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Genetic Analysis of the Chinese Cytochrome P4502D Locus: Characterization of Variant *CYP2D6* Genes Present in Subjects with Diminished Capacity for Debrisoquine Hydroxylation

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SUMMARY

Cytochrome P4502D6 (CYP2D6) catalyzes the oxidative metabolism of several clinically important classes of drugs. Many of these have lower metabolic clearance rates among Chinese, compared with Caucasians, and are prescribed at lower doses for Asian patients. We have now evaluated the molecular genetic basis for this interethnic difference in drug metabolism. The *CYP2D* loci from two Chinese subjects, one homozygous for the *Xba*I 44-kilobase haplotype and one homozygous for the *Xba*I 29-kilobase haplotype, were cloned and characterized. Sequence analysis revealed two variant *CYP2D6* genes, *CYP2D6Ch*$_1$ and *CYP2D6Ch*$_2$, having mutations yielding two and eight amino acid substitutions, respectively. Exon 9 of the *CYP2D6Ch*$_2$ gene contained a sequence of 49 bases originating from the pseudogene *CYP2D7P*. In addition, mutations in the 5′ flanking region common to both *CYP2D6Ch* genes were found. To evaluate the origin of the detrimental mutation in the genes, parts of the 5′ flanking regions were introduced into a Hep G2/simian virus 40 expression system with chloramphenicol acetyltransferase as a reporter gene, and transfected cells were analyzed for activity. The ability of the upstream regions to bind nuclear factors was also evaluated using gel-shift analysis. Furthermore, several chimeric constructs of the *CYP2D6wt* and *CYP2D6Ch* genes were made, inserted into pCMV2 vectors, and expressed in COS-1 cells. A part of the upstream region of base pairs −1407 to −1068 was found to constitute an enhancer element, but the *CYP2D6Ch*-specific mutations did not influence the chloramphenicol acetyltransferase activity in the expression system. In contrast, expression of the chimeric genes revealed that the detrimental mutation of the *CYP2D6Ch* genes was $C^{188}\rightarrow T$, causing a $Pro^{34}\rightarrow Ser$ amino acid substitution in a region that is a highly conserved in cytochromes P450 belonging to gene families 1 and 2. This substitution caused expression of a more unstable gene product, as evident from comparison of the relative levels of CYP2D6 mRNA, CYP2D6 protein, and bufuralol 1′-hydroxylase activities in pCMV2-*CYP2D6*-transfected COS-1 cells. Allele-specific polymerase chain reaction analysis of genomic DNA from 90 Chinese individuals revealed that the *CYP2D6Ch*$_1$ allele was the most common one and its distribution correlated well with a higher metabolic ratio for debrisoquine. These data demonstrate that important interethnic differences exist in the structure of the *CYP2D* locus, and they suggest that the frequent distribution of the $C^{188}\rightarrow T$ mutation among the *CYP2D6Ch* genes explains the lower capacity among Chinese to metabolize drugs that are substrates of CYP2D6, such as antidepressants and neuroleptic agents.

Interethnic differences in the disposition of xenobiotics are well recognized and include those seen in the acetylation and hydroxylation of drugs and the oxidation of ethanol (1, 2). Such differences have been described for debrisoquine hydroxylase, which catalyzes the oxidation of several clinically important groups of drugs, e.g., lipophilic β-blockers, antiarrythmic agents, antidepressants, and neuroleptic drugs. The deficient metabolism of debrisoquine affects about 7% of the Caucasian population in Europe and North America (3–6), whereas only 7 of 695 Chinese individuals (7) and none of 100 Japanese individuals (8) were found to be PM (MR for debrisoquine >12.6). Important interethnic differences have also been found with respect to *S*-mephenytoin hydroxylase, an enzyme that catalyzes the oxidation of, for example, proguanil, omeprazole, and diazepam (2, 7). In this case, 3% of Caucasians and 15–20% of Asians are deficient in this isozyme (7, 8).

The molecular basis for the interethnic differences in the debrisoquine hydroxylase deficiency is being clarified. Thus,

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ABBREVIATIONS: PM, poor metabolizer(s) of debrisoquine; CYP or P450, cytochrome P450; EM, extensive metabolizer(s) of debrisoquine; MR, metabolic ratio; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); bp, base pair(s).

the major Caucasian defective *CYP2D6* alleles (*CYP2D6A* and *CYP2D6B*) are rarely found among Chinese (9, 10), black Americans (11), and black Zimbabweans (12). In contrast, the detrimental mutations in the gene encoding *S*-mephenytoin hydroxylase, *CYP2C19* (13, 14), still remain obscure.

Chinese individuals, who by definition are EM (MR for debrisoquine <12.6), metabolize CYP2D6 substrates more slowly than do Caucasians, as revealed by the generally higher MR values for debrisoquine among Chinese EM (7). Accordingly, plasma clearance of desipramine (a CYP2D6 substrate) has been found to be lower among Chinese, compared with Caucasians (15), and Chinese also metabolize other antidepressants more slowly than do Caucasians (16). These findings are compatible with the fact that lower doses of antidepressants are prescribed for Asian, compared with Caucasian, patients (17). A similar relationship is also true for treatment with neuroleptic drugs (18). Chinese individuals have been found to have 52% higher plasma concentrations of haloperidol than American non-Asian individuals, when given a fixed amount of haloperidol orally (19), a finding also in line with the results of Lin *et al.* (16). It has also been suggested that Chinese individuals are more sensitive than Caucasians to the side effects of desipramine (15) and haloperidol (19), drugs that are metabolized by CYP2D6 (see Ref. 20).

