Sequence of the cDNA of a human dihydrodiol dehydrogenase isoform (AKR1C2) and tissue distribution of its mRNA

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Human liver contains three isoforms (DD1, DD2 and DD4) of dihydrodiol dehydrogenase with 20α- or 3α-hydroxysteroid dehydrogenase activity; the dehydrogenases belong to the aldo–oxo reductase (AKR) superfamily. cDNA species encoding DD1 and DD4 have been identified. However, four cDNA species with more than 99 % sequence identity have been cloned and are compatible with a partial amino acid sequence of DD2. In this study we have isolated a cDNA clone encoding DD2, which was confirmed by comparison of the properties of the recombinant and hepatic enzymes. This cDNA showed differences of one, two, four and five nucleotides from the previously reported four cDNA species for a dehydrogenase of human colon carcinoma HT29 cells, human prostatic 3α-hydroxysteroid dehydrogenase, a human liver 3α-hydroxysteroid dehydrogenase-like protein and chlordecone reductase-like protein respectively. Expression of mRNA species for the five similar cDNA species in 20 liver samples and 10 other different tissue samples was examined by reverse transcriptase-mediated PCR with specific primers followed by diagnostic restriction with endonucleases. All the tissues expressed only one mRNA species corresponding to the newly identified cDNA for DD2: mRNA transcripts corresponding to the other cDNA species were not detected. We suggest that the new cDNA is derived from the principal gene for DD2, which has been named AKR1C2 by a new nomenclature for the AKR superfamily. It is possible that some of the other cDNA species previously reported are rare allelic variants of this gene.

INTRODUCTION

Dihydrodiol dehydrogenase (DD; EC 1.3.1.20) catalyses the $NADP^+$-linked oxidation of *trans*-dihydrodiols of aromatic hydrocarbons to corresponding catechols. The enzyme has dual toxicological roles in the metabolism of polycyclic aromatic hydrocarbons: it suppresses the formation of their carcinogenic dihydrodiol epoxides [1,2] but is involved in producing cytotoxic *o*-quinones through auto-oxidation of the catechol metabolites [3,4]. In addition, DD in mammalian liver has been suggested to be implicated in the metabolism of xenobiotic carbonyl compounds, steroids and prostaglandins because of its broad substrate specificity [5–8], and the rat liver enzyme has been shown to be identical with a bile acid-binding protein [9].

We previously purified four multiple forms (DD1–DD4) of monomeric DD with molecular masses of approx. 36 kDa from human liver [10], of which the minor form (DD3) is aldehyde reductase and the other forms show broad but distinct specificity for 3α- and/or 20α-hydroxysteroids and prostaglandins [10–13]. The cloning and expression of cDNA species for DD1 and DD4 [13–15] revealed their identity with those for other proteins: the cDNA for DD1 is identical with that for an oxidoreductase (H-37) of ethacrynic acid-resistant human colon H-29 carcinoma cells [16], and the cDNA for DD4 is the same as those for human liver chlordecone reductase (CCDR12) [17] and 3α-hydroxysteroid dehydrogenase (3αHSD) type 1 (HAKRa) [18,19]. The analyses of the amino acid sequence and properties of DD2 [12,13] have suggested that this enzyme is identical with human hepatic bile acid-binding protein [20] and is encoded by another cDNA (c81) isolated from the H-29 carcinoma cells [21]. The amino acid sequence deduced from c81 shows sequence identities of 98 % and 83 % with DD1 and DD4 respectively, and belongs to the aldo–oxo reductase (AKR) superfamily [22], in which DD1, DD2 and DD4 have been named AKR 1C1, AKR 1C2 and AKR 1C4 respectively. However, three additional cDNA species with sequences quite similar to c81 have been reported: one is a cDNA clone (TypeIII) for 3αHSD type 3 of human prostate [23], and the others are MCDR2 and HAKRd, which have been isolated in studies of the cloning of CCDR12) [17] and HAKRa [18] respectively. The cDNA species and names of their encoded proteins, including those for the other human AKR superfamily members, are summarized in Table 1. The differences in nucleotide sequence between c81 and TypeIII, MCDR2 or HAKRd are three, six and five within their coding regions, although MCDR2 lacks a part of its 5′ region (Figure 1). It therefore remains unknown which of the four cDNA species reported encodes DD2 (i.e. AKR 1C2) and what is the significance of the existence of these similar genes in human tissues. To address the questions raised by these recent findings, we have cloned a cDNA for DD2 and examined the expression of mRNA species corresponding to DD2 cDNA and the c81-like cDNA species in human liver and other tissues.

