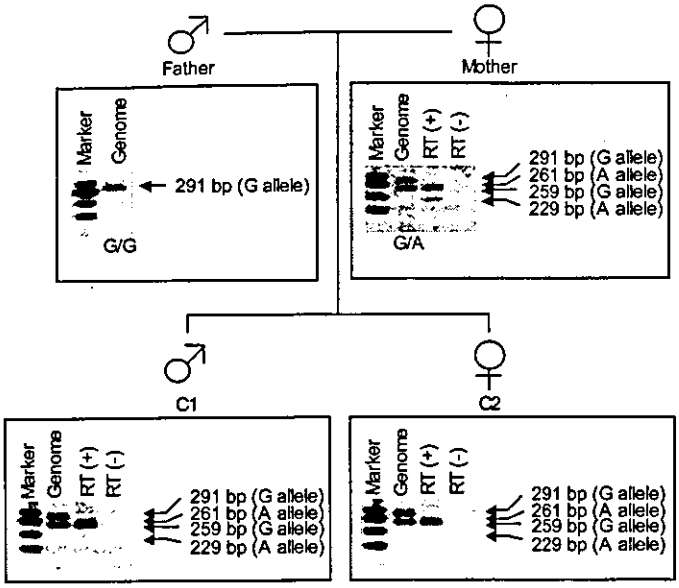

FIG. 4. Monoallelic paternal expression of the *BCRP* gene in informative lymphoblasts. Maternal inactive A34 allele was inherited by both siblings.

Brockmöller (2004), which demonstrated a hepatic transcriptional imbalance of the *CYP3A5* gene in heterozygous *CYP3A5*1A/*3* samples. In that case, *CYP3A5*1A* may act as a *cis*-acting SNP, which increases the expression of the *CYP3A5* transcript from the allele carrying it, resulting in an imbalance of mRNA expression. Thus, allelic imbalance can be used for the functional evaluation of SNPs that affect mRNA expression. If the C421A variant is a functional *cis*-acting polymorphism differentially affecting the expression level of each copy of a gene, a strong over- or under-shift in allelic expression bias will be expected. However, as shown in Fig. 3, although only two samples (19 and 62) exhibited significant allelic imbalance (i.e., preferential expression of one of the two alleles), the allelic expression bias in most samples was within the approximated 95% confidence interval (0.85~1.48). These results suggest that the predominant expression pattern of *BCRP* in placental samples is biallelic, and C421A is not a *cis*-acting SNP. The C421A variant was associated with a reduction in placental protein levels but not in mRNA levels (Fig. 2). These observations are in line with a finding by Imai et al. (2002), who demonstrated that murine PA317 cells expressing C421A had increased intracellular drug accumulation, coupled with reduced protein levels and a similar mRNA expression. Therefore, taking these observations into consideration, the reduction in protein expression levels in the A421-placental samples is considered to be a result of the translation efficiency of BCRP. Nevertheless, we cannot exclude the possibility that the *cis*-acting regulatory polymorphisms, which may form haplotypes with the C421A variant, responsible for the change in BCRP expression reside far up- and down-stream of the gene of the affected allele.

Since protein expression is known to be regulated not only by mRNA and/or DNA sequences but also by epigenetic modifications such as genomic imprinting, chromatin alteration, methylation, and acetylation, we introduced an epigenetic concept in this study. As shown in Fig. 4, we have demonstrated that human lymphoblasts showed a paternal expression of *BCRP*, in contrast with the findings in placental samples, in which a monoallelic expression was observed in only a minority of samples, suggesting a unique allele-specific expression profile of *BCRP*. Among known imprinted genes, the Wilms' tumor suppressor gene (*WT1*) has been reported to exhibit a unique expression profile (Mitsuya et al., 1997); cultured human fibroblasts and lymphocytes showed a paternal or biallelic expression of *WT1* in some cases, whereas a maternal or biallelic expression was observed in human placental villi and fetal brain tissue (Jinno et al., 1994; Nishiwaki et al., 1997). These results suggest that the allele-specific expression profile of certain genes depends on the tissue source. Thus, expression patterns in other BCRP-enriched human tissues such as liver and small intestine are of interest.

The data presented here suggest that the C421A variant in the *BCRP* gene, a common SNP in both Japanese and Caucasian populations, alters protein levels in the human placenta. In addition, a tissue-dependent allele-specific expression profile of *BCRP* is sus-

TABLE 3

Primer sequences used for the PCR-SSCP analysis of the human BCRP gene

Primer Pair	Forward Primer	Reverse Primer
Promoter-1	5'-GTACTAGGATTACAGGCGTGAGC-3'	5'-AATGACCCGAGATCCCACCACTG-3'
Promoter-2	5'-CGCAGATTTTTCAGTTTTTGC-3'	5'-TATTAATGGTTCACCCAATGC-3'
Promoter-3	5'-AGGGTCTTGAAACTGACAGAAAT-3'	5'-AAGGGGAGAAACTTACTGAATGA-3'
Promoter-4	5'-TTTCTAGCCTTTCCACACCATCG-3'	5'-ACGCAGGGACAAGCCAAACACTC-3'
Promoter-5	5'-GCCAGTGACGGCGACCAAACC-3'	5'-GCGCTGACACGAACTTCCTAAGC-3'
Exon 1-1	5'-GGCTCAGCGCGGCAGGACA-3'	5'-GACCCGGACATCCAGGGGACGAG-3'
Exon 1-2	5'-AGCGCGGCTTAGGAAGTTCGTGT-3'	5'-CGCGTCTCTCAATCTCAGTGG-3'
Exon 1-3	5'-CGTGCTGTGCCCACTCAAAAG-3'	5'-ATACACAACGCCCACCAACCT-3'
Exon 2	5'-ATTGTCACCTAGTGTTTGC-3'	5'-AAAAATGTTCATAGCCAGTTTCT-3'
Exon 3	5'-AAAATGGAATAGTAAATCAGTCT-3'	5'-CTGCTACATGTCAATCACCTTAT-3'
Exon 4	5'-ATAGCATGTGTTGGAGGGAAAAA-3'	5'-ATTGGTATCACTGTCCTTACAAG-3'
Exon 5	5'-AAACAGTCATGGTCTTAGAAAAG-3'	5'-TCTCATTGTTATGGAAAGCAACC-3'
Exon 6	5'-CAAATGATAATGACTGGTTGTTA-3'	5'-TTGTTTTTCTTGATAATGCTTTT-3'
Exon 7	5'-AGCAAACAATCTAAAGGCAAGAA-3'	5'-CCAAAGACCAAACAGCACTCCTG-3'
Exon 8	5'-AAGTGAGTTCTCTTTGTTTTCCA-3'	5'-GTTGACTGGTATCAGAAGACTGC-3'
Exon 9	5'-TGTTTGTGTTTCCTTTTTATCCA-3'	5'-CATTGTTCCCATTTGAGTATTTC-3'
Exon 10	5'-TTATCTCTAATTGAAACTCTTCC-3'	5'-AAATAAACTGACTCATCCTACCC-3'
Exon 11	5'-ACGCTTCCCTGTTCCAACCAGAA-3'	5'-TGTAATCAGTCTAACCAATAGCC-3'
Exon 12	5'-GACAAGTCTAGCCTGCCCTGTGG-3'	5'-GTTTGGTTTATAGTTTTGAGAAC-3'
Exon 13	5'-AATAAGCAATCCCAAACATACGG-3'	5'-TTATCAGAGCAAACACAGTTCAG-3'
Exon 14	5'-AGAGGAGAAGAGTTTAGTGAGTG-3'	5'-ACAGTGACAGACAAGGAAGACAT-3'
Exon 15	5'-ACATTAGTTGGTTTGGTGAGACA-3'	5'-ATTCAGTGCCCCTGGAAGGACTC-3'
Exon 16-1	5'-CTGAGTAACATTTGACGGATGCT-3'	5'-GTGATGGCAAGGGAACAGAAAAC-3'
Exon 16-2	5'-TTCAGTATGATTTATCCTCACAT-3'	5'-GCTACTAACCTACCTATTCATTT-3'
Exon 16-3	5'-GTCTCTGGCATTTGTTTCCTCAT-3'	5'-CTCTACTCTACCCACAGTTCCAA-3'

pected. In vivo human studies with regard to these findings are required for the establishment of individualized anticancer therapy.

Appendix

Primer sequences used for the PCR-SSCP analysis of the human *BCRP* gene are shown in Table 3.

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Haplotype-Oriented Genetic Analysis and Functional Assessment of Promoter Variants in the *MDR1* (*ABCB1*) Gene

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ABSTRACT

Recently, a number of nucleotide variants have been described in the multidrug resistance 1 (*MDR1/ABCB1*) gene; however, most studies have focused on the coding region. In the present study, we identified promoter variants of the *MDR1* gene and evaluated their phenotypic consequences using a reporter gene assay and the real-time polymerase chain reaction method. Ten allelic variants were detected in the promoter region (approximately 2 kilobases), seven of which were newly identified. Certain mutations occurred simultaneously, and a total of 10 haplotypes were observed. These promoter polymorphisms were found more frequently in Japanese than Caucasians. Some haplotypes were associated with changes in luciferase activity and placental and hepatic mRNA levels. We also determined DNA methylation status in the proximal promoter region of the *MDR1* gene. The promoter region around potential binding sites for transcription factors was found to be hypomethylated and thus likely to be independent of the gene expression. Nucleotide and/or haplotype variants not only in the coding region but also in the promoter region of the *MDR1* gene may be important for interindividual differences of P-glycoprotein expression.

Polymorphisms in the genes encoding membrane transporters have recently been reported to be associated with variations in the pharmacokinetic and pharmacological effects of clinically used drugs (Fromm, 2002; Kim and Tirona, 2002; Takane et al., 2003). Among various drug transporters, P-glycoprotein, the multidrug resistance 1 (*MDR1/ABCB1*) gene product, is one of the best studied. P-glycoprotein is expressed in various human tissues such as the intestine, liver, and kidney, and functions as a cellular efflux pump for foreign xenobiotics and endogenous substrates.

Although a number of nucleotide variants have been described in the *MDR1* gene, most studies in this area have focused on the association of single nucleotide polymorphisms (SNPs) in the coding region with the altered expression of P-glycoprotein or pharmacokinetics of clinically used drugs. Hoffmeyer et al. (1999) demonstrated that the synonymous C3435T polymorphism (Ile1145Ile) in exon 26 was associated with a low level of expression of P-glycoprotein in the duodenum, resulting in an increase in plasma concentrations after oral administration of digoxin, used as a probe for P-glycoprotein. In contrast, higher level of duodenum P-glycoprotein expression and lower level of serum digoxin after oral administration were observed in the subjects with this variant (Sakaeda et al., 2001; Nakamura et al., 2002). The association of the C3435T polymorphism with P-glycoprotein protein expression and function is controversial. Up to now, various investigators have reported that the variant is associated with decreased or increased expression, or it has no clearly discernible effect (Sparreboom et al., 2003; Ishikawa et al., 2004). In a recent report, an approach using gene-based haplotypes, which are specific combinations of SNPs located throughout the genome, proved superior to the use of individual SNPs for predicting the association between phenotypes and genomic variation (Judson et al., 2000). For example, Drysdale et al. (2000) reported that the bronchodilator response to β agonist is significantly related to haplotype pairs of the β_2-adrenergic receptor gene but not to individual SNPs. With regard to the *MDR1* gene, Johne et al.

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ABBREVIATIONS: SNP, single nucleotide polymorphism; CpG, cytosine-guanosine pair; PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; bp, base pair; MDR, multidrug resistance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay.

(2002) indicated that it was important to consider the variability in haplotype structure rather than in SNPs when characterizing the *MDR1* phenotype. However, the association of variants in the promoter region of the *MDR1* gene with the expression of P-glycoprotein has been not well investigated.

