSD/DD transcripts which vary by less than 200 bp in their 5′-UTRs. To identify the predominantly expressed 3α-HSD/DD transcript in rat liver, 5′-RACE was performed. In this experiment total RNA was extracted from male rat liver, 3α-HSD/DD mRNA was reverse transcribed, double stranded cDNA was synthesized and adpators were ligated to the cDNA. PCR amplification using adaptor specific primers from Clontech as a 5′ primer plus a 3′ primer complementary to +32–+11 bp of the cDNA gave a single band about 100 bp in size (Fig. 3). The DNA fragment was subcloned into the pCRII vector and subjected to dideoxysequencing. The 5′-RACE product contained only the −37 bp of the cDNA described by Stolz et al. [17]. This −37 bp fragment was found only in the type 1 3α-*HSD*/*DD* gene which by default must encode the major transcript in rat liver. The lack of multiple 5′-RACE products suggested that the other two species of 3α-HSD/DD cDNA reported are either not present or rarely expressed in rat liver.

RT-PCR of total RNA from male rat liver was also employed to amplify the 3′ end of the predominant transcript using (+1032–+1050 bp; 5′-GATATCTTGGATACTGGTG-3′) as a 5′ primer and oligo(dT)$_{12–18}$ as a 3′ primer. Dideoxysequencing of the resultant 3′-end of 3α-HSD/DD cDNA confirmed that the cDNA described by Stolz et al. [17] was full-length (data not shown). The 5′-RACE and the RT-PCR of the 3′-UTR using RNA as template set the exon-boundaries of the major gene of interest. However, despite these cloning efforts the genomic structure for the type 1 3α-HSD/DD gene eluded completion.

3.3. Identification of multiple 3α-HSD/DD genes in bacteriophage P1 clones

Since the presence of intron 2 was a unique feature or the type 1 3α-*HSD*/*DD* gene, PCR primers were prepared to amplify a 138 bp fragment of intron 2. The 5′ primer (5′-GTATTTTGTATATGGGATGC-TTG-3′) and a 3′ primer (5′-ACCCTAACTAAG-ACAGTGCAT-3′) from intron 2 were then used to PCR screen P1 genomic clones for type 1 3α-*HSD*/*DD* gene. A positive clone of 75 kb was obtained. Using 5′- and 3′-primers (Table 1), attempts were made to generate overlapping genomic fragments corresponding to sequential exons of the 3α-*HSD*/*DD* gene from the positive P1 clone. Using forward and reverse primers from exon 1 to exon 5 two bands were observed in the PCR reactions indicating either incomplete amplification or the presence of at least two genes. From the first series of PCR products it was found that the gene structure being determined did not correspond to either the type 1 or type 2 3α-*HSD*/*DD* genes. In this new structure exon 1 through exon 3 were contained within a 2.1-kb region, yet in the type 1 3α-*HSD*/*DD* gene, exon 1 through exon 2 were contained within a region that was greater than 4.3 kb in length. In the type 2 3α-*HSD*/*DD* gene exon-1 through exon-5 were fused. These data provided evidence for a type 3 3α-*HSD*/*DD* gene in the P1 clone which also contained an ORF identical to that reported in the cDNA's (Fig. 2C).

To amplify the full length type 1 3α-*HSD*/*DD* gene, a new set of primers were used in which the 5′-primer corresponded to −550 to −530 bp (5′-TCCCTACAGATGGTGTCTAGT-3′) of the 5′-flank-