The molecular basis for the interethnic difference between Chinese EM and Caucasian EM in the MR for debrisoquine is not clear. It has been found that Chinese EM individuals frequently carry a haplotype, characterized by a *Xba*I 44-kb *CYP2D* fragment, that is associated with the PM phenotype among Caucasians (9, 21). In view of the potentially important pharmacological consequences, we considered it of interest to investigate the differences in the *CYP2D* locus between Caucasians and Chinese. We now present data showing that the Chinese *Xba*I 44-kb haplotype is different from the Caucasian homologue and contains two variant *CYP2D6* genes, causing the formation of apparently unstable enzyme products that are ineffective in drug hydroxylation. One of these genes, $CYP2D6Ch_1$, is the most common one in the Chinese population studied and most likely represents an ancestor of the defective Caucasian *CYP2D6B* gene. The second gene is, in addition, a product of a gene conversion between a pseudogene and the $CYP2D6Ch_1$ gene.

Experimental Procedures

Isolation of the *CYP2D* genes. DNA samples from Chinese individuals were from a previous study (9). Leukocyte DNA from a Chinese individual homozygous for the 44-kb *Xba*I fragment and with a MR for debrisoquine of 3.7 (see Ref. 9) was completely digested with *Eco*RI and ligated into λEMBL4 for construction of a gene library. The DNA was packaged using Gigapack II Gold (Stratagene, La Jolla, CA). The DNA library was screened with [α-^{32}P]dCTP-labeled *CYP2D6* cDNA. Phages from positive clones were analyzed with *CYP2D6* allele-specific PCR amplification (22) and restriction maps after digestion with *Eco*RI, *Eco*RI plus *Bam*HI, and *Eco*RI plus *Kpn*I.

Sequencing analysis. Clones corresponding to the *CYP2D6* gene or the extra Chinese *CYP2D* gene were digested with *Eco*RI and *Kpn*I. The 5.8-kb fragments (23), containing all nine exons and 1531 bp of the upstream region, were subcloned into the pUC19 vector (Pharmacia Biotech, Uppsala, Sweden). Further subcloning into the pBluescript vector (Stratagene) was also performed. The *Eco*RI-*Kpn*I fragments were partly sequenced by the double-strand dideoxy chain termination method, using Sequenase version 2.0 T7 DNA polymerase (United States Biochemical, Cleveland, OH). Universal and reverse primers were used as well as synthesized oligonucleotides (17-mers).

Mutation-specific PCR amplifications. The mutations in the exons and in the 5′ flanking region were investigated with regard to their distribution in Caucasian and Chinese populations. For determination of the $C^{188}\rightarrow T$ mutation in exon 1, *CYP2D6*-specific PCR amplification was carried out with primers 9 and 10 (Table 1). About 13% of the Chinese alleles were of the *CYP2D6L* type (23) and contained a sequence in intron 1 originating from *CYP2D7P*. Amplification in these cases was carried out using primer 9 and primer 10B (23) (Table 1). The exon 1 mutation was determined by a second PCR of the amplified products using primer 9 and primer 11 or 12 (Table 1). Similarly, the $G^{4268}\rightarrow C$ mutation in exon 9 was determined by initial *CYP2D6*-specific amplification using primers 15 and 16, followed by mutation analysis using primer 15 and primer 17 or 18 (Table 1) in separate reactions. The $C^{-1338}\rightarrow T$ mutation in the 5′ flanking region was determined in a similar way by *CYP2D6*-specific amplification using primers 23 and 14, followed by mutation-specific amplification using primer 14 and primer 21 or 22 (Table 1). The pseudogenic origin of the clones carrying inserts corresponding to the *CYP2D8P* and *CYP2D7P* genes was determined by direct sequencing of PCR products obtained after amplification of cloned phage DNA using biotinylated primers and isolation of template using magnetic streptavidin-conjugated beads (Dynal AS, Oslo, Norway).

Construction of chimeric expression plasmids. Four chimeric expression clones containing the full length *CYP2D6* gene were constructed according to the method of Kagimoto *et al.* (24), with some modifications. The *Hind*II-*Bss*HII fragment of the *wt* allele was combined with the *Bss*HII-*Kpn*I fragment of the *wt*, Ch_1, and Ch_2 alleles, and thereby three clones were constructed, denoted pUC19-*wt*, -*mut 9:1*, and -*mut 9:2* respectively. The *Hind*II-*Bss*HII or the *Hind*II-*Ksp*I fragment from the Ch_2 allele was combined with the *Bss*HII-*Kpn*I or *Ksp*I-*Kpn*I fragments to obtain the pUC19-Ch_1 and pUC19-Ch_2 constructs, as outlined in Fig. 1. All chimeric genes were originally constructed in pUC19 vectors and then transferred to pCMV2 vectors (25) using the restriction enzymes *Eco*RI and *Xba*I. To verify the identity of all pCMV2 clones, they were subjected to allele-specific PCR analysis and restriction enzyme analysis with *Nco*I. The *CYP2D6wt* cDNA was used as a positive control. A negative control was constructed by deleting the *Sma*I fragment from the *CYP2D6wt* cDNA, yielding the vector alone except for 150 bp of exon 1.

Expression of chimeric genes. The chimeric genes were expressed in COS-1 cells. Cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL Life Technologies, Inchinnan, Scotland) containing 1000 mg/liter glucose, 10% fetal calf serum, and penicillin/streptomycin (100 IU/ml, 100 mg/ml), on 92-mm plates, to a confluence of approximately 70% and were transfected with 15 μg of plasmid DNA according to the DEAE-dextran method (26), using 500 μg/ml DEAE dextran (Pharmacia Biotech, Uppsala, Sweden). Treatment with DEAE-dextran was for 2 hr, and chloroquine (52 μg/ml) was then

TABLE 1
Primers used in the current investigation

Primer name	Position	Sequence
23	(−1523 to −1504)	5′-GACCAGCCTGGACAACTTGG-3′
14	(−982 to −1001)	5′-CTTTGTTCAGGATATGTTGC-3′
21	(−1352 to −1338)	5′-CTCTACTGAAAATAC-3′
22	(−1352 to −1338)	5′-CTCTACTGAAAATAT-3′
9	(−196 to −179)	5′-ACCAGGCCCCTCCACCGG-3′
10	(321 to 302)	5′-TCTGGTAGGGGAGCCTCAGC-3′
10B	(L320 to 302)	5′-GTGGTGGGGCATCCTCAGG-3′
11	(201 to 188)	5′-AGGGGGCCTGGTGG-3′
12	(201 to 188)	5′-AGGGGGCCTGGTGC-3′
15	(3623 to 3640)	5′-TCTAGTGGGGAGACAAAC-3′
16	(4810 to 4793)	5′-ATATAGCTCCCTGACGCC-3′
17	(4281 to 4268)	5′-ATAGGGGGATGGGC-3′
18	(4281 to 4268)	5′-ATAGGGGGATGGGG-3′

