Biochem. J. (2001) **355**, 537–544 (Printed in Great Britain)

Co-operative regulation of the transcription of human dihydrodiol dehydrogenase (*DD*)*4*/aldo–keto reductase *(AKR)1C4* gene by hepatocyte nuclear factor (HNF)-4α/γ and HNF-1α

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Human dihydrodiol dehydrogenase (DD) 4/aldo–keto reductase (AKR) 1C4 is a major isoform of hepatic DD that oxidizes *trans*-dihydrodiols of polycyclic aromatic hydrocarbons to reactive and redox-active *o*-quinones and that reduces several ketone-containing drugs. To investigate the mechanism of transcriptional regulation of the human *DD4* gene, the 5′-flanking region of the gene was fused to the luciferase gene. The results of luciferase assays using HepG2 cells and of 1,10-phenanthroline-copper footprinting indicated that two positive regulatory regions were located in regions from −701 to −684 and from −682 to −666. The former region contained a putative hepatocyte nuclear factor (HNF)-4 binding motif, and the latter region contained an HNF-1 consensus binding sequence. DNA fragments of the HNF-4 or HNF-1 motif gave a shifted band in a gel-shift assay with nuclear extracts from HepG2 cells. The formation of the DNA–protein complex was inhibited by the HNF-4 or HNF-1 motif of the α_1-antitrypsin gene. A supershift assay using antibodies to human HNF-4α, HNF-4γ and HNF-1α showed that HNF-4α and HNF-4γ bound to the HNF-4 motif, and that HNF-1α interacted with the HNF-1 motif. Introduction of mutations into the HNF-4 or HNF-1 motif lowered the luciferase activity to 10 or 8% respectively of that seen with the intact human *DD4* gene. These results indicate that HNF-4α, HNF-4γ and HNF-1α regulate co-operatively the transcription of the human *DD4* gene in HepG2 cells.

Key words: footprinting, liver, nucleotide sequence.

INTRODUCTION

Dihydrodiol dehydrogenase (DD; EC 1.3.1.20) catalyses the oxidation of *trans*-dihydrodiols of polycyclic aromatic hydrocarbons (PAHs) and alicyclic alcohols, the reversible oxido-reduction of 3α-hydroxysteroids and prostaglandins, and the reduction of xenobiotic carbonyl compounds. From the toxicological point of view, the rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD)/DD was found to suppress the formation of the carcinogenic *trans*-dihydrodiol epoxides of PAHs by the oxidation of the *trans*-dihydrodiols [1]. However, the auto-oxidation of the PAH catecols to yield PAH *o*-quinones is anticipated to generate reactive oxygen species (ROS: hydroxyl radical, H_2O_2 and superoxide anion radical) [2,3]. ROS can lead either to the formation of oxidatively damaged bases or to an $OH^\bullet$-mediated strand scission which yields base propenals [4,5]. On the other hand, the resultant *o*-quinones are highly reactive Michael acceptors which can form both stable and depurinating DNA adducts [6,7]. Recently, it has been reported that human DDs also catalyse the oxidation of PAH *trans*-dihydrodiols [8]. Furthermore, human DDs play an important role in drug metabolism, since they are major reductases of several ketone-containing drugs such as ethacrynic acid, ketoprofen and loxoprofen that are administered therapeutically [9].

At least four forms of DD (DD1–DD4) are expressed in human livers. According to catalytic properties, human DD3 has been confirmed to be identical with an aldehyde dehydrogenase, and the other DDs with 3α- or 3(20)α-HSD [10,11]. Analysing the nucleotide and amino acid sequences and the function of enzymes expressed in *Escherichia coli* cells transformed with human DD2 or DD4 cDNA [12,13], it has been shown that human DD2 and DD4 are identical with human bile-acid-binding protein [14] and human chlordecone reductase/3α-HSD [15,16] respectively. According to the new nomenclature for the aldo–keto reductase (AKR) superfamily, human DD1, DD2 and DD4 are termed AKR1C1, AKR1C2 and AKR1C4 respectively [17]. Evidence for the existence of an additional human DD isoform {type II dihydrodiol dehydrogenase (type II DDH) [18]/type II 3α-HSD [19] or AKR1C3 [17]}, which resembles human DD1 and DD2, has also been reported.

About 40-fold inter-individual difference in DD activities has been noted in human livers [20]. Since human DD4 is a major form of DD [10], the increase in the expression level of human DD4 may lead to the enhancement of the bioactivation of PAHs and the metabolism of drug ketones, suggesting inter-individual differences in susceptibility to cancer and in the effect of several drugs. For understanding of (a) causal factor(s) which determines the expression level of DD4 mRNA in the liver, the transcriptional mechanism of the human *DD4* gene should be clarified. In the present study we isolated and characterized the 5′-flanking

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Abbreviations used: AKR, aldo–keto reductase; DD, dihydrodiol dehydrogenase (DDH has been used for the specific isoform type II dihydrodiol dehydrogenase); HNF, hepatocyte nuclear factor; 3α-HSD, 3α-hydroxysteroid dehydrogenase; MEM, minimum essential medium; PAH(s), polycyclic aromatic hydrocarbon(s); ROS, reactive oxygen species; α_1-AT α_1-antitrypsin.

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The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, GSDB and GenBank® Nucleotide Sequence Databases under the accession number D89962.

region of the human *DD*4 gene. We provide lines of evidence that the transcription factors hepatocyte nuclear factor (HNF)-4α/γ and HNF1α are major determinants in the transcription of this gene.

MATERIALS AND METHODS

Sequence analysis of the 5′-flanking region of the human *DD4* gene

A λFIX® II (Stratagene) human genomic DNA library was prepared from the total genomic DNA of a Japanese subject. Approx. 1×10^6 plaques were screened with the *Eco*RI–*Pst*I fragments of human DD4 cDNA from plasmid pKKDD4 [21] as probes. Four positive clones were obtained. One of these clones, λ4, was digested with the restriction enzymes *Bam*HI, *Eco*RV, *Eco*T221, *Pst*I, *Pvu*II, *Sca*I or *Sma*I. After agarose-gel electrophoresis of the digests, the separated DNA fragments were blotted on to a nylon membrane (Nytran; Schleicher und Schüell, Dassel, Germany). The membrane was hybridized with the ^{32}P-labelled oligonucleotide +32/+52 (see Table 1 for the sequence) as a probe. The hybridized 2.2-kb *Sca*I fragment was subcloned into the *Sma*I site of pBluescript® II KS(−) (Stratagene). This clone (named 'λ4-*Sca*I BS') was used for subsequent analyses.