DNA methylation, referred to as the methylation of cytosine in a cytosine-guanosine pair (CpG), is the most common eukaryotic DNA modification and one of many epigenetic (an alteration in gene expression without a change in nucleotide sequence) phenomena (Singal and Ginder, 1999). Normally, both the core promoter and transcriptional start site are included within the CpG-rich region, and DNA methylation regulates gene expression by interfering with the binding of specific transcription factors to their recognition sites (Singal and Ginder, 1999; Jones and Takai, 2001). Interestingly, the human *MDR1* gene has a CpG-rich promoter region. Hypomethylation of *MDR1* during chemotherapy resulted in a high level of gene expression in recurrent tumors, and it had important consequences for clinical outcome in acute myeloid leukemias (Nakayama et al., 1998) and bladder cancer (Toda et al., 2000). However, there is currently no data available on the role of DNA methylation in transcriptional regulation in normal tissues.

The aim of this study was to describe variants in the promoter region of *MDR1* in Japanese and Caucasian populations and to evaluate their functional significance with regard to transcriptional activity and mRNA expression in placentas and livers obtained from Japanese subjects. Furthermore, we determined the methylation status of the promoter region of *MDR1* and its association with the interindividual variability in gene expression in normal tissue.

Materials and Methods

Isolation of Genomic DNA and RNA. Human full-term placentas (highly enriched placental trophoblast populations) and livers were obtained from 96 and 19 Japanese patients, respectively. These tissues were immediately frozen in liquid nitrogen and stored at −80°C for the preparation of DNA and RNA. Blood samples were obtained from 96 healthy Caucasian volunteers. Genomic DNA from the samples was prepared using the Toyobo blood kit on a Toyobo HMX-2000 robot (Toyobo, Osaka, Japan). The isolation of genomic DNA from tissues was performed using a DNA preparation kit (QIAamp DNA mini kit; QIAGEN GmbH, Hilden, Germany). Total RNA was extracted using ISOGEN (Nippongene, Tokyo, Japan), and reverse transcription was performed with random hexamers (Promega, Madison, WI) and reverse transcriptase (Invitrogen, Carlsbad, CA). This study was approved by the Tottori University Ethics Committee, and informed consent was obtained from all individuals.

Identification of Variants in the *MDR1* Promoter Region. The genotypes of *MDR1* such as A–41aG, C–145G, T–129C, and C3435T were identified by PCR-restriction fragment length polymorphism analysis as described previously (Tanabe et al., 2001). To identify unknown mutations in the *MDR1* promoter region, SSCP analysis was performed using the GenePhor system (Amersham Biosciences AB, Uppsala, Sweden) as described previously (Ieiri et al., 2000). PCR was performed in a total volume of 25 μl consisting of 50 ng of genomic DNA, 10× PCR buffer II, 1.5 mM $MgCl_2$, 1.25 U of Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 0.25 μM of each primer. The primer sets were designed to divide the promoter region (−1700a to Ex1/+88) of the *MDR1* gene (GenBank accession no. AC002457) into five fragments (~500 bp). After an initial denaturation at 94°C for 5 min, 45 cycles of 40 s at 94°C, 45 s at 50 to 59°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed.

Haplotype Analysis. A 2112-bp fragment, including the promoter region of *MDR1* (−1700a to Ex1/+88), was amplified by using gene-specific primers (5′-GGAGCAAAGAAATGGAATACAATA-3′ and 5′-TTCTCCCGTGAAGACCAAGTTC-3′). The PCR mixture was essentially the same as for the identification of mutations except for the *Taq* polymerase (LA *Taq*; Takara, Shiga, Japan). After an initial denaturation at 94°C for 5 min, 45 cycles of 40 s at 94°C, 15 s at 58.3°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed. The PCR fragments were subcloned into pGEM-T easy vector (Promega) and sequenced.

DNA Sequence. All PCR products were sequenced directly on an ABI 377 automatic sequencer (Applied Biosystems) using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Before the sequencing, reaction mixtures were purified with a DyeEx Spin kit (QIAGEN GmbH). The sequencing primers were those used in the PCR amplifications. The sequencing of both strands was performed for products from at least two independent PCR amplifications to ensure that the identified mutations identified were not PCR-induced artifacts.

Real-Time Quantitative PCR (TaqMan) Analysis. PCR was performed using a master mix based on the TaqMan universal PCR master mix (Applied Biosystems) and run on the ABI PRISM 7000 sequence detection system (Applied Biosystems). The following primers and TaqMan probe were used for determining the MDR1 mRNA: forward primer, 5′-TATCAGCAGCCCACATCATCA T-3′; reverse primer, 5′-CCAAATGTGACATTTCCTTCCA-3′); and probe, 5′-TACAGCACGGAAGGCCTAATGCCGA-3′. The endogenous reference gene was determined using the commercially available human GAPDH TaqMan Predeveloped Assay Reagent (Applied Biosystems). Each primer set and TaqMan probe were used at final concentrations of 200 and 100 nM, respectively. The reactions were run in duplicate. For all experimental samples, the amount of mRNA was determined from a standard curve (serial diluted samples from placental tissue expressed at higher levels of MDR1 and GAPDH mRNA). The mRNA level of MDR1 was expressed as a ratio to that of GAPDH.

Plasmid Construction. To obtain the first plasmid, a 2056-bp fragment (−1704a to Ex1/+28) of *MDR1*, including the promoter region and exon 1, was initially amplified from genomic DNA with gene-specific primers incorporating 5′-KpnI and 3′-NheI, for the 5′ end of inserts 5′-CGGGGTACCGGAGCAAAGAAATGGAATACA-3′ and for the 3′ end of inserts 5′-CTAGCTAGCAGTAGCTCCCAGCTTTGCGTG-3′. The PCR fragment was subcloned into the pGEM-T easy vector and then introduced into competent JM109 cells (Promega). The plasmids obtained were sequenced and digested with KpnI and NheI. The digested fragment was ligated into the KpnI/NheI site of the vector pGL3-enhancer (Promega). Manipulated DNA portions were sequenced again in their entirety.

Cell Culture and Transfection. HepG2 human hepatoma cells were incubated at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. One day before transfection, cells (5.5×10^5) were seed into culture plates (60 mm). The cells were washed two times with serum-free medium. The luciferase reporter gene constructs (5 μg) and the control reporter gene plasmid pRL-TK vector (0.5 μg) (Promega) were mixed with the Tfx-20 reagent (15 μl) (Promega), transferred to serum-free medium, and then incubated at room temperature for 15 min. The mixtures were added to the washed cells and incubated for 1 h at 37°C under 5% CO_2. After the incubation, the cells were cultured in growth medium and harvested after 48 h.

Assay of Luciferase Activity. Luciferase reporter gene activity was evaluated with the Dual luciferase reporter assay system (Promega). HepG2 cells were washed once with a phosphate-buffered saline solution and lysed in passive lysis buffer (400 μl). After incubation at 37°C for 15 min, lysates were mixed in a vortex blender for 15 s and centrifuged at 4°C for 30 s. Supernatants (20 μl) were mixed with the luciferase reagent (100 μl), and the luciferase activity was

measured with a luminometer (Turner Designs, Sunnyvale, CA). After background correction (activity in untreated cells), results were expressed as the level of pGL3 activity divided by pRL activity. The total cellular protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts from HepG2 cells were prepared as reported previously (Takeuchi et al., 2000). Oligonucleotides for the *MDR1* gene, including −1517a T (ACTGTTTAGGGAGGGTTTAAGGCCATTCAAA), −1517a C (ACTGTTTAGGGAGGGCTTAAGGCCATTCAAA), −1459a G (ATAAATGAAAGGTGAGATAAAGCAACAAAGC), −1459a A (ATAAATGAAAGGTGAAATAAAGCAACAAAGC), −1017a T (GAGGCAGGAGAATGGTGTGAACCCGGGAGGC), −1017a C (GAGGCAGGAGAATGGCGTGAACCCGGGAGGC), −145 C/−129 C (CTTTGCCACAGGAAGCCTGA GCTCATTCGAGTAGCGGCTCTTCCAAG), −145 G/−129 T (CTTTGCCACAGGAAGGCTGAGCTCATTCGAGTAGCGGCTCTTCCAAG) and −145 C/−129 C (CTTTGCCACAGGAAGCCTGAGCTCATTCGAGCAGCGGCTCTTCCAAG) were synthesized with both sense and antisense strands, the corresponding pairs of which were annealed and end-labeled with T4 polynucleotide kinase (Takara) and [γ-^{32}P]dATP (Amersham Biosciences AB) according to standard methodology. The γ-^{32}P-labeled probe (1×10^4 cpm) was incubated for 30 min at 0°C with nuclear protein (5 μg) in binding buffer (10 μl) containing 25 mM Hepes (pH 7.9), 40 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 7.5% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet-40, 5 μM ZnSO4, 0.2 μg of poly(dI-dC), and 1 μg of bovine albumin. Competitor oligonucleotides were added at 100-fold molar excesses. Reaction mixtures were electrophoresed on 5% polyacrylamide and 2.5% glycerol gel in a Tris-glycine-EDTA buffer (250 mM Tris, 1.9 M glycine, and 10 mM EDTA) at 4°C and visualized by a BAS-2500 Imaging Analyzer (Fuji Film, Tokyo, Japan).

Determination of Methylation. The methylation status of CpG sites within the proximal promoter region of *MDR1* was confirmed using the bisulfite sequencing method (Frommer et al., 1992). DNA was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen, Purchase, NY) according to the manufacturer's instructions. PCR (578 bp, −214a to Ex1/+40) was performed in a total volume of 25 μl consisting of 50 ng of bisulfite-modified genomic DNA, 0.625 to 2.5 U of Amplitaq Gold DNA polymerase, and 0.25 μM of each primer, 5′-AAGGTGTTAGGAAGTAGAAAGGT-3′ and 5′-AAACTATCCCATAATAACTCCCAA-3′. After an initial denaturation at 95°C for 5 min, 35 cycles of 45 s at 95°C, 45 s at 55°C, and 1 min at 72°C, as well as a final extension period of 5 min at 72°C, were performed. The PCR product was cloned into the pGEM-T easy vector according to the manufacturer's instructions. The CpG methylation status of individual DNA strands was determined based on a comparison with the sequence obtained from the genomic DNA without the addition of bisulfite modifications. The number of methylated CpGs at a specific site was divided by the number of clones analyzed ($N > 15$) to yield percentage of methylation for each site.

Statistical Analysis. The 95% confidence interval was calculated to compare the differences in genotype or haplotype frequencies between Japanese and Caucasians. Results of MDR1 mRNA expression and mutation (C3435T) were analyzed with a Kruskal-Wallis test. Comparisons between two groups were performed using a Mann-Whitney *U* test. A 5% level of probability was considered to be significant.

Results

Identification of Variants in the Promoter Region of the *MDR1* Gene. Ten variants were detected in the promoter region of the human *MDR1* gene by PCR-SSCP analysis using DNAs obtained from unrelated Japanese and Caucasian subjects (Fig. 1; Table 1). Seven variants, at positions −1517a, −1459a, −1423a, −1132a, −1017a, −824a, and −755a, were newly identified in this study. The most common mutation in Japanese was G–1459aA (allelic frequency 0.250), whereas A–41aG (0.106), T–1517aC (0.080), T–1017aC (0.080), T–129C (0.080) were found at low frequency. The 5-base deletion at position –1132a to –1128a, C–145G and T–824aC were detected at extremely low frequency (0.037, 0.032, and 0.005, respectively). The frequencies of T–1017aC and T–129C were significantly lower in Caucasians than Japanese ($P < 0.05$). The two-base deletion at position −1423a to −1422a and A–755aG were identified only in Caucasian subjects, but at frequencies below 0.010. In contrast, T–1517aC, G–1459aA, a five-base deletion at position −1132a to −1128a, T–824aC, A–41aG, and C–145C were not detected in Caucasian subjects. These results indicate that genotypic frequencies of variants in the promoter region of the *MDR1* gene seemed to be dependent on race.

Identification of *MDR1* Promoter Haplotypes. On the basis of a haplotype analysis using subcloning and direct sequencing, 10 haplotypes derived from all identified promoter variants were found to be present in both populations (Table 2). In Japanese, seven haplotypes were identified with a frequency ranging from 0.005 to 0.665. Unlike in Caucasians, three variants at −1517a, −1017a, and −129 occurred simultaneously in Japanese. In total, 13 different haplotype pairs were found in the subjects examined (Table 3). In Caucasians, the most common haplotype pair was 1/1 (0.923). In contrast, hetero- or homogenous combinations of haplotypes with one or more variant sites were found at a relatively high frequency in Japanese compared with Caucasians (0.553 versus 0.077).

