The *CYP2D* Gene Subfamily: Analysis of the Molecular Basis of the Debrisoquine 4-Hydroxylase Deficiency in DA Rats†

Eiji Matsunaga,‡ Ulrich M. Zanger,§ James P. Hardwick,∥ Harry V. Gelboin,‡ Urs A. Meyer,§ and Frank J. Gonzalez*,‡

Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, Department of Pharmacology, Biocenter, University of Basel, CH-4056 Basel, Switzerland, and Division of Biological and Medical Research, Argonne National Laboratories, Argonne, Illinois 60439

Received March 28, 1989; Revised Manuscript Received May 11, 1989

ABSTRACT: The DA rat has been proposed as an animal model for the human debrisoquine 4-hydroxylase/bufuralol 1′-hydroxylase genetic deficiency. To determine the mechanism of this deficiency, we isolated and sequenced five cDNAs in the *CYP2D* gene subfamily including a new IID1 allele and two cDNAs of novel P450s, designated IID3 and IID5. IID3 and IID5 cDNA-deduced amino acid sequences contained 500 and 504 residues with calculated molecular weights of 56 683 and 57 081, respectively. IID5 displayed 20 amino acid differences with the IID1, yet bore only 72% and 76% similarity to IID2 and IID3. Despite an overall nucleotide similarity of 80–98% between the 4 cDNAs, a region of 134 nucleotides of sequence exists that contains only 1 base difference. This region is probably the result of gene conversion events between the P450 IID genes. Although all IID cDNAs were expressed into immunodetectable proteins using the COS cell SV40-based expression system, only IID1 could effectively catalyze the oxidation of the prototype substrate bufuralol. Expression of a cDNA isolated in an earlier study [Gonzalez, F. J., Matsunaga, T., Nagata, K., Meyer, U. A., Nebert, D. W., Pastewka, J., Kozak, C. A., Gillette, J., Gelboin, H. V., & Hardwick, J. P. (1987) *DNA 6*, 149–161], previously called db1 and now designated IID1v, produced a protein with a drastically reduced activity as compared to cDNA-expressed IID1 despite only four amino acid differences between the two cDNA-deduced protein sequences. IID1 and IID1v appear to be allelic variants of the same gene. To determine the mechanism of the debrisoquine/bufuralol drug oxidation deficiency in DA rat, specific cDNA and oligonucleotide probes were used to quantitate levels of each mRNA in Sprague-Dawley and DA rat livers. The former rat strain expressed IID1, IID2, IID3, and IID5 mRNAs, whereas the DA rat expressed only IID2, IID3, and IID5 mRNAs; the IID1 gene was not expressed in this rat strain. Moreover, immunoinhibition studies using a strongly inhibitory antibody suggested a major contribution of IID1 to bufuralol metabolism in SD but not in DA rats. These results establish that the DA rat drug oxidation polymorphism is due to the absence of expression of the IID1 gene.

Cytochrome P450s[1] are the principal enzymes involved in the oxidation of drugs and other foreign compounds. In general, these reactions serve as the first step in eventual elimination of many foreign chemicals or xenobiotics from the body. The tremendous capacity of P450s to handle a wide and complex array of xenobiotics is due to both their multiplicity and their individual capacities to metabolize substrates of diverse structures. Another key feature of these enzymes is their overlapping substrate specificities.

The P450s have been grouped, on the basis of global amino acid sequence similarities, into a gene superfamily consisting of eight distinct mammalian families (Nebert et al., 1989). The P450 II family is composed of eight subfamilies designated IIA through IIH. Within each subfamily, from one to six genes exist, and the number of genes in a given subfamily may be different depending on the species. The molecular biology of P450s has recently been reviewed (Gonzalez, 1988).

The *CYP2D* P450 subfamily codes for an enzyme that is of clinical significance in the metabolism of numerous drugs. A human genetic deficiency, termed the debrisoquine/sparteine polymorphism, was discovered (Mahgoub et al., 1977; Eichelbaum et al., 1979) that is due to defective alleles in a IID1 P450 gene (Gonzalez et al., 1988; Skoda et al., 1988). About 5–10% of the Caucasian population in North America and Europe are incapable of metabolizing debrisoquine and several other drugs.

An animal model has been proposed for the human genetic defect. DA rats have an impaired ability to metabolize debrisoquine (Al-Dabbagh et al., 1980; Gonzalez et al., 1987) and Bufuralol (Boobis et al., 1986; Gonzalez et al., 1987). The female DA rat is particularly deficient in debrisoquine metabolism. Enzyme kinetic studies suggest that more than one enzyme carries out debrisoquine and bufuralol oxidation in rat liver microsomes and these enzymes may not be equivalent in different rat strains (Kahn et al., 1985; Boobis et al., 1986). In fact, a male-specific form of P450 in the rat IIC subfamily was also found to metabolize debrisoquine in rat in addition to IID1 (Larrey et al., 1984).

In an earlier study, we reported the isolation of a P450 from rat liver microsomes that catalyzed debrisoquine and bufuralol oxidation and the characterization of two cDNAs, designated db1 (IID1v)[2] and db2 (IID2), that code for P450s in the IID

† The nucleic acid sequences in this paper have been submitted to GenBank under Accession Numbers J02867, J02868, and J02869.

‡ National Institutes of Health.

§ University of Basel.

∥ Argonne National Laboratories.

