Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5240–5243, July 1988
Medical Sciences

Two mutant alleles of the human cytochrome P-450db1 gene (*P450C2D1*) associated with genetically deficient metabolism of debrisoquine and other drugs

(debrisoquine polymorphism/P450IID1/P-450db1/P-450bufI/restriction fragment length polymorphism)

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Communicated by Ronald W. Estabrook, March 25, 1988

ABSTRACT **The "debrisoquine polymorphism" is a clinically important genetic defect of drug metabolism affecting 5–10% of individuals in Caucasian populations. It is inherited as an autosomal recessive trait. A full-length cDNA for human cytochrome P-450db1, the deficient enzyme (also designated P450IID1 for P450 family II subfamily D isozyme 1), has recently been cloned. Leukocyte DNA from "extensive metabolizers" (EMs) or "poor metabolizers" (PMs) of debrisoquine was examined by Southern analysis. Two polymorphic restriction fragments were associated with the PM phenotype when DNAs from 24 unrelated PM and 29 unrelated EM individuals were probed with P-450db1 cDNA after digestion with *Xba* I restriction endonuclease and Southern blotting: a polymorphic 44-kilobase (kb) fragment was found in 58% of PMs but only in 3.4% of EMs, and a polymorphic 11.5-kb fragment was present in 33% of PMs but in none of the EMs. Seventy-five percent of PMs had either the 44-kb or the 11.5-kb fragment or both. Segregation of these restriction fragment length polymorphisms in the families of six PM probands demonstrated that each of the two fragments is allelic with the 29-kb fragment present in all EM individuals and suggests that they identify two independent mutated alleles of the P-450db1 gene (designated *P450C2D1*). At least a third mutated allele not detected by these restriction fragment length polymorphisms must be present in the population. The *Xba* I 44-kb fragment and 11.5-kb fragment were in linkage disequilibrium with restriction fragment length polymorphisms generated by four and five additional restriction endonucleases, respectively, which can be used to identify the same mutant alleles for the P-450db1 gene.**

Cytochrome P-450 enzymes are components of the microsomal multisubstrate monooxygenase system responsible for the oxidative metabolism of a large number of endobiotic and xenobiotic substances (1–3). In recent years, several genetically determined polymorphisms of P-450-mediated drug oxidation have been discovered. The term genetic polymorphism refers to a Mendelian or monogenic trait that exists in the population in at least two phenotypes, neither of which is rare—i.e., occurs at a frequency of more than 1–2% (4). Genetic polymorphisms of drug oxidation cause impaired biotransformation of certain drugs in so-called "poor metabolizer" (PM) subjects. Because of their frequency of occurrence, genetic polymorphisms of drug oxidation are major determinants of interindividual differences in the therapeutic and toxic responses to numerous clinically important drugs (5, 6).

One of the most extensively studied examples of a genetically determined variation in drug metabolism is the debrisoquine genetic polymorphism (7–9) which occurs in 5–10% of individuals in Caucasian populations and which affects the metabolism of >20 drugs, including the prototype drugs debrisoquine, sparteine, dextromethorphan, and bufuralol and also β-adrenegic blocking agents, antidepressants, antiarrhythmics, and other drugs widely used in clinical medicine (6, 9). Pedigree studies suggest that this defect is monogenically inherited as an autosomal recessive trait (10). This laboratory recently reported isolating from the liver of "extensive metabolizer" (EM) subjects an enzyme with high catalytic activity responsible for debrisoquine and bufuralol metabolism, which was designated P-450bufI (11, 12). Another laboratory has reported on a similar enzyme designated P-450DB (13). Antibodies recognizing this enzyme have been developed, and in immunoblots some of us have observed a marked decrease or absence of this protein in most PM livers (14). A cDNA probe for human P-450bufI (designated P-450db1) has been cloned, and several splicing mutants in human livers with low activity of debrisoquine or bufuralol hydroxylation have been described (14). The gene for P-450db1 has been localized on human chromosome 22 (15, 22).

In the present report, genomic DNA from *in vivo* phenotyped PM and EM individuals and from families of PM subjects was analyzed for restriction fragment length polymorphisms (RFLPs) with the P-450db1 cDNA to identify mutant alleles of the locus encoding P-450db1.

MATERIALS AND METHODS

The study population consisted of 53 healthy, unrelated Caucasian individuals: 29 EM (♂:20, ♀:9) and 24 PM (♂:15, ♀:9) were studied. In addition six PM families with a total of 29 family members were investigated.