Table 2
Exon–intron junction sequences of the rat type 1 3α-HSD/DD (AKR1C9) gene

Intron	Size (kb)	Donor Sequence (Consensus Sequence) AG|GTPuAGT	Acceptor Sequence (Consensus Sequence) (Py)nNCAG|G
		84	85
1	4.2	5′-GAGAAG-gtaacatatg-ggtcttgggc- -ttagaggttt-agaggtaaca-3′	-cattttggt-gctccttatg-GTTGCT-3′ 5′-atctttcatt-atcaatagat-
		252	253
2	3.8	5′-TCAAAG-gtattttgta-atgggatgct- tgtggactat-ttaatgtgat-3′	-taagcctatc-ttacttgcag-CTTTGG-3′ 5′-tccacaaaga-gacggcttag
		369	370
3	1.9	5′-TTGCAG-gtgagcaatt-gctatgcttaa- aattttctca-gtagtgatgc-3′	-aatgaatgat-tctattccag-CCTGGA-3′ 5′-nncttcagag-aggtctctaa-
		447	448
4	1.8	5′-TGGGAG-gtgagtcctt-ggaagaaaga- gcacagaagg-ggacactggg-3′	-atcccctctc-ttcatacgag-GCCATG-3′ 5′-tgacttcact-gtcacatctc-
		567	568
5	1.9	5′-TGCAAC-aagccgatat-ccagcacact- ggcggccgtt-atcagtggta-3′	-gtaatctgac-tgtcttttgt-CAGGTG-3′ 5′-tacgttataa-caagcttcgt-
		680	681
6	0.3	5′-AACATG-gtcagtacca-aaggtgacag- agcatctcaa-acatcatggc-3′	-cttttaacct-tcccttccag-GGTGGA-3′ 5′-tttcaggggt-tcagcatttt
		846	847
7	1.7	5′-ACACAG-gttgacgact-gggctgtcgg- ccttgaagtg-cttgtgaaat-3′	-aatttctact-tttgtgatag-GTTTTT-3′ 5′-tgacacatga-actcaactaa
		930	931
8	1.6	5′-AAAATA-gtgagtaact-ttgatcattg- cactacttca-atttgctttt-3′	5′-ctaattttag-TTTTGA-3′

ing region of type 1 3α-*HSD/DD* gene and a 3′ primer corresponded to exon 5. These primers gave a genomic fragment of ≫10.0 kb using the P1 clone as template. This fragment contained the −37 bp of the 5′-UTR detected by 5′-RACE and represented the gene which transcribes into the dominant 3α-HSD/DD mRNA in rat liver. The remaining half of the gene was PCR amplified from the P1 clone using the 5′ and 3′-primers listed in Table 1 for exons 5–9. These primers gave a series of overlapping genomic fragments corresponding to the remaining exon-introns of the type 1 3α-*HSD/DD* gene. With each primer set only single DNA fragments were obtained and their fidelity established by subcloning and sequencing. These data indicate the essential identity of the type 1 and type 3 genes from exon 5 to exon 9. This region also shared identity with exon 6 to exon 9 of the type 2 gene. Thus the type 1, 2 and 3 3α-*HSD/DD* genes are essentially identical from exon 6 to exon 9. The complete genomic structure of the type 1 3α-*HSD/DD* gene and its re-

Table 3
Genomic structures of mammalian *3α-HSD* genes

Rat type 1 *3α-HSD* gene (*AKR1C9*)					Human type 1 *3α-HSD* gene (*AKR1C4*)[a]					Human type 3 *3α-HSD* gene (*AKR1C2*)[b]				
Exon	Size (bp)	Nucleotide	Intron	Size (kb)	Exon	Size (bp)	Nucleotide	Intron	Size (kb)	Exon	Size (bp)	Nucleotide	Intron	Size (kb)
1	122	−37–84	1	4.2	1	115	−30–84	1	53	1	115	−30–84	1	2.1
2	168	85–252	2	3.8	2	168	85–252	2	82	2	168	85–252	2	0.9
3	117	253–369	3	1.9	3	117	253–369	3	119	3	117	253–369	3	1.3
4	79	370–447	4	1.8	4	78	370–447	4	74	4	78	370–447	4	0.5
5	120	448–567	5	1.9	5	123	448–570	5	79	5	123	448–570	5	3.1
6	113	568–680	6	0.3	6	110	571–680	6	48	6	110	571–680	6	0.3
7	166	681–846	7	1.7	7	166	681–846	7	59	7	166	681–846	7	3.0
8	84	847–930	8	1.6	8	83	847–929	8	75	8	83	847–929	8	1.7
9	1328	931–2258			9	265	930–1194			9	265	930–1194		

[a] Ref. [25]; human type 1 *3α-HSD (AKR1C4)* and human type 2 *3α-HSD (AKR1C3)* genes have identical genomic organization.
[b] Ref. [26].

