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Isolation of a cyp2b10-like cDNA and of a Clone Derived from a cyp2b10-like Pseudogene[1]

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By screening Balb/c male mouse liver cDNA library with a rat CYP2B1 cDNA probe, we have isolated a 1795 bp cyp2b10-like clone, referred to as P16. Its sequence exhibited 34 base differences (98% similarity) with the cyp2b10 published sequence, together with a 97% identity at the amino acid sequence level. By RT-PCR and PCR analyses with Balb/c female and male liver RNA and genomic DNA, using a region showing 8 base differences between the P16 and the cyp2b10 sequences, we have confirmed the identity of our cloned cDNA, and failed in finding a PCR product exhibiting a sequence 100% identical with that of cyp2b10. Our results therefore suggest that the P16 sequence is the authentic cyp2b10 sequence. We have also isolated a partial clone, P21, which 1609 bp sequence overlapped with that of P16, except for a T→G transversion, giving rise to a premature TGA stop codon, indicating that it was derived from a pseudogene.

Key Words: **cytochrome P450; cDNA; phenobarbital; mouse; liver.**

Cytochromes P450 (CYP) are heme-containing enzymes involved in the oxidative, peroxydative and reductive metabolism of numerous and diverse endogenous compounds such as steroids, fatty acids or prostaglandins (1). Many of these enzymes also metabolize a wide range of man-made chemicals, including drugs and pollutants which can, collectively, be responsible for birth defects and other forms of toxicity, as well as tumor initiation and progression.

CYPs belong to a large superfamily of at least 500 genes and/or pseudogenes described in 85 eucaryote and prokaryote species (2). They are classified into 74 families on the basis of amino acid homology (3). CYPs are further divided into subfamilies whose members share at least 68% sequence identity. One major peculiarity of a large number of CYP genes is their susceptibility to inducing compounds for which several prototypes have long been known, including polycyclic aromatic hydrocarbons, phenobarbital (PB), glucocorticoids, ethanol and clofibrate. Among these regulatory processes, much remains to be uncovered about the mechanism of action of PB which induces CYPs from subfamilies 2a, 2b, 2c and 3a in the mouse (4).

The cyp2b subfamily in mice is presumed to be one of the largest, with at least 10 members (5,6). This subfamily is extensively studied because expression of gene members is the most dramatically modified by PB. Until now, five cDNAs have been cloned: the cyp2b9, 10 and 13 from female mouse liver (7,8), the cyp2b19 from keratinocytes (9), and we recently isolated from male mouse liver cyp2b20, a hybrid cDNA between a cyp2b10-like and the NADPH CYP-oxydoreductase sequences, which was expressed in the liver as well as in kidney, lung and intestine but was not responsive to PB (10).

With the aim of characterizing putative new members of the mouse cyp2b subfamily, which might differ in their regulation by PB, we have screened a mouse liver cDNA library prepared from Balb/c male mice and we isolated 2 new cDNA clones: one is a full length coding nucleotide sequence highly similar to cyp2b10, and the second is a partial sequence lacking part of the 5′-end region and showing a distinctive T→G transversion which would lead to a premature stop codon, thereby indicating that this cDNA is derived from a cyp2b10-like pseudogene.

METHODS

Animals. Balb/c male and female mice were obtained from CERJ (Le Genest, St-Isle, France). The animals were housed in plastic cages on wood chip bedding and fed *ad libitum* with AO4 food

[1] The sequences of the P16 and P21 cDNAs have been submitted to the GenBank data base, and were given the AF128849 and AF129405 accession numbers, respectively.

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Abbreviations used: Cyp, cytochrome P450; PB, phenobarbital; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair.