Association of *MDR1* Promoter Haplotypes with mRNA Expression in Placenta and Liver. Before investigating the influence of *MDR1* promoter haplotype combinations on mRNA expression in placental and hepatic tissues, we determined whether the C3435T variant influences MDR1 mRNA expression. As shown in Fig. 2, the synonymous C3435T polymorphism in exon 26 was associated with a low level of placental MDR1 expression ($P < 0.05$; Kruskal-Wallis test). Next, we compared *MDR1* promoter haplotype pairs (haplotypes 1/1, 1/2, 1/3, 1/4, and 4/4) with corresponding placental and hepatic MDR1 levels in 29 and 11 samples with the 3435 C/C and C/T genotype, respectively (Fig. 2). The MDR1 expression in placental tissue with haplotype 1/2 or 1/3 tended to increase compared with that in 1/1 samples ($P = 0.091$; Mann-Whitney *U* test; Fig. 2). However, mean mRNA levels in hetero- and homogygous samples for haplotype 4 was comparable with those in 1/1 samples. Also, the MDR1 expression in hepatic tissue of haplotype 1/2 or 1/3 tended to increase compared with that in 1/1 samples ($P = 0.07$; Mann-Whitney *U* test; Fig. 2).

Luciferase Reporter Gene Assay. To investigate the influence of promoter haplotypes on the potential for transcriptional regulation, 10 reporter plasmids containing *MDR1* promoter sequences were transiently transfected in HepG2 cells, and then luciferase activities were measured. As shown in Fig. 3, haplotypes 2, 3, and 9 increased the luciferase activity by 41, 32, and 30%, respectively, compared with that by haplotype 1. In contrast, the haplotype 6 construct resulted in a 28% reduction in activity. Other haplotypes did not seem to influence the activity.

Binding of Nuclear Proteins to the Promoter Variant Sites. To determine whether the variants in the promoter

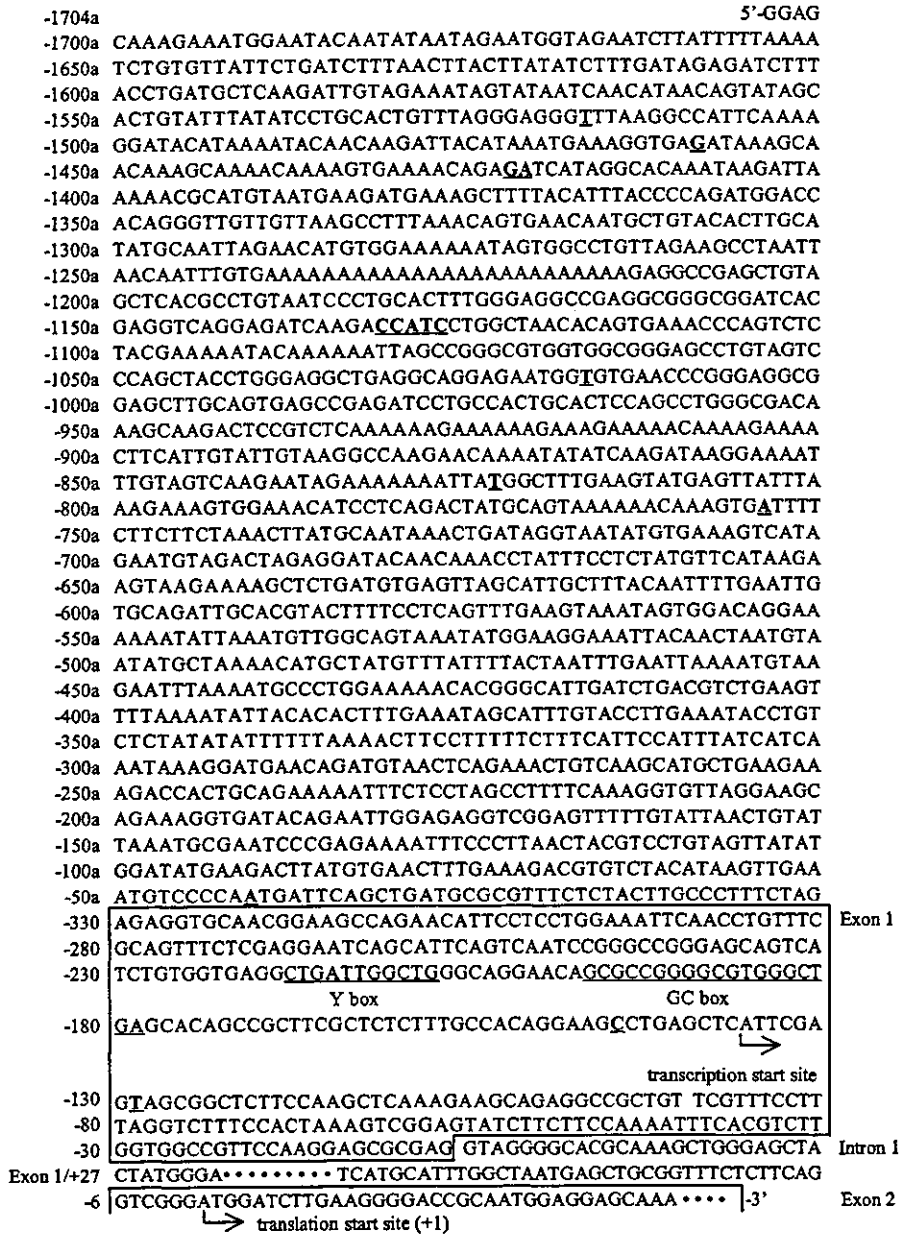

Fig. 1. Nucleotide sequence of the human *MDR1* gene promoter. Positions of nucleotide variants are in bold and are underlined. The exons are boxed. The location of variants in the coding region is relative to the initiation site for translation, which is defined as +1 based on the cDNA nucleotide sequence. The position in the promoter region is relative to the nucleotide sequence immediately preceding exon 1, which is defined as −1a.

region altered binding for transcription factors, we performed EMSA using nuclear extracts prepared from HepG2 cells. By competition assays using an excess of unlabeled probe, allele-specific binding of nuclear proteins was observed when the nuclear extracts were incubated with probes, including −1517a C (complex I) and −1459a G (complex II) (Fig. 4, A and B). Also, strong nuclear protein-DNA binding (complex III) was observed with the probe containing the −1017a T allele when compared with the −1017a C allele (Fig. 4C). The higher binding completely disappeared under an excess of unlabeled −1017a T probe, and weaker inhibition of the binding was observed with the −1017a C probe. With the −145/−129 C/T and G/T probes, nuclear protein-DNA binding (complex VI) was detected, but with the C/C probe it was not detected or was much weaker (Fig. 4D). The protein-DNA binding was completely inhibited by an excess of unlabeled C/T and G/T probes, and slightly competed with by an excess of unlabeled C/C probe. No nuclear protein-DNA binding was observed when the probes containing the −41a A/G and −145 G/C alleles were incubated with nuclear extracts.

Correlation between Placental MDR1 mRNA Expression and Methylation Status at CpG Sites in the *MDR1* Promoter Region. The proximal region of the human *MDR1* is rich in CpG (Fig. 5A). This region including the Y box and GC box elements is required for activation of the *MDR1* promoter. We focused on the CpG-rich proximal promoter region in the *MDR1* gene and determined the relationship between methylation status at each CpG site and MDR1 mRNA expression using placenta with promoter haplotype 1/1 and 3435 C/C genotypes. Results of a bisulfite sequencing analysis of 26 CpG sites in seven subjects, whose MDR1 levels varied considerably, are shown in Fig. 5B. In all samples, methylated CpG sites were found upstream of the promoter region. Moreover, an interindividual difference in methylation status was observed; however, no clear associa-

TABLE 1
Variants in promoter region of the *MDR1* gene in Japanese (n = 94) and Caucasian (n = 96) subjects

Location	Position	Allele	Nucleotide Sequence	Allele Frequency		Genotype	Frequency	
				Japanese	Caucasian		Japanese	Caucasian
Promoter	−1517a[a]	T[b]	agggTttaa	0.920 (0.881–0.959)[c]	1.000	T/T	0.840	1.000
						T/C	0.160	0.000
		C	agggCttaa	0.080 (0.041–0.119)	0.000	C/C	0.000	0.000
	−1459a[a]	G[b]	gtgaGataa	0.750 (0.688–0.812)	1.000	G/G	0.553	1.000
						G/A	0.394	0.000
		A	gtgaAataa	0.250 (0.188–0.312)	0.000	A/A	0.053	0.000
	−1423a[a]	GA[b]	cagaGAtcat	1.000 (0.985–1.005)	0.995	GA/GA	1.000	0.990
						GA/−	0.000	0.010
		Deletion	caga_tcat	0.000	0.005 (−0.027–0.015)	−/−	0.000	0.000
	−1132a[a]	CCATC[b]	aagaCCATCctgg	0.963 (0.936–0.990)	1.000	CCATC/CCATC	0.926	1.000
						CCATC/−	0.074	0.000
		Deletion	aaga_ctgg	0.037 (0.01–0.064)	0.000	−/−	0.000	0.000
	−1017a[a]	T[b]	atggTgtga	0.920 (0.881–0.959)	0.984 (0.966–1.002)	T/T	0.840	0.969
						T/C	0.160	0.031
		C	atggCgtga	0.08 (0.041–0.119)	0.016 (−0.002–0.034)	C/C	0.000	0.000
	−824a[a]	T[b]	attaTggct	0.995	1.000 (0.985–1.005)	T/T	0.989	1.000
						T/C	0.011	0.000
		C	attaCggct	0.005 (−0.027–0.015)	0.000	C/C	0.000	0.000
	−755a[a]	A[b]	agtgAtttt	1.000 (0.985–1.005)	0.995	A/A	1.000	0.990
						A/G	0.000	0.010
		G	agtgGtttt	0.000	0.005 (−0.027–0.015)	G/G	0.000	0.000
	−41a[a]	A[b]	cccaAtgat	0.894 (0.850–0.938)	1.000	A/A	0.798	1.000
						A/G	0.191	0.000
		G	cccaGtgat	0.106 (0.062–0.150)	0.000	G/G	0.011	0.000
	−145[d]	C[b]	gaagCctga	0.968 (0.943–0.993)	1.000	C/C	0.936	1.000
						C/G	0.064	0.000
		G	gaagGctga	0.032 (0.007–0.057)	0.000	G/G	0.000	0.000
Exon 1	−129[d]	T[b]	cgagTagcg	0.920 (0.881–0.959)	0.984 (0.966–1.002)	T/T	0.840	0.969
						T/C	0.160	0.031
		C	cgagCagcg	0.080 (0.041–0.119)	0.016 (−0.002–0.034)	C/C	0.000	0.000

[a] Position is relative to the initiation site of exon 1a, which is defined as +1a.
[b] Reference sequence GenBank accession no. AC002457.
[c] The 95% confidence intervals are given in parentheses.
[d] Position is relative to the initiation site of translation, which is defined as +1.

tion was observed. In addition, methylation was not observed around either the Y box or GC box element in most samples.

Discussion

Despite evidence supporting an association of coding SNPs with pharmacokinetics and pharmacodynamics, little is known about the presence or functional relevance of allelic variants in the promoter region of *MDR1*. We described 10 polymorphic variants in the *MDR1* promoter in Japanese and Caucasian populations. A–41aG, C–145G, and T–129C have been detected in the proximal promoter region of the *MDR1* gene as low frequency variants (Horinouchi et al., 2002; Tang et al., 2002; Kroetz et al., 2003). Here, we identified another seven variants. Their presence and frequency varied according to race. For example, C–145G was identified in Asian-Americans but not Caucasians (Kroetz et al., 2003). In the present study, this variant was observed only in Japanese. In addition, the new G–1459aA variant was the most frequent variant in Japanese (25.0%), but was not found in Caucasians.