EXPERIMENTAL

Materials

*Cfr*9I was obtained from Toyobo (Osaka, Japan); other restriction endonucleases and the specific PCR primers for human β-actin were from Takara (Kusatsu, Japan); Moloney murine

Abbreviations used: DD, dihydrodiol dehydrogenase; 3αHSD, 3α-hydroxysteroid dehydrogenase; AKR, aldo–oxo reductase; RT–PCR, reverse transcriptase-mediated PCR; TypeIII, cDNA clone for 3αHSD type 3 of human prostate.

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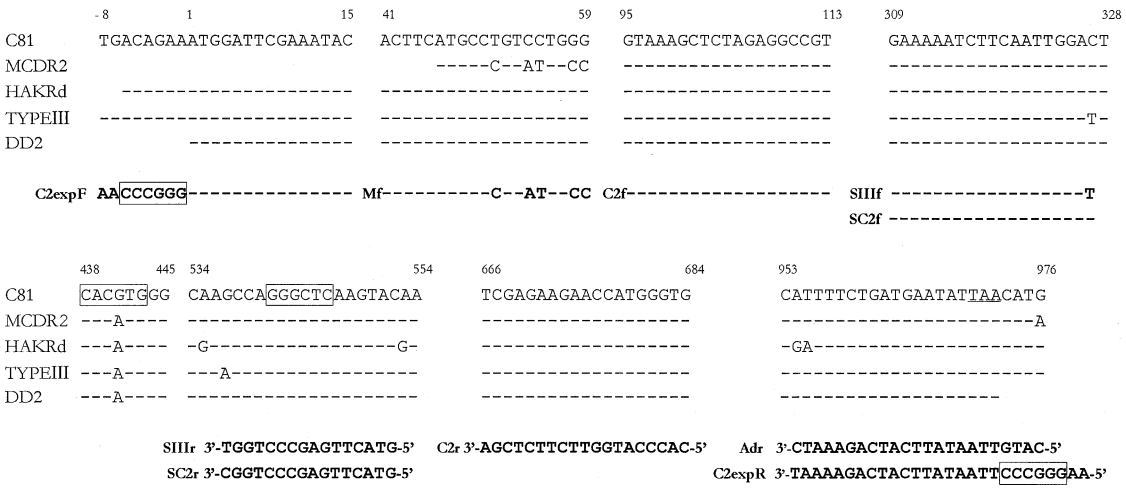


Figure 1 Alignment of nucleotide sequences of c81, MCDR2, HAKRd, TypeIII and the cloned DD2 cDNA, and the positions and sequences of primers used in this study

Partial sequences are shown that correspond to primer positions and contain nucleotide differences between the five cDNA species. Matching nucleotides between the c81 sequence and those of the other cDNAs or the primers are denoted by hyphens. MCDR2 lacks the 5′ region before position 45. The restriction sites of *Cfr*9I (CCCGGG), *Pma*CI (CACGTG) and *Ban*II (GGGCTC) are boxed, and the primer sequences are shown in bold letters. The sequences of the anti-sense primers (SIIIr, SC2r, C2r, Adr and C2expR) are complementary to those of the respective cDNA species.

Table 1 Multiple cDNA species for human DDs and related proteins

The cDNA species are grouped into four types by their sequence similarity in accordance with the nomenclature of the AKR superfamily proteins [22]. Abbreviation: n.s., not studied.

Type	Abbreviation of cDNA	Encoded protein	AKR designation	References
1	DD1 cDNA	DD1/H-37 protein	1C1	[13,16]
	HAKRc	n.s.		[18]
2	DD2 cDNA	DD2/bile acid-binding protein	1C2	This study
	c81*	n.s.	1C2	[21]
	TypeIII*	3αHSD type 3	1C2	[23]
	MCDR2	n.s.		[17]
	HAKRd	n.s.		[18]
3	HAKRb	3αHSD type 2	1C3	[18,19]
	KIAA0119	n.s.		[39]
4	DD4 cDNA†	DD4	1C4	[14,15]
	CCDR12†	Chlordecone reductase	1C4	[17]
	HAKRa†	3αHSD type 1	1C4	[18,19]
	CCDR33	n.s.		[17]

* The nucleotide sequences of the cDNAs are different from that of DD2 cDNA, but the amino acid sequences of the encoding proteins are identical to that of DD2.