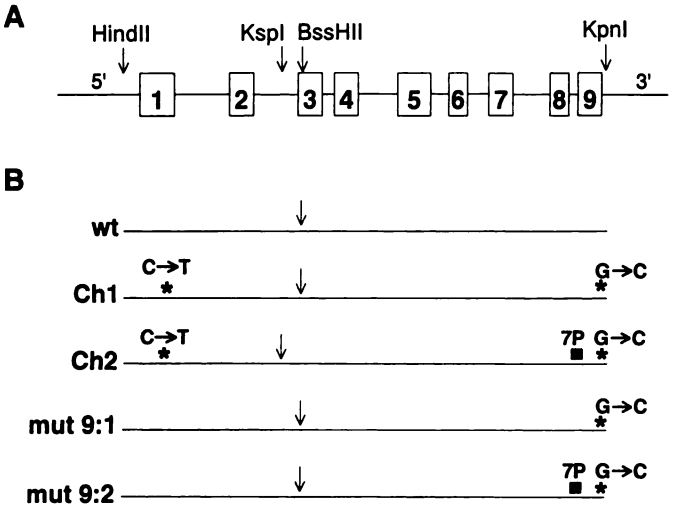

Fig. 1. Illustration of the chimeric *CYP2D6* genes constructed. A, Restriction enzyme sites used for construction of the chimeras. B, Schematic diagram of the chimeric genes. *, Presence of a functional single-base mutation in a coding region (exon 1, $C^{188}\rightarrow T$; exon 9, $G^{4268}\rightarrow C$). ■, Presence of the *2D7P* gene conversion in exon 9. *Arrows* show whether *BssHII* or *KspI* was used (see text).

present for 4–5 hr. After incubation for 54 hr, the cells were harvested in 0.1 M sodium phosphate buffer, pH 7.4.

Quantification of CYP2D6 mRNA and protein. Cells for mRNA analysis were lysed and total RNA was isolated according to the method of Okayama *et al.* (27). Northern blot analysis was carried out essentially as described by Johansson *et al.* (28). Cells for Western blot analysis and bufuralol hydroxylase determination were sonicated for 20 × 1 sec. Cell homogenates corresponding to 35 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels. The proteins were transferred to a Hybond nitrocellulose filter (Amersham, Buckinghamshire, England), incubated with a monoclonal mouse anti-CYP2D6 antibody (114/2; obtained from Dr. Urs A Meyer, Biozentrum, Basel, Switzerland) (29) and with a horseradish peroxidase-linked goat anti-mouse antibody (DAKO A/S, Glostrup, Denmark), and visualized by the enhanced chemiluminescence method (Amersham). The filters were scanned using a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

Bufuralol 1′-hydroxylation assay. Cell homogenate corresponding to 1.2 mg of protein was incubated at 37° in 0.1 M sodium phosphate buffer, pH 7.4, with 0.15 nmol of rat P450 reductase, purified as described by Ingelman-Sundberg and Glaumann (30), and 160 μM (+)-bufuralol, in a total volume of 125–175 μl. Every 60 min 80 μg of NADPH were added to the mixture. After 2 or 4 hr the incubation was terminated by addition of 14 μl of 70% perchloric acid. The samples were centrifuged at 1500 × *g* for 5 min and the supernatants were then subjected to reverse phase high performance liquid chromatography essentially as described by Kronbach *et al.* (31). 1′-Hydroxybufuralol was used as an external standard.

Mobility-shift DNA-binding analysis. Nuclear protein extracts were prepared from a human liver specimen active in debrisoquine hydroxylation, according to the protocol described by Nakabayashi *et al.* (32). Mobility-shift DNA-binding analysis was carried out by using high ionic strength electrophoresis according to the method of Chodosh *et al.* (33), using a labeled fragment of the 5′ upstream region of *CYP2D6wt* and $CYP2D6Ch_1$ (bp −1523 to −1087). Competition experiments were performed with fragments corresponding to the upstream region of the genes as well as with a double-stranded oligonucleotide (bp −1362 to −1331) covering the $C^{-1338}\rightarrow T$ mutation site.

Expression in the pCAT system. A 340-bp fragment from the upstream region (bp −1407 to −1068) of the plasmids containing *CYP2D6wt* and $CYP2D6Ch_1$ alleles, which was obtained by digestion with *Sau*3AI, was cloned into the *Bam*HI site of the pCAT promoter vector (Promega, Madison, WI). The pCAT promoter vector and pCAT control vector (Promega) were used as controls. The pCAT vectors were transfected into Hep G2 cells using the $CaCl_2$ precipitation method (34). The cells were grown for 52 hr before CAT assays were performed according to the manufacturer's protocol.

Results

Characterization of the *CYP2D* locus from Chinese individuals. A genomic library was constructed from a subject homozygous for the *Xba*I 44-kb fragment (21) and having a MR for debrisoquine of 3.7. As a control, another library was made from a Chinese subject homozygous for the *Xba*I 29-kb fragment and having a MR for debrisoquine of 0.33.

Screening of 800,000 clones from the *Xba*I 44-kb library with [^{32}P]dCTP-labeled *CYP2D6* cDNA resulted in 20 positive clones. *CYP2D6*-specific PCR (22) amplified DNA fragments from six of the clones. Phage DNA was isolated from the *CYP2D6* PCR-positive clones and the length of the inserted DNA was determined by Southern blotting after *Eco*RI digestion. Two different *Eco*RI fragments that hybridized to *CYP2D6* cDNA, of 9.4 kb and 13.7 kb, were obtained from different clones. Among Caucasians, the 9.4-kb fragment corresponds to the *CYP2D6* gene (35). The other type of insert had the same length (13.7 kb) as the fragment corresponding to *CYP2D7BP* present in DNA from Caucasian PM of the *Xba*I 44-kb haplotype (35). The two clones were designated to contain the genes $CYP2D6Ch_1$ (9.4 kb) and $CYP2D6Ch_2$ (13.7 kb), respectively.