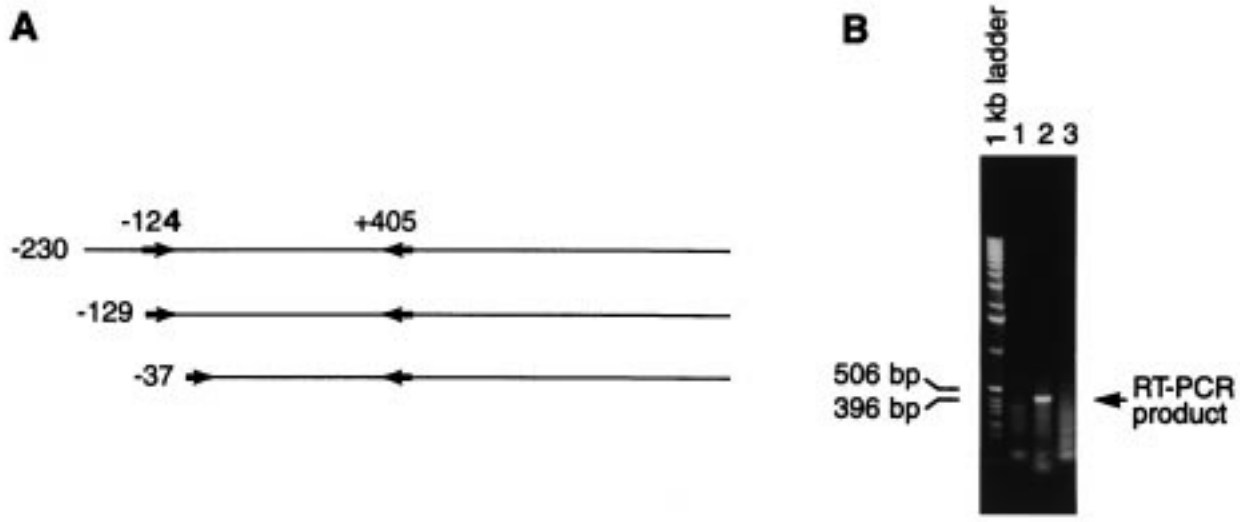

Fig. 4. Detection of 3α-HSD/DD mRNA expression in rat liver using RT-PCR. Total RNA was extracted from male rat liver, and first strand cDNA was reverse transcribed using oligo(dT)$_{12–18}$ primers. PCR reactions were performed using a 3′ primer complementary to +423––404 bp of rat 3α-HSD/DD cDNA and a 5′ forward primer corresponding to the 5′-UTR of the cDNA sequences reported by Pawlowski et al. [15] (lane 1), by Stolz et al. [17] (lane 2), and by Usui et al. [19] (lane 3), where +1 is the translation start site. PCR products were resolved on a 1.2% agarose gel and stained with ethidium bromide.

lationship to the type 2 and type 3 3α-*HSD/DD* genes is given (Fig. 2D). The gene is over 30.0 kb in length and contains 9-exons and 8-introns. Exon 9 is 1329 bp in length and contains +931–+966 bp of the ORF and 1293 bp of the 3′-UTR. The sequence of the 3'-UTR in exon-9 was identical to that obtained by RT-PCR amplification of this region using total RNA as template (see, earlier). It contained the polyadenylation signal and the discrepancy that exists between the predicted length of the transcript (=2.3 kb) and that observed (=2.7 kb) is most likely due to the presence of a long poly(A)$^+$ tail. Subsequent subcloning and sequencing of the fragments identified the donor/acceptor sequences of the exon–intron boundaries in the rat type 1 3α-*HSD/DD* gene and are listed in Table 2.