† The cDNAs are identical in both nucleotide and amino acid sequences.

leukaemia virus reverse transcriptase, ribonuclease H, ribonuclease inhibitor and $(dT)_{12-18}$ were from Gibco BRL. *Pfu* DNA polymerase and *Taq* DNA polymerase were obtained from Stratagene and Boehringer Mannheim respectively. The other chemicals, plasmids and *Escherichia coli* host strain JM109 used in this study are as specified elsewhere [11,12,14,24].

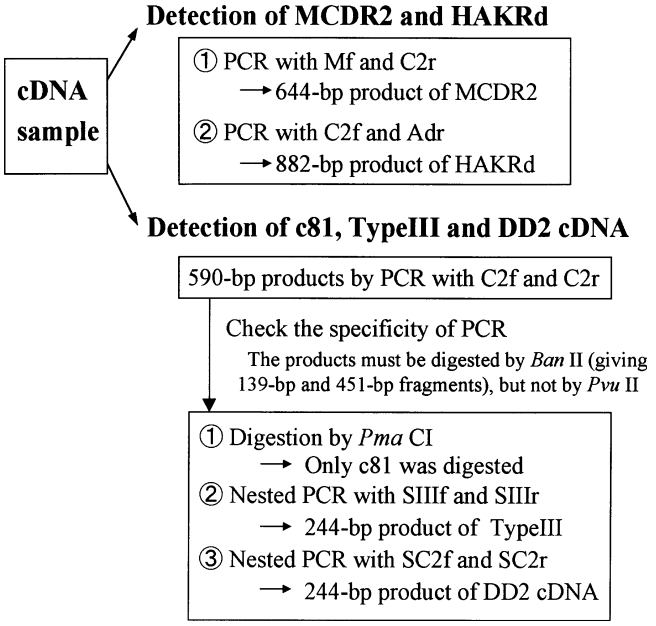
Human samples

Livers, kidneys, prostates, adrenal gland and testis were obtained with informed consent from patients during biopsy or surgery of these tissues for pathological examination, and a placenta was from a healthy volunteer after parturition. All the tissues were frozen immediately and stored in liquid N_2 until use. Total RNA species of the tissues (brain, lung, liver, heart, spleen and small intestine) of non-Japanese were purchased from Sawady Technology (Tokyo, Japan). The use of the human samples for this study had been approved by the University Ethics Committee. Hepatic DD2 and the cDNA species for DD1 and DD4, prepared in our previous studies [11,13,15], were used, and a cDNA (HAKRb) for 3αHSD type 2 [19] was prepared from the hepatic total RNA by reverse transcriptase-mediated PCR (RT–PCR) with primers corresponding to the N-terminal and C-terminal sequences of this enzyme (K. Matsuura, unpublished work).

cDNA cloning, and expression and purification of a recombinant enzyme

Since the 5′ and 3′ coding regions of c81 and TypeIII are the same and their deduced amino acid sequences also match the partial sequence of DD2, the cDNA for DD2 was cloned from total RNA of a Japanese liver sample by RT–PCR with the primers C2expF and C2expR (Figure 1). Total RNA was isolated from the liver (200 mg) with Isogen (Nippongene, Osaka, Japan) and was reverse transcribed with reverse transcriptase. PCR was performed for 30 temperature cycles (each cycle was 94 °C for 1 min/55 °C for 1 min/72 °C for 2 min; the last cycle included a 72 °C/10 min step) with *Pfu* DNA polymerase. The PCR products (approx. 990 bp) were subcloned into pBluescript II SK (−) plasmids at the *Cfr*9I restriction site. The constructions were transfected into *E. coli*; the colonies, which had the cDNA inserts, were selected by PCR with the primer pair of C2f and C2r (described below). The nucleotide sequences of the cDNA species were determined as described [14].

To construct the expression plasmid for recombinant DD2, the coding region of DD2 cDNA was amplified by PCR with DD2EN1 and C2expR as the primers. The 5′ sense primer, DD2EN1, contains an *Eco*RI site followed by nt 1–38 of DD1 cDNA [13], in which the amino acid sequences deduced from the corresponding sequences of the cDNA species for DD1 and DD2



Scheme 1 PCR and diagnostic restriction with endonucleases to discriminate MCDR2, HAKRd, c81, TypeIII and DD2 in the cDNA sample from a tissue

Although C2f and C2r are group-specific primers for amplifying all five cDNA species, the other primers specifically anneal to the sequences of the respective cDNA species as shown in Figure 1.