From the *CYP2D6* PCR-negative clones of the subject with *Xba*I 44-kb haplotype, phage DNA was isolated with two types of inserts, of 8.8 kb and 15.1 kb. These two fragments have the lengths previously described for *CYP2D8P* and *CYP2D7P/7AP*, respectively (35, 36). Their pseudogenic origin was analyzed by sequencing. Direct sequencing of PCR products obtained from exons 4 and 5 of *CYP2D8* unexpectedly revealed the absence of the stop codon in exon 4 seen in the corresponding gene from Caucasians (35). However, the extra inserted AT in exon 4 was present, as well as the stop codon in exon 5. The 15.1-kb fragment contained the inserted thymine in exon 1 described for *CYP2D7P/7AP* among Caucasians (36). These results verify the pseudogenic origin of these two genes present in the *CYP2D* locus in Chinese of the *Xba*I 44-kb haplotype.

Sequence analysis of the *CYP2D6Ch* genes. The two types of *Eco*RI inserts, 9.4 kb and 13.7 kb, containing the *CYP2D6Ch* genes were digested with *Eco*RI and *Kpn*I. Both phages yielded 5.8-kb *Eco*RI-*Kpn*I fragments, containing 1531 bp of the upstream region and all nine exons. These fragments were subcloned into pUC19 for sequence analysis. All nine exons in both genes and the major parts of the introns of $CYP2D6Ch_1$ were sequenced (Table 2). Both genes resemble *CYP2D6wt* but have important differences. $CYP2D6Ch_1$ has four mutations in the coding regions, two causing amino acid substitutions, that are also present in the deficient Caucasian *CYP2D6B* gene (35). The open reading frame of the $CYP2D6Ch_2$ gene is identical to that of $CYP2D6Ch_1$ except for exon 9, where 13 base substitutions were found (Table 2). Examination of the sequences of other genes in the *CYP2D* locus revealed that the part containing these substitutions (G^{4212}–C^{4260}) originates entirely from the pseudogene *CYP2D7P* (35), indicating a gene conversion event.

The 5′ flanking regions of the *CYP2D6Ch* genes were inves-

TABLE 2

Sequence analysis of 5′-flanking region and exons of the two Chinese variants of the *CYP2D6* gene, compared with the Caucasian *CYP2D6wt* allele

Region	*CYP2D6wt*	*CYP2D6Ch*$_1$	*CYP2D6Ch*$_2$	Amino acid substitution
Upstream	−1338 C	T	T	
	−1149/−1148	AA inserted	A inserted	
	−1147 A	G	G	
	−912 G	A	A	
Exon 1	188 C	T	T	Pro^{34}→Ser
Exon 2	1127 C	T	T	
Exon 3	1749 G	C	C	
Exon 9	4212 G		C	
	4216 C		G	Pro^{469}→Ala
	4219 A		G	Thr^{470}→Ala
	4221 T		C	Thr^{470}→Ala
	4243 C		T	His^{478}→Ser
	4244 A		C	His^{478}→Ser
	4246 G		C	Gly^{479}→Arg
	4252 T		G	Phe^{481}→Val
	4254 T		C	Phe^{481}→Val
	4255 G		A	Ala^{482}→Ser
	4256 C		G	Ala^{482}→Ser
	4257 T		C	Ala^{482}→Ser
	4260 C		T	
	4268 G	C	C	Ser^{486}→Thr

tigated. The cloned *Eco*RI-*Kpn*I fragments in pUC19 from both *CYP2D6Ch* genes were sequenced for 1514 bp upstream. As shown in Table 2, three differences, with respect to the *wt* sequence, common to both genes were found in the 5′ flanking region.

Sequence analysis of the Chinese *CYP2D6* gene from an individual of the *Xba*I 29-kb haplotype. From the genomic library of the Chinese individual of the *Xba*I 29-kb haplotype, a λ phage with a 9.4-kb insert was isolated. Sequence analysis was carried out with the 5.8-kb *Eco*RI-*Kpn*I 5′ fragment of the insert, after subcloning into pUC19. In all positions where differences in the *CYP2D6Ch*$_1$ and *CYP2D6Ch*$_2$ genes were found, compared with the Caucasian *CYP2D6wt* gene (Table 2), the Chinese *CYP2D6wt* gene was found to exhibit the *wt* sequence (data not shown).

Distribution analysis of *CYP2D6Ch*$_1$-specific mutations. Allele- and mutation-specific PCR methods were developed for the analysis of the C^{-1338}→T, C^{188}→T, and G^{4268}→C mutations present in *CYP2D6Ch*$_1$ (see Experimental Procedures). Ninety Chinese individuals who had been previously phenotyped (9), being homozygous or heterozygous for the *Xba*I 29-kb or *Xba*I 44-kb haplotypes, were genotyped with respect to these mutations. It was found that, with the exception of two subjects, all individuals with the C^{-1338}→T mutation exhibited the exon 1 and exon 9 mutations. The exceptions were one subject with the C^{-1338}→T and G^{4268}→C mutations but not the C^{188}→T mutation and one subject lacking the G^{4268}→C mutation. Similarly, all individuals except one with the exon 1 mutation carried the upstream mutation (data not shown). The results also revealed that the *CYP2D6Ch*$_1$ allele was frequently distributed among individuals of the *Xba*I 29-kb haplotype (Fig. 2).