[1] The nomenclature used in this report is that described by Nebert et al. (1989). The rat *CYP2D* gene subfamily consists of five known genes, *CYP2D1*, *CYP2D2*, *CYP2D3*, *CYP2D4*, and *CYP2D5*. These proteins and mRNAs are abbreviated IID1, IID2, IID3, IID4, and IID5 in the text.

0006-2960/89/0428-7349$01.50/0

subfamily (Gonzalez et al., 1987). The cDNA-deduced amino acid sequence of IID1v revealed amino-terminal sequence identity with the purified enzyme. In the present report, we describe the isolation and sequence of two new P450 cDNAs, designated IID3 and IID5, the latter of which displays 96% cDNA-deduced amino acid similarity with IID1. We also isolated another cDNA, designated IID1, that displayed only four amino acid differences from IID1v. Only cDNA-expressed IID1 and not IID1v, IID2, IID3, or IID5 was found to possess significant activities characteristic of the bufuralol/debrisoquine oxidation defect. We furthermore used specific oligonucleotide probes to establish that the IID1 gene is not expressed in the DA rat strain.

Materials and Methods

Animals. Sprague-Dawley (SD) rats were obtained from the Small Animals Section, Veterinary Research Branch, National Institutes of Health. DA (Dark Agouti) rats were purchased from Harlan Sprague Dawley, Madison, WI. For induction studies, rats were treated with either phenobarbital (100 mg/kg baby weight), 3-methylcholanthrene (25 mg/kg), or dexamethasone sulfate (500 mg/kg) by a single intraperitoneal injection 24 h prior to killing. The injection vehicle for phenobarbital and dexamethasone was 0.15 M NaCl and for 3-methylcholanthrene was corn oil.

Biochemical Procedures. Total liver RNA was isolated by the method of Chirwin et al. (1979) except that cesium trifluoroacetic acid (Pharmacia, Milwaukee, WI) was substituted for CsCl. RNA was electrophoresed on 1% agarose–2.2 M formaldehyde gels (Lehrach et al., 1977), transferred to Nytran membranes (Schleicher & Schuell Inc.), and hybridized with either nick-translated cDNA probes or oligonucleotide probes labeled with ^{32}P using T4 polynucleotide kinase. We chose the region between residues 692 and 711 to make oligonucleotides specific for IID1 and IID5 (Figure 1). The oligonucleotide probes are as follows: the first oligonucleotide, oligo 1, has the sequence 5′-ACTTCTGTCAAACTCTCTTC-3′ that is complementary to IID1 mRNA, and the second oligonucleotide, oligo 5, has the sequence 5′-ACTTCTATCAAACTTTCTTC-3′ complementary to IID5 mRNA. Oligonucleotides were synthesized by using an Applied Biosystems Model 380B synthesizer. Hybridization was performed according to the technique of Church and Gilbert (1984) at a temperature of 65 °C for cDNA probes and according to the technique described by Devlin et al. (1988) at temperatures of 48 and 50 °C for oligonucleotide probes oligo 1 and oligo 5, respectively. For Northern blot analysis of the IID2 and IID3 mRNAs, fragments were isolated from their respective cDNAs by digestion with *Pst*I and *Ban*II, which cleave at nucleotides 204 and 1015, respectively (Figure 1). These fragments specifically hybridize with each mRNA under our conditions.

Poly(A) RNA, derived from an adult male SD rat and isolated by oligo(dT) chromatography (Aviv & Leder, 1972), was used to prime first-strand synthesis for construction of a λgt11 cDNA library (Young & Davis, 1983). The library was made by using the strand replacement method by a procedure similar to that described by Watson and Jackson (1985). The library consisted of 2×10^6 independent clones, and approximately 1×10^5 phage were screened with the IID1v cDNA (Gonzalez et al., 1987) by use of plaque hybridization. Plaques containing positive signals were identified, and phage clones were then amplified. DNA was purified by phenol extraction, digested with *Eco*RI, electrophoresed on 1% agarose gels, and was then transferred to nylon membranes (BioTrace RP, Gelman Sciences). The membranes were hybridized with the nick-translated IID1v cDNA, and clones containing the longest inserts of about 1.6 kbp were processed further by plaque purification. The inserts were then subcloned into pUC9 and completely sequenced by shotgun cloning into m13 (Deininger, 1983) and the dideoxynucleotide sequencing strategy (Sanger et al., 1977).

Genomic DNA was isolated from SD and DA rat livers by the method of Blin and Stafford (1976). Both DNAs were digested with *Eco*RI and *Bam*HI, electrophoresed on 0.5% agarose gels, and transferred to nylon filters. Hybridization and washing were performed as described by Southern (1975).

The cDNAs were expressed into their respective proteins by using the COS cell expression system and the SV40 adenovirus derived vector p91023(B) (Wong et al., 1985), and the pCMV4 vector constructed and provided by Dr. David W. Russell, Department of Biochemistry, University of Texas Southwestern Medical Center Dallas, TX. This vector yields at least a 5-fold higher level of expression than the p91023(B). COS-1 monkey kidney cells were transfected with the cDNA-containing expression plasmids using the DEAE-dextran method (Luthman & Magnusson, 1983) and harvested after 72 h. The cells were lysed by sonication and then analyzed by Western immunoblotting (Towbin et al., 1979). Polyclonal antibodies to rat IID1 (Gonzalez et al., 1987) and a human autoantiserum (anti-liver/kidney microsome antibody or anti-LKM1) which specifically binds to human IID1 (Zanger et al., 1988a) were used as Western blotting probes. SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970).