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(U.A.R., VilleMoisson-Sur-Orge, France). Animals were sacrificed by cervical dislocation; liver and spleen samples were rapidly removed and frozen in liquid nitrogen. All experiments involving animals were conducted according to French regulations.

cDNA isolation. cDNA clones were isolated from a commercial cDNA library prepared from liver RNA of male Balb/c mice, constructed in the λgt11 vector (CLONETECH Laboratories, Palo Alto, CA). The screening was made by the plaque hybridization method as recommended by the supplier, using a 1250 bp rat cDNA probe corresponding to the 5′-end of the coding region of CYP2B1. cDNAs were further sub-cloned into the Pgem3zf(+) plasmid (Promega, Madison, WI).

Sequencing. Double strand cDNAs were sequenced by the dideoxy method (11) with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Cleveland, Ohio), and also in part at the Genome Express facility (Paris, France). The nucleotide sequences were then compared with sequences present in the Genbank database using the BLAST algorithm from the network service at the National Center of Biotechnology Information.

RNA and genomic DNA extraction, reverse transcription and PCR. Total RNA was extracted from fresh male and female liver biopsies by the one-step guanidinium thiocyanate-phenol-chloroform extraction method according to Chomezynsky and Sacchi (12). From 1 μg of total RNA (treated with DNAse I to remove any contaminating genomic DNA) an oligo-dT primer was used for reverse transcription. High molecular weight DNA was prepared from spleen tissue according to Blin and Stafford (13).

Freshly synthesized cDNAs or 50 ng of genomic DNA were used to perform PCR reactions in 50 μl of PCR buffer: 1 X Promega PCR buffer, 500 nmoles of each primer, 1 mM $MgCl_2$, 125 μM dNTPs and 0,5 U of Taq DNA polymerase. The sequence and the position of the primers used were: forward primer 5′ CCCAAGGAGAGTGGTATTGGA 3′ (+1450) and reverse primer 5′ AGCCCTGGAGATTTGGAGAC 3′ (+1628). Samples were subjected to 30 amplification cycles consisting of: 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The PCR products were gel-purified and sub-cloned into the PgemT-easy plasmid (Promega) and sequenced using a T7 primer.

RESULTS

Isolation and Sequence Analysis of 2 New cyp2b cDNA Members

We have screened a male liver cDNA library with a rat CYP2B1 cDNA probe: 22 independent clones were obtained from approximately 10^6 hybridization plaques (Fig. 1). Among them, we identified the cyp2b9 cDNA (100% nucleotide identity with the published sequence) and have characterized the cyp2b20, a hybrid cDNA between a cyp2b10-like sequence and the NADPH Cyp-oxydoreductase mRNA (10). Nineteen clones had different sizes but had strictly identical sequences throughout their regions of overlap, and 1 clone—designated P21—appeared to be the product of a pseudogene (see below). Among the 19 clones, P16 was the largest: its nucleotide sequence consisted of 1473 bp of open-reading frame, and 10 and 312 bp of 5′- and 3′-non-coding regions, respectively. It exhibited 34 base differences with the cyp2b10 published sequence, spread all along the sequence, which represented an homology of 98% (Fig. 2). The first 1348 nucleotides of this sequence were 100% homologous with the cyp2b10-like portion of cyp2b20. This new sequence also shared 87% and 91% identity with the mouse cyp2b9 and the rat CYP2B1 and CYP2B2 mRNAs, respectively (Table I). The similarity between the deduced amino acid sequences of P16 and cyp2b10 was 97%, corresponding to 13 distinct amino acids (Fig. 3, Table I).

The P21 nucleotide sequence lacked 188 bp from the 5′-end. The rest of the sequence overlapped with that of P16, except for a T→G transversion at position +196, which would give rise to a TGA codon (Figs. 2 and 3), thereby indicating that P21 was derived from a pseudogene.

RT-PCR Analyses

In order to confirm the identity of the P16 clone, we performed a RT-PCR analysis with Balb/c female and male liver RNA, using a sub-region with the largest possible number of differences (8 base differences) between our sequence and the cyp2b10 sequence published by Noshiro *et al.* (7) (Fig. 1). The PCR products had the expected 178 bp size. In order to avoid any PCR artifact, we sub-cloned 4 and 5 independent PCR products from female and male RNA, respectively. Upon sequencing, the PCR products were found to be 100% identical with our sequence whatever the sex of the animals.