Table 1 Synthetic oligonucleotides used for screening, chimaeric plasmid construction, gel-shift assay and mutagenesis

Oligonucleotide*	Sequence†
+32/+52	5′-ATCCCAAATATCAGCGTGTAG-3′
−373/−352	5′-GGGATATCATCATGGCATGAAC-3′
+11/+28 *Hin*dIII	5′-CCCAAGCTTGCTTGCCACTTCTTTC-3′
Foot A	
Sense	5′-TGATGTCCAAAGTCCAAACATT-3′
Antisense	3′-ACTACAGGTTTCAGGTTTGTAA-5′
Foot Am	
Sense	5′-TGATGTCCttAGTCgAAACATT-3′
Antisense	3′-ACTACAGGaaTCAGcTTTGTAA-5′
α_1-AT-A	
Sense	5′-GCCAGTGGACTTAGCCCCTG-3′
Antisense	3′-CGGTCACCTGAATCGGGGAC-5′
Foot B	
Sense	5′-ACATTGTTAATAATTAATACTCC-3′
Antisense	3′-TGTAACAATTATTAATTATGAGG-5′
Foot Bm	
Sense	5′-ACATTGTTAggAAggAATACTCC-3′
Antisense	3′-TGTAACAATccTTccTTATGAGG-5′
α_1-AT-B	
Sense	5′-CCTTGGTTAATATTCACCAGCA-3′
Antisense	3′-GGAACCAATTATAAGTGGTCGT-5′
Foot A+B	
Sense	5′-TGATGTCCAAAGTCCAAACATTGTTAATAATTAATACTCC-3′
Antisense	3′-ACTACAGGTTTCAGGTTTGTAACAATTATTAATTATGAGG-5′
Foot Am+B	
Sense	5′-TGATGTCCttAGTCgAAACATTGTTAATAATTAATACTCC-3′
Antisense	3′-ACTACAGGaaTCAGcTTTGTAACAATTATTAATTATGAGG-5′
Foot A+Bm	
Sense	5′-TGATGTCCAAAGTCCAAACATTGTTAggAAggAATACTCC-3′
Antisense	3′-ACTACAGGTTTCAGGTTTGTAACAATccTTccTTATGAGG-5′
Foot Am+Bm	
Sense	5′-TGATGTCCttAGTCgAAACATTGTTAggAAggAATACTCC-3′
Antisense	3′-ACTACAGGaaTCAGcTTTGTAACAATccTTccTTATGAGG-5′
M13 −21	5′-GTAAAACGACGGCCAGT-3′
M13 reverse	5′-GGAAACAGCTATGACCATG-3′

* α_1-AT-A and α_1-AT-B were synthesized according to the sequence of the human α_1-antitrypsin gene; m, mutant; sense, coding strand; antisense, complimentary strand.

† Additional *Hin*dIII site is underlined; the mutated nucleotide sequences are depicted with lower-case letters; the reported transcriptional start site [19] is assigned as +1 for the human *DD4* gene.

Sequencing reactions were performed with the ABI PRISM™ Dye Primer Cycle Sequencing Kit (Perkin–Elmer) according to the dideoxy chain-termination method [22]. The sequences were analysed by an ABI PRISM™ 377 DNA sequencer (Perkin–Elmer).

Construction of reporter plasmids

A plasmid pDD4 −2220/+28, containing human *DD4* gene sequences from −2220 to +28 and the luciferase gene, was constructed by ligation of the following three DNA fragments: (i) an *Eco*RV–*Hin*dIII fragment (from −368 to +28) obtained by means of PCR using oligonucleotide primers, −373/−352 and +11/+28 *Hin*dIII (Table 1), and the λ4-*Sca*I BS as a template; (ii) a 1861-bp *Pst*I–*Eco*RV fragment from the λ4-*Sca*I BS, whose *Pst*I site was blunt-ended and ligated with an *Xho*I linker (d(pCCTCGAGG); New England Biolabs, Beverly, MA, U.S.A.); and (iii) a 4799-bp *Xho*I–*Hin*dIII fragment from a luciferase reporter plasmid, PicaGene™ Basic Vector 2 (Toyo Ink, Tokyo, Japan). A plasmid pDD4 −980/+28 was constructed as follows. First, the pDD4 −2220/+28 was digested with *Sty*I and blunt-ended with T4 DNA polymerase (Takara, Osaka, Japan). Then this fragment was ligated with an *Xho*I linker. This intermediate plasmid was digested with *Xho*I and self-ligated. A series of 5′-deletion constructs, pDD4 −703/+28, pDD4 −692/+28, and pDD4 −667/+28, was generated from the pDD4 −980/+28 by the nested deletion method [23]. Another series of constructs, pDD4 Foot A+B:−95/+28, pDD4 Foot Am+B:−95/+28, pDD4 Foot A+Bm:−95/+28 and pDD4 Foot Am+Bm:−95/+28, was constructed by ligation of the following three DNA fragments: (i) each of the double-stranded oligonucleotides, Foot A+B, Foot Am+B, Foot A+Bm and Foot Am+Bm (Table 1); (ii) a 123-bp *Nsp*I–*Hin*dIII fragment from the pDD4 −667/+28, whose *Nsp*I site was blunt-ended; and (iii) a 4765-bp *Sma*I–*Hin*dIII fragment from PicaGene™ Basic Vector 2. A plasmid pDD4 −95/+28 was constructed by ligation of fragments (ii) and (iii) above. All plasmids were verified by restriction-enzyme mapping and DNA sequencing.

Cell culture

HepG2 cells were maintained at 37 °C in 5% CO_2 with Eagle's minimum essential medium (MEM; Nissui Pharmacy, Tokyo, Japan) containing 10% (v/v) fetal-bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1 × MEM non-essential amino acids (ICN) and 1 mM sodium pyruvate (Gibco BRL).