Bufuralol 1′-hydroxylase activity was determined with 500 μM (+)-bufuralol in whole cell extracts obtained by sonication. Cumene hydroperoxide (CuOOH)-mediated bufuralol 1′-hydroxylation was performed as described by Zanger et al. (1988b). NADPH-dependent bufuralol 1′-hydroxylase activity was determined in the presence of 3 mM CHAPS and 2 μM NADPH–P450 oxidoreductase that was purified from rat liver according to Yasukochi and Masters (1976). The presence of CHAPS detergent during the assay was found to stimulate the effect of exogenous rat NADPH–P450 oxidoreductase on bufuralol 1′-hydroxylase activity. Immunoinhibition studies were performed with anti-LKM1 IgGs as described (Zanger et al., 1988a).

Results

An adult male SD rat λgt11 library was screened with the IID1v cDNA probe. Three new cDNAs, designated IID1, IID3, and IID5, coding for P450s in the rat P450 IID gene subfamily were isolated and sequenced. A total of 12 cDNAs containing the complete protein coding regions were isolated. The number of cDNAs corresponding to IID1, IID2, IID3, and IID5 were three, four, one, and four, respectively. The nucleotide sequences of these cDNAs are displayed in Figure 1. We believe that IID1 and IID1v are allelic variants of the same gene (see Discussion). A fifth cDNA, predicted from the sequence of a genomic clone and designated IID4,[3] was not found; however, a partial IID4 cDNA was recently isolated

[2] In any earlier report, we described the isolation of rat db1 and the sequence of its cDNA clone. This cDNA coded for an enzyme that displayed an altered enzymatic activity (see text). We therefore refer to this cDNA as IID1v or IID1 variant. A new cDNA, isolated and sequenced in the present report, has four amino acid changes as compared to IID1v and is able to code for an enzyme that has catalytic activities characteristic of the purified rat IID1. We refer to this cDNA and its expressed P450 as IID1.

[3] E. Matsunaga and F. J. Gonzalez, unpublished results.

