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Cloning and Expression of Complementary DNAs for Multiple Members of the Human Cytochrome P450IIC Subfamily[†,‡]

Marjorie Romkes,[§] Michael B. Faletto,[§] Joyce A. Blaisdell,[§] Judy L. Raucy,[∥] and Joyce A. Goldstein[*,§]
Laboratory of Biochemical Risk Analysis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709, and College of Pharmacy and Toxicology Program, University of New Mexico, Albuquerque, New Mexico 87131

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ABSTRACT: The present study characterizes the profile of cDNAs from the human P450IIC subfamily in a library from one individual, and it describes three new members of this subfamily (IIC17, IIC18, and IIC19) isolated from two human cDNA libraries. cDNA libraries were constructed from two human livers which differed phenotypically in the hepatic content of P450 HLx (IIC8). The library from the phenotypically low HLx individual was screened by using a cDNA for rat liver P450IIC13 and an oligonucleotide probe for human IIC8. One clone, 254c, was isolated which clearly represents a new member of the human P450IIC subfamily (IIC17). This clone lacked the first 358 nucleotides at the N-terminus but was only 91% homologous in its nucleic acid sequence to IIC9 and 79% homologous to IIC8. Near-full-length clones for IIC9 were also isolated from this library, but no clones for IIC8 were found. Northern blots indicated that the mRNA for IIC8 was low or absent in this individual. A second cDNA library (from a liver phenotypically high in HLx) was then screened. Eighty-three essentially full-length (>1.8 kb) clones belonging to the IIC subfamily were isolated from this library. These include full-length clones for two additional new members of the IIC subfamily. Clones 29c and 6b appear to be allelic variants (IIC18), differing by one nucleotide (one amino acid change) in the coding region. Clone 11a represents a full-length clone for a third new P450 (IIC19). Both IIC18 and IIC19 are most homologous to IIC17 (87% and 95%, respectively). Full-length clones were isolated for two allelic variants of IIC9 (clones 25 and 65) which differed by three nucleotides, resulting in one amino acid difference. Hybridization analysis and partial sequencing indicated that of the 83 clones in this library, 50 were 1 of the 2 allelic variants of IIC9, 29 were IIC8, 3 were the 2 allelic variants of IIC18, and 1 was IIC19. cDNAs for IIC9, IIC18, and IIC19 were expressed in COS-1 cells. Antibody to IIC9 recognized the proteins in cells transfected with cDNAs for IIC9; however, neither antibody to IIC9 or IIC8 recognized the other two cytochromes, suggesting that these cytochromes differ immunochemically from IIC8 and IIC9. COS-1 cells transfected with IIC18 showed an increase in metabolism of mephenytoin, but no metabolism of mephenytoin was detected in COS-1 cells transfected with IIC9 or IIC19.

Cytochrome P450[1] enzymes are important in the oxidative metabolism of both endogenous substrates and xenobiotics. Genetic polymorphisms of P450 enzymes result in distinct subpopulations which differ in their ability to perform particular drug biotransformation reactions. We have recently found that a polymorphism in rat P450g (IIC13) in outbred rats is due to the presence of a few single base mutations in the mRNA resulting in simple amino acid changes (Yeowell et al., 1990). Human P450 HLx, which is immunochemically

[†] A preliminary report of a portion of this work was presented at the 81st Annual Meeting of the American Society for Biochemistry and Molecular Biology (Romkes et al., 1990).
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05326.
[*] To whom correspondence should be addressed.
[§] National Institute of Environmental Health Sciences.
[∥] University of New Mexico.

[1] We have used the recommended P450 gene nomenclature system (Nebert et al., 1991) throughout this paper. All new P450IIC sequences were submitted to Dr. Nebert for inclusion in the 1991 update to this nomenclature system, and the numbering system is consistent with this update. Abbreviations: SSC, standard sodium citrate; SDS, sodium dodecyl sulfate.

related to rat P450g (IIC13), is phenotypically variable in humans as determined by immunoblot analysis (Wrighton et al., 1987). Okino et al. (1987) isolated a cDNA (IIC8) which appears to encode the protein for human HLx, on the basis of its N-terminal sequence. Other research groups have isolated cDNAs MP12/MP20 (Ged et al., 1988) and IIC2 (Kimura et al., 1987) which are presumably allelic variants of IIC8. However, the basis of the polymorphism has not yet been explained.

At least three or more forms of P450 belonging to the human IIC subfamily have been isolated by different laboratories, including three proteins termed $P450_{MP-1}$, $P450_{MP-2}$, and $P450_{MP-3}$ (Shimada et al., 1986; Lasker et al., 1987; Kawano et al., 1987; Gut et al., 1986; Beaune et al., 1985; Ged et al., 1988). There are also other polymorphisms in this subfamily. For example, there is a polymorphism in the metabolism of the *S* enantiomer of mephenytoin in humans. Guengerich and co-workers (Shimada et al., 1986) have isolated two forms of P450 (MP-1 and MP-2) which appear similar in their (*S*)-mephenytoin hydroxylase activity. They then isolated the cDNAs for the highly related MP-8 (IIC10) and MP-4 (IIC9) (which varied by only two bases in the coding region) using antibody to $P450_{MP-1}$ (Umbenhauer et al., 1987; Ged et al., 1988). These may represent allelic variants of the same protein but have seen classed at present as separate genes on the basis of differences in the 3′-noncoding region. Several other groups have also isolated cDNAs which appear to be allelic variants of IIC9 (Kimura et al., 1987; Shephard et al., 1989; Yasumori et al., 1987). However, the majority of studies from a number of laboratories using different cDNA expression systems indicate that various IIC9 variants do not appear to metabolize (*S*)-mephenytoin. For example, when the cDNA isolated by Kimura et al. (1987) was expressed in HepG2 cells, it metabolized racemic mephenytoin but had no (*S*)-mephenytoin hydroxylase activity, suggesting that some other isozyme is responsible for the polymorphism in the metabolism of (*S*)-mephenytoin. Similarly, when a complete MP-8 clone (constructed by using the five N-terminal amino acids determined from amino acid sequencing of MP-1) was expressed in yeast, it metabolized tolbutamide but did not 4-hydroxylate (*S*)-mephenytoin (Brian et al., 1989). However, Yasumori et al. (1987) isolated a variant of IIC9 differing by only two amino acids from MP-8 (positions 358 and 417) and by only three amino acids (positions 4, 6, and 144) from the IIC9 cDNA isolated by Kimura et al. (1987). His laboratory reported that the protein encoded by this cDNA metabolized (*S*)-mephenytoin when expressed in yeast (Yasumori et al., 1989). The role of the various allelic variants of IIC9 in (*S*)-mephenytoin metabolism remains controversial at this time.