Transfection and luciferase assay

HepG2 cells were seeded at a density of 2×10^6 cells/60-mm-diameter tissue-culture dish 18 h prior to transfection. The cells were transfected with a test plasmid (5 μg) and a β-galactosidase expression plasmid (1 μg), pCH110 (Amersham Pharmacia Biotech), using the calcium phosphate method [24]. At 4 h after transfection, the cells were shocked with 20% (v/v) glycerol for 3 min, and then fed on culture medium (4 ml). After incubation for 40 h, the cells were harvested. The luciferase activity in the cell lysates was assayed using the PicaGene™ luciferase assay system (Toyo Ink) according to the manufacturer's instructions. The light output was measured for 10 s by a Lumat LB9501 luminometer (Berthold, Pforzheim, Germany). β-Galactosidase

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ACTTCAAAAG GGAGAAAGGT GGAGACAGGG GCAAAGATGA CAAAACTTCC TATTGGGTAC AATGTTCACT ATTTAAGTGA TGTGTACACC AGAGGCCCAA -2121
ACCTCACCAG TATGTAATGT GTCTATATAA CAAACCTACA CATGTGCCTA TTTCAATCTA AAATAATTTT TAAGACTTTA ATAAGAAATA AAAATCCGAA -2021
AGTCTTTCCT TACTAATATC AATGCCTGTA ATTCTTCACA GTTTGTCAGT TTATTTTCTT CCTCATAATG AACGCTGTTT TCCTGTTTCT TTGTAGCTTT -1921
GTTATTGTTT TTATCGCACA TTTGAAAAAC AGCCACATTT CATAATCTAT ATATATTCTG TGTCATGACA ATGATCAGCT AATATGCTTG GCATATTCTT -1821
AAGTCTCGAC ATCAGCCCTA CATGAAAACT TAAGGTCTTC TCAGTTCTTT TCTGAACGTG CATCTGCCTA GACTCTGTGT GCCCTATTTG ATTTCTCCAA -1721
ATACCCAATG GTTTTGAATA CCTTATCATG TCAAAAATTC ACAGCTCAGC TTCTCCTAAG TGCCATAGAT GGCCTATTGT ATGTCTCTTC CCCCTAATCT -1621
CTTGCCAGTG GCATCTGTGC ATCTATAGTC ACCCTGCATC TCTACTGAGC CACTCTATAA ATAAATGAGT TTATTTTAGT TCATTTCAGA TTGTTTTTTG -1521
ATAATTCCAT GGGAGATTTG AGAGCTTTCT AATCTACCAT CCTGCTGATG ACACTCTGTG TGAAAATTTT TAAATGGCTC TATTATATTC CATTGCTTCA -1421
ATGTATAGTA ATTTACCTAA TTATTTTCTT TTATTGTTCC TTTTAGGAAG AAGTGAATAG AAGAAAAGAG GGTGTTGCAA ATCAAAAGCA CATAGAGATA -1321
                                                                                                       EcoR V
TCATCTTACA CCAGTCAGAA TGGCTATTCT TAAAATGTCA GACAACAACA GATATTGGTA TGGATTCTTC ACAGATGTTA ATCATCTTCG TTTTTTGTTT -1221

TTTGTTTTTT TTTTTTTTTT GAGACAGTCT TGCTCTGTCG CCCAGGCTGG AGTGCAGTGA CCCAATCTTG GCTCACTGCA ACCTCTGCCT CCTGAGTTTA -1121

AGCAATTGTC TTGCCTCAGC CTCCTGAGTA ACTGGGATTA CAGGTATCCA CTACCACTCC AGACTAATTT TTGTATTTTT CGTAGAGACA AGATTTCACT -1021

TGATTGGTCT CAAACTCCTG ACCTCAAGTA AACCTACCGC CTTGGCCTTC CAAAGTGCTG GGGTTACAGG CGTGAGCCAC TGCCCCCGGC CTAATCATCG  -921
                                             Sty I
TGTTCTAGTC TGCCCTCCAA TGGGAGAAAC AAGTAAAATT ATGCCATGTG AGGATTATTC ACCAATTTAT TTTAATTACT TTTTTCTTTA TAACATTTAA  -821

TAAGATCACA AATTATATAA AATAGTTATC AGCTTTTTGG GAAGTTACTT TTGCTGGTTT CTTATAAAAC TGATGGAAGA TACAAACACT ATTAAAGAAC  -721
                                   A                  B
TGTTTGCATG TTGCAAATGA TGTCCAAAGT CCAAACATTG TTAATAATTA ATACTCCAAT AAACATCATG TCAGAATTTC TGTTTTCTTT TCCCTTTGAA  -621
                                 HNF4                HNF1
CCTTTGCAGG ATTACCACAT CATCAGGACC ACACCTTCAT CAGGAATGAA TATTCCTACT ACAATTAAAG AAGAAACAAA ATTAATTTGT TGGTGAAAAA  -521

CGTAAAAAGA GAAATTTTCT TTGGTTTTGT TTAATTTCTT TATTGAGGGT CACCACTAAA AAAAATGCTC ACTGGTCATT CTTTTGAATA CTGTCTGAGA  -421
                                                   Eco065 I
GAAAGATGTA AGATGGTTGA TTATTTCAAA TGACAGAAGA TAAAGATGGG ATATCATCAT GGCATGAACA AAAACAAGAT TTGTAGCTGG AGGTATTTTA  -321
                                                     EcoR V
TAGTCTAACA TGATCACCAA TCATTCTATA AACCTGTTGG ATGAGTTTAT CAGACAGACA GAGAGAGAGA TTGATTGATT CTGAATAGAA AATTTCACTT  -221

TAGAAAAAAA TATTTTGACT ATATAATAAT GTATGTAAAA ATTCTCTTTG ATAAGAAACG AGTGAACTGG ATTCAATTTT CCTCACAGCC TGTGTAATAC  -121

ACCATCACTT GCTTCCTCCT ACATGCCATT GATTAGCCCC AGGGAGCAGT GCAGCACTGC CTGCCCATGT TTTACATAAC CCCTGAATAT AAATGCCAGA   -21
                       Nsp I                                                                 TATA BOX