The promoter region of the *MDR1* gene has been isolated and sequenced (Ueda et al., 1987; Madden et al., 1993). The promoter has an initiator sequence at the transcriptional start site, without a TATA box (van Groenigen et al., 1993). Numerous studies have shown that the Y box (inverted CCAAT box) and GC box, recognized by the transcription factors NF-Y and Sp1, respectively, are required for efficient transcriptional regulation of the *MDR1* promoter (Goldsmith et al., 1993; Sundseth et al., 1997). Several studies suggest that a region upstream of the Y box negatively regulates the *MDR1* promoter activity (Ogura et al., 1992; Cornwell and Smith, 1993), although the exact positions of the negative element differ. However, our variants are not located within those *cis*-elements.

We identified 10 different *MDR1* promoter haplotypes using subcloning and direct sequencing methods. A comparison of *MDR1* haplotype pairs with placental and hepatic expression showed that haplotypes 1/2 and 1/3 were associated with increased mRNA expression, independent of the C3435T mutation in the coding region. Interestingly, haplotypes 2 and 3, in which T–1517a C, T–1017aC, and T–129C mutations occurred simultaneously in both populations, were associated

TABLE 2
Localization of variants and identification of the *MDR1* promoter haplotypes in Japanese and Caucasian subjects

Nucleotide	−1517a	−1459a	−1423a	−1132a	−1017a	−824a	−755a	−41a	−145	−129	Frequency	
	T/C	G/A	GA/delete	CCATC/delete	T/C	T/C	A/G	A/G	C/G	T/C	Japanese	Caucasians
											n = 94	n = 96
Haplotype 1	T	G	GA	CCATC	T	T	A	A	C	T	0.665 (0.598–0.732)*	0.964 (0.937–0.991)
2	C	G	GA	CCATC	C	T	A	G	C	C	0.043 (0.014–0.072)	0.000
3	C	G	GA	delete	T	T	A	G	C	C	0.037 (0.010–0.064)	0.000
4	T	A	GA	CCATC	T	T	A	A	C	T	0.191 (0.135–0.247)	0.000
5	T	A	GA	CCATC	T	T	A	G	C	T	0.027 (0.004–0.050)	0.000
6	T	A	GA	CCATC	T	T	A	A	G	T	0.032 (0.007–0.057)	0.000
7	T	A	GA	CCATC	T	C	A	A	C	T	0.005 (−0.005–0.015)	0.000
8	T	G	delete	CCATC	T	T	A	A	C	T	0.000	0.010
9	T	G	GA	CCATC	C	T	A	A	C	C	0.000	0.016
10	T	G	GA	CCATC	T	T	G	A	C	T	0.000	0.010

* The 95% confidence intervals are given in parentheses.

TABLE 3
Haplotype configurations in promoter region of the *MDR1* gene in Japanese and Caucasian subjects

Promoter Haplotype Pair	Frequency	
	Japanese (n = 94)	Caucasian (n = 96)
1/1	0.447 (0.376–0.518)*	0.923 (0.885–0.961)
1/2	0.043 (0.014–0.072)	0.000
1/3	0.053 (0.021–0.085)	0.000
1/4	0.234 (0.173–0.295)	0.000
1/5	0.043 (0.014–0.072)	0.000
1/6	0.064 (0.029–0.099)	0.000
1/8	0.000	0.038 (0.011–0.065)
1/9	0.000	0.038 (0.011–0.065)
2/4	0.021 (0.001–0.0041)	0.000
2/5	0.011 (−0.004–0.026)	0.000
2/7	0.011 (−0.004–0.026)	0.000
3/4	0.021 (0.001–0.0041)	0.000
4/4	0.053 (0.021–0.085)	0.000

* The 95% confidence intervals are given in parentheses.

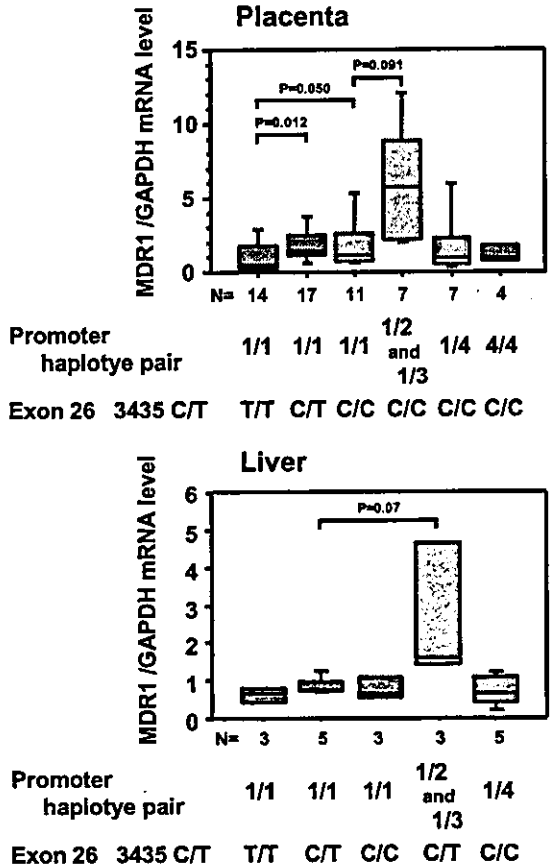

Fig. 2. Influence of *MDR1* promoter haplotypes and SNPs in the coding region on placental and hepatic MDR1 mRNA levels in Japanese. Statistical significance between the two genotypes was analyzed with the Mann-Whitney *U* test.

with an increase in transcriptional activity in human hepatoma cell line. Moreover, we showed that the T–1517aC, T–1017aC, and T–129C variants affected putative transcriptional protein-DNA binding. Heterozygosity for the –129C allele is associated with a high level of transport activity of P-glycoprotein in hematopoietic stem cells (Calado et al., 2002). The tacrolimus oral dose requirement is higher in renal transplant recipients with the T/C allele than T/T allele at position –129 (Anglicheau et al., 2003). Although these findings are not significant because the T–129C variant was

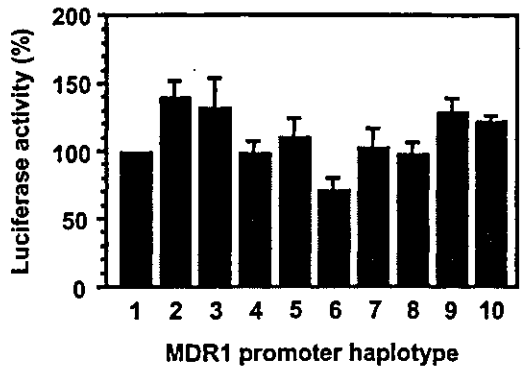

Fig. 3. *MDR1* promoter region reporter gene constructs and their relative luciferase activity levels. The value for the haplotype 1 construct was set at 100%. Each value is the mean ± S.D. of relative luciferase activity from four to five experiments.

rare, the C allele at position –129 may be associated with high level of expression of P-glycoprotein, resulting in an increase in transport activity and then a decrease in tacrolimus bioavailability after oral administration. In contrast to these haplotypes, haplotype 6 (G–1459aA and C–145G variant) was associated with a low level of transcriptional activity in hepatoma cells. We found that the G–1459aA variant inhibits the binding of unknown transcriptional factor to DNA. Therefore, allelic variants not only in the coding region but also in the promoter region of the *MDR1* gene, may be important to the interindividual difference in P-glycoprotein expression.

We determined the interindividual difference in methylation status in the proximal promoter region of the *MDR1* gene using seven placentas whose mRNA levels varied considerably. Extensive methylation of the *MDR1* gene assembled into chromatin enriched with methylated CpG binding protein interfered with the binding of transcription factors to their elements, resulting in transcriptional repression of the gene (Jin and Scotto, 1998; El-Osta et al., 2002). In this study, the region upstream of the promoter region was relatively well methylated, but individual differences in methylation status were unlikely to cause large variation in MDR1 mRNA expression. Nakayama et al. (1998) reported that methylation at CpG sites near the Y box was important for *MDR1* gene expression and was associated closely with clinical outcome in acute myeloid leukemia. However, we did not find any methylation around either the Y box or GC box element. Similar results for methylation status in the promoter region have been reported in CD8-positive cells (Fryxell et al., 1999). The element Sp1 protects the promoter from de novo methylation (Brandeis et al., 1994). Macleod et al. (1994, 1998) have suggested that the presence of a functional element within the GC-rich domain maintains the methylation-free status. Accordingly, although the proximal promoter of the *MDR1* gene is important for regulating basal gene expression in normal human tissues, epigenetic status

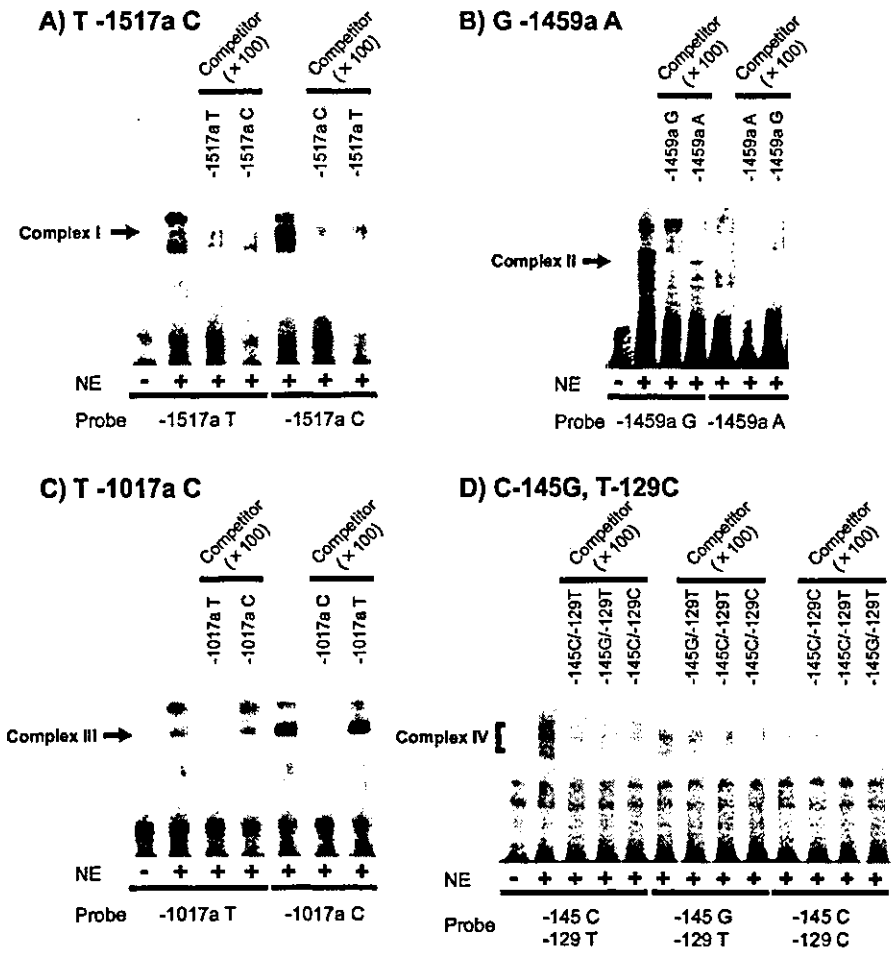

Fig. 4. EMSA analysis with oligonucleotides corresponding to variants (A, T-1517aC; B, G-1459aA; C, T-1017aT; and D, C-145G and T-129C) in the *MDR1* gene promoter. The nuclear extracts (NE; 5 μg of protein) from HepG2 cells were incubated with ^{32}P-labeled oligonucleotides. Specificity of nuclear protein binding was demonstrated by a 100-fold molar excess of unlabeled oligonucleotide. Data are representative of three similar experiments.