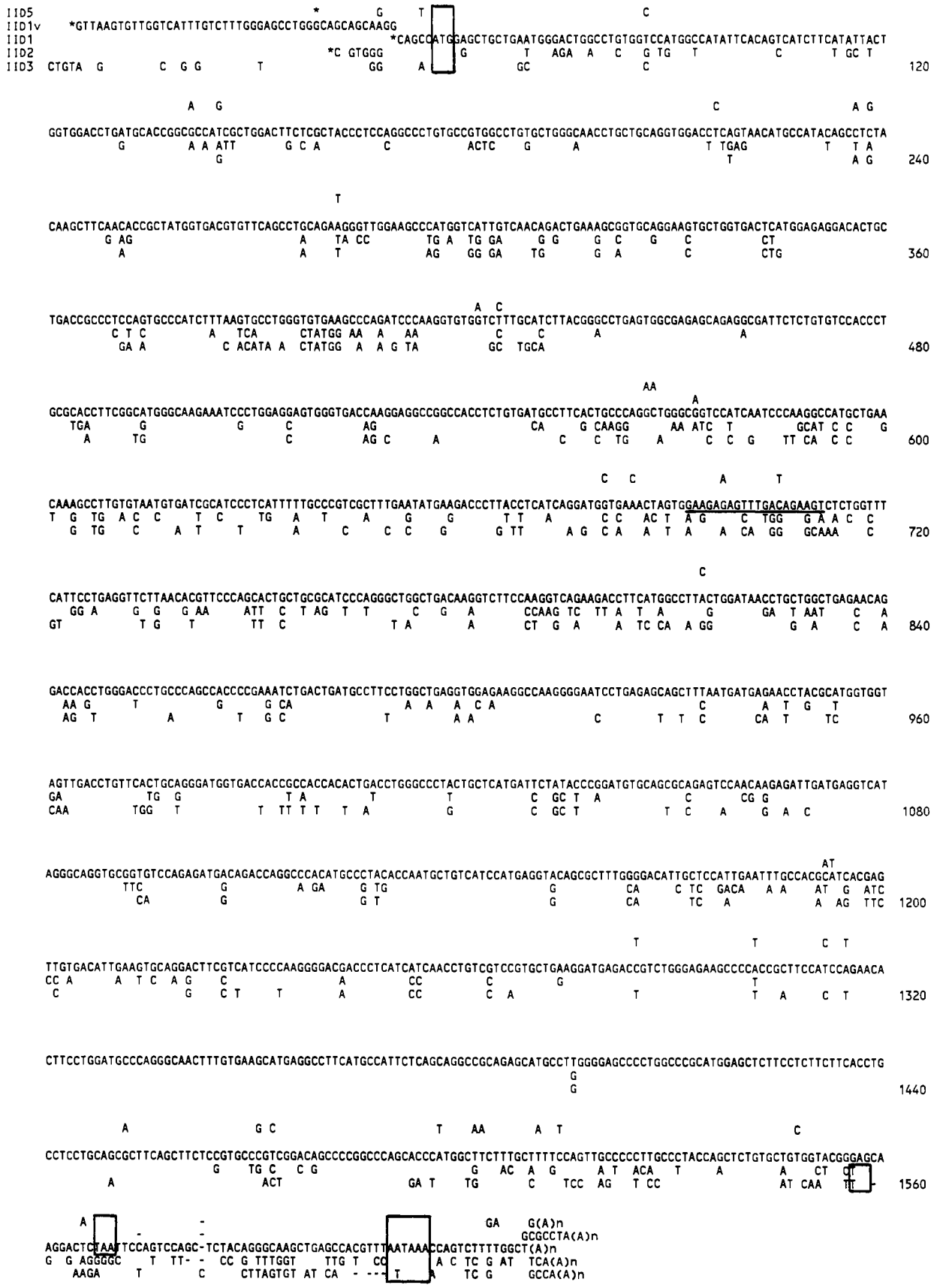

FIGURE 1: Alignment of the nucleotide sequences between IID1, IID1v, IID2, IID3, and IID5. The complete nucleotide sequence of IID1 is displayed and aligned with those of IID2, IID3, and IID5, and only residues that do not match IID1 are shown. The ATG initiator methionine, the termination codon, and the poly(A) addition signal are enclosed by boxes. Asterisks indicate the range that each cDNA is extended at the 5′ end. Oligonucleotide probes used for Northern blot analysis are shown underlined.

by others (Ishida et al., 1988). The reason we did not find IID4 in our libraries is probably due to its low mRNA content in SD rat.[3]

The global nucleotide similarities of the five IID cDNAs were compared and ranged from 79% to 99%. IID5 displayed 98% similarity with IID1 but only 79% and 84% similarity with

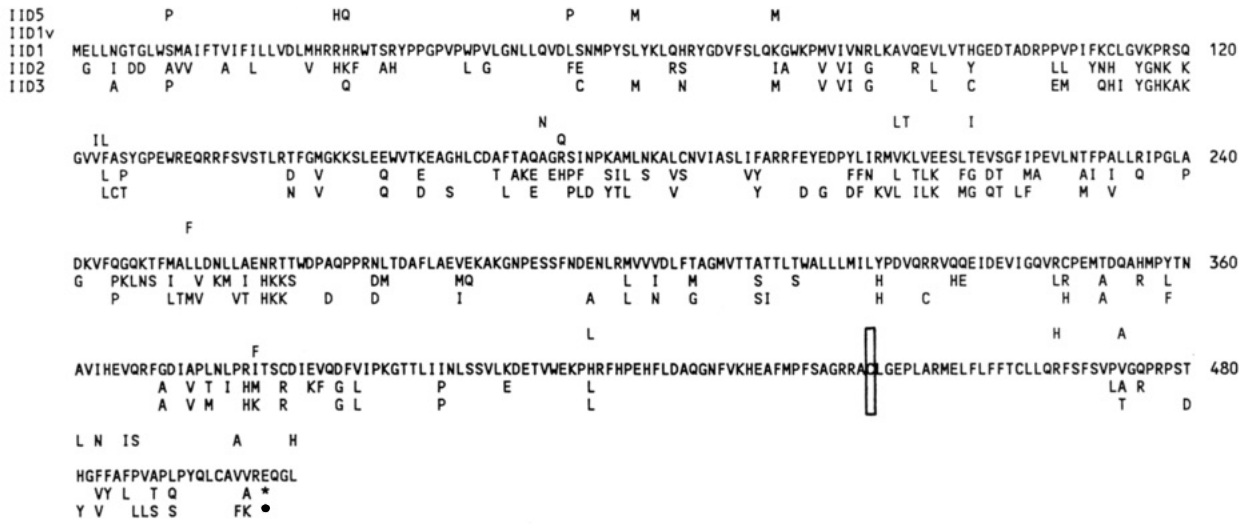

FIGURE 2: Alignments of the cDNA-deduced amino acid sequences of IID1, IID1v, IID2, IID3, and IID5 cDNAs. The amino acid sequences were aligned by using the program of Pearson and Lipman (1988). Only the nonmatched residues are displayed. The heme-binding cysteine is enclosed by a box.

IID2 and IID3, respectively. IID3 displayed 83% and 80% similarities with IID1 and IID2, respectively. In general, IID1, IID2, and IID3 displayed near to equal nucleotide similarity with each other. These data indicate that three IID genes existed for a long period of time during evolution and the IID1 and IID5 genes were formed via a recent gene duplication from a common ancestor. Interestingly, the regions of nucleotide dissimilarities among the IID1, IID2, IID3, and IID5 cDNAs are randomly dispersed except for a region of remarkably high nucleotide similarity between residues 1316 and 1449 (Figure 1). In this region of 134 nucleotides, only a single base difference is found. A segment of such high nucleotide similarity surrounded by areas of low nucleotide similarities is indicative of earlier gene conversion events.

The cDNA-deduced amino acid sequences of IID1, IID1v, IID2, IID3, and IID5 were aligned (Figure 2) and found to display between 72% and 99% similarities. As predicted from the DNA alignment data (Figure 1), the amino acid sequence similarities were high between IID1 and IID5 and much less similar among these P450s and the IID2 and IID3. The amino acid dissimilarities are also dispersed except for a region between residues 417 and 471 at the carboxy-terminal third of the protein sequence where all of the P450s have the identical sequence (Figure 2). A fifth gene, designated IID4, also coded for a protein that was identical with IID1 through IID5 between amino acid residues 417 and 471, indicating that this gene was also involved in gene conversion activity.[3]

Gene conversions have also been seen in other P450 gene families (Atchison & Adesnik, 1986; Gonzalez et al., 1986); however, no consistent area of sequence along these P450s has been conserved by these conversion events. Surprisingly, in the IID gene cluster, gene conversions have maintained a region of high nucleotide similarity surrounding a critical region of the P450 protein that contains the highly conserved cysteine-containing peptide. This cysteine is the fifth thiolate ligand to the heme iron at the P450 active site (Gotoh et al., 1983; Gonzalez, 1988). It appears that gene conversion events have maintained a region of these P450s which is important for catalytic activity. The possibility that the area of high nucleotide similarity between these cDNAs was conserved to maintain some unusual RNA structure or stability is ruled out by our findings of intron involvement in the putative gene conversion events. The eighth introns of four of the IID genes that were sequenced were found to be strikingly conserved.[3]