TGTTGCTGAA GGAAACAGGA TCTGCTTAGT GAAAGAAGTG GCAAGCAATG GATCCCAAAT ATCAGCGTGT AGAGCTAAAT GATGGTCACT TCATGCCCGT    80
                                                   Met AspProLysT yrGlnArgVa lGluLeuAsn AspGlyHisP heMetProVa    18

ATTGGGATTT GGCACCTATG CACCTCCAGA GGTAATAATC ACATTTTCAG CATTGAGCAT TTAAAAGAGC AAAGCTAGAA TAAGTGAACG ATGACCTGGG   180
lLeuGlyPhe GlyThrTyrA laProProGl u                                                                              28

TTGTTCAGCT TTGTGTTTCT GTTACCCTGA GTGACTCACG TGGTCTGTCT TACTGGGCTA GAGCTATTCT ATGTTCAAAG AGAAAAGGTA GTCAATGTTT   280

GTTTTGCACT GAGGTCTGTC ATATGGTAAT TTACTGCTTA TTTTTATTTT ATACACTGTT TTTATATGCT GTTTCTGTGT ATTGCCCAGC TTGGCAGAAT   380

ATATAAAACT CAACAGTGAA GAACACTGCC TGGCAGTTCC CTTTCTAGGA GATGACTGCA G                                            441
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Figure 1 Nucleotide sequence of the 5′-flanking region of the human DD4 gene from −2220 to +441

The sequence of the 2661-bp *Sca*I–*Pst*I fragment of the human *DD4* gene is shown. Nucleotides and amino acid position numbers are shown on the right. The reported transcriptional start site [19] is assigned as +1 (➤). The coding region begins from +28, and is shown as the translated amino acid sequence (*italic*) under the nucleotide sequence. Restriction-enzyme sites used in this study are underlined. Putative *cis*-acting elements and TATA box are boxed: HNF-4, a putative HNF-4-binding site; HNF-1, a putative HNF-1-binding site. The spans of regions A and B are overlined (see Figure 3).

activity in the cell lysates was determined in duplicate as a control for transfection efficiency [25].

Preparation of nuclear extracts and 1,10-phenanthroline-copper footprinting

Nuclear extracts were prepared from HepG2 cells according to the method of Dignam et al. [26]. 1,10-Phenanthroline-copper footprinting was carried out as described in [27,28], with minor modifications. Briefly, DNA fragments were labelled at a unique end using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (185 TBq/mmol; Amersham). DNA binding reactions were carried out in a total volume of 50 μl containing 22 mM Hepes/NaOH (pH 7.9) 60 mM KCl, 1 mM $MgCl_2$, 0.12 mM EDTA, 1.3 mM dithiothreitol, 0.3 mM PMSF, 12% (v/v) glycerol, salmon sperm DNA (80 μg) (Nippon Chemical Feed, Tokyo, Japan), and the nuclear extracts from HepG2 cells (180 μg). After incubation on ice for 15 min, a probe DNA (2.5×10^5 c.p.m.) was added, and then the mixture was incubated at 24 °C for 30 min. DNA–protein complexes and free probes were separated on a non-denaturing 4%-(w/v)-polyacrylamide gel in 0.5×TBE (25 mM Tris/borate/1 mM EDTA). The gel was electrophoresed at 150 V for 1.5 h at room temperature. The wet gel was then immersed in 200 ml of 10 mM Tris/HCl, pH 7.5, containing 45 μM $CuSO_4$, 0.2 mM 1,10-phenanthroline and 4.7 mM 3-mercaptopropionic acid for 30 s at room temperature. The reaction was quenched by addition of 2,9-dimethyl-1,10-phenanthroline (final concn. 2.3 mM), and the resulting mixture was incubated for 2 min. The wet gel was exposed to an X-ray film followed by development. The gel pieces containing the free and bound probes were excised and eluted overnight at 37 °C in 10 ml of an elution buffer [100 mM Tris/HCl (pH 7.5)/100 mM NaCl/1 mM EDTA]). DNA fragments thus eluted were purified by a DE52 (Whatman) column chromatography [29] and ethanol precipitation, and then resuspended in a loading buffer containing 95% formamide, 15 mM EDTA, 0.095% Bromophenol Blue and 0.095% Xylene Cyanol. The samples were electrophoresed at a constant power of 50 W on a denaturing

6 %-(w/v)-polyacrylamide gel followed by autoradiography. Electrophoresis marker (G+A) was prepared by the Maxam–Gilbert method [30].

Gel-shift assay

The DNA binding reaction was performed in a total volume of 20 μl as described above, except that poly[d(I-C)] (0.5 μg) (Boehringer Mannheim) instead of salmon sperm DNA and the nuclear extracts (6 μg) from HepG2 cells were used. A 100-fold molar excess of an unlabelled probe DNA was added for competition assays. After incubation on ice for 15 min, end-labelled probe DNA (2.0×10^4 c.p.m.) was added, and then the reaction mixture was incubated at 24 °C for 30 min. The samples were electrophoresed on a non-denaturing 4 %-(w/v)-polyacrylamide gel in 0.5 × TBE at 150 V for 1 h at room temperature, and the gel was autoradiographed.

Supershift assay

Antibodies to human HNF-4α, HNF-4γ, HNF-1α and HNF-1β were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The supershift assay was performed as follows: after incubation of probe DNA with nuclear extracts form HepG2 cells, antibodies were added to the reaction mixture and further incubated at 4 °C for 1 h. The products were then analysed by a gel-shift assay.