were identical. Because the nucleotide sequences (positions 784–972) of the two cDNA species are also the same and have an *Eco*RI site at position 851, the expression plasmid was prepared by isolation of a long-digested *Eco*RI cDNA fragment (860 bp) of the amplified DD2 cDNA followed by ligation of the cDNA fragment into a pKKDD1-L plasmid containing the 3′ terminal region (nt 852–972) of DD1 cDNA in the expression plasmid pKK 223-3 [24]. The identity of the entire coding region of the cDNA in the expression plasmid with that of the cloned DD2 cDNA was confirmed by DNA sequencing, except for the one nucleotide at position 15, which did not affect the deduced amino acid sequence as described above. Expression of the recombinant DD2 in *E. coli* and its purification were performed by the methods described for recombinant DD4 [15] and hepatic DD2 [10] respectively.

Enzyme assay

The dehydrogenase activity of DD2 was assayed fluorometrically by recording the production of NADPH as described [10]. The standard reaction mixture consisted of 0.1 M potassium phosphate, pH 7.4, 0.25 mM $NADP^+$, 1 mM *S*-indan-1-ol and enzyme, in a total volume of 2.0 ml. One unit of the enzyme activity was defined as the amount catalysing the formation of 1 μmol of NADPH/min at 25 °C. Kinetic constants for the substrates were determined by Lineweaver–Burk analyses with five different substrate concentrations at a saturating $NADP^+$ concentration of 0.25 mM, and IC_{50} values for inhibitors were estimated in the standard reaction mixtures containing five different inhibitor concentrations. These values are means of duplicate or triplicate determinations.

RT–PCR and diagnostic restriction with endonucleases

Total RNA isolation from the tissue (10 mg for biopsy sample) and cDNA synthesis from the RNA (2 μg) in the presence of ribonuclease inhibitor (10 units) were performed as described above. After the remaining RNA in the cDNA solutions (each 12 μl) had been removed by the addition of ribonuclease H (2 units), 1 μl portions of the solutions were subjected to PCR in 25 μl of 10 mM Tris/HCl, pH 8.3, containing 1.5 mM $MgCl_2$, 50 mM KCl, 50 μM deoxyribonucleotide triphosphates, 0.5 unit of *Taq* DNA polymerase, and 50 nM forward and reverse primers (Scheme 1). PCR amplification consisted of an initial denaturation step at 94 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at a temperature (indicated in the parentheses for the respective primer pairs) for 1 min, and extension at 72 °C for 2 min: Mf and C2r (61 °C); C2f and Adr (55 °C); C2f and C2r (58 °C); SIIIf and SIIIr (55 °C); SC2f and SC2r (58 °C). A final extension step at 72 °C for 10 min was also performed in a Biometra thermocycler. With each sample β-actin cDNA was amplified as an internal control with the specific primers as described by the manufacturer (Takara) and negative controls for each PCR included reverse transcription template with no RNA or with no reverse transcriptase. To prepare the positive control cDNA fragments for the amplification with the primer pairs Mf and C2r, C2f and Adr, or SIIIf and SIIIr, PCR was performed with DD2 cDNA as the template and one of the primer pairs at 5 °C below the annealing temperatures indicated for the respective primer pairs, and the formed DNA fragments were confirmed to contain the sequences of the respective primers at both ends of the DD2 cDNA fragments by DNA sequencing.

For diagnostic restriction with endonucleases to discriminate the quite similar cDNA species, the PCR products amplified with C2f and C2r as the primers were precipitated by the addition of ethanol, and a portion (approx. 0.2 μg) of the products was incubated at 37 °C for 2 h with 10 units of *Pma*CI, *Ban*II or *Pvu*II. The digested DNA fragments were analysed by agarose electrophoresis.

Other methods

Protein concentration was determined with BSA as the standard by the method of Bradford [25]. SDS/PAGE on a 12.5 % (w/v) slab gel and isoelectric focusing on a 7.5 % (w/v) polyacrylamide disc gel were performed as described by Laemmli [26] and Hara et al. [27] respectively.

RESULTS

Nucleotide sequence of DD2 cDNA and properties of the recombinant enzyme

To examine which of c81 and TypeIII encodes DD2, we cloned the cDNA from the total RNA of a Japanese liver sample by RT–PCR with C2expF and C2expR, and isolated five positive clones, which contained plasmid cDNA species amplified by PCR with the primer pair C2f and C2r. The nucleotide sequences of the cloned cDNA species were all identical, but they differed by one nucleotide at position 441 from that of c81, and by two nucleotides at positions 327 and 537 from that of TypeIII (Figure 1). Because the cloned cDNA also differs by four and five nucleotides from those of HAKRd and MCDR2 respectively within the comparable coding regions, it was designated DD2 cDNA to distinguish it from the other similar cDNA species.