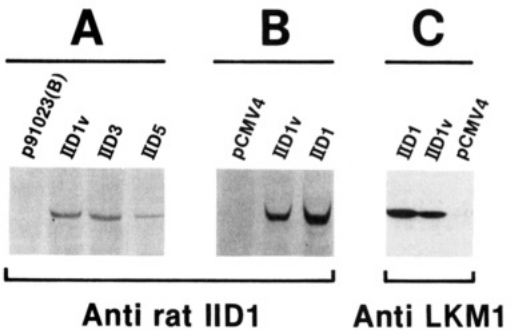

FIGURE 3: Analysis of COS-1 cells transfected with expression plasmids containing the IID cDNAs. Lysates (100 μg) from cells infected with vector alone or vector-containing cDNAs were subjected to immunoblotting analysis and stained by using anti-rat IID1 (panels A and B) and anti-LKM1 antibody (panel C). The vector p91023(B) was used in panel A, and pCMV4 was used in panels B and C.

Intron involvement strongly supports the likelihood of gene conversions.

cDNA-Directed Expression of the IID P450s. Of note was the finding of a second P450, IID5, that shared high amino acid similarity with IID1. The amino-terminal sequence of IID1 purified from rat liver, that efficiently metabolizes bufuralol in a reconstituted system (Gonzalez et al., 1987), is indistinguishable from the sequences derived from the IID1, IID1v, and IID5 cDNAs. These data suggest that any of these P450 cDNAs could code for the bona fide bufuralol metabolizing enzyme. To determine which cDNA corresponds to the P450 purified earlier that metabolizes bufuralol (Gonzalez et al., 1987), cDNA expression was carried out using the vector p91023(B). Proteins, synthesized from IID1v, IID3, and IID5 cDNAs, were detected on Western immunoblots using antibody against rat IID1 that had similar mobilities on SDS–polyacrylamide gels (Figure 3A). Preliminary studies revealed that none of these expressed P450s could catalyze bufuralol 1′-hydroxylation. After the isolation of IID1, we continued these experiments using the human cytomegalovirus-derived vector pCMV4. This vector yielded a substantially higher level of expression than p91023(B) as shown in Figure 3B. The human autoantibody anti-LKM1, an antibody that recognizes human IID1 (Zanger et al., 1988a), also recognized expressed IID1 and IID1v (Figure 3C). Although all cDNAs produced P450s detectable on SDS–polyacrylamide gels, only IID1 could effectively catalyze the oxidation of bufuralol (Table I). IID2,

Table I: Expression of IID1 and IID1v cDNAs Using the COS Cell System and the pCMV4 Vector[a]

	(+)-bufuralol 1′-hydroxylase act. [nmol of product (60 min)$^{-1}$ (mg of protein)$^{-1}$]	
	NADPH	CuOOH
IID1	4.1	13.9
IID1v	0.4	1.2
control	<0.02	<0.02

[a] COS cells were transfected with IID1 or IID1v cDNAs in pCMV4. (+)-Bufuralol 1′-hydroxylase activities were determined in cell homogenates in the presence of rat NADPH–P450 oxidoreductase (NADPH) or cumene hydroperoxide (CuOOH). Results are means of double incubations obtained in a typical experiment.

IID3, and IID5 were incapable of carrying out bufuralol 1′-hydroxylation.[4] IID1v could oxidize this substrate with drastically reduced activity (Table I) when it was expressed at comparable levels to IID1 (Figure 3B,C). Interestingly, in the absence of exogenously added rat NADPH–P450 oxidoreductase, little NADPH-dependent activity was found, indicating that the rat IID1 P450 could not interact well with the monkey oxidoreductase.[4] The metabolism of bufuralol in situ was also analyzed in COS-1 cells transfected with pCMV4 containing IID1, IID1v, and IID5. 1′-OH-Bufuralol was only produced at significant levels (10–40 pmol mL^{-1} h^{-1}) when 200 μM bufuralol was included in the media of cells that had been transfected with the IID1 cDNA. Cells transfected with pCMV4 containing the IID1v and IID5 cDNAs produced less than 1–4 pmol of 1-OH-bufuralol mL^{-1} h^{-1}. This level of metabolite production was about the same as that produced by cells transfected with vector alone.

To investigate whether the enzymatically active IID1 could be responsible for the described DA rat deficiency, we performed immunoinhibition studies using anti-LKM1 antibodies. This human autoantibody has been demonstrated to specifically and potently inhibit the enzymatic activity of the human liver IID1 (Zanger et al., 1988a). Bufuralol 1′-hydroxylase activity of COS cells transfected with the rat IID1 cDNA was strongly inhibited by anti-LKM1 IgGs. Significant inhibition of the total microsomal activity was also observed in male (60%) and in female (67%) SD rat livers. By contrast, in both male and female DA rat livers, which displayed approximately 50% of the respective SD microsomal activities, no significant inhibition by anti-LKM1 was observed. These data suggest a major contribution of IID1 to the total microsomal bufuralol 1′-hydroxylase activity of SD rats, whereas other P450 isozymes must be responsible for the residual activity of DA rats.