Phenotyping Procedure. Clinical test data and blood from EM and PM subjects were collected according to protocols approved by the ethics review boards of the institutions providing samples. Most subjects were phenotyped with debrisoquine [10 mg oral administration (p.o.)], except for seven subjects with sparteine (100 mg p.o.) and six subjects with dextromethorphan (20 mg p.o.). It has been established that these three test drugs provide identical information on the phenotype (16–18). PM individuals were identified by a urinary metabolic ratio of >12.6 for debrisoquine/4-OH-debrisoquine (16), >20 for sparteine/2- and 5-dehydrosparteine (16, 18), and >0.3 for dextromethorphan/dextrorphan (17).

Nucleic Acid Procedures. Blood (30–50 ml) was obtained from each individual, and DNA was isolated from peripheral leukocytes as described (19). Human leukocyte DNA (5 μg)

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Abbreviations: EM, extensive metabolizer; PM, poor metabolizer; RFLP, restriction fragment length polymorphism.

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was digested to completion with restriction endonucleases, and Southern blotting (20) was done by using Biotrace RP membranes (Gelman) and hybridization probes prepared by nick-translations with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq) to a specific activity of 1–3 × 10^8 dpm/μg of DNA.

RESULTS

RFLPs Generated by *Xba* I Associated with the PM Phenotype. The RFLPs observed with nine restriction endonucleases in leukocyte DNA from 24 unrelated PMs and 29 unrelated EMs are shown in Fig. 1. *Xba* I RFLPs define four allelic forms of the gene for P-450db1: one allele is characterized by the presence of a 29-kb fragment (patterns A, B, and D), a second allele is identified by the presence of a 44-kb fragment (patterns B, C, and E), and a third allele is defined by the presence of a 11.5-kb fragment (patterns D and E). Patterns A and C are the homozygous states for the 29-kb and 44-kb alleles, respectively, and patterns B, D, and E represent the heterozygous states for the three combinations of the 29-, 44-, and 11.5-kb alleles. A fourth allele is defined by the presence of 16- and 9-kb fragments. This allele was found in the heterozygous state only in combination with the 29-kb allele (pattern F).

The distribution of alleles identified by *Xba* I RFLPs between unrelated EM and PM individuals is shown in Table 1. The most frequent polymorphic fragment was the 29-kb fragment. It can signify a normal allele because all EMs were homo- or heterozygous for this 29-kb fragment. However, it can also signify a mutant allele because it was present in 13 PMs in the heterozygous and 6 PMs in the homozygous state. Thus, the 29-kb fragment is not informative for predicting the debrisoquine phenotype. In contrast, the 44-kb and 11.5-kb fragments were clearly associated with the PM phenotype. The 44-kb allele was present in 54% of PMs in the heterozygous state and in 4% of PMs in the homozygous state, whereas only 1 of 29 EMs (3.4%) was heterozygous ($P < 0.001$, Fisher's exact test). Furthermore, the 11.5-kb fragment was present in 33.3% of individuals in the PM group in the heterozygous state, no homozygous individual for the 11.5-kb fragment was detected, and none of 29 EMs tested showed an 11.5-kb fragment ($P < 0.001$).

Segregation of the *Xba* I Fragments in Families of Six PM Individuals. The data presented in Fig. 2 and Table 2 confirm that the 44-kb and 11.5-kb fragments are allelic with the 29-kb fragment and segregate as two independent mutated alleles of the P-450db1 gene. No inconsistency between genotype and phenotype for autosomal recessive inheritance was observed.

Thus, the presence of a 44-kb or 11.5-kb fragment in combination with a 29-kb fragment (Fig. 1, *Xba* I pattern B or D) predicts that this individual is either heterozygous EM phenotype (if the 29-kb fragment represents a normal allele)