Site-directed mutagenesis

Mutagenesis was performed by PCR as described [31]. An *Eco*RV fragment from the pDD4 −2220/+28 (from −1321 to −369 of the human *DD4* gene) was subcloned into the *Sma*I site of pBluescript® II KS(−), and the resulting plasmid was used as a template. Mutations in the region A (see Figures 1 and 3) were introduced by a sequential PCR. Two products were amplified by the first PCR using two pairs of primers: Foot Am (sense) and M13 Reverse, and Foot Am (antisense) and M13 −21 (Table 1). Then a fragment (from −1321 to −369 with mutations in region A) was generated by a second PCR using the two PCR products obtained as described above and a pair of primers, M13 −21 and M13 Reverse. This fragment was digested with *Bam*HI and *Pst*I, and then subcloned into the *Bam*HI–*Pst*I fragment of pBluescript® II KS(−) (Foot Am BS). To construct pDD4 −2220/+28 Foot Am, an *Eco*O65I–*Sty*I fragment of pDD4 −2220/+28 (from −976 to −473) was replaced by a 504-bp *Eco*O65I–*Sty*I fragment of Foot Am BS. The same procedure was employed to construct pDD4 −2220/+28 Foot Bm, using two pairs of primers, Foot Bm (sense) and M13 Reverse and Foot Bm (antisense) and M13 −21 (Table 1). A plasmid pDD4 −2220/+28 Foot Am+Bm was constructed by the introduction of mutations to region B of the Foot Am BS. All plasmids were verified by DNA sequencing.

RESULTS

Analysis of the sequence of the 5′-flanking region of the human *DD4* gene

Approx. 1×10^6 plaques from λFIX® II human genomic DNA library were screened with the 513-bp fragment of human DD4 cDNA containing exons from 1 to 5 as a probe. Four positive clones (λ1–4) were obtained. Southern-blot analysis with an oligonucleotide +32/+52 revealed that the 2.8-kb *Sca*I fragment of clone λ4 contained the exon 1 and the 5′-flanking region of the human *DD4* gene (results not shown). Therefore this clone was used for further experiments.

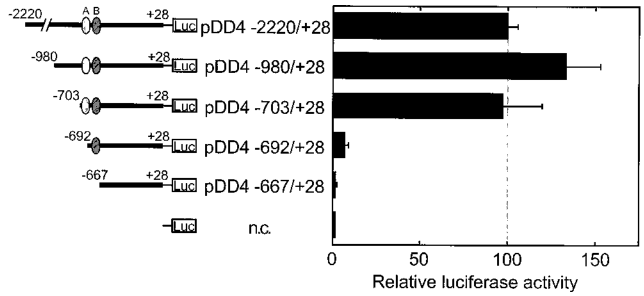

Figure 2 Transcriptional activity of the 5′-flanking region of the human *DD4* gene in HepG2 cells

A schematic representation of the human *DD4* 5′-flanking regions that are fused with the luciferase gene is depicted on the left. The lighter and darker shaded ellipses shown in the diagrams on the left indicate the footprinted regions A and B respectively (Figure 3). The histogram on the right shows the relative luciferase activity of each deletion construct which is normalized against a β-galactosidase activity and indicated as the percentage of the activity with pDD4 −2220/+28. The data shown are from three independent transfections (means ± S.D.). Luc, luciferase gene; n.c., negative control (Basic Vector2).

The nucleotide sequences of the 5′-flanking region, exon 1 and intron 1 were analysed (Figure 1). The sequence of exon 1 was completely identical with the corresponding region of human DD4 cDNA [12,15]. The 5′-flanking sequence of the human *DD4* gene was 96.7 % identical with that of the human *type I* 3α-*HSD* gene (up to −425) [19]; there were 15 nucleotide differences in these regions.

5′-Deletion analysis of the human *DD4* promoter in HepG2 cells

To identify the sequences responsible for the transcriptional activity of the human *DD4* gene, a series of 5′-truncated human *DD4* promoter–luciferase reporter plasmids was constructed, and then transfected into human hepatoma HepG2 cells. The transcriptional activities of these deletion mutants were measured by the activity of transiently expressed luciferase (Figure 2). The elimination of sequences from −2220 to −704 did not affect the luciferase activity in HepG2 cells, although the removal of sequences from −703 to −693 resulted in the 90 % reduction of the transcriptional activity. Sequential deletion down to −668 reduced the activity close to a basal activity seen with the negative control plasmid (Basic Vector 2).

1,10-Phenanthroline-copper footprinting in a region from −703 to −570 of the human *DD4* gene

Deletion analysis indicated that the two regions (from −703 to −693 and from −692 to −668) contained *cis*-acting elements. In addition, we detected two shifted bands (band U, the upper band; band L, the lower band) by a gel-shift assay using ^{32}P-labelled probes containing sequences from −703 to −570 of the human *DD4* gene (−703/−570) (Figure 3A). Therefore, we investigated the interaction of the fragment −703/−570 with a nuclear protein(s) present in HepG2 cells by 1,10-phenanthroline-copper footprinting (Figure 3B). In lane L (derived from the band L), a region A (from −701 to −684) was protected from 1,10-phenanthroline-copper digestions. This region A contains a sequence that resembles the putative HNF-4 binding site (Table 2) [32]. In lane U (derived from the band U), a nuclear protein(s) bound to a region B (from −682 to −666). This region B includes a sequence that shows considerable similarity to the consensus sequences of HNF-1 (Table 2) [33]. The spans of these footprints are schematically shown in Figure 1 (overlined regions

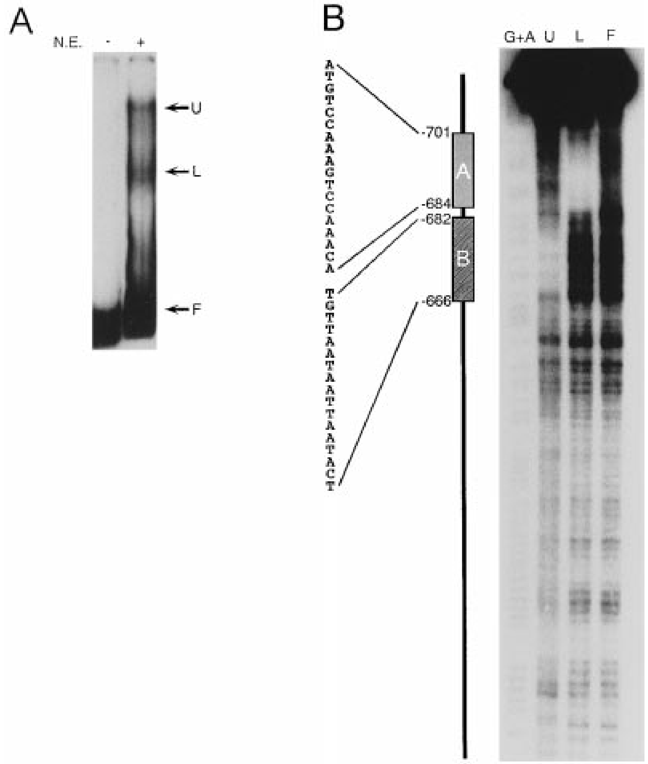

Figure 3 1,10-Phenanthroline-copper footprinting of the 5′-flanking region of the human *DD4* gene with nuclear extracts prepared from HepG2 cells