The *CYP2D6L*$_1$ gene, which is commonly seen in Caucasian populations (23) and has a mutation in exon 6 (Arg^{296}→Cys) and the Ser^{486}→Thr substitution, was also frequent among Chinese individuals (Table 3). Additional *CYP2D6* alleles were also identified, including those in two individuals with the *Xba*I

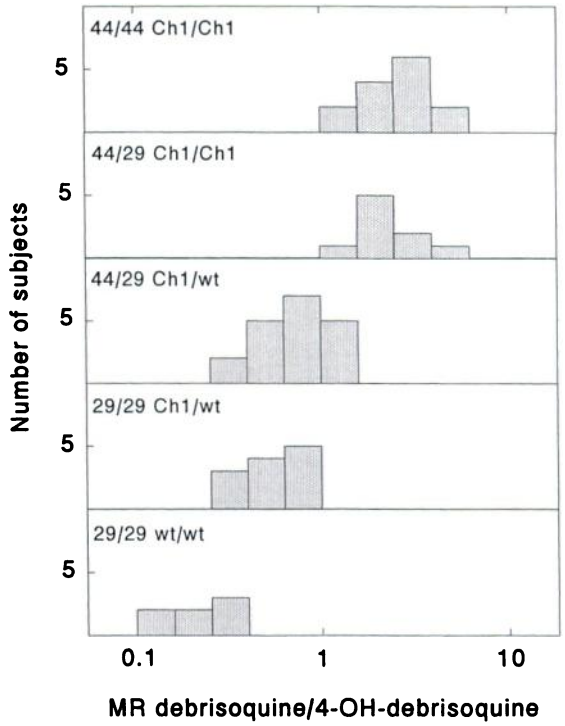

Fig. 2. MR for debrisoquine as a function of the *Ch*$_1$ and *wt* genotypes among Chinese subjects of the *Xba*I 29-kb and/or 44-kb haplotype. *Ch*$_1$ is defined as the allele with the C^{-1338}→T, C^{188}→T, and G^{4268}→C mutations present, according to mutation-specific PCR amplification. In the *wt* allele these mutations, as well as the exon 6 mutation, C^{2938}→T, were absent. Shown is the MR for debrisoquine among individuals carrying two, one, or none of the *Ch*$_1$ alleles, compared with the *wt* allele. The data distinguish between subjects of the *Xba*I 44-kb and *Xba*I 29-kb haplotypes, because in the former case the *Ch*$_2$ gene is also present in the locus. The similar MR values obtained for both haplotypes thus suggest that *Ch*$_2$ does not contribute to the hydroxylase activity *in vivo*.

42-kb haplotype, indicative of gene duplication of the *CYP2D6L* gene and correlating with ultrarapid debrisoquine metabolism (23). Furthermore, one individual with a *Xba*I 58-kb haplotype, indicative of five *CYP2D* genes, was identified. However, as is evident from Table 3, the *CYP2D6Ch*$_1$ gene was the most common one in the population studied.

The MR for debrisoquine among the Chinese population studied correlated very well with the distribution of the mutated *CYP2D6Ch*$_1$ variant when subjects carrying the *Xba*I 29-kb and *Xba*I 44-kb haplotypes, including those with the *CYP2D6L*

TABLE 3

Xba*I haplotypes and allele frequencies of *CYP2D6* in the Chinese population studied (n = 113)

Type	Frequency total	*Xba*I haplotype					
		44-kb	29-kb	11.5-kb	42-kb	16- + 9-kb	58-kb
	%				%		
CYP2D6Ch$_1$	50.7	37.1	13.6				
CYP2D6Ch$_2$	37.1	37.1					
CYP2D6wt	26.9		26.9				
CYP2D6L$_1$[a]	13.4		12.5		0.9		
CYP2D6D[b]	5.7			5.7			
CYP2D6L$_2$[a]	0.9				0.9		
Others	3.3		2.5			0.4	0.4

[a] See Ref. 23.

[b] This represents a deletion of the *CYP2D6* gene, as evident from *Xba*I restriction fragment length polymorphism. These data are from the report of Johansson *et al.* (9).

allele, were considered (Table 4). Thus, despite the presence of an extra *CYP2D6* gene (*CYP2D6Ch*$_2$) with an open reading frame among subjects of the *Xba*I 44-kb haplotype, these subjects did not have lower MR values, i.e., more rapid metabolism. When subjects of the two haplotypes having only *wt* and *Ch*$_1$ alleles were compared, a strict relationship between increased MR for debrisoquine and increasing number of *Ch*$_1$ alleles was obtained (Fig. 2).

Analysis of the 5′ upstream region. The almost complete linkage between the C^{-1338}→T mutation in the upstream region and those in exons 1 and 9 made it impossible to identify the detrimental mutation causing lower MR values among subjects carrying the *CYP2D6Ch*$_1$ allele. Parts of the 5′ flanking region from the *wt* and *Ch*$_1$ genes were therefore subcloned, their abilities to bind nuclear factors were analyzed with DNA mobility-shift assays, and their contents of putative enhancer or repressor elements were analyzed in a pCAT system. The region between bp −1523 and −1087 bound nuclear factor(s), as evident from the gel-shift assay (data not shown). This binding was competed with by an unlabeled fragment but could not be competed away with an oligonucleotide covering the area around the C^{-1338}→T mutation (data not showm). Similar data were obtained when fragments from the *wt* or *Ch*$_1$ alleles were used either for binding or for competition (data not shown). Other fragments used in the gel-shift analysis excluded high affinity binding of nuclear factors to the region of bp −981 to −576. The competitive fragments used could not establish the importance of the mutations at bp −1149/48 (two extra adenines inserted) and A^{-1147}→G. However, because these mutations are also present in the *CYP2D6L*$_1$ allele[1] and because the metabolic capability of this allele is equal to that of the *wt* allele (23), we could exclude these mutations as being important for the lower MR seen among subjects with the *CYP2D6Ch*$_1$ allele.