Genetic analysis has suggested the presence of ≥7 genelike sequences in the IIC subfamily. Therefore, it seems likely that this subfamily may include other members. In this study, we constructed two cDNA libraries from individuals phenotypically high and low in HLx, to examine whether a variant mRNA for IIC8 was responsible for the polymorphic expression of HLx and to identify additional members of the IIC subfamily. No clones for IIC8 were isolated from the individual phenotypically low in HLx, while several full-length cDNAs for IIC8 were isolated from the phenotypically high individual. Two allelic variants for IIC9 were isolated. In addition, a partial cDNA for a new member of the IIC subfamily (IIC17) and full-length cDNAs for two additional new members (IIC18 and IIC19) were isolated. These new members of the IIC subfamily were expressed in COS-1 cells and shown to be immunochemically distinct from HLx and IIC9, and IIC18 metabolized racemic mephenytoin.

Materials and Methods

Materials. Human liver samples were obtained from organ donors through the National Disease Research Interchange in Philadelphia, PA, and from the Human Liver Research Facility, Stanford Research Institute, Life Sciences Division, Menlo Park, CA. Restriction endonucleases were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) and [α-^{35}S]dATP (650 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest quality available.

Purified Proteins and Western Blots. Cytochromes P450IIC8, P450IIC9, and NADPH:P450 reductase were purified and antibodies to IIC8 and IIC9 prepared in rabbits as previously described (Lasker et al., 1987). Human hepatic microsomes were prepared from several different liver organ donor samples as previously described (Goldstein et al., 1982). The microsomal protein was determined by the method of Bradford (1976). SDS–polyacrylamide gel electrophoresis (7.6% acrylamide) of hepatic microsomes was performed according to Laemmli (1979), and the proteins were transferred to nitrocellulose. Western blots were performed as previously described (Yeowell et al., 1985) using polyclonal antibodies to IIC8 and IIC9 (Raucy et al., 1989).

Construction and Screening of Human Liver cDNA Libraries. Total human liver RNA was prepared by the guanidine hydrochloride method (Cox, 1968) from two human livers either low (860624) or high (S33) in HLx as identified by immunoblot analysis. Poly(A+) RNA was then isolated by two passages over an oligo(dT)–cellulose column (Aviv et al., 1972). The low-HLx cDNA library was prepared by Stratagene Cloning Systems (La Jolla, CA), and the double-stranded cDNA was treated with S1 nuclease. Following the addition of *Eco*RI linkers, the double-stranded cDNA was size-fractionated on a CL-4B Sepharose column. The largest fraction was ligated into λZAPII and then transfected into XL1-Blue. The high-HLx cDNA library was constructed in our laboratory following the methods of Watson and Jackson (1985). Double-stranded cDNA was ligated to *Eco*RI linkers, size-fractionated on an agarose gel (1.8–2.4 kb), and then ligated into λZAPII (Stratagene) and transfected into XL1-Blue.

The low-HLx library was screened under conditions of low stringency with a ^{32}P-labeled rat P450IIC13 cDNA probe and with oligonucleotides for human IIC8 (T300R) (5′-TTAGTAATTCTTTGAGATAT-3′) and IIC9 (M300R) (5′-CTGTTAGCTCTTTCAGCCAG-3′). The high-HLx library was screened under conditions of low stringency using a ^{32}P-labeled 254c (IIC17) cDNA probe and M300R (IIC9). Positive clones were isolated, transfected into XL1-Blue, and excised into the plasmid Bluescript, according to Stratagene's excision protocol.

Sequence Analysis. The Bluescript plasmids containing the positive cDNA inserts from the low-HLx library were purified by CsCl gradients, while the plasmids containing cDNA inserts from the high-HLx library were purified by using Qiagen plasmid purification kits (Qiagen, Inc., Studio City, CA). The double-stranded cDNA inserts were sequenced by the dideoxy chain termination method for Sanger et al. (1982) using Sequenase kits (U.S. Biochemical Corp., Cleveland, OH). The nearly full-length clones 186 and 254c and the full-length clones 65, 25, 7b, 35g, 11a, 29c, and 6b were sequenced completely in both directions with primers spaced approxi-

mately 200 bases apart. The remaining positive clones from the high-HLx cDNA library were sequenced in both directions through both the 5′ and 3′ ends and through all the regions which would identify any of the known allelic variants.

Human RNA Blot Analysis and Hybridization Conditions. Poly(A+) RNA (10 μg) was electrophoresed in a 1% agarose gel under denaturing conditions and transferred to a Nytran filter (Micron Separation, Inc., Westboro, MA), and filters were then baked for 2 h at 80 °C. The filters were prehybridized for 2 h, then hybridized overnight with a ^{32}P-labeled specific oligonucleotide probe for IIC8 (T300R) at 42 °C, washed 3 × 5 min at room temperature and 1 × 5 min at 42 °C with 2 × SSC/0.1% SDS, and radioautographed. Filters were then stripped with 5 mM Tris (pH 8.0), 0.2 mM EDTA, 0.05% sodium pyrophosphate, and 0.1 × Denhardt's for 2 h at 65 °C and rehybridized with a random-primed actin cDNA (Oncor, Gaithersburg, MD) at 50 °C using 6 × SSC, 4 × Denhardts, and 0.5% SDS. These filters were washed 1 × 5 min at room temperature, 1 × 10 min at 48 °C, and 4 × 15 min at 48 °C and radioautographed as before. The IIC8 mRNA band was quantitated by scanning with an LKB Ultrascan laser densitometer, and the values of the integrated peaks were divided by those of the actin peaks.

Hybridization and washing conditions for screening libraries with random-labeled cDNAs for IIC13 (g) or IIC17 used the same solutions as described for actin, but were performed at nonstringent temperatures (42 °C). Conditions for hybridization of clones with T300R were identical with those described above. Hybridization of cDNA clones with M300R (recognizes IIC9, IIC10, and IIC19), 254c-351R (IIC17) (5′-ACTTTTCAATGTAAGCAAAT-3′), and M1724R (IIC9) (5′-CATATTAATAACTCAACATT-3′) was identical except that for each oligomer the hybridization temperature and the high-stringency wash were 5 °C below the calculated melting temperatures.