The probe containing a human *DD4* sequence from −703 to −570 was ^{32}P-labelled at the 5′-terminus of the complementary strand and was used in a gel shift assay (**A**). *In situ* cleavage was performed as described in the Materials and methods section, then the products were electrophoresed (**B**). Lane G+A is a chemical G+A sequencing ladder using the footprinting probe DNA. Lanes U, L, and F are the products of footprinting reactions derived from the upper band (U), the lower band (L), and the free DNA probe (F) in an electrophoresed gel matrix respectively. Footprinted regions A and B are portrayed alongside the autoradiograph, with spans and nucleotide sequences (coding strand).

Table 2 Comparison of the sequences among putative *cis*-acting elements in the human *DD4*, *type II DDH* and α_1-AT genes

Gene*	Position	Sequence†
Human *DD4*	−698/−687	`TcCAAAGTCCAa`
Human *type II DDH*	−658/−647	`TcCAAAcTCCAa`
Human α_1-AT	−111/−122	`GGCTAAGTCCAC`
HNF-4 consensus		`GGCAAAGGCCAT` `T  T G TT  C`
Human *DD4*	−681/−669	`GTTAATAATTAAt`
Human *type II DDH*	−641/−629	`GTTAATAATTAAC`
Human α_1-AT	−74/−63	`GTTAAT-ATTcAC`
HNF-1 consensus		`GTTAATNATTAAC`

* The consensus sequences of the HNF4 and HNF1 recognition sites were derived from reported data [32,33].

† The nucleotide which differs from the consensus sequence is indicated with a lower-case letter.

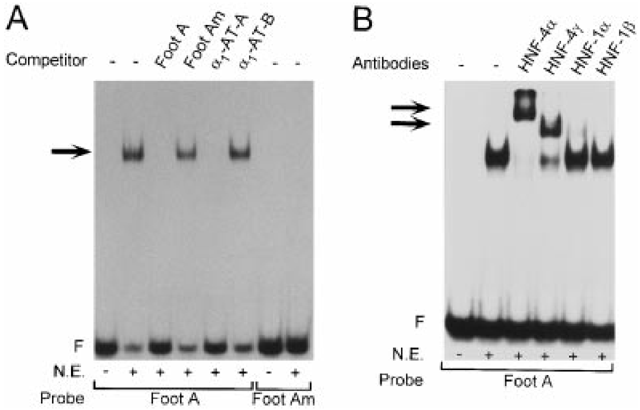

Figure 4 Gel-shift and supershift assays with oligonucleotides derived from region A of the human *DD4* gene

(**A**) Approx. 2.0×10^4 c.p.m. of double-stranded oligonucleotides, Foot A (5′-TGATGTCCA-AAGTCCAAACATT-3′) and Foot Am (5′-TGATGTCCttAGTCgAAACAT T-3′, in which the mutated nucleotide sequences are depicted with lower-case letters) were used as probes in gel-shift assays with or without a 100-fold molar excess of a competitor as indicated. Arrow indicates the DNA–protein complex. (**B**) ^{32}P-labelled double-stranded Foot A was incubated with nuclear extracts from HepG2 cells in the presence or absence of antibodies as indicated in the Figure. Arrows indicate the supershifted band generated with antibodies to HNF-4α and HNF-4γ. The oligonucleotides used in the gel shift assays are shown in Table 1. F, free DNA probes; N.E., nuclear extracts from HepG2 cells.

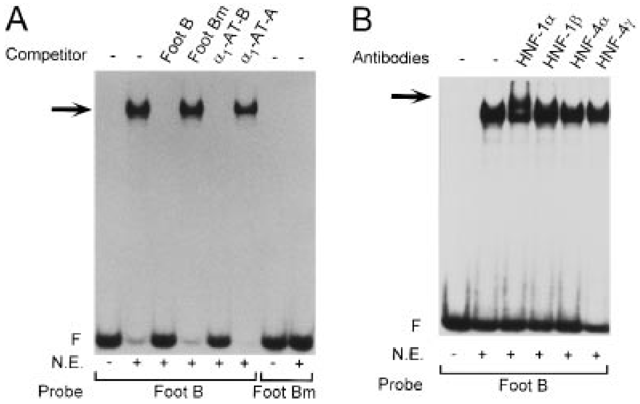

Figure 5 Gel-shift and supershift assays with oligonucleotides derived from region B of the human *DD4* gene

(**A**) Approx. 2.0×10^4 c.p.m. of double-stranded oligonucleotides, Foot B (5′-ACATTGTTAA-TAATTAATACTCC-3′) and Foot Bm (5′-ACATTGTTAggAAggAATACT CC-3′, in which the mutated nucleotide sequences are depicted with lower-case letters) were used as probes in the gel-shift assays with or without a 100-fold molar excess of a competitor as indicated. The arrow indicates the DNA–protein complex. (**B**) ^{32}P-labelled double-stranded Foot B was incubated with nuclear extracts from HepG2 cells in the presence or absence of antibodies as indicated in the Figure. Arrows indicate the supershifted band generated with antibodies to HNF-1α. The oligonucleotides used in gel shift assays are shown in Table 1. F, free DNA probes; N.E., nuclear extracts from HepG2 cells.

A and B). We designated the region from −703 to −682, which included region A, as Foot A, and named the region from −686 to −664, which contained region B, as Foot B (Table 1). We used these regions for further experiments as oligonucleotide probes.