The deduced amino acid sequence of DD2 cDNA was the same as those of c81 and TypeIII, but it differed by one and three

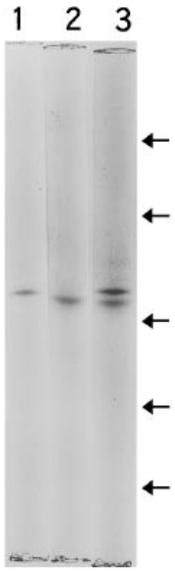


Figure 2 Isoelectric focusing of hepatic and recombinant DD2

The hepatic (lane 1) and recombinant (lane 2) enzymes (5 μg of each) and their mixture (lane 3) were run on 7.5% (w/v) polyacrylamide disc gels containing 0.1% ampholyte (pH 3.5–10), and stained for protein with Coomassie Brilliant Blue G. Arrows indicate the positions of marker proteins with pI values of 4.9, 6.4, 8.3, 9.7 and 10.6, listed from the top downwards.

amino acid residues from those of HAKRd and MCDR2 respectively. Because some data on the substrate specificity for oxosteroids of the recombinant enzyme expressed from TypeIII have been reported with the crude extract of the transfected cells [23], we expressed the recombinant enzyme from DD2 cDNA and compared its properties with hepatic DD2. The recombinant enzyme was purified 270-fold, with an overall yield of 36% (1 mg) from the extract of the *E. coli* cells cultured in 1 litre of medium. The purified enzyme migrated as a single band on SDS/PAGE and isoelectric focusing analyses (Figure 2). The molecular mass of the enzyme (36 kDa) was similar to that of hepatic DD2, although their pI values differed by 0.2 pH unit. The substrate specificity and inhibitor sensitivity of the recombinant enzyme were essentially identical with those of hepatic DD2 (Table 2). In particular the susceptibility to inhibition by bile acids has been described as a representative characteristic of hepatic DD2 but not of other hepatic DD isoforms [11,13]. The results, together with the isolation of one cDNA species, indicated that DD2 is encoded by the cloned DD2 cDNA at least in this liver sample.

mRNA species expressed in liver and other tissues

The present cDNA was isolated from one Japanese liver sample, whereas HAKRd, MCDR2, TypeIII and c81 have been cloned from cDNA libraries of livers and prostate of non-Japanese and from a RNA sample of human colonic carcinoma cells. It remains unknown whether these cDNA species are due to allelic variations or are derived from different genes. To clarify this point, we first examined the expression of the different mRNA species corresponding to the five cDNA species in liver samples of 18 Japanese and 2 non-Japanese by RT–PCR followed by diagnostic restriction with endonucleases (Scheme 1), and the representative results are shown in Figures 3 and 4. The PCR with C2f and C2r amplified the expected 590 bp DNA fragments for all the liver samples and control DD2 cDNA, but not for the cDNA species encoding DD1, DD4 and 3αHSD type 2 (Figure 3A). The specificity of the primers for detection of all the five c81-like cDNA species was also confirmed by the digestion of the PCR products with *Pvu*II and *Ban*II. The *Pvu*II restriction site is present in the cDNA species for DD1, DD4 and 3αHSD type 2 but not in the five c81-like cDNA species, whereas the *Ban*II

Table 2 Comparison of substrate specificity and inhibitor sensitivity between recombinant and hepatic DD2

The activities towards the substrates were determined at pH 7.4 except for 9α,11β-prostaglandin F_2 dehydrogenase activity, which was assayed at pH 10.0. The apparent kinetic constants for hepatic DD2 are taken from [11], but the values for 5α-androstan-3α-ol-17-one were determined in the present study.