PCR Analyses on the Genomic DNA

In order to look for the genomic counterparts of cyp2b10 and P16, we amplified by PCR the same region as above from Balb/c genomic DNA. Had 2 distinct genes existed—potentially among others—we would have expected to obtain 2 different classes of PCR products in similar proportions, one with a sequence identical with that of cyp2b10, and another one identical with our sequence. Eight independent clones were derived following PCR and sub-cloning, and showed, upon sequencing, 100% identity with the P16 sequence.

DISCUSSION

Restriction and Southern blot analyses previously reported have suggested that the mouse cyp2b gene subfamily contained more than 10 members (5,6). In a previous study, 21 mouse cyp2b cDNA clones for mouse cyp2b were isolated from a cDNA library constructed from Balb/c female liver mRNA (7). This library screening was performed using a rat CYP2B2 cDNA probe. Full length nucleotide sequences were obtained for two types of cDNAs now known as cyp2b9 and cyp2b10. In addition, cyp2b13 was isolated from liver mRNA of PB-treated DBA2/J male mice (8).

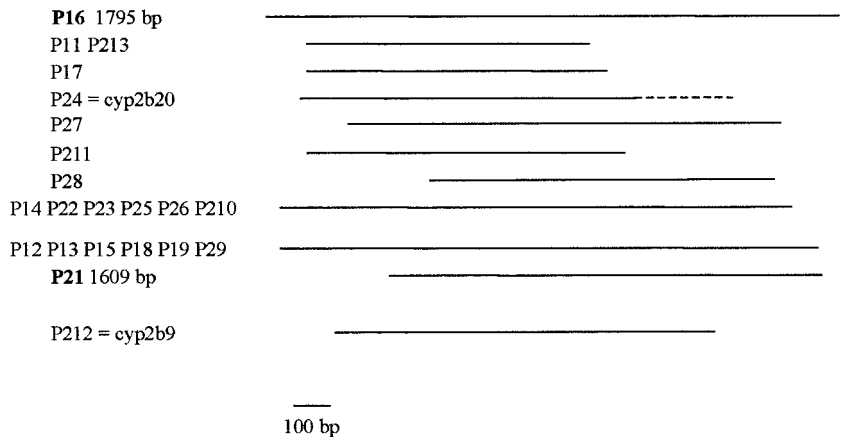

FIG. 1. Alignment of the 22 cyp2b clones isolated. Twenty two independent clones have been isolated by screening a Balb/c male liver cDNA library with a rat CYP2B1 cDNA probe. Among these clones, we have isolated one cyp2b9 clone (P212), the cyp2b20 clone, a hybrid cDNA between a cyp2b10-like sequence and the NADPH Cyp-oxydoreductase (10) (P24: the broken line corresponds to the NADPH Cyp-oxydoreductase portion) and 20 cyp2b10-like clones, including one pseudogene, P21 (1609 bp). The largest clone is P16 (1795 bp) and the smallest ones are P11 and P213 (1000 bp). P16 and P21 appear as bold faced.

In the present study, we have screened a Balb/c male liver cDNA library with a rat CYP2B1 cDNA probe. Twenty-two independent clones were obtained. Among them, we identified one clone 100% identical with the cyp2b9 cDNA, whereas all of the 21 other clones exhibited a novel sequence. The sequence of the P24 clone—now called cyp2b20—, which is a hybrid form between a cyp2b10-like and the NADPH Cyp-oxydoreductase cDNAs, has already been published (10). Nineteen clones had the same nucleotide sequence over their regions of overlap, close to that of cyp2b10 (34 base differences, which represents 98% identity), and the last one was derived from a pseudogene. The fact that we have found only two types of cyp2b clones (one cyp2b9 clone and 21 cyp2b10-related clones) was quite surprising since the murine cyp2b sub-family is believed to be a large one suggested to comprise 10-16 members (5, 6). Either the size of this sub-family has been overestimated, or the other members may be expressed only in certain murine strains, in specific tissues as, for example, the cyp2b19 characterized as a keratinocyte-specific CYP (9), in particular physiological conditions or on the action of chemical inducers distinct from PB. However, our analysis of genomic cyp2b PCR products, although based on a limited DNA region, argues against the occurrence of multiple, closely similar members.