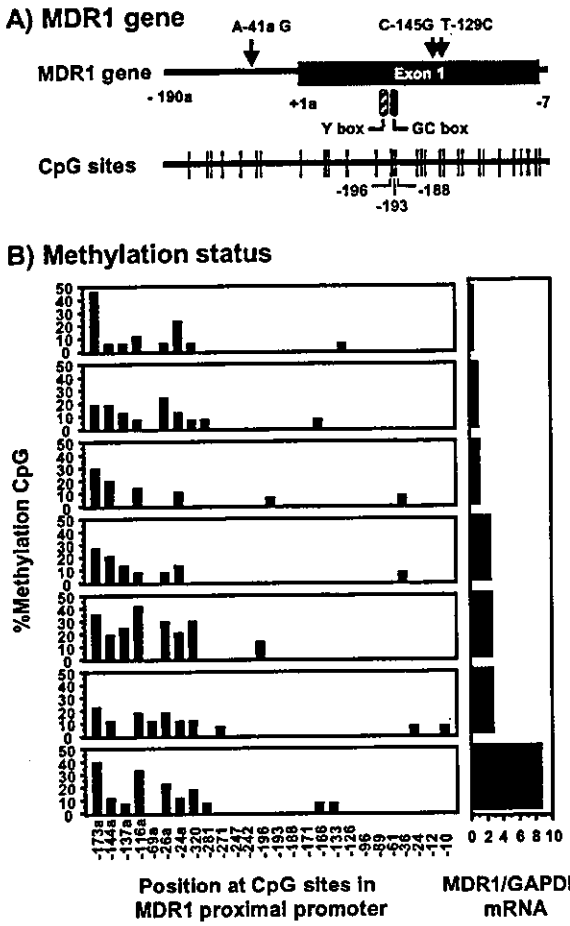

Fig. 5. A, location of CpG sites in the *MDR1* promoter region. The CpG sites are represented by short vertical bars. B, methylation status of individual sites of the *MDR1* promoter region and mRNA expression in placental tissues with the promoter haplotype 1/1 and 3435 C/C genotypes.

seems not to be associated with variability in the transcriptional activity of the *MDR1* gene.

In conclusion, we identified various variants in the promoter of the *MDR1* gene and investigated their effects on transcriptional activity and mRNA expression in the placenta. Among these variants, the promoter haplotypes containing T–1517aC, T–1017aC, and T–129C were particularly associated with high level of transcriptional activity and mRNA expression but independently of the coding SNP C3435T. Because these promoter haplotypes or SNPs are found at a relatively low frequency in Japanese and Caucasian populations, further study is needed to establish the impact of their allelic variants on drug disposition and responses in clinical situations. Interestingly, one study has shown a possibility of regulation by tissue-specific factors for the *MDR1* gene expression (Kohno et al., 1990). Also, Sundseth et al. (1997) have reported that an element just upstream of the Y box had opposite effects in different human carcinoma cell lines. Their results suggest that allelic variants in the promoter region of the *MDR1* gene contribute to the polymorphic expression of P-glycoprotein in a tissue-specific manner. Future studies may need to estimate the influence of *MDR1* promoter variants on transcriptional activity in various P-glycoprotein-expressing tissues.

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Allelic expression imbalance of the human *CYP3A4* gene and individual phenotypic status

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The human cytochrome P450 3A4 (CYP3A4) plays a dominant role in the metabolism of numerous clinically useful drugs. Alterations in the activity or expression of this enzyme may account for a major part of the variation in drug responsiveness and toxicity. However, it is generally accepted that most of the known single nucleotide polymorphisms in the coding and 5′-flanking regions are not the main determinants for the large inter-individual variability of CYP3A4 expression and activity. We show that the allelic variation is critically involved in determining the individual total hepatic CYP3A4 mRNA level and metabolic capability. There exists a definite correlation between the total CYP3A4 mRNA level and allelic expression ratio, the relative transcript level ratio derived from the two alleles. Individuals with a low expression ratio, exhibiting a large difference of transcript level between the two alleles, revealed extremely low levels of total hepatic CYP3A4 mRNA, and thus low metabolic capability as assessed by testosterone 6β-hydroxylation. These results present a new insight into the individualized CYP3A4-dependent pharmacotherapy and the importance of expression imbalance to human phenotypic diversity.

INTRODUCTION

Among the human cytochrome P450 (CYP) proteins, the members of the CYP3A subfamily occupy an important position owing to their abundance in liver and gut and to their collective large substrate spectrum (1). CYP3A proteins account for up to 50% of total CYP activity in the liver, and they metabolize up to 60% of all drugs currently in use (2,3). Cytochrome P450 3A4 (CYP3A4) is the major form of CYP in human liver; accounting for 30% of total CYP protein content (4). A wide inter-individual variation exists in CYP3A4 activity as assessed by direct analysis of liver microsomes (4) and through the use of *in vivo* probe drugs (5). The basis of this variation is not yet understood but may be due to genetic factors. A clinical study with CYP3A4 substrates suggested that ~60–90% of the inter-individual variability in hepatic CYP3A4 activity is genetically determined (6). The coding and 5′-flanking regions of the *CYP3A4* gene have been isolated and sequenced, and some single nucleotide polymorphisms (SNPs) have been identified; however, their allelic frequencies and/or the available functional experiments indicate a limited role for these variants in the inter-individual variability of CYP3A4 expression and activity (7–12).

In addition to SNPs, various gene expression mechanisms have recently been reported to determine phenotypic variability; these patterns include genomic imprinting (13,14), X-chromosome inactivation (15) and other mechanisms (16,17). Among them, genomic imprinting is an epigenetic phenomenon where parental alleles are genetically marked, leading to the differential expression of paternal and maternal alleles in somatic cells (13,14). Imprinting genes are often clustered in chromosomal domains and are thought to

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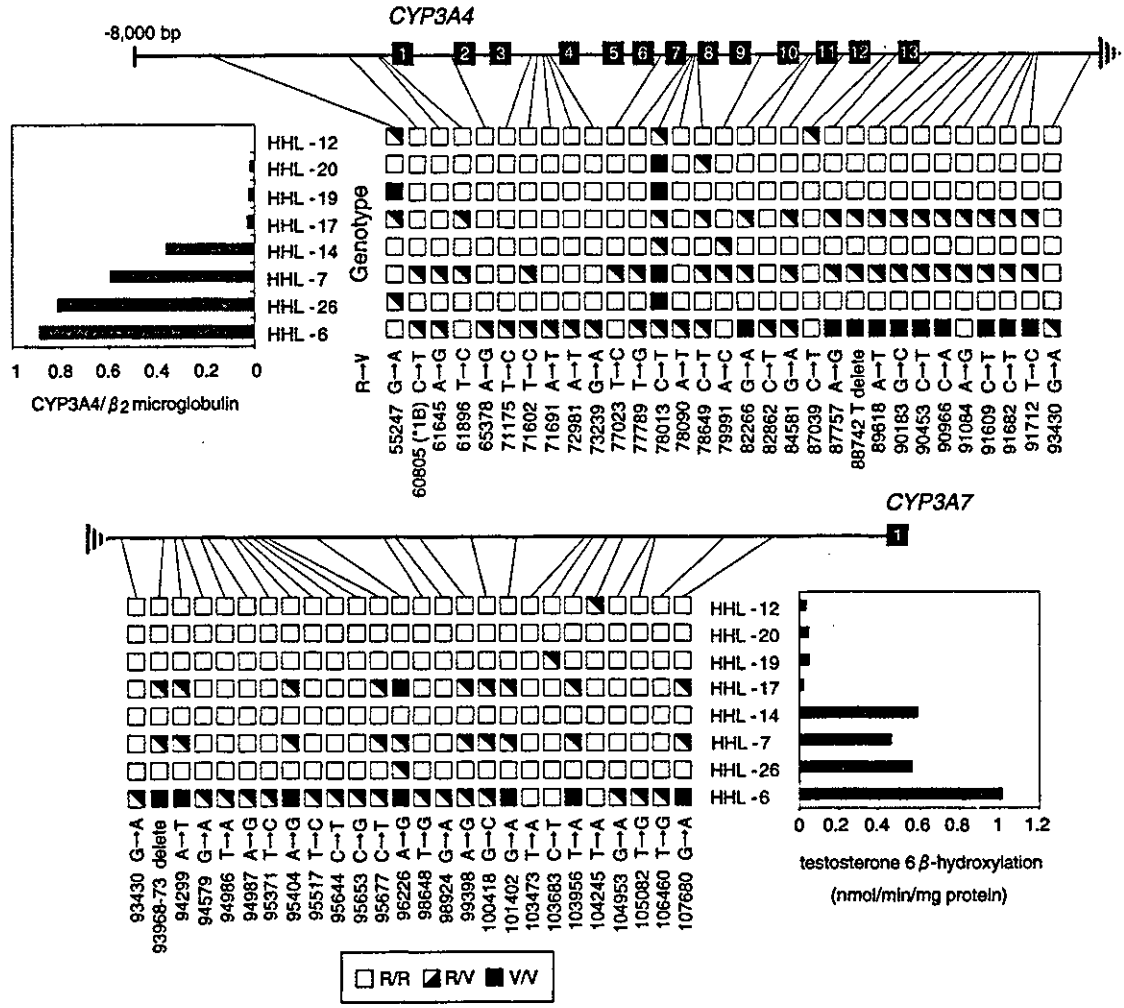

Figure 1. Relationship between CYP3A4 phenotypes and *CYP3A4* genotype in Caucasian livers. Open, partially filled and closed squares correspond to liver homozygous for the reference sequence (R), and heterozygous and homozygous for the variant sequence (V).

be coordinately regulated by imprinting control centers (13,18). Interestingly, three members of the CYP3A subfamily, *CYP3A4*, *CYP3A5* and *CYP3A7*, are localized in tandem on the long arm of chromosome 7 at q21–q22.1 (19,20), where several imprinted genes are clustered (21). In addition, differences in expression levels between the two alleles of the same gene (i.e. preferential expression of one of the two alleles), which were not consistent with parental imprinting, have been reported in various genes such as *PKD2*, *p73* and *Calpain-10* (16,22). Some investigators indicated that such allelic variation in gene expression was common in the human genome (17,22). The individual allelic expression status may result in a change in the expression level of the gene (23,24), leading to phenotypic variability in the pharmacokinetic and pharmacodynamic outcomes of drug therapy. On screening the entire *CYP3A4* gene, we found two common non-functional and racially independent intronic SNPs, C78013T (C → T at nt 78013) and C78649T (C → T at nt 78649), and used them as markers in subsequent gene expression analyses. Using these SNPs, we elucidated that the allelic variation is critically involved in the hepatic CYP3A4 mRNA expression. Individuals with a low expression ratio, exhibiting a large difference of transcriptional level between the two alleles, revealed extremely low hepatic CYP3A4 levels and thereby reduced metabolic activity. These findings explain the substantial inter-individual differences in CYP3A4 expression and activity.

RESULTS

Screening of the marker SNPs for assessment of allelic variation

To screen the marker SNPs for assessment of allelic variation and to evaluate the presence of functional *CYP3A4* gene variants, we amplified and sequenced the whole *CYP3A4* gene, *CYP3A4* 5′-flanking region and 3′-untranslated region (UTR), spanning ~60 kb, using genomic DNAs extracted from eight Caucasian liver samples with high (HHL-6, -7, -14 and -26) or low (HHL-12, -17, -19 and -20) levels of total CYP3A4 mRNA (Fig. 1). The former and latter samples also indicated high and low testosterone 6β-hydroxylation

activity, respectively. In the present study, testosterone 6β-hydroxylation capability was used as an index for individual CYP3A4 activity. We identified 4, 35 and 17 polymorphisms in the 5′-flanking region, 3′-UTR and intronic region, respectively, but did not find any coding SNPs. Although three samples, HHL-6, HHL-7 and HHL-17, had many SNPs in the regions analyzed, the other samples had only 2–4 SNPs. Among them, frequencies of the C78013T (8/8) and C78649T (4/8) polymorphisms were relatively high. Thus, we used these two intronic SNPs as markers in the subsequent gene expression study.

We next examined the association of the allelic pattern with total hepatic CYP3A4 mRNA levels. Although the CYP3A4 level was comparable between HHL-6 and HHL-26, the SNP patterns were clearly different. In contrast, there were remarkable differences in the CYP3A4 levels between HHL-7 and HHL-17 even though the SNP pattern was similar. In addition to the total mRNA levels, a similar trend was observed in the testosterone 6β-hydroxylation activity. As has previously been demonstrated (7–12), these results suggest that SNPs in these regions are not involved in the large variability in the *CYP3A4* gene expression and metabolic activity.