Binding of HNF-4α and HNF-4γ to region A

We performed a gel-shift assay to identify (a) factor(s) that binds to the sequence within region A (Figure 4A). We detected a shifted band when a probe Foot A was incubated with nuclear

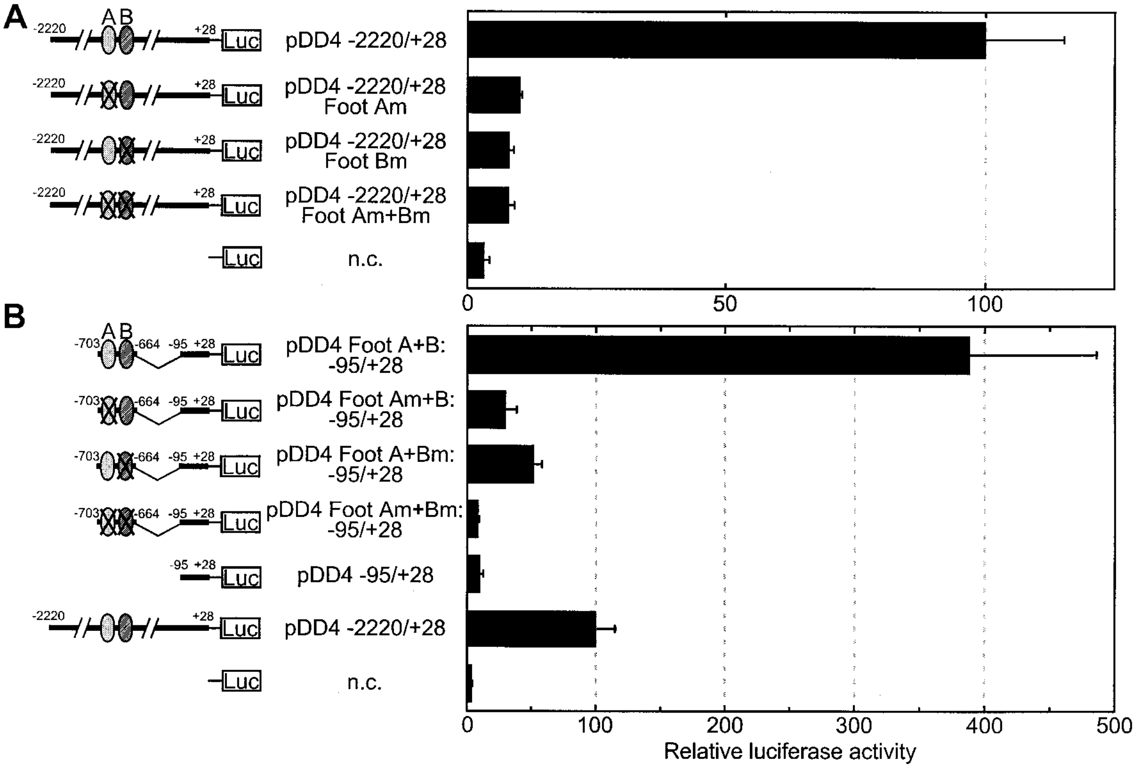

Figure 6 Effects of mutations in regions A and B on the transcriptional activity of the 5′-flanking region of the human *DD4* gene assayed as a luciferase activity using HepG2 cells

A construct which contained the wild-type or the mutated sequences of the 5′-flanking region of the human *DD4* gene from −2220 to +28 (5 μg) was co-transfected with pCH110 (1 μg) into HepG2 cells (**A**). The same experiments were performed using a series of constructs in which a wild-type or mutated sequence of the region from −703 to −664 (Foot A+B, Foot Am+B, Foot A+Bm or Foot Am+Bm; the sequences are shown in Table 1) was placed in front of the human *DD4* promoter region (−95 to +28) (**B**). The luciferase activity of each construct is normalized against a β-galactosidase activity and indicated as a percentage of the activity seen with pDD4 −2220/+28. The lightly and darkly shaded ellipses shown in the diagrams on the left indicate the footprinted regions A and B (Figure 3), and the cross indicates a region to which a mutation was introduced. Results are from three independent transfections (means±S.D.). Luc, luciferase gene; n.c., negative control (Basic Vector2).

extracts prepared from HepG2 cells. The formation of this complex was inhibited by the presence of a 100-fold molar excess of either unlabelled Foot A or α_1-AT-A (the HNF-4 binding site of the human α_1-antitrypsin gene) [34], while a 100-fold molar excess of unlabelled α_1-AT-B (the HNF-1 binding site of the human α_1-AT gene) [34] did not compete with Foot A for the protein binding. We synthesized a mutated probe, Foot Am (the positions of the mutations are indicated in Table 1) and examined the effects of the introduction of mutations. In the gel-shift assays with the labelled probe Foot A, the formation of the complex was not inhibited by the presence of a 100-fold molar excess of unlabelled Foot Am. Furthermore, a labelled probe Foot Am did not form any complexes with nuclear extracts of HepG2 cells (Figure 4A). A supershift assay using antibodies to human HNF-4α, HNF-4γ, HNF-1α and HNF-1β showed that this Foot A–nuclear protein complex contained HNF-4α and HNF-4γ (Figure 4B). Neither HNF-1α nor HNF-1β was a component of this complex.

Binding of HNF-1α to region B

We carried out a gel-shift assay using a probe Foot B, and detected a shifted band when the probe was incubated with nuclear extracts of HepG2 cells (Figure 5A). Foot B was efficiently out-competed with a 100-fold molar excess of unlabelled Foot B or α_1-AT-B for the binding to the nuclear protein(s), but did not compete with the unlabelled α_1-AT-A. Using a mutated probe, Foot Bm (the positions of the mutations are indicated in Table 1), the Foot Bm did not form any complexes with nuclear extracts of HepG2 cells (Figure 5A). This Foot B–nuclear protein complex was partially supershifted by the presence of antibodies to human HNF-1α (Figure 5B). Antibodies to human HNF-1β, HNF-4α or HNF-4γ did not affect the electrophoretic mobility of this complex, indicating that these factors were not bound to region B.