In our study, we did not isolate any cDNA close to cyp2b13. Three basic hypotheses could account for this observation. Firstly, the homology level between rat CYP2B1/2 probe and mouse cyp2b13 might have been too low, although this is rather unlikely (the nucleotide identity level is above 80%). Secondly, the mice used to construct the library from which cyp2b13 was isolated had been treated by PB, whereas our library was from untreated animals. Thirdly, cyp2b13 could be more strongly expressed in DBA2/J mice (the strain from which it was isolated) than in Balb/c mice. Indeed, it is well known that CYP expression can be strain-specific: in a previous report, we have found that the basal expression of the cyp2b9 gene was higher in the A/J and DBA2/J strains than in the Balb/c and SWR/J strains, and was barely detectable in C57BL/6J mice (14).

We did not find any clone showing 100% homology with the cyp2b10 sequence published by Noshiro *et al.* (7), but we have characterized a unique cyp2b10-related nucleotide sequence. The high homology level (98%) between cyp2b10 and the P16 sequence might not have looked so surprising since, for example, rat CYP2B1 and CYP2B2 share 98% identity in their mRNA sequences (15). In addition, some cyp2b10-related cDNAs have already been described (7). However, the P16 clone corresponds neither to the reported pf46 clone (there is no 27 bp insertion at position +1306), nor to pf11 (its polyadenylation signal does not exhibit the 99 bp extension) from this study. The difference in nature of the rat CYP2B cDNA probes (larger than 1200 bp) used distinctly by Noshiro and by us (CYP2B2 and CYP2B1, respectively) could not explain the observations, since, as said above, these 2 rat CYP cDNAs display 98% identity with one another. In addition, the differences found in P16 are extremely unlikely to correspond to sequencing errors, since we found the same 34 base differences at the same positions spread all over the sequence among all of our cyp2b10-like clones. Finally, it is noteworthy that the cyp2b10-like portion of our previously reported cyp2b20 clone is 100% identical with that of P16.

In order to try to reconcile our data with those of Noshiro, we first looked into the possibility of a sex