Correlation between total hepatic CYP3A4 mRNA levels and CYP3A4 hnRNA levels

We next examined whether tissue levels of heterogeneous nuclear RNA (hnRNA), the unprocessed precursor of the mature, functional mRNA, can be used as a surrogate for gene transcription. CYP3A4 hnRNA and total mRNA levels were determined in a total of 18 hepatic samples. Quantification of total CYP3A4 mRNA and hnRNA was performed by the real-time PCR method. As shown in Figure 2, CYP3A4 mRNA showed significant regression ($r = 0.775$; $P < 0.001$). These results validated the use of CYP3A4 hnRNA as an estimate of CYP3A4 gene activity in human liver samples.

Allelic variations in human liver samples

Prior to evaluating the functional significance of allelic variation in total hepatic CYP3A4 mRNA levels and metabolic activity, we estimated allelic expression ratios, defined as a measure of the expression of the less-abundant allele divided by that of the more-abundant allele, in order to confirm inter-individual variation in the allelic expression of *CYP3A4*. As described in more detail in the Materials and Methods, allelic variation was determined on the basis of difference in band intensities between the two alleles using a fluorescence image analyzer. When an individual is heterozygous for a marker SNP, it is possible to detect the relative abundance of allelic transcripts. Generally, both copies of human autosomal genes are assumed to be co-dominantly expressed in equal proportions. The detection of allelic variation is based on a quantitative analysis of RNA transcripts in order to detect deviations from the expected equimolar ratio between two alleles in a heterozygous sample. After the screening of genomic DNA from all 40 Caucasian liver samples, it was possible to identify 18 individuals who were heterozygous for either the *Mnl*I (C78013T) or *Aci*I (C78649T) site.

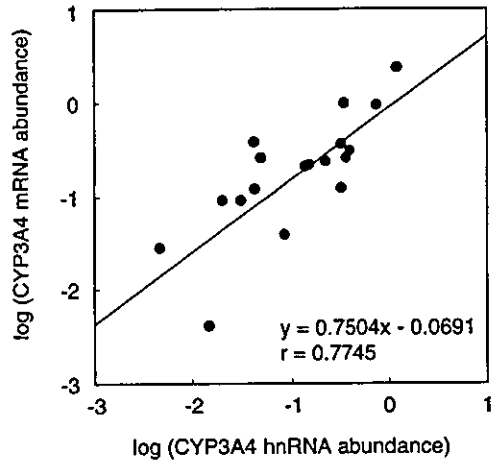

Figure 2. Relationship of total CYP3A4 mRNA and hnRNA levels in human liver samples. A simple linear regression analysis was performed.

The difference in expression between the two alleles varied among samples (Fig. 3), and some of the 18 individuals had fractional allelic expression values lower than the 95% confidence interval for the mean (0.70 ± 0.20; 95% confidence interval, 0.60–0.80). Notably, the values in HHL-12 (0.28), HHL-16 (0.45), HHL-20 (0.46) and HHL-36 (0.49) were extremely low, being well outside the intervals, indicating monoallelic expression. Preferentially expressed allele in an individual sample was also shown in Figure 3. Predominantly expressed allele was different among samples, suggesting that both marker SNPs have no significant effects on the expression of the *CYP3A4* gene.

Allelic expression pattern in informative lymphoblasts

To determine whether the two alleles of the human *CYP3A4* gene are differentially expressed according to parental origin, we used reverse transcription–polymerase chain reaction (RT–PCR) of total RNA extracted from Epstein–Barr (EB) virus-transformed lymphoblasts, followed by PCR–RFLP. The parental origin of alleles expressed in children was identified by RFLP analysis. Lymphoblasts were obtained from a panel of 22 healthy Japanese individuals who were members of six distinct families. These samples allowed the precise determination of the parental origin of alleles in the heterozygous children. Of all the cases, two siblings were heterozygous for a polymorphism at either the *Mnl*I site (C78013T in intron 7) or the *Afa*I site (G82266A in intron 10) (Fig. 4A). As the SNPs used here are in intronic regions of *CYP3A4*, the cDNA analyzed represents unspliced, hnRNA (25). Thus, all RT reactions in the present study included a negative control to ensure that genomic DNA did not contaminate the subsequent PCR. We firstly determined the parent's genotypes using genomic DNA samples. At the *Mnl*I site (for C1 in Fig. 4B and C), although the maternal genotype was homozygous for the T78013 allele, the paternal genotype was heterozygous for the C78013 allele. At the *Afa*I site (for C2), the corresponding genotypes were homozygous for the G82266 allele and heterozygous for the A82266 allele, respectively. In the RT–PCR products, in contrast to C1, who showed a

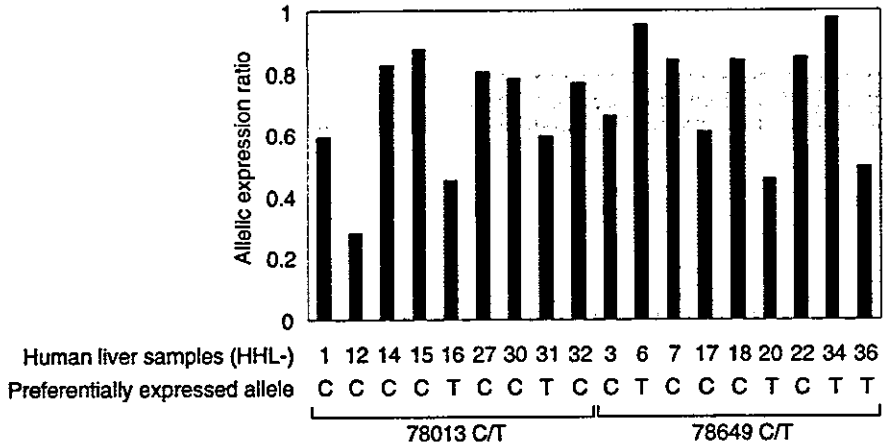

Figure 3. Allelic expression ratio of the *CYP3A4* gene in Caucasian livers. The expression ratio (*y*-axis) was estimated on the basis of the average less-/more-abundant ratios (replicated data-points for each sample) at either the C78013T or the C78649T polymorphism, and corrected using the average genomic ratio. The shaded box represents ~95% confidence interval and the red bars indicate individuals displaying significant variation.

monoallelic maternal (T78013) expression, the sibling C2 showed a biallelic expression, but the paternal allele (A82266) was preferentially expressed.

It is interesting to know whether allelic variation is inherited. In order to address this issue, we further analyzed allelic expression patterns using paternal RT–PCR products, because paternal genotype was heterozygous for both polymorphic sites (at genomic DNA-based genotypes). As shown in Figure 4B, paternal alleles were inherited by the two siblings; the paternal inactive allele (i.e. unexpressed C78013 allele at the *MnlI* site) and active allele (i.e. expressed A82266 allele at the *AflI* site) were inherited by siblings C1 and C2, respectively. These results suggest that allelic variation is inherited, at least in B virus-transformed lymphoblasts.

Methylation status in the most proximal 5′-CpG island of the *CYP3A4* gene

We focused on the most proximal 5′-CpG island (covering ~450 bp), which is ~30 kb upstream of the translational start codon, and examined the association of the methylation status with total CYP3A4 mRNA levels using six liver samples; samples HHL-6, -7 and -14 had high and HHL-12, -19 and -20 had low mRNA levels (Fig. 1). The percent methylation of the 31 CpG sites analyzed in the *CYP3A4* CpG island was calculated. Although differentially methylated CpG sites were found, most of the sites analyzed were largely methylated (Fig. 5). No clear association between the methylation status and total mRNA levels was observed.

Allelic variation and CYP3A4 phenotypes

Correlations between the allelic expression ratio and phenotype indexes are shown in Figure 6. The total hepatic CYP3A4 mRNA level correlated strongly with the allelic ratio (Fig. 6A; $r^2 = 0.786$, $P < 0.001$). Of these 18 samples, we could measure CYP3A4 activity using testosterone as an enzyme-specific substrate in 10. As expected, 6β-hydroxylation capability also correlated strongly with the allelic ratio (Fig. 6B; $r^2 = 0.541$, $P < 0.05$). These results indicate that the individuals with a low ratio, who exhibited a large difference in hnRNA expression level between the two alleles, have extremely low total hepatic CYP3A4 mRNA levels, and consequently poor metabolic capability.

Frequency of the two marker SNPs in different racial populations

We determined frequencies of heterozygous carriers for C78013T and C78649T polymorphisms, which were relatively common among the eight liver samples, using genomic DNA from three racial populations (Table 1). We found that the frequency of heterozygous carriers of the C78013T allele in Caucasians was 0.35; in Japanese, 0.39; and in African Americans, 0.56. The corresponding values for the C78649T allele were 0.21, 0.16 and 0.36, respectively. When excluding individuals having two SNPs simultaneously, 50.0% of Caucasians, 41.7% of Japanese and 61.5% of African Americans were heterozygous for either SNP.

DISCUSSION

Variations in gene sequence and expression underlie much of human variability. To decide individual gene expression status, we first sequenced the whole *CYP3A4* gene, *CYP3A4* 5′-flanking region and 3′-UTR using liver tissues from eight Caucasians, and then examined the association of SNP patterns with total CYP3A4 mRNA levels and testosterone 6β-hydroxylation capability. Although the observed nucleotide diversity in the *CYP3A4* promoter was 1 in 7246 bp in Caucasians (26), two recent reports provided evidence for the existence of a specific *cis*-acting element, 8000 bp distal to the transcription start point, which plays an important role in the transcriptional induction of CYP3A4 (27,28). Thus, we analyzed the *CYP3A4* 5′-flanking region, spanning 8 kb. As has been expected, none of the SNPs in these regions was clearly associated with differences in CYP3A4 levels and metabolic capability. These findings raise the possibility

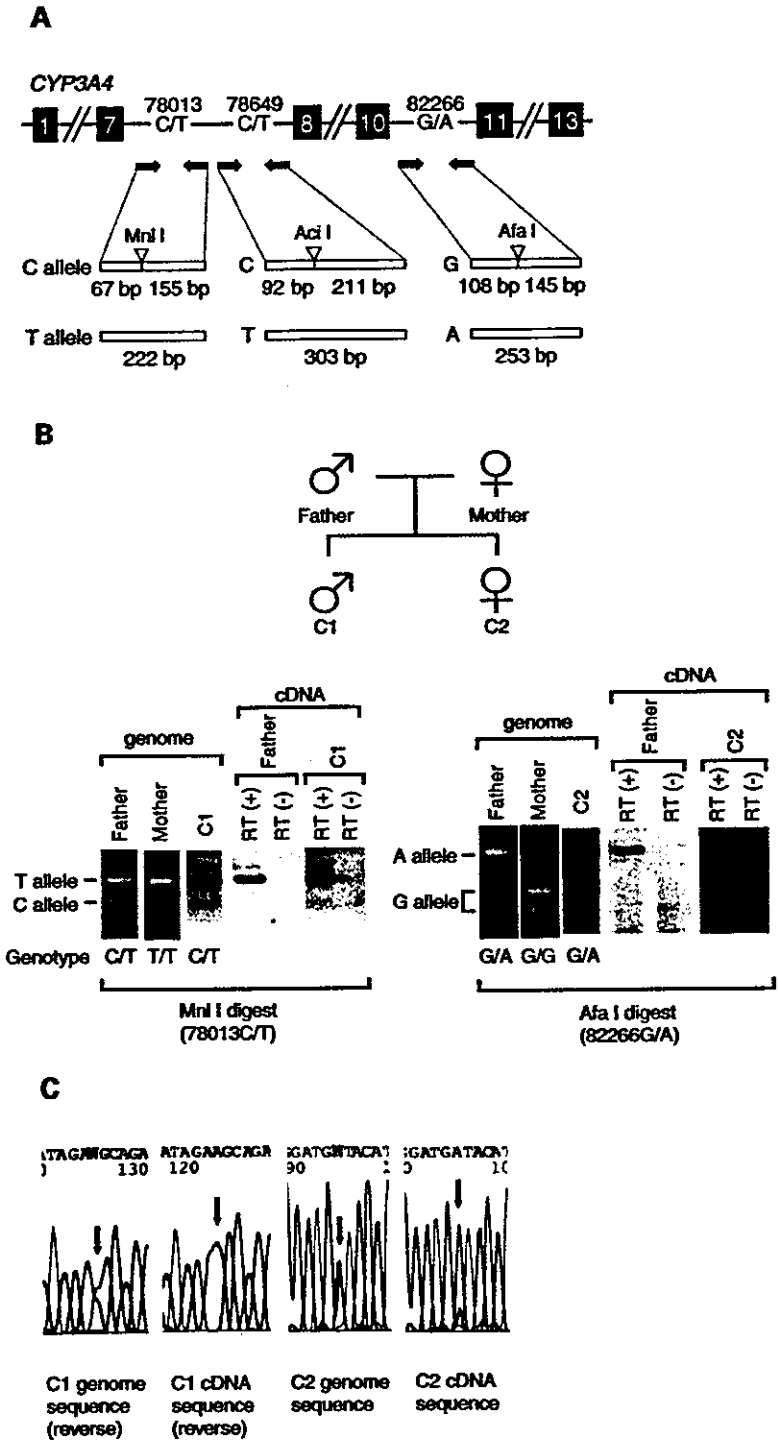

Figure 4. Identification of allelic variation of the *CYP3A4* gene. (A) Schematic of the PCR–RFLP for the three polymorphisms. Black squares denote *CYP3A4* exons. Locations of PCR primers are indicated by arrows. Predicted RFLP products of each polymorphism are drawn below the schematic. (B) Actual expression patterns in lymphoblasts obtained from two informative siblings. (C) Validation of the allelic variation by sequencing.

that other mechanisms such as an epigenetic gene alteration affect CYP3A4 levels more frequently than SNPs (7–12).