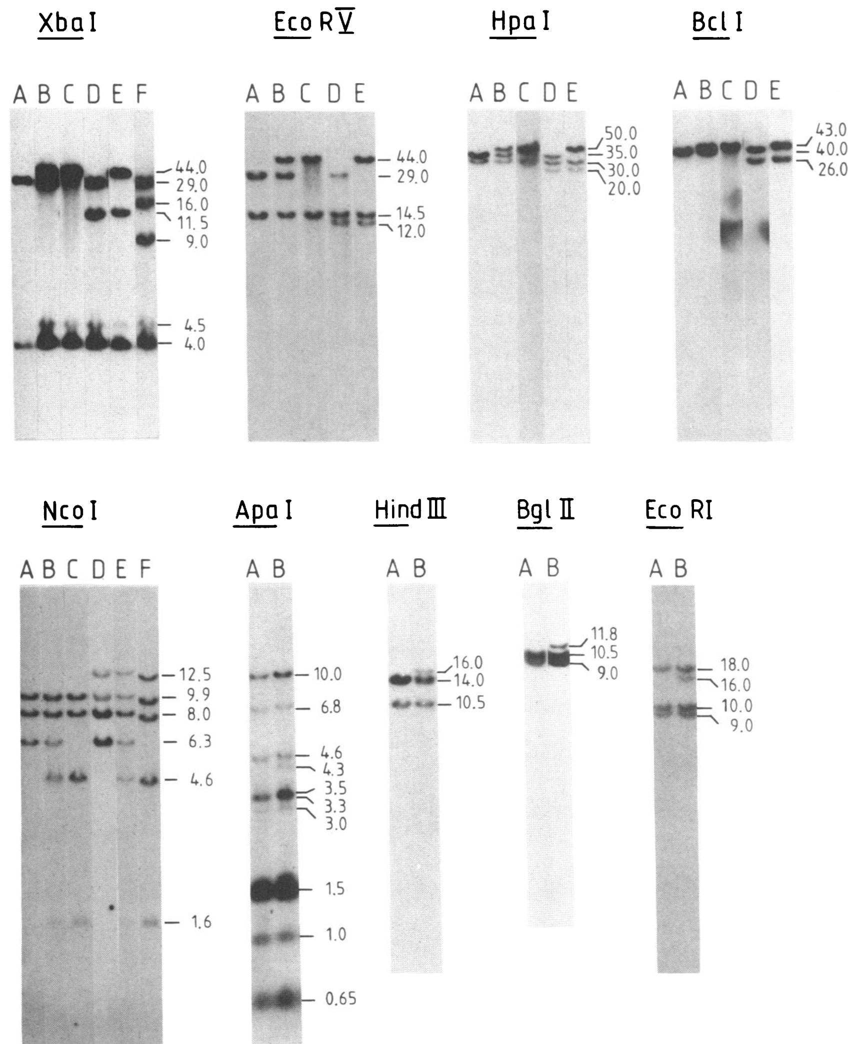

FIG. 1. Southern blot analysis of human leukocyte DNA after digestion with nine restriction endonucleases and hybridization with the ^{32}P-labeled full-length human P-450db1 cDNA probe. One example for each observed restriction pattern (53 individuals examined) is shown. Numbers indicate fragment size in kb.

Table 1. Analysis of *Xba* I RFLPs in unrelated individuals of EM and PM phenotype

Xba I pattern (Fig. 1)	Polymorphic fragments, kb	EMs (n = 29)			PMs (n = 24)		
		n	Genotype†	Pattern frequency	n	Genotype†	Pattern frequency
A	29	26	D/D or D/d	0.9	6	d/d	0.25
B	44/29	1	d°/D	0.03	9	d°/d	0.37
C	44	—	—	—	1	d°/d°	0.04
D	29/11.5	—	—	—	4	d/d^*	0.17
E	44/11.5	—	—	—	4	d°/d^*	0.17
F	29/16 + 9	2		0.07	—	—	—

†D designates the normal 29-kb allele; d (29 kb), d° (44 kb), and d^* (11.5 kb) designate mutant alleles. The expected genotypes for the EM phenotype are: D/D, D/d, D/d°, and D/d^*; for the PM phenotype, they are: d/d, d/d°, d/d^*, d°/d^*, d°/d°, and d^*/d^*.

or PM phenotype (if the 29-kb fragment represents a mutant allele). Individuals heterozygous for two mutated alleles of 44 kb and 11.5 kb (Fig. 1, *Xba* I pattern E) and subjects homozygous for the 44-kb allele (Fig. 1, *Xba* I pattern C) or 11.5 kb (not yet observed) are predicted to be PM phenotype.