The *Sau*3AI fragment (bp −1407 to −1068) of the 5′ flanking region of both the *CYP2D6wt* and *CYP2D6Ch*$_1$ alleles was introduced into the pCAT vector system. Analysis of CAT activity after transfection into Hep G2 cells revealed that the region contained an enhancer element but no differences between the *wt* and *Ch*$_1$ allelic forms could be registered (pCAT control, 6.1 ± 2.2; pCAT promoter, 1.0 ± 0.7; pCAT promoter plus *wt*, 2.0 ± 0.8; pCAT promoter plus *Ch*$_1$, 2.0 ± 0.9; (U) mean ± standard deviation, four experiments). Taken together, these results indicate that the mutations seen in the 5′ flanking region are not important with respect to impaired function of the *CYP2DCh*$_1$ allele.

TABLE 4

Values of log MR mean ± standard deviation for debrisoquine among Chinese subjects of various *Xba*I haplotypes

Data are shown for subjects who are homozygous (*Ch*$_1$/*Ch*$_1$) or heterozygous (*Ch*$_1$/*wt*), compared with subjects who are homozygous for *wt* alleles. For comparison, the corresponding MR values based on the log MR data are given.

*Xba*I haplotype	Genotype	*n*	log MR	MR
29/29	*wt*/*wt*[a]	17	−0.59 ± 0.22	0.26
29/29	*Ch*$_1$/*wt*	16	−0.28 ± 0.13[b]	0.50
44/29	*Ch*$_1$/*wt*	28	−0.10 ± 0.24	0.82
44/29	*Ch*$_1$/*Ch*$_1$	9	+0.37 ± 0.16[c]	2.4
44/44	*Ch*$_1$/*Ch*$_1$	14	+0.42 ± 0.19	2.6

[a] *wt* includes the *L* allele.
[b] Statistically significantly different from 29/29, *wt*/*wt*, $p < 0.001$.
[c] Statistically significantly different from 44/29, *Ch*$_1$/*wt*, $p < 0.001$.

[1] E. Lundqvist, unpublished observations.

Expression of chimeric *CYP2D6* genes. The data presented above indicate that the detrimental mutations in the *CYP2D6Ch* genes are located in the open reading frame. To evaluate the relative functional importance of the mutation in exon 1 (Pro^{34}→Ser) and the two types of mutations in exon 9, Ser^{486}→Thr (*mut 9:1*) and the *CYP2D7* gene conversion (*mut 9:2*) (Table 2), four chimeric genes from the *CYP2D6wt*, *CYP2D6Ch*$_1$, and *CYP2D6Ch*$_2$ alleles were constructed (see Fig. 1), inserted into pCMV2 vectors, and expressed in COS-1 cells. For comparison, cDNA alone and the vector containing only a short fragment of exon 1 (negative control) were used. The amount of CYP2D6 mRNA was determined using Northern blotting and the expression of enzyme was quantified by Western blotting. Furthermore, the rate of CYP2D6-dependent bufuralol 1′-hydroxylase activity was determined in homogenates of the transfected cell systems.

COS-1 cells with *CYP2D6* cDNA expressed the highest bufuralol hydroxylase activities and CYP2D6 protein levels (Fig. 3). All chimeras except the negative control caused expression of CYP2D6 mRNA (Fig. 3A) and expression of immunodetectable enzyme, but at a lower level, compared with the cDNA (Fig. 3B). The relative mRNA level was about the same for all allelic genes evaluated, but the level of immunodetectable CYP2D6 protein in cells transfected with *CYP2D6Ch*$_1$ or *CYP2D6Ch*$_2$ was about 40% of that obtained in cells transfected with the *wt* gene. In contrast, transfection with the *mut 9:1* and *mut 9:2* chimeras caused 2–3-fold more CYP2D6 protein to be expressed.

The rate of CYP2D6-dependent 1′-hydroxylation of bufuralol in homogenates was very low when cells were transfected with *CYP2D6Ch*$_2$. Homogenates from cells transfected with *CYP2D6Ch*$_1$ had significant hydroxylase activity, but this was only 2.5% of that obtained in homogenates from cells with the *CYP2D6wt* gene (Fig. 3C) when compared on a per-milligram basis and 6.25% when compared with the amount of CYP2D6. In contrast, both chimeras (*mut 9:1* and *mut 9:2*) with the mutations only in exon 9 caused catalytic activity in the cellular homogenates at a rate similar to that seen in cells transfected with the pCMV2-*CYP2D6wt* construct.

These results suggest that the *CYP2D6Ch*$_2$ gene, containing a large part of *CYP2D7P* sequence in exon 9 as well as the Pro→Ser mutation in exon 1, causes the formation of an unstable enzyme product that is catalytically almost inactive. The two types of exon 9 mutations studied appear to be of little importance for enzymatic activity, compared with the *wt* gene. Thus, the results strongly suggest that the exon 1 mutation is the important one in *CYP2D6Ch*$_1$, causing less enzyme to be expressed. The data obtained, when related to the amount of CYP2D6, indicated that the CYP2D6 products of chimeras with the exon 1 mutation had lower specific activity, compared with the *wt* alleles (Fig. 3B). This indicates that the enzyme with this mutation is more unstable or has an improper conformation, although a diminished rate of translation cannot be excluded.

Discussion

Our investigation reveals that the interethnic differences in the structure of the *CYP2D6* alleles and the *CYP2D* locus between Caucasian and Chinese individuals are considerable