	Recombinant DD2		Hepatic DD2	
Substrate	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})
S-Indan-1-ol	490	11	520	9.5
S-1,2,3,4-Tetrahydronaphth-1-ol	140	12	260	16
5α-Androstan-3α-ol-17-one	2.2	0.4	2.0	0.4
5β-Pregnane-3α,20α-diol	1.0	0.1	1.0	0.2
9α,11β-Prostaglandin F_2	220	5.0	208	25

	IC_{50} (μM)	
Inhibitor	Recombinant DD2	Hepatic DD2
Lithocholic acid	0.08	0.07
Ursodeoxycholic acid	0.06	0.08
Chenodeoxycholic acid	0.15	0.13
Flufenamic acid	0.7	0.9
Medroxyprogesterone acetate	1.6	1.7
Hexestrol	3.5	2.8
Ibuprofen	10	6.9
Phenolphthalein	18	12
Indomethacin	70	75

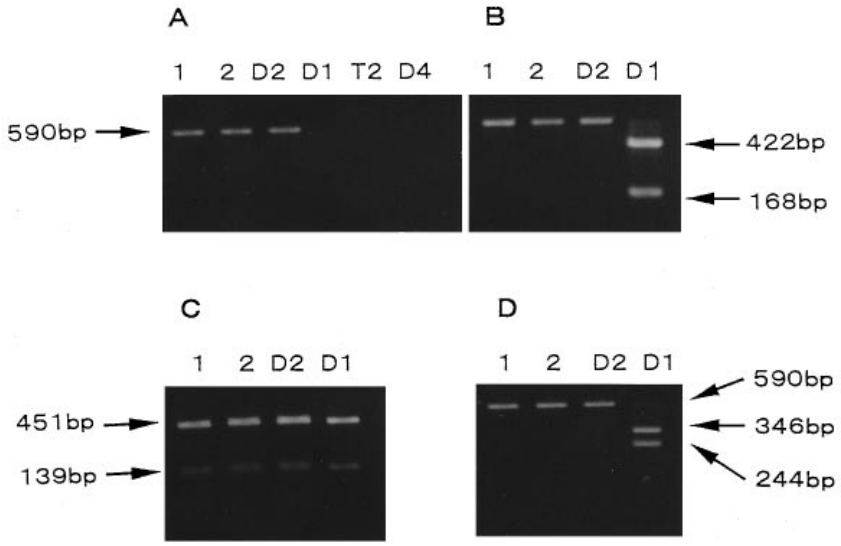


Figure 3 RT–PCR analysis for expression of mRNA species corresponding to the c81-like cDNA species, and restriction digestion diagnosis of the amplified DNA fragments

(**A**) PCR with C2f and C2r primers for the representative cDNA samples of two liver specimens (lanes 1 and 2), and cDNA species for DD2 (lane D2), DD1 (lane D1), 3αHSD type2 (lane T2) and DD4 (lane D4). (**B**–**D**) Digestion of the PCR products from the specimens (lanes 1 and 2) and the cDNA species for DD2 (lane D2) and DD1 (lane D1) by *Pvu*II (**B**), *Ban*II (**C**) and *Pma*CI (**D**). The PCR products and DNA fragments were subjected to electrophoresis on 2% (w/v) agarose gels and revealed after staining with ethidium bromide.

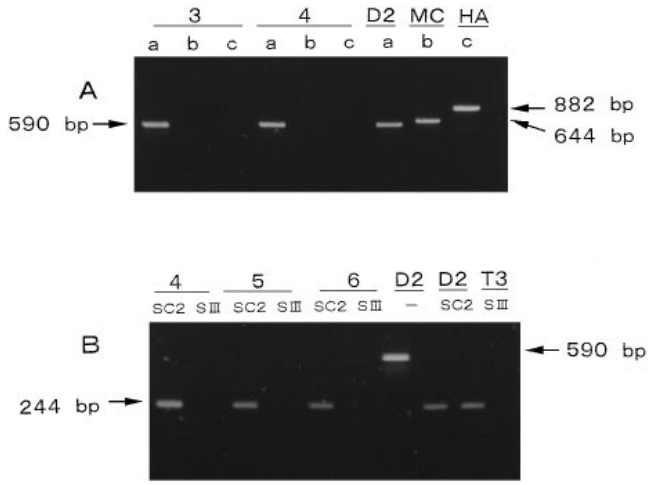


Figure 4 RT–PCR with a primer pair specific for MCDR2 or HAKRd for liver samples, and nested PCR with a primer pair specific for DD2 cDNA or TypeIII

(**A**) The two cDNA samples from liver specimens (lanes 3 and 4) were subjected to PCR with one of the primer pairs, C2f and C2r (lanes a), Mf and C2r (lanes b) and C2f and Adr (lanes c). As the positive controls, DD2 cDNA (lane D2) and the cDNA fragments for MCDR2 (lane MC) and HAKRd (lane HA) were included. (**B**) Nested PCR with SC2f and SC2r (lanes SC2) or with SIIIf and SIIIr (lanes SIII) as the primer pair was performed for the DNA fragments amplified from liver specimens (lanes 4, 5 and 6) by the first PCR with C2f and C2r primers. DD2 cDNA (lane D2) and the TypeIII cDNA fragment (lane T3) were included as the positive controls, and the DNA fragment amplified from DD2 cDNA without the nested PCR (lane D2 —) was run on the electrophoresis. Detection of DNA fragments was done as described in the legend to Figure 3.