Mutation analysis of regions A and B

To investigate the effects of modification of the sequence of regions A and B on the luciferase activity, we constructed three reporter plasmids, pDD4 −2220/+28 Foot Am, pDD4 −2220/+28 Foot Bm and pDD4 −2220/+28 Foot Am+Bm. Plasmids, pDD4 −2220/+28 Foot Am and pDD4 −2220/+28 Foot Bm contained the region from −2220 to +28 of the human *DD4* gene with the same mutations introduced in Foot Am and Foot Bm respectively. The plasmid pDD4 −2220/+28 Foot Am+Bm possessed the mutations in both regions A and B. As shown in Figure 6(A), mutations within region A or B resulted in a 90 or 92% decrease in the transcriptional activity relative to the activity of pDD4 −2220/+28. A similar result was obtained when the plasmid pDD4 −2220/+28 Foot Am+Bm was transfected into HepG2 cells. Thus, to elucidate whether or not

HNF-4α, HNF-4γ and HNF-1α activate the transcription of the human *DD4* gene, we constructed another series of reporter plasmids containing regions A and B, which was fused to the promoter region of the human *DD4* gene (the region from −95 to +28). Luciferase assays were performed with these constructs to determine the function of regions A and B as an enhancer (Figure 6B). A plasmid pDD4 −95/+28 yielded transcriptional activity close to basal activity seen in HepG2 cells transfected with the negative control plasmid (Basic Vector 2). When the fragment Foot A+B (the region from −703 to −664) was inserted upstream of the promoter, the transcriptional activity was increased 40-fold relative to pDD4 −95/+28. Introduction of a mutation within Foot A or Foot B (Foot Am+B or Foot A+Bm was fused to the promoter region of the human *DD4* gene) resulted in a reduction of the transcriptional activity to 8 or 13% of the activity with pDD4 Foot A+B:−95/+28. Furthermore, mutations within both regions A and B reduced the activity to the level seen with pDD4 −95/+28. On the basis of these lines of evidence, it seemed reasonable to assume that liver-enriched nuclear factors, HNF-4α, HNF-4γ and HNF-1α, modulate co-operatively the expression of the human *DD4* gene.

DISCUSSION

To date, the results of analysis for the promoters of the human *type II DDH/type II 3α-HSD* and the rat *3α-HSD/DD* genes have been reported [18, 35]; the expression of these two DDs is not restricted within the liver [19,36]. To our knowledge, no information has been reported on the mechanism of the transcriptional regulation of the human *DD4* gene. Thus this is the first study to characterize the 5′-flanking region of the human *DD4* gene.

The sequence of the clone λ4 showed 15 nucleotide differences in the 5′-flanking region (up to −425) compared with the published sequences of the genomic clone λKQ8 [19], which was believed to encode the human *type I 3α-HSD/DD4* gene. Since the existence of at least one pseudogene of DD4 has been reported (as a human chlordecone reductase) [15], we cannot conclude whether these two clones are derived from different genes or are variants of the same gene. However, λKQ8 has three substitutions at positions 42 (C → T), 405 (A → G), and 406 (A → T) (the first base of the initiation codon, ATG, is assigned +1) compared with human DD4 cDNA. Moreover, the substitution at position 406 causes an in-frame TAA (nonsense) triplet in exon 4. Therefore λKQ8 might be derived from another pseudogene.

The deletion analysis and 1,10-phenanthroline-copper footprinting revealed that nuclear factors interacted with the regions from −701 to −684 (region A) and from −682 to −666 (region B) (Figures 2 and 3). Regions A and B contained sequences that were similar to the consensus sequences of liver-enriched factors, HNF-4 and HNF-1, respectively (Table 2). Our supershift assay indicated that region A was recognized by HNF-4α and HNF-4γ (Figure 4B). In addition, the Foot B–nuclear factor complex contained HNF-1α (Figure 5B). However, the bands were only partially supershifted by the presence of antibodies to HNF-1α when Foot B was used as the probe. Thus another factor(s) is assumed to contribute to the regulation of the human *DD4* gene in addition to the HNFs identified here.

We found that both HNF-4α/γ and HNF-1α are necessary factors for the transcriptional activation of the human *DD4* gene. So far, positive interactions between HNF-4 and HNF-1 units have been seen in the rat *HNF-1α* gene promoter [37]. Deletion of the HNF-4 binding sequence or the introduction of mutation within the HNF-1 binding site resulted in a 98 or 53% decrease in the transcriptional activity relative to the intact 5′-flanking region of the rat *HNF-1α* gene [37]. Unlike the rat *HNF-1α* gene, mutation in the HNF-1-binding region of the human *DD4* gene reduced the transcriptional activity to 8% (Figure 6). Thus it seems that the synergistic interactions among HNF-4α, HNF-4γ and HNF-1α occurred in regions A and B of the human *DD4* gene, but not in that of the rat *HNF*-1α gene. There are differences in the structure of the human *DD*4 and rat *HNF-1α* genes in their HNF-4- and HNF-1-binding sequences: (i) the HNF-4 and HNF-1 units of the human *DD4* gene are located in the distal promoter region, whereas those of the rat *HNF-1α* gene are present in the proximal promoter; and (ii) the distance between the HNF-4 and HNF-1 recognition sites of the human *DD4* gene is shorter than that of the rat *HNF-1α* gene (5 bp and 19 bp respectively). Further experiments will be required to confirm if these differences are responsible for the difference in the function of adjacent HNF-4- and HNF-1-binding sequences between the two genes.

Interestingly, regions A and B are highly conserved in the 5′-flanking region of the human *type II DDH/type II* 3*α-HSD* gene (Table 2) [18]. In the human *type II DDH* gene, the sequence from −658 to −647 is almost identical with the HNF-4 consensus sequences, and the sequence in the region from −641 to −629 is completely identical with the HNF-1 consensus sequences [32, 33]. This observation suggests that HNF-4α/γ and HNF-1α are also involved in the regulation of the expression of human type II DDH. Ciaccio et al. [18] demonstrated that the deletion of sequences from −666 to −590 of the human *type II DDH* gene resulted in a 78% reduction in the transcriptional activity in HepG2 cells. Thus it appears that the HNF-4 consensus-like sequences (and the HNF-1 putative binding sequences) also contribute to the transcriptional activity of the human *type II DDH* gene.

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, and by a Grant-in-Aid from the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan.

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Received 22 November 2000/5 February 2001; accepted 14 February 2001