COS-1 Cell Expression Studies. cDNA inserts were ligated into the cloning region of the expression plasmids pSVL (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) or pcD (Okayama & Berg, 1983) and used to transform COS-1 cells. COS-1 cells were plated at (1–2) × 10^6 cells per 10-cm dish and grown for 24 h in Dulbecco's-modified Eagle's medium with 10% fetal bovine serum (DMEM). The cells were then washed with Dulbecco's phosphate-buffered saline (PBS) and transfected with recombinant plasmid (3 μg per dish) in DEAE-dextran (500 μg/mL) for 30 min–1 h at 37 °C. The transfected cells were then treated with chloroquine (52 μg/mL) in DMEM for 5 h (Luthman & Magnusson, 1983), washed with PBS, refed with DMEM, and incubated for 72 h prior to harvest. Typically, 15–20 dishes were transfected with each recombinant plasmid. For Western blot analysis of the recombinant transformed COS-1 cells, cells were scraped from the dishes into buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, and 1 mM EDTA) and lysed with 3 × 5 s bursts with a polytron. A portion of each lysate was centrifuged at 9000*g* and then 10000*g* for the preparation of a microsomal fraction. Western blots were then performed as described above. Total RNA was isolated from transfected COS-1 cells, and Northern blots were performed as described for human samples. The filters were hybridized with a ^{32}P-labeled oligonucleotide probe which hybridizes with all IIC clones isolated (IIC500R) (5′-GGAGCACAGCCCAGGATGAA-3′) at 55 °C overnight, then washed 3 × 5 min at room temperature and 2 × 5 min at 37 °C, and radioautographed.

Mephenytoin 4-Hydroxylation Assay. The methods for determining the enzymatic hydroxylation of mephenytoin were performed by using the general incubation conditions and HPLC assay previously described by Shimada et al. (1986). The microsomal fractions prepared from the COS-1 cells, containing 200 μg of microsomal protein, 1000 units of purified P450 reductase, and 0.2 nmol of cytochrome b_5, were incubated with 0.5 mM racemic mephenytoin and 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 mL. The reactions were carried out at 37 °C, were initiated by the addition of 20 μL of 50 mM NADPH, and stopped 15 min later by the addition of 100 μL of 1 N HCl. The samples were extracted with water-saturated ethyl acetate and dried on low heat under nitrogen. The residue was reconstituted in 25 μL of 50% methanol, and 10 μL was injected onto the HPLC with UV detection at 204 nm using a Rainin 10-cm Microsorb C_{18} octyldecylsilyl "short one" column. The mobile phase was 55% methanol/45% H_2O with a flow rate of 1 mL/min. Statistical significance was assessed by a Kruskal–Wallis test for overall differences between groups, and pairwise comparisons were made with a Mann–Whitney U test.

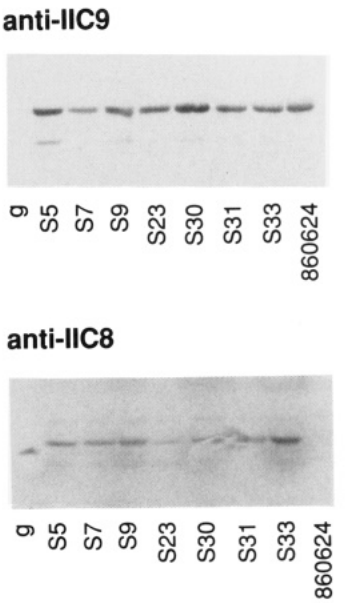


FIGURE 1: Western blots of human liver microsomal proteins. Microsomal proteins were separated by SDS–polyacrylamide gel electrophoresis. Blot A was performed using polyclonal antibody to IIC9 and blot B with anti-IIC8 (HLx). Each lane represents 20 μg of microsomal protein from an individual liver. The IIC8 antibody also recognized purified rat P450IIC13 (g). cDNA libraries were constructed from livers 860624 (low HLx) and S33 (high HLx).

Results

Expression of cytochromes P450IIC8 and IIC9 was examined in human liver microsomes by Western blot analysis (Figure 1). Polyclonal antibodies to both IIC8 and IIC9 exhibited some cross-reactivity to related proteins. Both of these P450s are expressed constitutively; however, P450IIC8 (HLx) is phenotypically variable as described by Wrighton et al. (1987). The level of P450IIC9 did not vary appreciably in these tissues. Microsomes from liver 860624 contained IIC9 but were essentially lacking HLx, while liver microsomes from S33 contained high amounts of both proteins.

Two cDNA libraries were constructed from human livers 860624 and S33, which expressed phenotypically low or high amounts of HLx, respectively. Screening the cDNA library constructed from a low-HLx individual with a cDNA for rat IIC13 under nonstringent conditions and with oligonucleotide probes specific for IIC8 and IIC9 yielded several clones for IIC9 and a novel P450IIC cDNA. None of the clones iden-

Table I: Percent Homology for Nucleotide and Amino Acid Sequences of P450IIC cDNAs[a]

clone	IIC8	IIC9	254c (IIC17)	29c (IIC18)	11a (IIC19)
254c (IIC17)	79	91	100	87	95
	78	90	100	86	92
29c (IIC18)	81	85	87	100	82
	77	82	86	100	81
11a (IIC19)	81	90	95	82	100
	79	92	92	81	100

[a] For each comparison, the upper value represents percent nucleotide homology, and the lower value represents percent amino acid homology. The nucleic acid comparisons include both the coding and noncoding regions. The IIC9 sequence used in this comparison was the cDNA sequence for human form 2 (Yasumori et al., 1987).

Table II: Distribution of P450IIC cDNA Clones from Human Liver S33[a]

	no. of clones	% distribution
IIC8	29	35
IIC9		
65	39	47
25	11	13
IIC10	0	0
IIC18		
29c	1	1.2
6b	2	2.5
IIC19 (11a)	1	1.2
total	83	100

[a] Clones were classified by hybridization with specific oligonucleotide probes and partial sequencing.

tified in this library were full-length. Clone 186 was identical with but 25 base pairs longer than MP-4, a IIC9 clone previously described by Ged et al. (1988). Clone 254c lacked 358 nucleotides at the N-terminus but clearly represents a new member of the IIC subfamily (IIC17), since it is only 90% homologous in amino acid sequence to IIC9 and 78% homologous to IIC8 (Table I). The cDNA sequence for 254c is shown in Figure 2 (bottom line) along with the sequences of other cDNAs from the IIC subfamily sequenced later in this study. No clones for IIC8 were identified in this library, despite the screening of >90 000 plaques with a specific oligoprobe for IIC8.