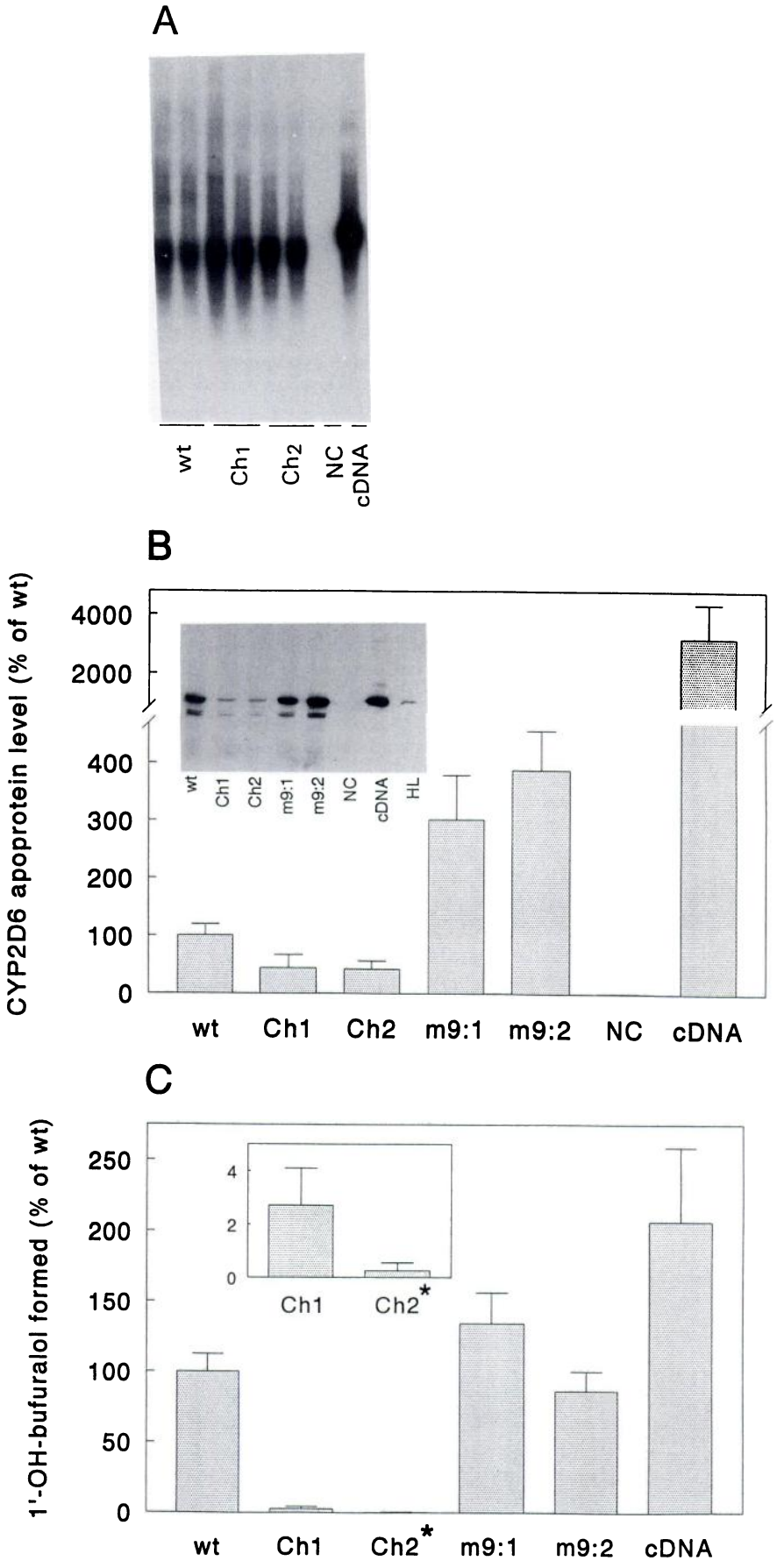

Fig. 3. Expression of chimeric *CYP2D6* genes. A, Northern blot analysis. CYP2D6 mRNA levels were measured in cells transfected with different plasmids. Seven micrograms of total RNA were used for Northern blot analysis. B, Western blot analysis, with densitometric quantification of CYP2D6 apoprotein levels in the cell homogenates. *Inset*, Western blot analysis of the amount of CYP2D6 in homogenates (35 μg for *wt*, *Ch*$_1$, *Ch*$_2$, *mut 9:1*, *mut 9:2*, and the negative control and 2.5 μg for cDNA) from transfected COS-1 cells. Human liver microsomes (2 μg) were also analyzed. The lower molecular mass band seen in the expression system appears to represent a degradation product of CYP2D6 (24). C, Catalytic activities. Bufuralol 1′-hydroxylation assays were performed with cell homogenates corresponding to 1.2 mg of protein. The reaction was linear with time for 3 hr, and the experiments were run for 2 hr, with the exception of *Ch*$_2$, where data were obtained from 4-hr experiments (*) (shown on another scale in the *inset*) due to the limited amount of product formed. The very small amount of product formed in samples transfected with the negative control has been subtracted. The catalytic activity is expressed as a percentage of the *wt* activity (0.1 nmol/mg/hr). The results in B and C represent data (mean ± standard deviation) from three independent experiments performed in duplicate. In A one representative experiment of two is shown. Plasmids from two independent preparations were used for transfection. *wt*, pCMV2-*CYP2D6wt*; *Ch*$_1$, pCMV2-*CYP2D6Ch*$_1$; *Ch*$_2$, pCMV2-*CYP2D6Ch*$_2$; *m9:1*, pCMV2-*mut 9:1*; *m9:2*, pCMV2-*mut 9:2*; *cDNA*, pCMV2-*CYP2D6* cDNA; *NC*, pCMV2 containing 150 bp of exon 1 (negative control); *HL*, human liver microsomes.

and constitute an important explanation for interethnic differences in drug metabolism. The sequence analysis of the Chinese *CYP2D* locus from the subject with the *XbaI* 44-kb haplotype showed the presence of two very similar genes, one (*CYP2D6Ch*$_1$) with two amino acid substitutions, compared with *CYP2D6wt*, and one (*CYP2D6Ch*$_2$) with the same substitutions as well as a gene conversion from the pseudogene *CYP2D7P* in exon 9, yielding six additional amino acid substitutions (Table 2). Expression of the 5′-flanking regions in the pCAT vector/Hep G2 cellular system and construction of chimeras of the genes with subsequent expression in COS-1 cells revealed that the detrimental mutation, causing diminished capacity for debrisoquine hydroxylation, is C^{188}→T, causing a Pro^{34}→Ser amino acid change. Among the Chinese population investigated, this mutated allele (*CYP2D6Ch*$_1$) was found to be the most common one. This apparently explains the lower capacity among Chinese, compared with Caucasians, to metabolize several drugs that are CYP2D6 substrates, such as neuroleptic agents and tricyclic antidepressants. All different functional *CYP2D* loci found among the Chinese population investigated are illustrated in Fig. 4.