Expression of the IID P450 Genes. In an earlier study, the full-length IID1v cDNA was used to measure mRNA levels in rats of different strains, during development and treated with various monooxygenase inducing agents. It is now apparent that in these studies both the IID1 and IID5 were being quantitated simultaneously. IID1 and IID5 mRNA levels were reexamined by using specific oligonucleotide probes. On the basis of the cDNA characterization in this report and unpublished data on the *CYP2D* genomic clones,[3] we believe that there are five genes in this subfamily in rat. We used the sequence data to generate specific mRNA probes to study expression of the *CYP2D* genes. Neither mRNA was induced in liver by phenobarbital, 3-methylcholanthrene, or dexamethasone (Figure 4, panel A). In fact, a slight decrease in IID1 mRNA was noted with these inducing agents. Both mRNAs were absent in newborn male and female SD rats and

[4] E. Matsunaga, U. M. Zanger, U. A. Meyer, and F. J. Gonzalez, unpublished results.

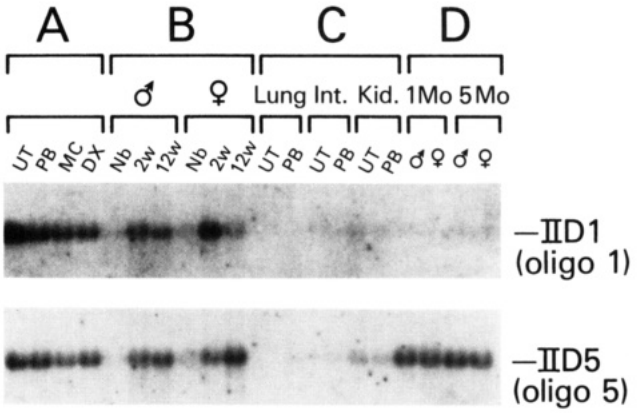

FIGURE 4: Northern blotting analysis of RNA from Sprague-Dawley (panels A–C) and DA (panel D) rats. Total RNA (10 μg) from the livers of rats either untreated (UT) or treated with phenobarbital (PB), 3-methylcholanthrene (MC), and dexamethasone (DX) (panel A); male and female rats of various ages (panel B); lung, small intestine (int), and kidney (panel C); and livers from 1-month-old and 5-month-old DA rats (panel D) were subjected to Northern blot analysis and hybridized with oligonucleotide probes specific for IID1 and IID5 mRNA. The filters were exposed to autoradiographic film for 5 days at −80 °C using a Dupont Lightning Plus intensifying screen.

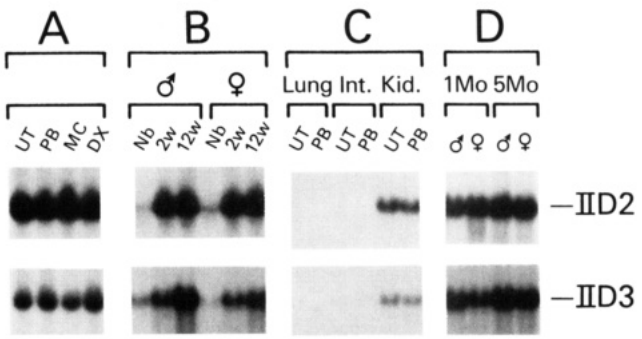

FIGURE 5: Analysis of IID2 and IID3 mRNAs in Sprague-Dawley (panels A–C) and DA (panel D) rats. IID2 and IID3 cDNA fragments were used as probes after being labeled with α-^{32}P by nick translation. For details, see the legend to Figure 4.

were elevated by 2 weeks of age (Figure 4, panel B). In females, however, the IID1 mRNA decreased in 12-week-old animals relative to its level at 2 weeks, whereas the IID5 mRNA remained elevated in adult rats. These results indicate that the IID1 and IID5 genes are differentially regulated during development. Both mRNAs were present in kidney at levels considerably less than those in liver but were undetectable in lung and small intestine (Figure 4, panel C). When the levels of IID1 and IID5 mRNA in both 1-month-old and 5-month-old male and female DA rats were examined using oligonucleotide probes, only IID5 mRNA, but not IID1 mRNA, was detected (Figure 4, panel D), in contrast to the presence of both mRNAs in SD rat liver (Figure 4, panels A and B). These results therefore suggest an explanation of the observed absence of immunoinhibition by anti-LKM1 in DA rat liver microsomes.

The regulation of the IID2 and IID3 mRNAs was also examined using fragments derived from the cDNAs that only react with these two mRNAs (Figure 5). Both mRNAs were unaffected by inducer administration, and their genes were activated by 2 weeks of age in males and females (Figure 5, panels A and B). Both IID2 and IID3 mRNAs were also found in kidney but absent in lung and small intestine (Figure 5, panel C). Finally, these mRNAs were expressed more in old DA rats than in young rats.