Other RFLPs Associated with the PM Phenotype. In all 53 unrelated individuals and in the six families, the same allele as the one defined by the *Xba* I 44-kb fragment could also be identified by polymorphic *Eco*RV 44-kb, *Hpa* I 50-kb, *Bcl* I 43-kb, *Nco* I 12.5-kb, *Apa* I 4.3-kb, and *Eco*RI 16-kb fragments (Fig. 1). The only exception was two individuals lacking the

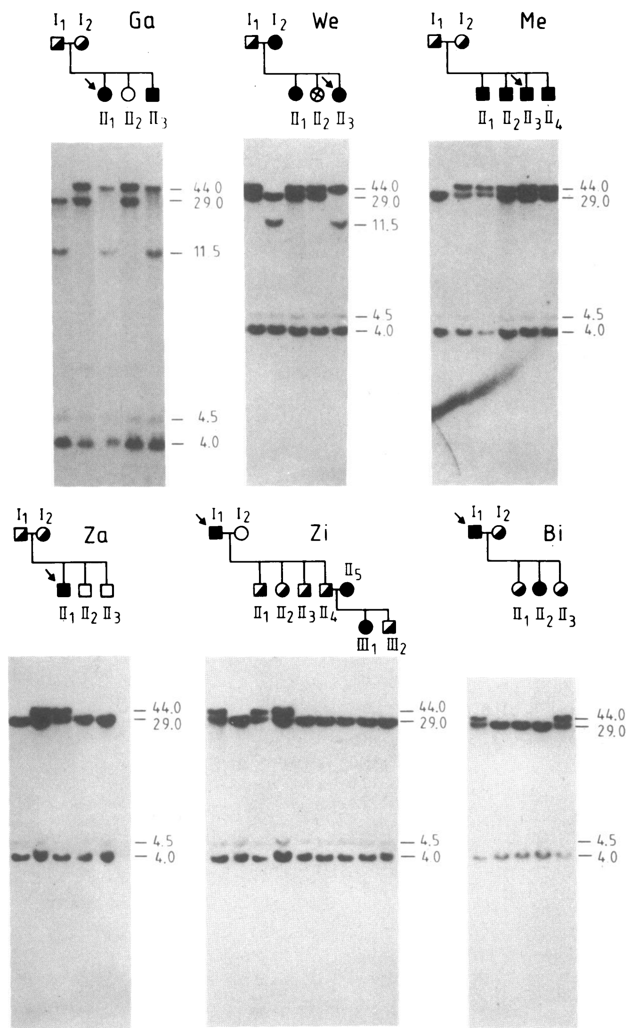

FIG. 2. Segregation of polymorphic *Xba* I fragments in six families of PM propositi. Numbers indicate size of *Xba* I hybridizing fragments in kb. The individuals within a pedigree are placed above the corresponding lane. Arrows identify the proband; □ and ○, male and female subjects, respectively, with EM phenotype; ◪ and ◑, EM phenotype, obligate heterozygote by pedigree analysis; ■ and ●, male and female subjects with PM phenotype; ⊗, phenotype not tested.

Table 2. Phenotypes and genotypes in families of 6 PM individuals

	MR	Phenotype	Genotype
Family GA			
I_1	Dx 0.1	EM	D/d^*
I_2	Dx 0.1	EM	D/d°
II_1	Dx 9.7	PM	d^*/d°
II_2	Dx 0.3	EM	D/d°
II_3	Dx 9.1	PM	d^*/d°
Family We			
I_1	3.4	EM	D/d°
I_2	Dx 2.2	PM	d/d^*
II_1	69.0	PM	d°/d
II_2	ND	ND	d°/d
II_3	56.3	PM	d°/d^*
Family Me			
I_1	1.0	EM	D/d
I_2	0.5	EM	D/d°
II_1	22.4	PM	d/d°
II_2	41.8	PM	d/d°
II_3	21.5	PM	d/d°
II_4	68.8	PM	d/d°
Family Za			
I_1	0.7	EM	D/d
I_2	1.6	EM	D/d°
II_1	23.4	PM	d/d°
II_2	0.2	EM	D/D or d/D
II_3	0.8	EM	D/D or d/D
Family Zi			
I_1	34.3	PM	d/d°
I_2	0.2	EM	D/D or D/d
II_1	0.3	EM	d°/D
II_2	0.2	EM	d°/D
II_3	0.4	EM	d/D
II_4	0.4	EM	d/D
II_5	78.6	PM	d/d
III_1	332.0	PM	d/d
III_2	0.5	EM	D/d
Family Bi			
I_1	45.0	PM	d/d°
I_2	1.1	EM	D/d
II_1	1.3	EM	d/D
II_2	55.0	PM	d/d
II_3	0.9	EM	d°/D

The phenotype was determined in urine collected after administration of debrisoquine except in six individuals, where dextromethorphan was used (data indicated by Dx). The number of the family member refers to the position in the pedigree given in Fig. 2. For explanation of genotype symbols, see the legend to Table 1. MR, metabolic ratio; ND, not determined.