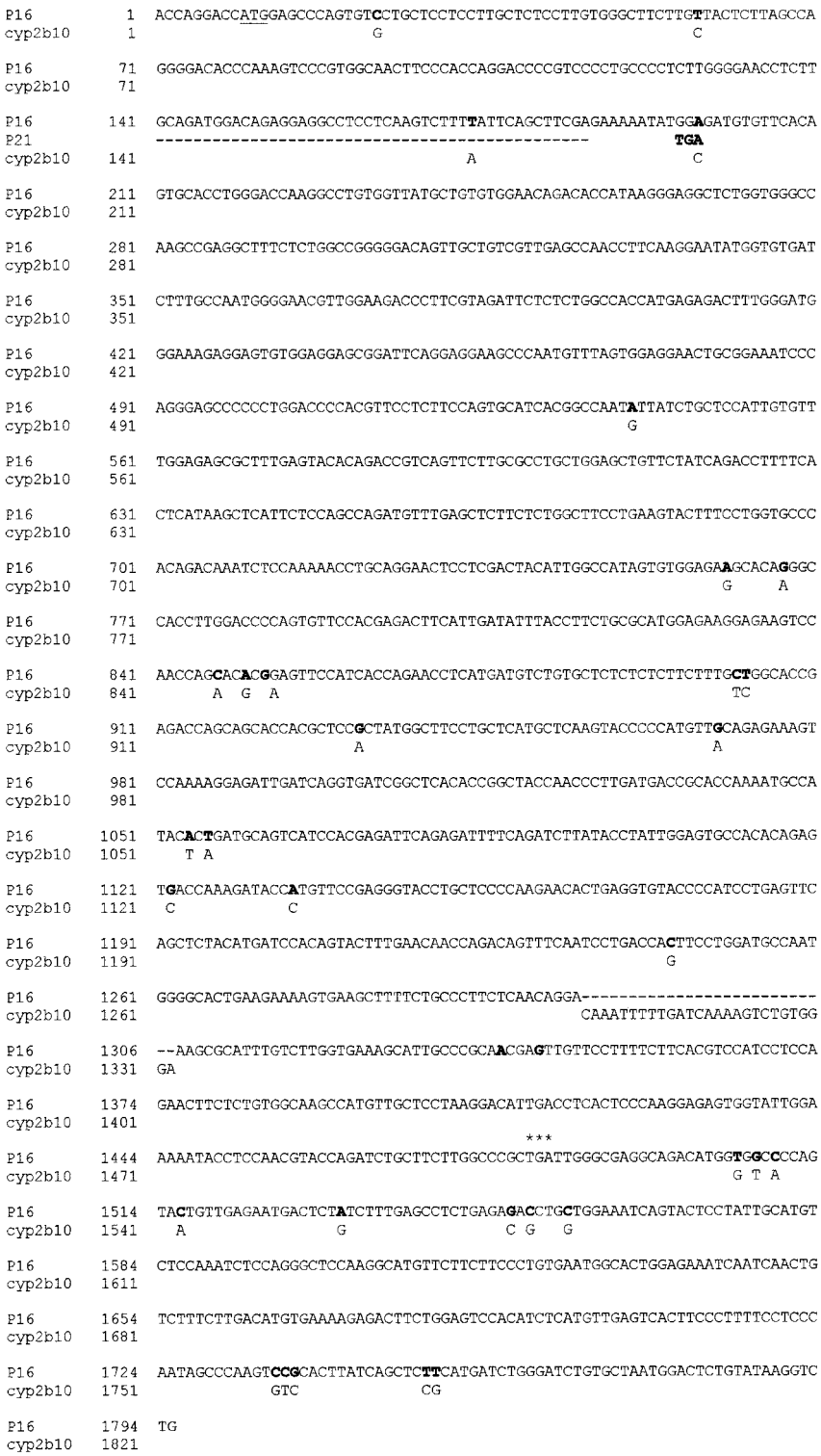

FIG. 2. Nucleotide sequences of P16 and P21 compared with cyp2b10 (the pf46 (7) cloned sequence is represented, with its 27 bp insertion at position +1306. The 1795 bp of P16 are shown. The initiation codon is underlined and the stop codon is indicated with asterisks. The broken line indicates a deletion. The premature stop codon (TGA) at position +196 of P21 appears as bold faced.

difference since cyp2b10 was isolated from a female library whereas our clones were from a male mouse library. Indeed the expression of cyp2b has been shown to be sex-specific, under the control of the Rip locus in female liver (7) and the Ripr locus in male liver (16). These loci are regulated by sex hormones as well:

TABLE I

Nucleotide and Amino Acid Sequence Identity Levels between P16 and Mouse cyp2b10, cyp2b9, cyp2b13 and Rat CYP2B1 and CYP2B2

	Cyp2b10	Cyp2b9	Cyp2b13	CYP2B1	CYP2B2
Nucleotide	98%	87%	81%	91%	91%
Amino acid	97%	84%	85%	92%	90%

Note. The P16 sequence was compared to these sequences, which are present in the Genbank database, using the BLAST algorithm from the network service at the National Center of Biotechnology Information.

castration of males increases expression of cyp2b mRNAs and proteins to female levels, while administration of testosterone restores male levels (17). Sex hormones also induce cyp2b expression in cultured mouse hepatocytes (18). From the proportion of cyp2b9 and cyp2b10 clones isolated, Noshiro *et al.* suggested that cyp2b9 was more expressed than cyp2b10 in Balb/c females (7). By the same sort of indirect estimate, our results suggest the opposite concerning Balb/c males, since cyp2b10-related clones were largely predominant compared to the single occurrence of a cyp2b9 clone.