restriction site exists in all the c81-like cDNA species and DD1 cDNA. The PCR products from all the liver samples were not digested by *Pvu*II (Figure 3B), but resulted in two DNA fragments of 451 bp and 139 bp by digestion with *Ban*II (Figure 3C). The PCR products were also resistant to the digestion with *Pma*CI (Figure 3D). This excludes the possibility of expression of mRNA corresponding to c81 because only c81, of the c81-like cDNA species, has this restriction site (Figure 1). More specific PCR with one of the primer pairs, C2f and Adr or Mf and C2r, was performed to examine the expression of HAKRd and MCDR2 in the liver samples, but no amplified DNA fragment was observed for all the hepatic samples and DD2 cDNA (Figure 4A). When the PCR products obtained with the group-specific primers C2f and C2r were further amplified by nested PCR with the primer pair SC2f and SC2r or SIIIf and SIIIr, DNA fragments (244 bp) were detected only with the primers specific for DD2 cDNA (Figure 4B). Therefore only one mRNA species for DD2 was expressed in all the samples of Japanese and non-Japanese analysed.

Secondly, we performed the series of PCR analyses for cDNA samples from 10 extra-hepatic tissues to examine whether mRNA species corresponding to the five c81-like cDNA species are differently expressed depending on the tissue. The 590 bp DNA fragments were amplified by PCR with the group-specific primers C2f and C2r but not by that with the specific primers C2f and Adr or Mf and C2r (Figure 5). In addition, the 244 bp DNA fragments were also detected by the nested PCR with SC2f and SC2r primers, but not with SIIIf and SIIIr primers, for the 590 bp PCR products amplified with C2f and C2r, which were also not digested by *Pma*CI (results not shown). The same results were obtained by the analyses for two further prostate samples and one kidney sample. Furthermore the cDNA fragments were amplified from the prostatic cDNA samples by PCR with the group-specific primers and *Pfu* DNA polymerase, and confirmed to have identical sequences with the that of the corresponding region of DD2 cDNA by direct DNA sequencing. Although the expression of the mRNA for DD2 was observed in all the tissues, relatively high levels of the message were found in liver, heart, lung, testis, brain and prostate.

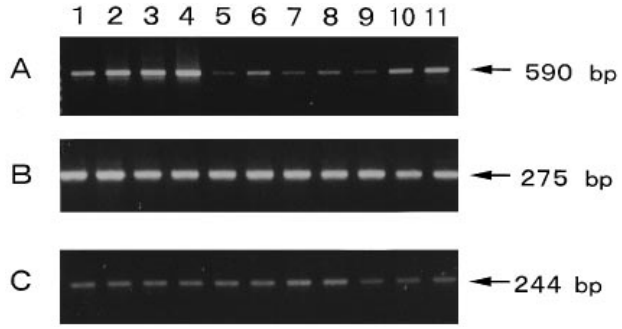


Figure 5 Expression of mRNA for DD2 in human tissues

RT–PCR with C2f and C2r (**A**) or the specific primers for β-actin cDNA (**B**) was performed for the total RNA from human tissues. (**C**) Nested PCR with SC2f and SC2r for the cDNA fragments obtained by the amplification with C2f and C2r. Electrophoresis and detection of DNA fragments were done as described in the legend to Figure 3. Lane 1, brain; lane 2, lung; lane 3, heart; lane 4, liver; lane 5, spleen; lane 6, adrenal gland; lane 7, kidney; lane 8, placenta; lane 9, small intestine; lane 10, prostate; lane 11; testis.