Southern Blotting Analysis of SD and DA Rats. To determine if the lack of expression of IID1 in the DA rat is due

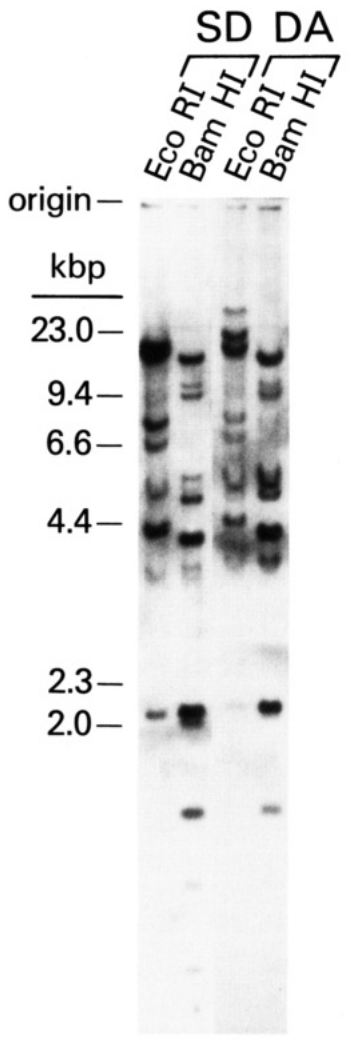

FIGURE 6: Southern blotting analysis of SD and DA rat DNA using the total IID1 cDNA fragment as a probe. DNA was digested with *Eco*RI and *Bam*HI, electrophoresed on 0.5% agarose gels, transferred to nylon membranes, and hybridized with the IID1 cDNA probe. The filters were exposed to autoradiographic film for 24 h at −80 °C with the aid of an intensifying screen.

to gene deletion, Southern blotting experiments were carried out. SD and DA rat liver DNAs were digested with *Eco*RI and *Bam*HI, blotted to nylon filters, and hybridized with the IID1 cDNA probe. This probe should detect DNA fragments that contain the IID1 and IID5 exons. Indeed, multiple fragments are found in both digestions (Figure 6). The number of fragments detected in the digests is similar between strains of rats. Although the longest *Eco*RI fragment in SD rat appears as a single broad band, it was found to contain two *Eco*RI fragments, by using a lower concentration of agarose gel.[3] The 23 kbp fragment in DA rat is probably derived from a restriction fragment length polymorphism (RFLP) between these two strains that results from loss of an *Eco*RI site. On the basis of our Southern blotting result, it does not appear that the DA rat contains any large gene deletions that could account for the absence of the IID1 mRNA. It remains a possibility that a subtle mutation exists in the DA IID1 gene that abolishes its transcriptional activity.

Discussion

In an earlier report, we isolated two P450s from SD rats, one of which carried out the efficient metabolism of debrisoquine and bufuralol in a reconstituted monooxygenase system (Gonzalez et al., 1987). Two cDNAs were also characterized and designated db1 (IID1v) and db2 (IID2). The IID1v cDNA possessed a deduced amino acid sequence that was identical with that of the amino-terminal protein sequence determined from the purified enzyme and yet distinct from that of the IID2 cDNA-deduced amino acid sequence. On the basis of Western immunoblot analysis using an antibody to the purified debrisoquine 4-hydroxylase and RNA analysis using a IID1v cDNA probe, we found similar levels of immunodetectable proteins and apparent IID1 mRNA in SD and DA rat livers (Gonzalez et al., 1987). This led to the hypothesis that the DA rat polymorphism was due to an altered catalytically inactive IID1 protein in the DA strain. This conclusion, however, was erroneously based on the assumption that no other enzyme or gene possessed high amino acid sequence similarity with IID1. The isolation and characterization of additional IID cDNAs from SD rats in the present study confirmed that the antibody and probes used in our earlier experiments had detected several IID proteins and mRNAs. Genomic cloning studies[3] also revealed the existence of the IID2, IID3, IID4, and IID5 genes coding for proteins sharing high amino acid similarity with IID1. In fact, the cDNA-deduced amino acid sequence of IID5 was found to be 96% similar to IID1, and the amino-terminal sequence of this protein was also compatible with that determined from the pure enzyme (Gonzalez et al., 1987), suggesting that the IID5 could have been the bona fide debrisoquine 4-hydroxylase. On the basis of these findings, we reevaluated the DA rat polymorphism through the use of cDNA expression and mRNA analysis using oligonucleotide probes.

cDNA expression confirmed that only IID1 was capable of metabolizing bufuralol. IID2, IID3, and IID5 showed no activity. The original db1 (IID1v) cDNA was also examined for its ability to code for bufuralol 1′-hydroxylase activity, and, surprisingly, we detected only low levels of activity with this cDNA in transfected COS cell extracts in the presence of NADPH. However, when cumene hydroperoxide was used as an electron donor, about 3-fold higher activity was found. The IID1 cDNA characterized in the present report was able to catalyze bufuralol 1′-hydroxylation at much higher rates than IID1v in the presence of NADPH or cumene hydroperoxide. The defect in IID1v may be due to one or more of the four amino acid substitutions between IID1v and IID1. This possibility is currently being investigated.

Taking into consideration that only six nucleotide differences were found between IID1 and IID1v cDNAs and that neither mRNA was detected in DA rat liver, we believe that IID1v is an allelic variant in the outbred SD rat strain. Indeed, allelic variants of the IIB1 gene have also been found in rats (Rampersaud & Walz, 1983, 1987). However, we cannot exclude the possibility that IID1v is derived from a separate gene. Alternatively, it also remains a possibility that IID1v is the result of a cloning artifact. In any case, this cDNA should be useful in structure–function studies to determine amino acids critical for IID1 enzymatic function.