In order to investigate whether our clone could be a male-specific derivative of a cyp2b10-like cDNA, we amplified by RT-PCR and sequenced a small region chosen to be distinctive between our sequence and that of cyp2b10, using Balb/c male and female RNAs. We failed in finding two distinct sequences: whatever the sex of origin of the RNAs, all the PCR products exhibited a sequence 100% identical with our sequence. These results suggested that our cyp2b10-like clone was not male-specific in Balb/c mice.

In order to look for the genomic counterparts of cyp2b10 and P16 sequences, we amplified the same exonic region using Balb/c genomic DNA: once again, sequencing of 8 independent clones obtained from the PCR products showed 100% sequence identity with the P16 sequence. Had two distinct genes existed—possibly among others—we should have observed an equal distribution of the two sequences for our PCR products (Chi square test, $p < 0.005$), which was not the case. Although it is formally possible that Noshiro's cyp2b10 sequence was derived from a variant of the Balb/c mouse strain distinct from ours, this seems extremely unlikely, since estimates indicate that genotypic variation (base differences) between distinct inbred mouse strains occurs only every 300-500 bp, which would amount to at most 5 differences over 1500 bp (19). It is of course, anticipated that different variants from the same inbred mouse strain should not display such variation. Moreover, such a divergence should presumably have also have occurred for the cyp2b9 sequence. Therefore, these genotypic data strongly suggest that the P16 sequence is the authentic cyp2b10 sequence. When we compared the deduced amino acid sequences of P16 and cyp2b10, the similarity was 97% (13 amino acid difference). Upon protein motif analysis, we noted, however, no major differences between the two sequences: neither phosphorylation, N-myristoylation, or N-glycosylation sites, nor the cysteine heme-iron ligand signature were altered. Enzyme sites important for the catalytic activity and the substrate specificity, although not described for the murine cyp2b proteins, did not appear to be altered in P16, as suggested by analogy with those described for the rat CYP2Bs. Nevertheless, it is possible that other enzyme characteristic features might be distinctive between cyp2b10 and P16-derived protein sequences.

Finally, we have also isolated one clone, P21, which sequence exhibited a single base difference with our 19 cyp2b10-like other clones at position +196. This divergence leads to a premature stop codon, thereby indicating that this mRNA cannot direct the synthesis of a full-sized protein, and thus this clone has the property of a pseudogene. This is the first pseudogene described in the murine cyp2b subfamily, although in rats two pseudogenes belonging to the CYP2B subfamily have been previously isolated: the CYP2B14P and the CYP2B16P (20), whereas in humans, one has been described, the CYP2B7PX (21). In addition, pseudogenes are not specific to the CYP2B subfamily since, up to now, 22 pseudogenes have been described among the CYP superfamily, including, for example, the CYP2C6P1 in rats or the human CYP3A5P1/P2 (2).