We then screened approximately 40 000 plaques from the library from liver S33 with the cDNA for 254c under nonstringent conditions and with an oligonucleotide probe specific for IIC9. Eighty-three essentially full-length IIC clones (>1.8 kb) were isolated, purified, and partially or completely sequenced (Table II). Of these, 29 clones were found to encode cytochrome P450IIC8. We isolated two variant clones (7b and 35g) of IIC8 which were completely identical with Hpl-1 and Hpl-2 reported by Okino et al. (1987). Clone 35g is identical with 7b except that it contains an additional 39 bases of a presumed intron which results in the addition of two early stop codons. Both cDNAs were full-length, but 35g contained a longer 5′ leader (81 vs 77 base pairs).

The majority of the clones (50) isolated from the library from liver S33 coded for IIC9. Interestingly, all of the 50 clones appeared to be 1 of 2 IIC9 allelic variants, typified by the full-length clones 65 and 25. All of these clones were sequenced through the 5′ and 3′ ends and through regions which would identify known allelic variants. Thirty-nine of the IIC9 clones were identical with clone 65, and 11 were identical with clone 25. The nucleotide sequence for clone 65 is shown in Figure 2, and the differences for clone 25 are displayed in the line above (boxed). Clones 25 and 65 were identical in the 5′- and 3′-noncoding regions but contained three single-base changes at positions 842, 1075, and 1425. Two of these base changes were conservative, but the third would result in one amino acid difference at position 359 (isoleucine versus leucine). Figure 3 compares the amino acid sequences for the variants of IIC9 isolated by various laboratories. Clone 65 is identical in amino acid sequence with human form 2, although it differs by two silent changes in the coding region and four differences in the noncoding region (Yasumori et al., 1987). Clone 65 contained a leucine instead of a isoleucine at position 4, a valine instead of a serine at position 6, and an arginine instead of a cysteine at position 144 compared to the IIC9 sequenced by Kimura et al. (1987). The IIC9 reported by Meehan et al. (1988) has substitutions at positions 144, 175, and 238 compared to our clones.

The remaining clones characterized from the human liver S33 cDNA library encode several novel P450IIC cDNAs. Their DNA sequences are shown in Figure 2. Two of these clones, 29c and 6b, differ by one nucleotide in the coding region (position 1154), which would result in a single amino acid change (threonine vs methionine at position 385). Clone 29c had a very long (198 bp) 5′-noncoding sequence and a polyadenylation signal 21 bases from the poly(A) tail. Clone 6b did not have a polyadenylation signal at the same site as clone 29c, but instead had an unusually long 3′-noncoding region containing three possible polyadenylation signals with no poly(A) tail. The differences in the 3′-noncoding region could represent alternate splicing, allelic variants, or possibly separate genes. However, these clones are designated as allelic variants of (IIC18) because they differ by only one base in the coding region. They are most similar to 254c (IIC17) (86% amino acid homology) and less similar to IIC9 (82%) and IIC8 (77%) (Table I).

A third unique P450IIC cDNA, clone 11a (designated IIC19), was also identified. IIC19 is most homologous to 254c (IIC17) (95% nucleic acid homology), but base differences were found throughout both the coding and noncoding sequence (Figure 2 and Table I). It is 92% homologous in its amino acid sequence to both IIC17 and IIC9, 81% homologous to IIC18, and 79% homologous to IIC8. Clone 11a had a short 5′-leader sequence and contained the stop codon, but did not have a polyadenylation signal or poly(A) tail. Interestingly, no clones for IIC10 (MP-8) were isolated from either library, despite the sequencing of the 3′ region of all 50 putative IIC9 clones.

Figure 4 shows the alignment comparisons for the deduced amino acid sequences of all known members of the human *CYP2C* family, including the three new P450s identified in the present study. The 7 proteins, along with the consensus sequence, can be aligned with no gaps, and each is predicted to be 490 amino acids long. The amino acid sequences show marked similarities with many regions of absolute conservation. Regions of marked conservation are noted from 131 to 180, and from 302 to 460. These human P450IIC protein sequences also demonstrate hypervariable regions which may be important for interactions between the enzyme and substrate. These include the region from 181–210 and 220–248 as well as 283–296 and a short region near the carboxy terminus at 461–479. Notably, a putative recognition site for phosphorylation of P450 by cAMP-dependent kinase for P4502B1 (Muller et al., 1985) (Arg-Arg-Phe-Ser) at positions 124–127 was conserved in IIC8, IIC9, and 11a (IIC19), suggesting that these cytochromes might be regulated by phosphorylation. However, neither IIC17 nor IIC18 contained a serine at this site. The overall percent homology for both nucleic acid and protein sequences is summarized in Table I.

Table III: Mephenytoin Hydroxylase Activity by P450IIC cDNAs Expressed in Microsomal Fractions of COS-1 Cells[a]

cDNA transfected	racemic mephenytoin 4-hydroxylase act. [pmol min^{-1} (mg of protein)$^{-1}$]
controls	14 ± 5
65 (IIC9)	12
25 (IIC9)	19
11a (IIC19)	16
29c (IIC18)	86 ± 22[b]
6b (IIC18)	46 ± 6[b]

[a] Cells were transfected with the cDNA indicated. Incubations were performed with 200 μg of microsomal protein, 0.5 mM substrate, 1000 units of reductase, 0.2 nmol of cytochrome b_5, and 2 mM NADPH in a final volume of 0.5 mL. All values are the mean ± SE of 6–9 determinations except for 65, 25, and 11a which are the average of duplicate values. [b] Significantly different from controls, $P < 0.01$ by the Mann–Whitney U test. These values compare with values of 239 ± 51 pmol min^{-1} (mg of protein)$^{-1}$ for human liver microsomes.

cDNA Expression in COS-1 Cells. The two variant cDNAs for IIC9, the two variant cDNAs for IIC18, and the cDNA for IIC19 were inserted into expression vectors and transfected into COS-1 cells. Cell lysates were prepared and immunoblotted by using antibody to HLx and P450IIC9. The results are shown in Figure 5. Transfection of COS-1 cells with the two variants of IIC9 (25 and 65) resulted in the expression of a protein with a molecular weight equal to that of pure IIC9. In contrast, neither IIC18 (either variant) nor IIC19 was detected by antibody to HLx or IIC9. However, Northern blot analysis indicated that all three cDNAs had been successfully transfected into these cells (Figure 6). The sizes of the transcripts were those expected for the constructs. The somewhat lesser hybridization of the IIC oligoprobe with RNA from cells transfected with 11a reflects a lower amount of RNA in this sample as shown by the hybridization with the actin probe.