In the present study, we used unspliced hnRNA as the template and used intronic SNPs as the marker to assess the allelic variation, because there were no exonic SNPs that can be used to test for allelic variation in mRNA levels. Thus, we secondarily examined whether tissue levels of hnRNA, the unprocessed precursor of the mature, functional mRNA, can be used as a surrogate for gene transcription. To ensure precise determinations for hnRNA and mRNA levels separately,

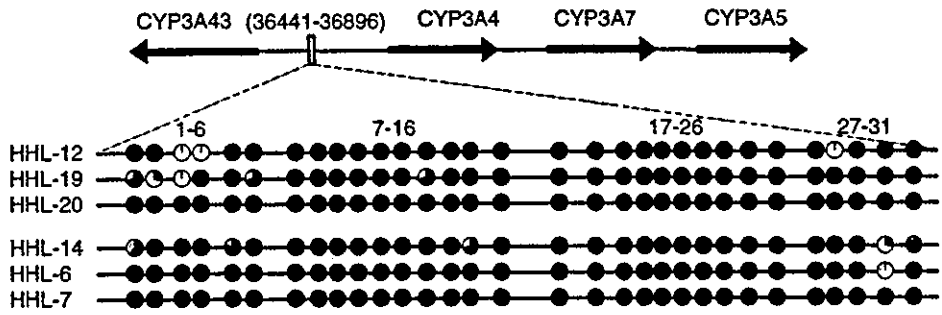

Figure 5. Methylation analysis of the CpG island of the *CYP3A4* gene. Bisulfite PCR products were subcloned and sequenced. The degree of methylation on each CpG dinucleotide was obtained from 20 individual clones. Open and closed areas represent unmethylated and methylated CpG dinucleotides, respectively.

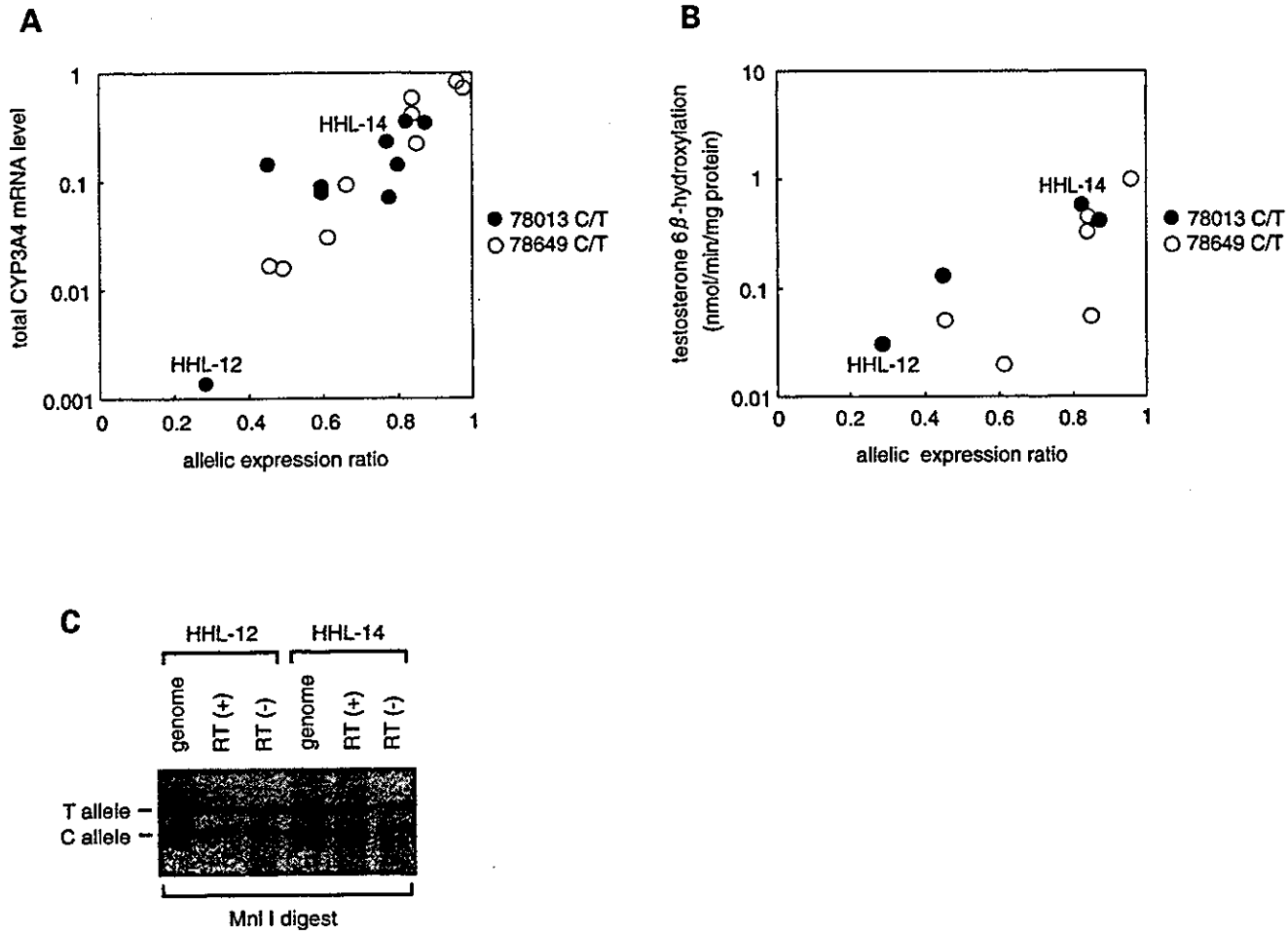

Figure 6. Allelic expression ratio and CYP3A4 phenotypes in Caucasian livers. (A) Relationship between total mRNA level (*y*-axis) and allelic ratio. The ratio was estimated based on the average less-/more-abundant ratios at either the C78013T or the C78649T polymorphism. (B) Relationship between testosterone 6β-hydroxylation capability and allelic ratio. The numbers are identical with the sample numbers in Figures 1 and 3. (C) Actual expression patterns in the livers.

we have designed intron- and exon-specific oligonucleotide primers, respectively. As shown in Figure 2, the two exhibited a significant correlation. These results validated the use of CYP3A4 hnRNA as an estimate of *CYP3A4* gene activity in human liver samples, and suggest that total CYP3A4 mRNA levels are controlled at a point upstream of the precursor by the transcriptional generation of hnRNA (29,30).

Recently, in order to determine allelic variation, an allele-specific quantitative PCR method with allele-specific probes has been developed. Such an analytical method provides the direct expression level of each allele separately by measuring the fluorescent intensity (22,31). In the present study, we had also tried to develop a real-time quantitative PCR method; however, we were unsuccessful owing to a technical limitation,

Table 1. Heterogeneous carriers for *CYP3A4* mutations in different racial populations

Position	Reference allele (R)	Variant allele (V)	Population	Genotype			Frequency of heterogeneous carriers
				R/R	R/V	V/V	
78013	ctgcCtcta	ctgcTtcta	Caucasian	8	34	54	0.35 (0.25–0.45)
			Japanese	6	37	53	0.39 (0.29–0.49)
			African American	9	54	33	0.56 (0.46–0.66)
78649	gtagCggtg	gtagTggtg	Caucasian	74	20	2	0.21 (0.13–0.29)
			Japanese	80	15	1	0.16 (0.09–0.23)
			African American	4	35	56	0.36 (0.26–0.46)

Values in parentheses indicate 95% confidence interval.

low expression levels of CYP3A4 hnRNA, especially in samples indicating monoallelic expression. Thus, we determined allelic variations on the basis of difference of band intensities between the two alleles using a fluorescence image analyzer. With such an analytical method, in addition to DNA contamination, splicing variants are drawbacks for the accurate estimation of allelic variation. In the *CYP3A4* gene, one variant, *CYP3A4*6*, with an insertion of adenine at position 17776, has been reported to create premature mRNA because the variant causes a frame shift and an early stop codon in exon 9 (32). However, the *CYP3A4*6* variant was not observed in our liver samples (data not shown), and this finding was in keeping with previous reports that it has not been observed in Caucasian populations (33,34).

Differential allelic gene expression resulting from genomic imprinting has been a focus of cancer research. Among known imprinted genes, the Wilms' tumor suppressor gene (*WT1*) has been reported to exhibit a unique allele-specific expression profile (35); cultured human fibroblasts and lymphocytes showed a paternal or biallelic expression of *WT1* in some cases, whereas a maternal or biallelic expression was observed in human placental villi and fetal brain tissue (36,37). These results suggest that the allele-specific expression profile (e.g. allele switching) of certain genes depends on the tissue source. Indeed, although somatic allele switching is not a common feature of imprinted genes, this unusual phenomenon is also observed in human *H19* (38) and *IMPT1* (39) genes. However, in the present study, the degree of difference in the expression between the two alleles varied among samples, and large variations were observed in only a minority of samples (Fig. 3). In addition, informative lymphoblast samples indicated opposite-directional expression; both paternal and maternal preferential expressions were observed in the two siblings (Fig. 4). Thus, taking these findings into consideration, it is feasible that, at least in human liver, *CYP3A4* is not an imprinting gene.

Although allelic variation in gene expression is common in the human genome (17,22), its pharmacokinetic and pharmacodynamic significance has not been reported. One study has demonstrated that the allelic variation in *APC* gene expression plays a critical role in colon cancer (16). The present study, however, is the first to demonstrate that variations in CYP3A4 phenotypes are caused by changes in allelic expression levels. The strong correlation between the allelic ratio and phenotypic indexes (e.g. total hepatic mRNA level and testosterone 6β-hydroxylation activity) indicates that individuals having low ratios, who exhibit a large difference in hnRNA expression levels between the two alleles, have extremely low levels of total CYP3A4 mRNA and thereby reduced metabolic activity. Human CYP3A activity reflects the heterogeneous expression of at least two CYP3A family members, CYP3A4 and CYP3A5. Although the role of CYP3A5 in testosterone 6β-hydroxylation *in vivo* has not been defined (40), this heterogeneity is a possible reason for the lower correlation observed between the allelic ratio and hydroxylation activity.