DISCUSSION

The present cloning and expression of the cDNA for DD2 confirmed the previous suggestion that the amino acid sequence of DD2 is identical with that deduced from c81 [13]. DD2 shows 3αHSD activity but is strongly inhibited by bile acids [11]. Although the identity of DD2 with a human liver bile acid binding protein has been suggested [13], its role in hepatic steroid metabolism is obscure, because of strong inhibition by bile acids and the presence of DD4 with high 3αHSD activity for various 3α-hydroxysteroids including bile acids. Recently, it has been reported that the expression of the mRNA for DD4 is liver-specific, and the mRNA for 3αHSD type 2 is expressed in various tissues [19]. The recombinant 3αHSD type 2 purified to homogeneity showed much lower dehydrogenase activity and high K_m values for some 3α-hydroxyandrostanes, compared with those of DD2 and DD4 with broader substrate specificity (K. Matsuura, unpublished work). The present results revealed that DD2 is identical with prostatic 3αHSD type 3 encoded by TypeIII [23], and that the mRNA for DD2 is expressed in various extrahepatic tissues. These findings suggest that DD2 is able to act as the major 3αHSD in peripheral steroid-producing and steroid-target tissues, where inhibitory bile acids are not present. 3αHSD has been reported to be involved in the regulation of intracellular concentrations of 5α-dihydrotestosterone, 5α-androstane-3α,17β-diol and 3α-hydroxy-5α-dihydroprogesterone, which have key roles in prostate growth [28], parturition [29] and modulation of the activity of γ-aminobutyric acid receptor [30] respectively.

Multiple cDNA species for human DD and/or 3αHSD isoforms with high degrees of structural similarity have been found (Table 1). PCR with specific primers has been recommended to distinguish the expression of the genes for the two types of 3αHSD [19]. To discriminate the five c81-like cDNA species with more than 99 % sequence identities from one another, our PCR method had to include two steps of a diagnostic restriction with *Pma*CI and a nested PCR for the cDNA fragments amplified by the first PCR, in addition to the two allele-specific PCRs for the amplification of HAKRd and MCDR2 (Scheme 1). Our method is tedious but has provided definitive results for the expression of the respective similar genes in tissues, as demonstrated by the specificity of the amplification with the respective cDNA species as positive controls and the cDNA species for DD1, DD4 and 3αHSD type 2 as negative controls. It should be noted that we observed the expression of mRNA species for DD1, DD4 and 3αHSD type 2 in all the present liver samples by PCR with other specific primers, although their tissue distributions were different (H. Shiraishi, unpublished work).

Polymorphisms of the human aldose reductase gene have been reported in the AKR superfamily proteins [31]. For human DD and/or 3αHSD isoforms, distinct genes for DD1 [32], DD4 [19], 3αHSD type 2 [19] and HAKRd [33] have been characterized and mapped to chromosome 10p14–15 [32–34]. In addition, multiple cDNA species strongly similar to those for the respective genes have been isolated from human liver and cultured cells (Table 1). For cDNA species similar to that for HAKRd, there are five by the present study. Among the five cDNA species, c81, TypeIII and DD2 cDNA encode an identical protein. The deduced amino acid sequence of DD2 differs at positions 179, 185 and 319 from that of HAKRd. MCDR2 is a partial cDNA that lacks 5′ coding region from nt 45, but its deduced amino acid sequence is identical with the corresponding region (positions 16–323) of DD2, except for one amino acid substitution at position 20. The amino acid residues, which differ between the three deduced amino acid sequences, do not correspond to those in the binding sites for the substrates and coenzymes of the crystal structures of several enzymes in the AKR family [35–38]. They are also equivalent to residues outside the substrate-binding and coenzyme-binding sites on the tertiary structure of rat liver 3αHSD (68 % sequence identity with DD2) when their positions are predicted on the basis of the rat enzyme structure [37]. Therefore the proteins encoded by HAKRd and MCDR2 might have properties almost identical with DD2. Jez et al. [22] have proposed that in the AKR superfamily proteins with more than 97 % amino acid sequence identity are alleles of the same gene. Furthermore the present RT–PCR analysis of the total 33 tissue samples from Japanese and non-Japanese showed that only one mRNA species corresponding to DD2 cDNA was expressed regardless of both individual difference and tissue specificity. This suggests the existence of a gene for DD2, but not for the other four cDNA species, at least in the present samples, in contrast with the isolation of the gene for HAKRd and the four cDNA species slightly distinct from DD2 cDNA by other researchers. In our preliminary PCR analyses for 20 genomic DNA samples no product of the expected size was amplified with specific primers for the sequences on the exon 9 and 3′ untranslated regions of HAKRd gene (H. Shiraishi, unpublished work). We suggest that DD2 cDNA might represent the principal AKR1C2 allele, and the other cDNA species might be derived from its rare variants or could be sequencing errors, although further analyses of both cDNA species and genomic DNA species for more samples will be necessary to establish the proposed genetic polymorphisms of AKR1C2.

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