3.4. Comparison of the genomic structure of type 1 3α-HSD/DD gene with human 3α-HSD/DD isoform genes

A comparison the exon–intron boundaries for the rat type 1 3α-*HSD/DD* (*AKR1C9*), human type 1 3α-*HSD/DD4/chlordecone reductase* (*AKR1C4*), human type 2 3α-*HSD* (*AKR1C3*), and human type 3 3α-*HSD/bile acid binding protein* (*AKR1C2*) genes is shown in Table 3. The genomic structures for these four genes are highly conserved with 9-exons and 8-introns; and positions of exon–intron boundaries are conserved relative to their cDNA sequences.

3.5. RT-PCR fails to provide evidence for the expression of more than one gene in rat liver

In this study, we have provided evidence that there are three *3α-HSD/DD* genes that have the potential to encode for three 3α-HSD/DD transcripts. 5′-RACE indicates that the dominant transcript in rat liver is encoded by the type 1 3α-*HSD/DD* gene. There are three rat liver 3α-HSD/DD cDNA's with identical ORF's and 3′-UTR but they differ in their 5′-UTR. We have shown that cDNA described by Stolz et al. [17] corresponds to the type 1 3α-*HSD/DD* gene. No information exists to relate the remaining cDNA's reported by Pawlowski et al. [15] Usui et al., [19] to the type 2 and type 3 3α-*HSD/DD* genes. To determine whether the remaining cDNAs were derived from 3α-HSD/DD transcripts that were poorly expressed from their respective genes we took advantage of RT-PCR. In this experiment, first strand cDNA was reverse transcribed from total RNA extracted from male rat liver using oligo d(T)$_{12–18}$ primers and AMV reverse transcriptase. A subsequent PCR reaction was performed using the single-stranded cDNA as a template in the presence of unique 5′-primers corresponding to −129–−107 bp of the cDNA reported by Pawlowski et al. [15], to −124––101 bp of the cDNA reported by Usui et al. [19] and to −37––17 bp of the cDNA reported by Stolz et al. [17] plus a 3′ primer complementary to +426 bp +405 bp of the cDNA. A single PCR product corresponding to a size of 450 bp was observed only when the 5′ primer derived from cDNA sequence

reported by Stolz et al. [17] was used (Fig. 4). Oligonucleotide primers derived from the cDNAs reported by Pawlowski et al. [15] and Usui et al. [19] failed to give PCR products of the expected sizes. These results confirm that these two cDNA's are not abundantly expressed in rat liver.

4. Discussion

In this study, we have shown that there are three rat 3α-*HSD*/*DD* genes in the rat genome. Classic genomic cloning identified two λ genomic clones in which exon 2 overlapped but the size of these exons were widely different. These clones correspond to the type 1 and type 2 3α-*HSD*/*DD* genes. 5′-RACE was used to identify the dominantly expressed 3α-HSD/DD mRNA in rat liver. Nucleotide sequence of the 5′-RACE products from rat liver mRNA matched the 5′-UTR of the cDNA reported by Stolz et al. [17] and the 5′-flanking region of a genomic clone previously reported by us [21]. This cDNA was encoded by type 1 3α-*HSD*/*DD* gene. The genomic organization of the type 1 3α-*HSD*/*DD* gene is now given. The type 2 and type 3 3α-*HSD*/*DD* genes were contained within the second λ genomic clone and the P1 clone, respectively. The entire type 1 3α-*HSD*/*DD* gene was also present in the P1 clone. Based on the size of the P1 clone (70–100 kb) it is likely that the type 1 and type 3 3α-HSD genes are located on the same chromosome.

Although three 3α-HSD/DD cDNAs have been reported with variable 5′-UTR's, there is only a single species of 3α-HSD/DD mRNA observed in rat liver using a cDNA probe corresponding to the coding region of 3α-HSD/DD [17,20]. However, different species of 3α-HSD/DD mRNA of similar size cannot be distinguished by Northern analysis. Previously three 3α-HSD/DD mRNA's had been detected in rat liver using specific 5′-UTR as probes on Northern analysis [19]. However, we were unable to detect a second species of 3α-HSD/DD mRNA using either 5′-RACE or RT-PCR analysis. We conclude that these is a single dominant species of 3α-HSD/DD mRNA present in rat liver. The discrepancies in the 5′-UTR sequences reported by Pawlowski et al. [15] and Usui et al. [19] versus that reported by Stolz et al. [17] may result from either cloning artifacts or these two species may be so rarely expressed in rat liver that their detection is not possible.