Although not much is known about mechanisms regulating the constitutive basal expression of the *CYP3A4* gene in human tissues, currently available data indicate that numerous transcriptional factors such as hepatocyte nuclear factor-1 (HNF-1), HNF-2, HNF-4 and CCAAT/enhancer-binding protein (C/EBP) regulate the constitutive expression of CYP genes (41). Liver-enriched transcription factors C/EBP and HNF-3γ, which are involved in the regulation of numerous liver-specific genes (42), *trans*-activate and cooperatively regulate hepatic-specific *CYP3A4* gene expression (43,44). Because these liver-enriched *trans*-activating factors play an important roles in the constitutive expression of *CYP3A4*, variations in their expression could ultimately be responsible for the different expression levels of *CYP3A4* found in various human tissues; *CYP3A4* is expressed primarily in liver and intestine and at very low and physiologically insignificant levels in lymphoblasts (45). Although transcriptional elements in lymphoblasts are suggestive of a limited expression (low or absent), allelic variation was clearly observed in lymphoblasts as well as in liver samples (Figs 4 and 6). Thus, these results suggest that it is unlikely that the transcriptional elements described earlier are involved in the allelic imbalance.

Initially in the present study, we could not find any functional SNPs in the *CYP3A4* gene. These results suggest that the post-transcriptional regulation of *CYP3A4* gene expression is likely to be similar for both alleles. As a majority of the differentially expressed genes have a virtually identical sequence in their mRNAs (46), transcriptional initiation by *cis*-acting components is one of the most important controls in regulating the variation in allelic gene expression. The deviation of allelic variation varied among samples (Fig. 3), which also suggests that the variation could result from the allelic heterogeneity of one or more *cis*-acting regulatory polymorphisms (47) or from epigenetic factors such as DNA methylation (48) and the existence of non-coding RNAs (49). In this regard, we examined methylation status in the CpG island,

which is ~30 kb upstream of the translational start codon, using six Caucasian liver samples with high and low levels of total CYP3A4 mRNA by cloning and sequencing bisulfite-treated DNA. However, unfortunately, we did not observe allele-specific differential methylation (Fig. 5).

Recently, Lo *et al.* (22) reported that 326 of 602 genes showed a preferential expression of one allele, and 170 of those showed greater than a 4-fold difference between the two alleles. Interestingly, some of the genes that showed a skewed allelic expression were located next to each other (i.e. clustered), and a subset of these genes is located in known imprinting domains. *ASB4*, *DLX5*, *SGCE* and *PEG10*, which have previously been reported to be imprinted, are located in the imprinting domain at 7q21–q31 (21). The human *CYP3A* genes, *CYP3A43*, *CYP3A4*, *CYP3A7* and *CYP3A5*, consist of a cluster spanning 231 kb within this domain, at 7q21–q22.1 (19,20). In addition, two *CYP3A* pseudogenes are formed in two intergenic regions (*CYP3A4–CYP3A7* and *CYP3A7–CYP3A5*) (50). These unique gene-structural features suggest that the allelic variation in hepatic CYP3A4 expression can be attributed to unknown non-coding RNA(s). The paternally expressed non-coding RNA (*Air* RNA) overlapping one of three imprinted, maternally expressed protein-coding genes (*Igf2r/Slc22a2/Slc22a3*) has been reported to play an important role in repression of all three genes on the paternal expression (49). Nevertheless, we cannot exclude the possibility that the cis-acting regulatory polymorphisms (47) responsible for the change in CYP3A4 expression reside far up- and downstream of the gene of the affected allele. Indeed, Wojnowski and Brockmoller (51) have recently indicated a hepatic transcriptional imbalance of the *CYP3A5* gene in *CYP3A5*1A/*3* heterozygous samples, and the *cis*-acting *1A variant, which increases the expression of the *CYP3A5* gene transcript from the allele carrying the variant, is a possible mechanism for the imbalance.

One report provided evidence that allelic variation can be transmitted by Mendelian inheritance (16). As shown in Fig. 4B, our results indicated that the allelic variation of *CYP3A4* expression was also inherited, as has been observed in the genes *Calpain-10* and *PKD2* (16). Yan *et al.* (16) identified three informative families and found that altered expression of the genes *CAPN10* and *PKD2* was consistently inherited with a single haplotype defined by at least two adjacent microsatellite markers. In contrast to the findings by Yan *et al.* (16), although the allelic variation of *CYP3A4* expression was consistent with Mendelian inheritance, allelic expression ratio appeared to be independent from genotypes. In the present study, we had tried to find the useful haplotypes within the regions we had analyzed; however, we were unsuccessful owing to large inter-individual variability in the SNP pattern (Fig. 1). The mechanism that generates allelic variation between two alleles remains unclear. However, as the expression of *CYP3A4* is unlikely to be regulated by imprinting, these results also suggest the existence of unknown, unidentified *cis*-acting inherited variations influencing gene expression. If *cis*-acting inherited variations in gene expression are common among normal populations, an insight into how this occurs in individuals may help us to understand the large variability in CYP3A4 phenotypes.

MATERIALS AND METHODS

Population samples

We examined allelic frequencies of C78013T and C78649T mutations in genomic DNA samples from unrelated Caucasian, Japanese and African American volunteers (96 subjects each) (Tennessee Blood Services, Memphis, TN, USA). We also obtained samples from the following sources: 22 lymphoblast samples for which the parental origin of the *CYP3A4* alleles was determined (35); 18 livers (selected from 40 Caucasian donors, National Disease Research Interchange, Philadelphia, PA, USA) for which allelic expression variations and total hepatic CYP3A4 mRNA and hnRNA levels were determined, and 10 of the 18 liver samples in which testosterone 6β-hydroxylation capabilities had been analyzed. We sequenced the *CYP3A4* gene, 5′-flanking region and 3′-UTR in eight of the 18 liver samples whose total CYP3A4 mRNA and hnRNA levels had been determined. We also determined methylation status in six of the 18 liver samples. EB virus-transformed lymphoblast cultures were obtained using standard procedures. This study was approved by the Ethical Board of the Faculty of Medicine, Tottori University and informed consent was obtained from all individuals.

Primers and sequencing

We designed 72 primer sets based on a published sequence (GenBank accession no. AF280107.1) to amplify the *CYP3A4* gene, *CYP3A4* 5′-flanking region and 3′-UTR; the amplicons were ~900 bp long (sequence available on request). Primer pairs were used for 30–40 cycles to amplify genomic DNA. The following conditions were used in each cycle: 95°C for 40 s, 52°C for 45 s, and 72°C for 1 min. PCR products were sequenced either directly or after subcloning using BigDye Terminator (Applied Biosystems, Foster City, CA, USA) sequencing. The sequencing primers were those used in the PCR amplifications. The sequence of both strands was analyzed for products from at least two independent PCR amplifications to ensure that the identified mutations were not PCR-induced artifacts.

cDNA synthesis

Total RNA was extracted with an RNAeasy Kit (Qiagen, Hilden, Germany) from EB virus-transformed lymphoblasts and liver samples. Prior to RT, total RNA samples were first treated with RNase-free DNase I, and then digested with *Hae*III and *Mbo*II (Takara, Kyoto, Japan). *Hae* III and *Mbo*II digest the potential DNA template which would lead to the amplification of both alleles and thus mask allelic variation. The RNA samples were then reverse-transcribed into first strand cDNA with 1 μg of total RNA, 4 μl of 5 × first strand buffer, 4 μl of 0.1 mM DTT, 1 μl of 500 μg/ml random primer (Promega, Madison, WI, USA), 4 μl of 10 mM dNTP mixture and 200 U of SuperScript II RNase H^- reverse transcriptase (Life Technologies, Rockville, MD, USA). The reaction was incubated at 42°C for 60 min. RT reactions were always carried out in the presence or absence of reverse transcriptase to ensure that genomic DNA did

not contaminate the subsequent PCR. In all experimental procedures, no amplification was detected in the absence of RT, excluding DNA contamination.

Assessment of allelic variation (estimation of allelic expression ratio)

To assess allele-specific expression of *CYP3A4*, an *MnlI* RFLP and an *AciI* RFLP in intron 7 (for liver samples), and an *MnlI* RFLP in intron 7 and an *AflaI* RFLP in intron 10 (for lymphoblast samples) were analyzed. Primer sequences for cDNA amplification were as follows: *MnlI* RFLP, 5′-TATCAG CCCCCTGTCACAAAC-3′ (forward) and 5′-TTCATGCCA CAACATAGTAAA-3′ (reverse); *AciI* RFLP, 5′-CAATAGA TAAAGGCAAAGAGA-3′ (forward) and 5′-GAAAGACTG CTGTAGGAAAAA-3′ (reverse); *AflaI* RFLP, 5′-GCAGTG TTCTCTCCTTCATTATGTA-3′ (forward) and 5′-CTAT GTTTCTTTCTTTTTCTTTTCA-3′ (reverse). All PCR products contain either a *HaeIII* or *MboII* restriction site. PCR was carried out under the same conditions for the screening of the variants, but only for 24–30 cycles. RFLP products were electrophoresed on a 3% agarose gel, then stained with SYBR green I (Takara). The relative expression of each allele was quantified on the basis of the difference in band intensities between the two alleles with a fluorescence image analyzer (Hitachi, Tokyo, Japan) using Analysis Version 6.0 software. As a control, genomic DNA PCR–RFLP products were included and ratios of the allele-specific band intensities were taken as a 1 : 1 allelic representation. In order to eliminate sampling or measurement error, we conducted the experiment for each sample with three replicates.

Methylation analysis

The methylation status of the CpG island which is ~30 kb upstream of the translational start codon (nt 36441–36896, GenBank accession no. AF280107), was confirmed by the bisulfite sequencing method (52). DNA was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen, Purchase, NY, USA) according to the manufacturer's instructions. PCR was performed in a total volume of 25 μl consisting of 50 ng of bisulfite modified genomic DNA, 0.625 U of DNA polymerase and 0.25 μM of each primer; 5′-GGGTTTTATTTAGTTTGAGTTT-3′ (forward) and 5′-TAACCCCTCCTCTACATTCTAT-3′ (reverse). After an initial denaturation at 95°C for 9 min, 43 cycles of 40 s at 95°C, 45 s at 52°C and 1 min at 72°C, as well as a final extension for 5 min at 72°C, were performed. The PCR product was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) then transformed into JM-109 (Promega), and plasmid DNA was collected by QIAprep Spin Mini-prep kit (Qiagen,). The CpG methylation status of individual DNA strands was determined on the basis of a comparison with the sequence obtained from the genomic DNA without the addition of bisulfite modifications. Percent methylation of each site was determined by dividing the number of methylated CpGs at a specific site by the total number of clones analyzed ($n = 20$ in all cases).

Quantitative real-time PCR

Quantification of total hepatic CYP3A4 mRNA and hnRNA was performed by real-time PCR detection using an ABI PRISM 7700 sequence detector (Applied Biosystems) with SYBR green detection of amplification products. Amplification mixtures contained 12.5 μl of 2 × SYBR green I Universal PCR Mix (Applied Biosystems), 0.5 μl of cDNA synthesis mixture, 5 pmol each of the forward and reverse primers and distilled water in a total volume of 25 μl. All primers were designed using the PrimerExpress program (Applied Biosystems). Primers for CYP3A4 mRNA were directed to a sequence that spans the junction of exons 12 and 13, corresponding to open reading frame 1405–1465; 5′-AAAGAAAC ACAGATCCCCCTGAA-3′ (forward) and 5′-CGGGTTTTTC TGGTTGAAGAAGT-3′ (reverse). CYP3A4 hnRNA primers were directed to a sequence located at bases +2253 to +2351 within intron 12 of the *CYP3A4* gene sequence; 5′-CACAGG TTTCCATGAATTTGTCT-3′ (forward) and 5′-AAGATTGG ACAGTGAGAGCATTC-3′ (reverse). The copy number of the transcript was measured against a copy-number standard curve of cloned target templates consisting of serial 10-fold dilution points. β_2-Microglobulin mRNA was used as the reference message for both CYP3A4 mRNA and hnRNA.

Testosterone 6β-hydroxylation capability in human liver samples

All incubations were performed in duplicate in solutions containing potassium phosphate (0.1 M, pH 7.4) and human liver microsomes (0.05 mg). Testosterone (final concentration, 30 μM) was added to the incubation mixture at a final methanol concentration of 1%, and the mixtures were incubated at 37°C for 10 min. We added a NADPH-generating system to initiate the reaction. Reactions were terminated by the addition of 100 μl of ice-CH_3CN. We used high-performance liquid chromatography (HPLC) to measure the quantities of extracted compounds (53).

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