Table III compares the metabolism of racemic mephenytoin in COS-1 cells transfected with the two allelic variants of IIC9, two variants of IIC18, and IIC19. Only COS-1 cells transfected with cDNAs for IIC18 (both allelic variants) exhibited a significant increase in the metabolism of mephenytoin over that of nontransfected cells. Values for IIC9 metabolism represent duplicate samples of both clones. However, when the four values for both clones are pooled, they are not significantly different than metabolism in untransfected COS-1 cells.

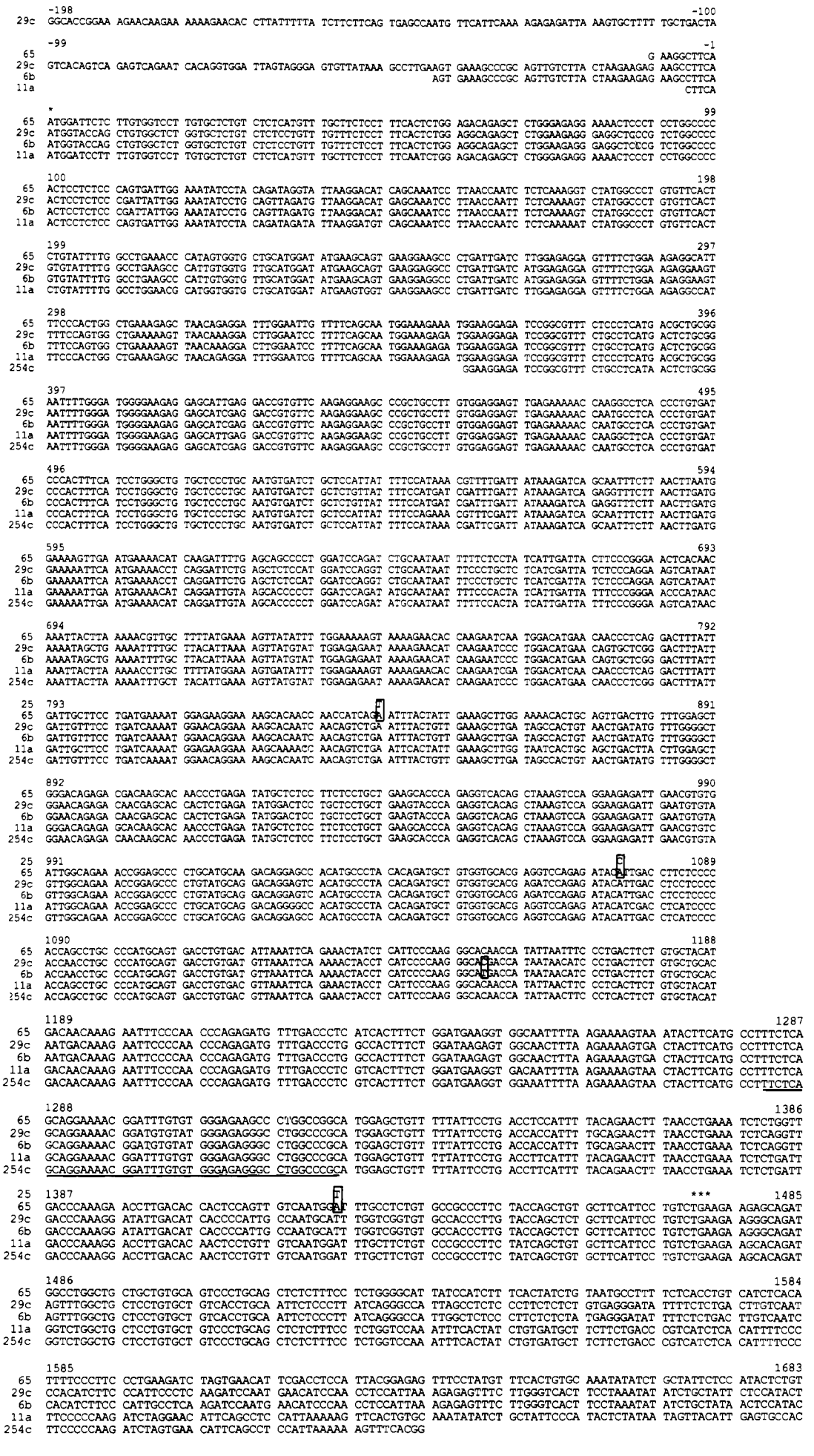
Human Tissues. Expression of P450IIC8 was examined in mRNA from various human hepatic tissues using Northern blot analysis with the specific oligoprobe for IIC8 (T300R). Hybridization with T300R was negligible in mRNA from 860624 compared to S33 and a number of other liver samples (Figure 7). When corrected for hybridization with the actin probe, the amounts of IIC8 mRNA were consistent with the relative amounts of HLx observed in Western blot analysis. Laser scans of the radioautographs indicated that IIC8 mRNA levels in sample 860624 were at least 70-fold lower than S33 and 3–15-fold lower than any of the remaining samples.

Discussion

Genetic analysis of the *CYP2C* subfamily indicates the presence of ≥7 genes. The present study characterizes the profile of cDNAs from this subfamily in a library from a single individual. The majority of the cDNAs characterized in the high-HLx library (60%) were one of two allelic variants of IIC9, while 35% represented IIC8. Two new genes were identified (two allelic variants of IIC18 and IIC19), and a third new gene (IIC17) was identified in a library from a second individual. A comparison of the P450IIC cDNAs and their predicted amino acid sequences shows that 70% of the amino acids are absolutely conserved among the human P450IIC subfamily. Regions of the human P450IIC protein sequence have particularly enhanced conservation, and these areas may participate in common P450 functions. The hypervariable regions may be responsible for substrate specificity. However, changes of only one amino acid have been shown to alter the substrate specificity of P450coh from coumarin hydroxylase to P45015 α-steroid hydroxylase activity (Lindberg & Negishi, 1989). Therefore, small changes in amino acid composition can completely reverse substrate specificity. A putative recognition site for phosphorylation of P450 by cAMP-dependent kinase (Muller et al., 1985) was found in IIC8, IIC9, and IIC19, suggesting that catalytic activity of these cytochromes might also be regulated by phosphorylation. In contrast, neither IIC17 nor IIC18 contained the phosphorylatable serine at position 127.