In this study, we identified three possible 3α-*HSD*/*DD* gene structures; they differed in their exon–intron organizations and may have different promoter regions which may be responsible for deregulated expression of these genes. The presence of multiple 3α-*HSD*/*DD* genes in rat may govern tissue specific expression of this gene. Ideally RT-PCR could be used to measure the potential expression of the type 2 and type 3 genes across rat tissues. Because these genes have identical ORF's and 3′-UTRs, unique 5′-UTR sequence would be required to conduct this study. At the present time we have been unable to generate this sequence for the type 2 and type 3 3α-*HSD*/*DD* genes. Also, we do not know whether the 5′-UTRs present in the different cDNAs are coded for by these three genes. Interestingly, the presence of multiple 3α-HSD/DD mRNA transcripts has been reported in rat ovary [20]. However, because HSDs in the AKR1C subfamily have high sequence identity it is also possible that these transcripts may code for different but related AKRs.

It was found that exon–intron junctions are conserved between the rat type 1 3α-*HSD*/*DD* gene (*AKR1C9*) and the human hepatic type 1 3α-*HSD*, type 2 3α-*HSD*, and type 3 3α-*HSD* genes in relation to their respective cDNA sequences. Rat liver 3α-HSD/DD and each human isoform possess both 3α-HSD and DD activity, and it is likely that since the genes that encode for these enzymes are structurally conserved they are evolutionarily related. This genomic organization is maintained across the AKR1C and AKR1B sub-families of the AKR superfamily. By comparing the genomic structures of the rat type 1 3α-*HSD*/*DD* gene and other AKRs we show that intron–exon junctions are conserved in five members of the AKR superfamily, including those for human placental aldose reductase (AKR 1B1) [24], human hepatic type 1 3α-HSD (AKR1C4) [25], human hepatic type 2 3α-HSD (AKR1C3) [25], human hepatic type 3 3α-HSD (bile acid binding protein; AKR1C2) [26,27] and mouse major vas deferens protein (AKR1B7) [28].

Comparative anatomy of proteins of the AKR superfamily shows that they all adopt an $(\alpha/\beta)_8$ barrel motif [29]. At the base of the barrel a highly conserved catalytic tetrad exists which corresponds to Tyr 55, Lys 84, Asp 50 and His 117. The first three residues are located on exon-2 and the remaining one is located on exon 3. Structures of family members also indicate that loops at the back of the barrel define substrate specificity, these loops are loop A, B and C. In the rat 3α-HSD structure, loop A extends from residues 119–144, loop B extends from 217–238 and the C-terminal tail residues extend from 304–322 [30]. These would correspond to nucleotides +357–+432, +651–+714 and +912–+966, respectively and do not correspond to single exons. Thus it is unlikely that substrate specificity arose by the formation of splice variants of exons encoding loop structures.

A human orthologue of the rat type 1 3α-*HSD*/*DD* gene, the human type 3 3α-*HSD* gene (bile-acid binding protein and dihydrodiol dehydrogenase 2, or DD2), has been cloned and sequenced [26,27]. The gene for a related human enzyme, DD1 (also 20α(3α)-

HSD), has also been cloned [26,31]. The human DD1 and DD2 cDNA's predict that the corresponding proteins differ by only seven amino acids. Because of their similarity the original cDNA for human DD2 was misassigned and when it was expressed it was found to encode for a recombinant protein whose properties resembled DD1 [26,27]. Nucleotide sequence comparisons were performed on the 5'-flanking region of the rat type 1 3α-*HSD/DD* gene (AKR1C9) and human *DD1* gene (AKR1C1) [21,26]. It was found that there was conservation of *cis*-elements between these two genes, including multiple steroid hormone response elements and AP-1 sites. Conserved transcription factor consensus sequences between these two genes combined with their similar genomic organization suggest that the two genes may share conserved mechanisms for their constitutive and regulated expression.

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