The effect of the C^{188}→T mutation in the expression system was quite dramatic, whereas the mutations in exon 9 were of limited importance when introduced alone in the chimeras. The *CYP2D6Ch*$_1$ gene exhibited 1/40th of the activity of the *wt* gene, whereas the *CYP2D6Ch*$_2$ gene product was almost completely inactive. The MR for debrisoquine correlated well with the presence of the *CYP2D6Ch*$_1$ gene among the subjects (Fig. 2; Table 4). The difference in MR between subjects homozygous for the *wt* and *Ch*$_1$ alleles is, however, less, i.e., about 10-fold, than the difference in catalytic activity in the expression system (40-fold). This might be explained by a shorter half-life for the CYP2D6Ch$_1$ enzyme, which is compensated for by a higher steady state level of the mutated enzyme *in vivo*.

The crucial amino acid substitution of Pro^{34}→Ser is localized in a very well conserved region, consisting of Pro-Pro-Gly-Pro, of all P450s belonging to gene families 1 and 2 (37). Some recent reports suggest that this region is important for P450 enzyme stability. Thus, Szczesna-Skorupa *et al.* (38) showed that deletion of the conserved peptide PPGP of *CYP2C2* caused a loss of enzyme activity and increased degradation of the enzyme but did not change the endoplasmic reticulum membrane location of the isozyme. Similarly, substitution of Pro^{34} with leucine in CYP21A2 also reduced the P450 activity in transfected cells (39).

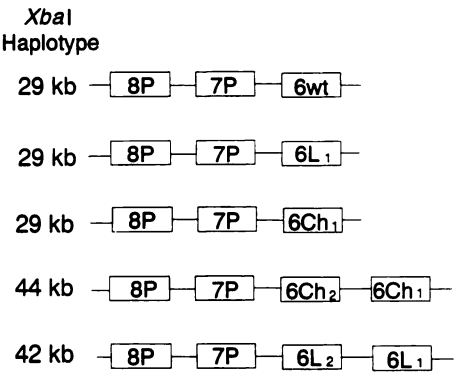

Fig. 4. Illustration of the various functional allelic *CYP2D* loci found among the Chinese population studied. The relative positions of the *CYP2D6Ch*$_1$ and *CYP2D6Ch*$_2$ genes were evident from *Hind*III and *Eco*RV restriction fragment length polymorphism, as described by Heim and Meyer (35) and E. Lundqvist (unpublished observations) and, in addition, by the *Eco*RI restriction fragment length polymorphism (see text).

The defective Caucasian *CYP2D6* gene variant *CYP2D6B* has seven mutations in coding regions, with a detrimental splicing defect in the intron 3-exon 4 junction (24). The *CYP2D6Ch*$_1$ gene contains three of these mutations, indicating that they may originate from the same allele. Kagimoto *et al.* (24) constructed chimeric genes and cDNAs between *CYP2D6B* and the *wt* gene and expressed the genes in COS-1 cells. It was then evident that the cDNA construct containing only the Pro^{34}→Ser mutation produced a more unstable enzyme, whereas larger amounts of enzyme were produced in cells transfected with chimeras containing only the Ser^{486}→Thr mutation. Our results reveal that when both mutations are present in the same allele, as in the *CYP2D6Ch*$_1$ gene, the negative influence of the exon 1 mutation is more important than the "enhancing" effect of the Ser^{486}→Thr mutation, giving strong evidence for the fact that the exon 1 mutation is the detrimental one. In the case of the *CYP2D6Ch*$_2$ gene, it was evident that this gene product exerted essentially no enzymatic activity in the transfection system. It might be that the additional six amino acid substitutions in exon 9, not far from the heme-binding cysteine, together with structural changes as a consequence of the exon 1 mutation cause the formation of an even more unstable enzyme product than that of *CYP2D6Ch*$_1$.

The mutations in exons 1 and 9 of the *CYP2D6* gene have recently been described, but it has not yet been possible to identify the detrimental one or to correlate the presence of these mutations with the *in vivo* MR for debrisoquine (10, 40, 41). One of the reasons for this discrepancy appears to be connected to the frequent presence of the *CYP2D6L*$_1$ gene (23) in Asian populations. In the *CYP2D6L*$_1$ gene, a gene conversion event in intron 1, where a part from the *CYP2D7P* gene has been introduced (see Experimental Procedures), makes amplification of exon 1 impossible when primers for the *CYP2D6wt* gene are used (10, 41). This means that individuals who are heterozygous for the *L*$_1$ allele cannot be identified with respect to their correct allelic distribution of the exon 1 mutation, giving a false result regarding the correlation between MR and this mutation (10). In the study of Yokota *et al.* (41), an indication for higher MR for the CYP2D6 substrate sparteine was seen among four of 28 Japanese studied who were homozygous for the exon 1 and exon 9 mutations, whereas no correlation with MR was apparent among the heterozygous subjects. Similar data were recently presented also by Amstrong *et al.* (42), where six individuals homozygous for the exon 1 mutation had higher MR values for debrisoquine than did subjects having a *wt* allele. Also in that study, a difference between individuals homozygous for the *wt* allele and those heterozygous for the exon 1 mutation could not be seen.

In conclusion, we have shown that the most common allelic form of the Chinese *CYP2D6* gene contains a mutation in an important and well conserved region of the enzyme, causing a more unstable product with lower catalytic activity. This appears to provide the explanation for impaired metabolism, among Chinese, of drugs that are CYP2D6 substrates. For drugs having a narrow therapeutic range, it might be considered of value in some cases to carry out genotype analysis with respect to the presence of this mutation. The interethnic differences in the *CYP2D* locus are pronounced, and in other

ethnic groups it is probable that additional allelic *CYP2D6* variants, with interesting properties regarding the molecular biological consequences, could be found.

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