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P16          1    MEPSVLLLLALLVGFLLLLARGHPKSRGNFPPGPRPLPLLGNLLQMDRGG
CYP2B10      1
P16         51    LLKSFIQLREKYGDVFTVHLGPRPVVMLCGTDTIREALVGQAEAFSGRGT
P21                       *
CYP2B10     51        L
P16        101    VAVVEPTFKEYGVIFANGERWKTLRRFSLATMRDFGMGKRSVEERIQEEA
CYP2B10    101
P16        151    QCLVEELRKSQGAPLDPTFLFQCITANIICSIVFGERFEYTDRQFLRLLE
CYP2B10    151                              V
P16        201    LFYQTFSLISSFSSQMFELFSGFLKYFPGAHRQISKNLQELLDYIGHSVE
CYP2B10    201              V
P16        251    KHRATLDPSVPRDFIDIYLLRMEKEKSNQHTEFHHQNLMMSVLSLFFAGT
CYP2B10    251    R K                          NA                V
P16        301    ETSSTTLRYGFLLMLKYPHVAEKVQKEIDQVIGSHRLPTLDDRTKMPYTD
CYP2B10    301           H           T                           S
P16        351    AVIHEIQRFSDLIPIGVPHRVTKDTMFRGYLLPKNTEVYPILSSALHDPQ
CYP2B10    351                              L
P16        401    YFEQPDSFNPDHFLDANGALKKSEAFLPFSTG---------KRICLGESI
CYP2B10    401                 Q                    QIFDQKSVG
P16        442    ARNELFLFFTSILQNFSVASHVAPKDIDLTPKESGIGKIPPTYQICFLAR 491
CYP2B10    451      S                                                500
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FIG. 3. Comparison of the deduced amino acid sequences of P16 and cyp2b10: 13 amino acid differences are evidenced. The broken line indicates a deletion (this region corresponds to the insertion of 9 amino acids as described by Noshiro *et al.* for the pf46 allele (7). The premature stop codon in P21 is indicated with an asterisk.

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REFERENCES

1. Gonzalez, F. J. (1989) *Pharmacol. Rev.* **40,** 243–288.
2. Nelson, D. R., Koymans, L., Kamatak, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C., and Nebert, D. W. (1996) *Pharmacogenetics* **6,** 1–42.
3. Nebert, D. W., and Gonzalez, F. J. (1987) *Annu. Rev. Biochem.* **56,** 945–993.
4. Corcos, L. (1992) *Drug. Metab. Dispos.* **20,** 797–801.
5. Lakso, M., Masaki, R., Noshiro, M., and Negishi, M. (1991) *Eur. J. Biochem.* **195,** 477–486.
6. Honkakoski, P., Moore, R., Gynther, J., and Negishi, M. (1996) *J. Biol. Chem.* **271,** 9746–9753.
7. Noshiro, M., Lakso, M., Kawajiri, K., and Negishi, M. (1988) *Biochemistry* **27,** 6436–6443.
8. Stupans, I., Ikeda, I., Kessler, D. J., and Nebert, D. W. (1984) *DNA* **3,** 129–137.
9. Keenay, D. S., and Skinner, C. (1995) 9th International Conference on Cytochrome P450, Zurich, Switzerland, p. 109.
10. Damon, M., Fautrel, A., Marc, N., Guillouzo, A., and Corcos, L. (1996) *Biochem. Biophys. Res. Com.* **226,** 900–905.
11. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Chomezynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
13. Blin, N. and, Stafford, D.W. (1996) *Nucleic Acids Res.* **3,** 2303.
14. Damon, M., Fautrel, A., Guillouzo, A., and Corcos, L. (1996) *Biochem. J.* **317,** 481–486.
15. Mizukami, Y., Sogawa, K., Suwa, Y., Muramatsu, M., and Fuji-Kuriyama, Y. (1983) *Biochemistry* **80,** 3958–3962.
16. Noshiro, M., and Negishi, M. (1988) *Biochemistry* **27,** 6444–6448.
17. Honkakoski, P., Kojo, A, and Lang, M.A. (1992) *Biochem. J.* **285,** 979–983.
18. Nemoto, N., and Sakurai, J. (1995) *Arch. Biochem. Biophys.* **319,** 286–292.
19. Silver, L. M. (1995) Mouse Genetics, Concepts and Applications (Silver, L. M., Ed.), Oxford University Press, New York.
20. Trottier, E., Dubois, S., Jean, A., and Anderson, A. (1996) *Biochem. Pharmacol.* **52,** 963–965.
21. Miles, J. S. (1990) *Nucleic Acids Res.* **18,** 189.