There are a number of polymorphisms in the human *CYP2C* subfamily. These include variations in the hepatic levels of HLx (Wrighton et al., 1987) and metabolic variations in the hepatic metabolism of (*S*)-mephenytoin. The molecular basis for these polymorphisms has not been characterized. IIC8 appears to encode the protein for HLx on the basis of its N-terminal amino acid sequence (Okino et al., 1987; Wrighton et al., 1987; Lasker et al., 1987). In the present study, 29 IIC8 clones were identified (~40 000 clones screened) in an individual who was phenotypically high for hepatic HLx expression. In contrast, no IIC8 clones were identified in the library from the individual who expressed extremely low amounts of HLx (>90 000 clones screened). These results were consistent with those of Northern analysis which indicate that IIC8 mRNA was low or absent in this individual. This suggests that the low amounts of HLx may result from a gene defect which results in defective expression of the mRNA in the low phenotype and not from a defective mRNA.

At least two P450 proteins which metabolize (*S*)-mephenytoin have been isolated from human liver (MP-1 and MP-2). cDNAs for IIC10 and IIC9 (which vary by only two amino acids in the coding region) were originally isolated by using antibody to $P450_{MP-1}$. However, neither the hepatic content of $P450_{MP-1}$ or $P450_{MP-2}$ nor the expression of mRNAs for MP-8 (IIC10), MP-4 (IIC9), or IIC8 could be correlated with mephenytoin hydroxylase activity (Ged et al., 1988). A number of allelic variants of IIC9 have been characterized by several different laboratories. We have isolated two additional full-length allelic variants of IIC9. One of these clones is identical with MP-4, but is full-length. It varies from the almost full-length human form 2 isolated by Yasumori et al. (1987) by only two silent base changes in the coding region and by four changes in the noncoding region. The number of differences in the nucleic acid sequences of the presumed allelic variants isolated by different laboratories range from 4 to 17 and the amino acid changes vary from 0 to 4, as illustrated in Figure 3. Two of the amino acid differences occur within the first six N-terminal residues, the others occurring singly throughout the sequence. The effect of these changes on catalytic activity has not been systematically studied. Thus far, Relling et al. (1990) found that when the cDNAs for IIC8 and IIC9 isolated by Kimura et al. (1987) were expressed in HepG2 and TK^- cells, both cytochromes metabolized tolbutamide (IIC9 > IIC8) and IIC9 4-hydroxylated racemic mephenytoin but did not metabolize (*S*)-mephenytoin. However, Yasumori, et al. (1990) found that the form of IIC9 isolated in his laboratory (human form



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      1684                                                                                                1780
 65   AACAGTTGCA TTGACTGTCA CATAATGCTC ATACTTATCT AATGTTGAGT TATTAATATG TTATTATTAA ATAGAGAAAT ATGATTTGTG TATTATAAT
29c   CTGTATCACT TGTATTGACC ACCACATATG CTAATACCTA TCTACTGCTG AGTTGTCAGT ATGTTATCAC TAGAAAACAA AGAAAAATGA TTAATAAAT
 6b   TCTGTATCAC TTGATTGACC ACCACAGATT GCTAATACCT ATCTACTGCT GAGTTGTAGA GTTATTACTA TAAACAAAGA AAAATGATTA ATAATGCAA
11a   ATAATGCTGA TACTTGTCTA ATGTTGAGTT ATTAACATAT TATTATTAAA TAGA

      1781                                                                                                1881
 65   TCAAAGGCAT TTCTTTTCTG CATGTTCTAA ATAAAAAGCA TTATTATTTG CTGAAAAAAA
29c   GACAATTCAG AGCCAAAAAA AAAAAAAA
 6b   TTAAAGGCAG AAAGGTCAAG AGGTAGAAAG CTGTTTAGCT AAATGCCACC TAGAGTTATT GGAGGTCTGA ATTTGAAAAA AAAACTATGT CCAGGAGCA

      1882                                                                                                1980
 6b   GCTGTAACCT GTAGGGAAAT AATGGAACAA TCATCCATAA GAGGGATGAA CATTAAGTGT TTGAATTCAT GCTCTGCTTT TGTGTTACTG TAAACACAA

      1981                                                                  2053
 6b   GATCAAGATT TGGATAATCT TTTTCCTTTG TGTTTCCAAC TTAGATCATG TCTAAATATA TGCTTTCATA TGG
```

FIGURE 2: Nucleotide sequences of human P450IIC cDNAs. Sequences were determined by the dideoxy chain termination method. The differences observed for clone 25 are listed above the sequence for 65 and are boxed. The initiation and termination codons are starred. The heme binding region and polyadenylation signals are underlined. The one-base difference between 29c and 6b is also boxed.

```
          1                                                                                                100
CONSEN    MDSlVvLVLC LSCLLLLSLW RQSSGRGKLP PGPTPLPVIG NILQIGIKDI SKSLTNLSKV YGPVFTLYFG LKPIVVLHGY EAVKEALIDL GEEFSGRGIF
  IIC1      I S
  Hum2    XXX
Meehan
    65
    25
  MP-8    XXXXX

          101                                                                                              200
CONSEN    PLAERANRGF GIVFSNGKKW KEIRRFSLMT LRNFGMGKRS IEDrVQEEAR CLVEELRKTK ASPCDPTFIL GCAPcNVICS IIFHKRFDYK DQQFLNLMEK
  IIC1                                              C
  Hum2
  MP-4    XXXXXXX
Meehan                                              C                                   Y
    65
    25
  MP-8
        201                                                                                                300
CONSEN    LNENIKILSS PWIQICNNFS PIIDYFPGTH NKLLKNVAfM KSYILEKVKE HQESMDMNNP QDFIDCFLMK MEKEKHNQPS EFTIESLENT AVDLFGAGTE
  IIC1
  Hum2
  MP-4
Meehan                                        L
    65
    25
  MP-8