Apa I 4.3-kb fragment, when the *Xba* I 44-kb band was present. Moreover, all individuals with an *Xba* I 11.5-kb allele also had a polymorphic *Eco*RV 12-kb, *Hpa* I 35-kb, *Bcl* I 25-kb, *Hin*dIII 16-kb, and *Bgl* II 11.8-kb band. Thus, these restriction enzymes can be used interchangeably to define the three allelic states of the P-450dbl gene.

RFLPs not associated with PM phenotype were observed with *Bam*HI, *Pst* I, *Bgl* I, and *Sph* I. No RFLPs were observed with seven additional restriction endonucleases—namely, *Pvu* I, *Taq* I, *Sst* I, *Nde* I, *Xmn* I, *Nae* I, and *Kpn* I.

DISCUSSION

Several RFLPs define two mutated alleles of the P-450dbl gene associated with the PM phenotype for debrisoquine hydroxylation. This is deduced from the frequency of occurrence of these alleles in unrelated PMs vs. EMs, the segregation in six PM families, and the agreement of the observed genotype with the phenotype.

At least three different mutant alleles of the P-450dbl gene locus associated with the PM phenotype must be present in the population studied—namely, the two alleles identified by the *Xba* I 44-kb and 11.5-kb fragments, which occur singly or in combination. At least a third allele must account for 25% of the PMs in which no *Xba* I RFLPs were detected. In fact, we suspect that several additional mutations will be found in this group. In this context, we recently have described three splicing mutations of the P-450dbl pre-mRNA in livers with low activity of microsomal debrisoquine 4-hydroxylation *in vitro* (14). The *Xba* I 44-kb allele was found in the heterozygous state with the 29-kb fragment in one of the three livers investigated. In this liver two splicing mutations were detected (variants b and b′ in ref. 14), and both were considered to represent variants of the same allele. The *Xba* I 44-kb allele could thus be the second mutated allele in this liver. No RFLPs with *Xba* I were found in the other two livers, which exhibit a different type of splicing mutation (variant a in ref. 14).

Because neither the normal nor the mutant P-450dbl gene has yet been sequenced, the mechanisms accounting for these RFLPs remain hypothetical. The linkage disequilibrium of RFLPs with an increase of fragment size of ≈15 kb for *Xba* I, *Eco*RV, and *Hpa* I could be explained by an insertion mechanism, whereas a decrease of ≈15 kb in fragment length as observed with *Xba* I, *Eco*RV, *Hpa* I, and *Bcl* I could be the result of a deletion. However, other mechanisms such as gene conversion, unequal crossovers, etc., are also possible. It also cannot be decided yet if the same mutation causes the RFLPs observed and the deficiency of the P-450dbl enzyme or if two different but linked mutations are involved.

The P-450dbl gene locus is highly polymorphic. Of 20 restriction enzymes tested, only 7 displayed no RFLPs, and with the remaining 13 enzymes, 14 allelic forms of the P-450dbl gene can be described. Only 2 of these alleles are linked to the PM phenotype.

These RFLPs will be useful as polymorphic markers of chromosome 22. This is of particular interest because of the vicinity of the *SIS* oncogene on the long arm of chromosome 22 (22) and recent reports on the decreased frequency of certain lung cancers in PM phenotypes (21). The relationship between these cancer studies and the mutations of the P-450dbl gene is unclear, since P-450dbl is not known to metabolize identified environmental carcinogens. However, P-450dbl may activate as-yet-undescribed carcinogens. The genotyping of patients with the RFLPs described here will allow an approach to some of these questions.

Dr. Christoph Zekorn and Dr. Michel Eichelbaum (Univ. of Bonn, and Robert-Bosch-Krankenhaus, Stuttgart, F.R.G.), Dr. Walter Ziegler (University of Zürich), Dr. Pierre Dayer and Dr. Marie-Paule Gascon (Univ. of Geneva), and Dr. Johannes Bircher (Univ. of Göttingen, F.R.G.) contributed blood samples and clinical data to this study. Our research is supported by Swiss National Science Foundation Grant 3.806.84.

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