          301                                                                                              400
CONSEN    TTSTTLRYAL LLLLKHPEVT AKVQEEIERV IGRNRSPCMQ DRSHMPYTDA VVHEVQRyiD LLPTSLPHAV TCDIKFRNYL IPKGTTILIS LTSVLHDNKE
  IIC1
  Hum2
  MP-4
Meehan
    65
    25                                                    L
  MP-8                                                    C

          401                                                                                              490
CONSEN    FPNPEMFDPH HFLDEGgNFK KSKYFMPFSA GKRICVGEAL AGMELFLFLT SILQNFNLKS LVDPKNLDTT PVVNGFASVP PFYQLCFIPV *
  IIC1                                                                                                     *
  Hum2                                                                                                     *
  MP-4                                                                                                     *
Meehan                                                                                                     *
    65                                                                                                     *
    25                                                                                                     *
  MP-8                    D                                                                                *
```

FIGURE 3: Alignment of human IIC9 proteins. The deduced amino acid sequences for hum2 (human form 2; Yasumori et al., 1987), MP-8, and MP-4 (Ged et al., 1988) are not complete, and the alignment begins following the "X". The other previously published IIC9 sequences are IIC1 (Kimura et al., 1987) and PB-1 (Meehan et al., 1988). Invariant residues are indicated in the consensus sequence as capitals, while residues which differ are indicated below and are also listed in lower case letters in the consensus sequence. The termination codon is starred.

2) metabolized (*S*)-mephenytoin preferentially when expressed in yeast. These forms differed by only three amino acids. In contrast, Brian et al. (1989) found that when a full-length MP-8 (constructed with the first 15 nucleotides predicted from the known amino acid sequence of $P450_{MP-1}$) was expressed in yeast, it did not metabolize (*S*)-mephenytoin. This form would differ from human form 2 by only two amino acids. Thus, the role of IIC9 in (*S*)-mephenytoin metabolism remains controversial.

In the present study, expression of the two variant full-length clones (65 and 25) of IIC9 in COS-1 cells resulted in expression of a protein that reacts with antibody to a purified form of P450 which metabolizes mephenytoin in a reconstituted system (anti-IIC9). However, neither of these transfected cells appeared to metabolize racemic mephenytoin at a rate appreciably above that of nontransfected COS-1 cells [14 pmol min^{-1} mg of protein)$^{-1}$]. Yasumori et al. (1990) reported that the cDNA for human form 2 (differing from clone 65 by only two silent base changes in the coding region) metabolized (*S*)-mephenytoin when expressed in yeast. However, expression of the P450 IIC9 protein in COS-1 cells was estimated to be ~13 pmol/mg of microsomal protein, while the expression of IIC9 in yeast was ~10 times higher. Therefore, we would not expect to detect an increase in mephenytoin metabolism in IIC9-transfected COS-1 cells equivalent to the low turnover numbers reported by Yasumori et al. (1990) for IIC9 in yeast [14 pmol min^{-1} (nmol of P450)$^{-1}$].

However, two allelic variants of IIC18 did 4-hydroxylate racemic mephenytoin when inserted into COS-1 cells in the present study at a rate significantly higher than controls, suggesting that these cytochromes may be important in mephenytoin metabolism. Although we could not measure the concentration of IIC18 in COS-1 cells in the absence of an antibody for this cytochrome, if we assume that the concentration is similar to that of IIC9 in COS-1 cells transfected with that cDNA (13 pmol/mg of protein), the rate of racemic mephenytoin metabolism by IIC18 would be approximately 4000 pmol min^{-1} (nmol of P450)$^{-1}$. This estimated rate is ~100 times higher than the rate of (*S*)-mephenytoin metabolism reported for IIC9 (Yasumori et al., 1990) when expressed in yeast. Additional studies in a cDNA expression system which results in higher levels of expression of P450 protein will be required to resolve the exact relationship of the various allelic variants of IIC9, IIC18, and other *CYP2C* proteins to the metabolism of mephenytoin (particularly the

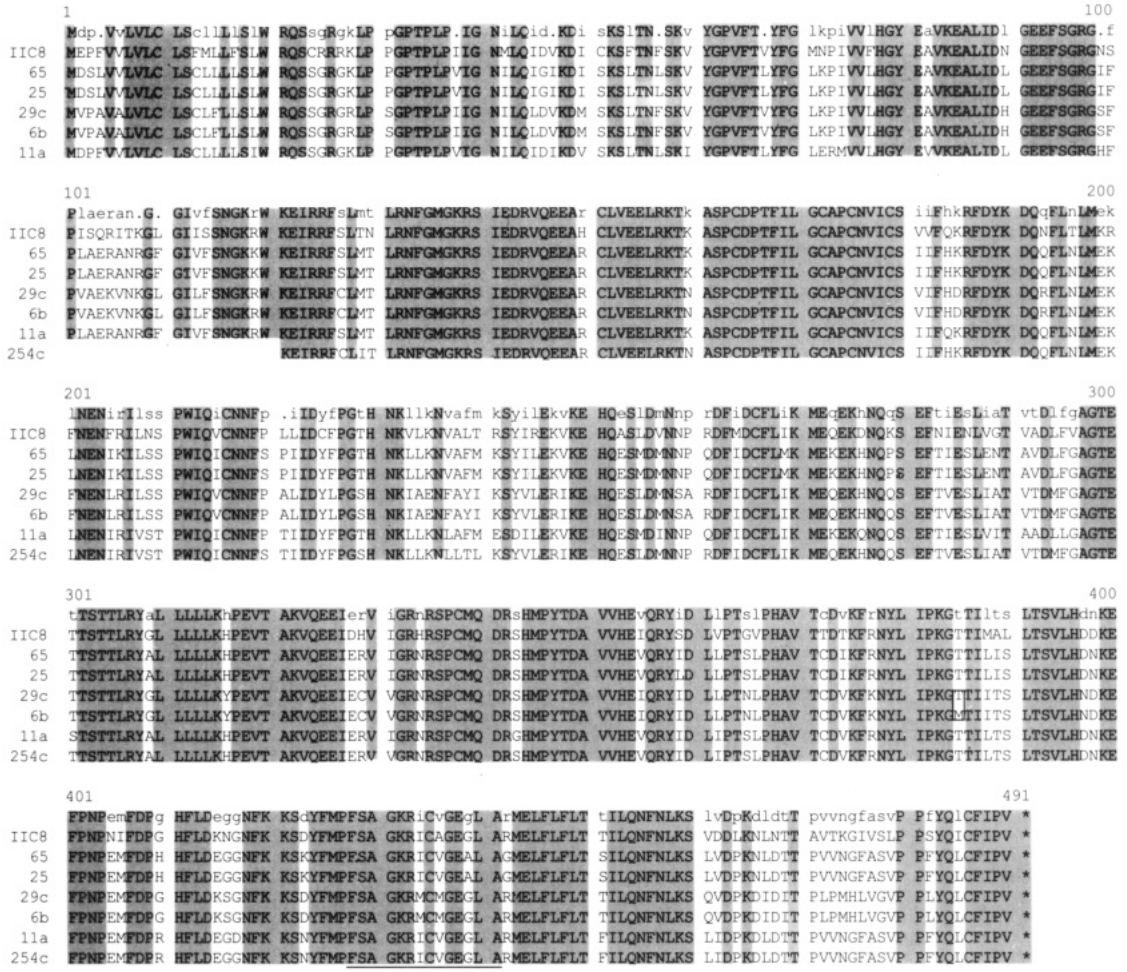

FIGURE 4: Alignment of human P450IIC proteins. The consensus sequence is indicated in the top line, where conserved residues are capitalized and shaded in gray and variable residues are indicated by dots or listed as lower case. The heme binding region is underlined. The termination codon is starred.

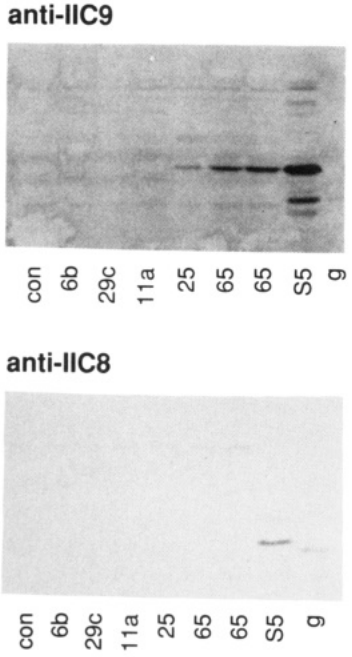

FIGURE 5: Western blot of recombinant transformed COS-1 cells. Each lane represents microsomal protein (50 μg) from an independent transformation with the indicated P450IIC cDNA, mock-transfected cells (CON), 20 μg of human liver microsomal protein (liver S5), or 2 pmol of pure P450g (IIC13).

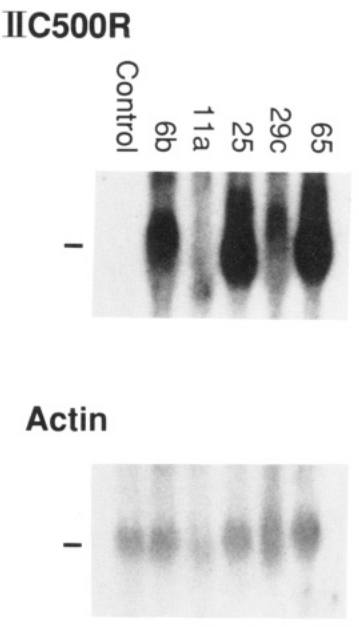

FIGURE 6: Northern blot of RNA from the recombinant transformed COS-1 cells. Each lane represents 20 μg of total RNA from mock-transfected cells (control) or cells transfected with the P450IIC clone indicated. The blot was probed with ^{32}P-end-labeled IIC500R (an oligoprobe which hybridizes with all IIC clones isolated) (top), then stripped, and reprobed with ^{32}P-actin cDNA (bottom). A 2.4-kb size marker is indicated at the side of each blot.

S enantiomer).

The two IIC18 clones (29c and 6b) represent a second pair of presumed allelic variants, differing by one predicted amino acid change. The 3′-noncoding DNA sequences are similar

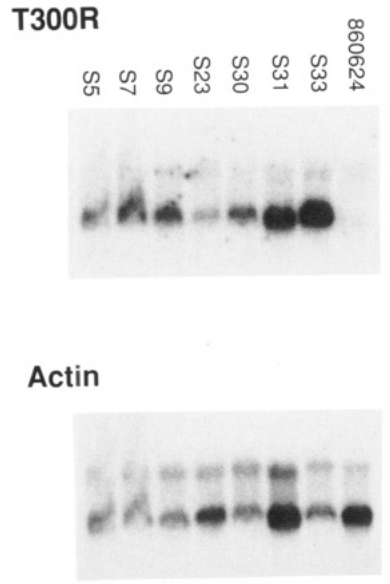

FIGURE 7: Northern blot of human mRNAs. Each lane represents 10 μg of mRNA, and the blot was probed with end-labeled T300R, an oligoprobe specific for IIC8 (top), stripped, and reprobed with ^{32}P-actin cDNA (bottom).

(a few base differences) for approximately 300 bases, at which point the 2 sequences diverge. At this site, 29c has a polyadenylation signal followed by a poly(A) tail, while clone 6b extends 243 nucleotides further, containing a total of 3 unusual variant polyadenylation signals and no poly(A) tail. These differences could suggest differential splicing, allelic differences, or different genes. At this point, we suggest that 29c and 6b are allelic variants. By the same reasoning, MP-4 and MP-8, which vary by only two amino acids and are now classified as IIC9 and IIC10 on the basis of divergent 3′ sequences, may also be only allelic variants of IIC9.

In conclusion, our results indicate there are at least five members of the human P450IIC subfamily. We have examined the profile of cDNAs for this subfamily in a human liver cDNA library from one individual. The predominant members of the IIC subfamily were two allelic variants of IIC9 and IIC8. IIC8 mRNA was low or absent in a second individual phenotypically low in HLx, and no clones for IIC8 were isolated from this individual. Three new members of this subfamily were also identified; IIC17, IIC18, and IIC19. cDNAs for IIC9, IIC18, and IIC19 were expressed in COS-1 cells. Only the two allelic variants of IIC18 4-hydroxylated racemic mephenytoin at a rate higher than untransfected COS-1 cells, suggesting that IIC18 may be important in mephenytoin metabolism. Additional future studies will address the relative importance of the members of the IIC subfamily in metabolism using other cDNA expression systems.

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