The studies described herein have established that a major cause for the DA rat deficiency in debrisoquine and bufuralol metabolism is the lack of expression of the IID1 gene. Three other IID genes are expressed in the DA rat strain, but they cannot catalyze bufuralol oxidation. It is noteworthy that DA rat microsomes still retain debrisoquine and bufuralol oxidizing activities and these activities are more prevalent in male- than in female-derived microsomes (Boobis et al., 1986; Larrey et al., 1984; Gonzalez et al., 1987). In this connection, a male-specific P450, designated P450 UT-A (IIC11), was able to catalyze debrisoquine 4-hydroxylation (Larrey et al., 1984),

suggesting that the low level of debrisoquine and bufuralol hydroxylating activity in DA rat liver is due to one or more P450s other than IID1. This interpretation is also supported by the lack of inhibition of bufuralol 1′-hydroxylase activity in DA rat microsomes by anti-LKM1, an antibody that effectively inhibits this activity in SD rats and in COS cells transfected with IID1 cDNA. Of interest is the fact that DA rats do not metabolize debrisoquine when the drug is given as an oral dose (Al-Dabbagh et al., 1980). These data indicate that the other debrisoquine metabolizing P450s in DNA rat may not play a role in in vivo metabolism, probably due to low affinities for the substrate.

On the basis of our cDNA expression data, it would appear that the rat IID1 cDNA corresponds to the IID1 cDNA that was previously characterized from man (Gonzalez et al., 1988). Both cDNAs code for enzymes with high bufuralol 1′-hydroxylase activity. It is interesting to note, however, that the human IID1 displays similar amino acid sequence similarities with all of the rat IID proteins. One cannot, therefore, conclude, on the basis of sequence data, that the rat and human debrisoquine 4-hydroxylase genes are orthologous counterparts. These findings suggest that the five rat genes were homogenized by gene conversions, thereby making the designation of rat and man orthologs difficult. In any case, the rat and human IID1 proteins have retained those amino acid residues required for their similar substrate specificities.

It should be noted that the DA rat deficiency is probably the result of a mutation that blocks gene transcription, whereas mutations in the human gene are heterogeneous. Defective alleles of the human IID1 gene have been found that are the result of base changes that result in faulty pre-mRNA splicing (Gonzalez et al., 1988). Other mutant alleles have been identified by RFLPs that have not yet been characterized by sequencing (Skoda et al., 1988). The main difference between the DA rat and human debrisoquine 4-hydroxylase deficiency, therefore, is that the former is probably the result of a single mutant allele while man has multiple defective IID1 alleles. Since the net result is a lack of expression of the IID1 protein in both species, we believe that the DA rat can serve as suitable a model for the metabolic aspects of this human genetic deficiency. However, the mechanisms generating the mutant phenotype in rat and man are quite distinct.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Therese Catin, Basel. We also thank Dr. David W. Russell, Department of Molecular Genetics, University of Texas Southwestern Medical Center, for supplying the pCMV4 vector.

References

Al-Dabbagh, S. G., Idle, J. R., & Smith, R. L. (1980) *J. Pharm. Pharmacol. 33*, 161–164.

Atchison, M., & Adesnik, M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2300–2304.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Blin, N., & Stafford, D. W. (1976) *Nucleic Acids Res. 3*, 2303–2308.

Boobis, A. R., Seddon, C. E., & Davies, D. S. (1986) *Biochem. Pharmacol. 35*, 2961–2965.

Chirwin, M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5300.

Church, G. M., & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1991–1995.

Deininger, P. L. (1983) *Anal. Biochem. 129*, 216–223.

Devlin, P. E., Ramachandran, K. L., & Cate, R. L. (1988) *DNA 7*, 499–507.

Eichelbaum, M., Spannbrucker, N., Steinke, B., & Dengler, H. J. (1979) *Pharmacology 16*, 183–187.

Gonzalez, F. J. (1988) *Pharmacol. Rev. 40*, 243–288.

Gonzalez, F. J., Song, B.-J., & Hardwick, J. P. (1986) *Mol. Cell. Biol. 6*, 2969–2979.

Gonzalez, F. J., Matsunaga, T., Nagata, K., Meyer, U. A., Nebert, D. W., Pastewka, J., Kozak, C. A., Gillette, J., Gelboin, H. V., & Hardwick, J. P. (1987) *DNA 6*, 149–161.

Gonzalez, F. J., Skoda, R. C., Kimura, S., Umeno, M., Zanger, V. M., Nebert, D. W., Gelboin, H. V., Hardwick, J. P., & Meyer, U. A. (1988) *Nature 331*, 442–446.

Gotoh, O., Tagashira, Y., Itzuka, T., & Fujii-Kuriyama, Y. (1983) *J. Biochem. 93*, 807–817.

Ishida, N., Tawaragi, Y., Inuzuka, C., Sugita, O., Kubota, I., Nakazato, H., Noguchi, T., & Sassa, S. (1988) *Biochem. Biophys. Res. Commun. 156*, 681–688.

Kahn, G. C., Rubenfield, M., Davies, D. S., Murray, S., & Boobis, A. R. (1985) *Drug Metab. Dispos. 13*, 510–515.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Larrey, D., Distlerath, L. M., Dannan, G. A., Wilkinson, G. R., & Guengerich, F. P. (1984) *Biochemistry 23*, 2787–2795.

Lehrach, H., Diamond, D., Wozney, J. M., & Boedtker, H. (1977) *Biochemistry 16*, 4743–4751.

Luthman, H., & Magnusson, G. (1983) *Nucleic Acids Res. 11*, 1295–1308.

Magoub, A., Idle, J. R., Dring, L. G., Lancaster, R., & Smith, R. L. (1977) *Lancet 2*, 584–586.

Nebert, D. W., Nelson, D. R., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, L. C., Johnston, E. F., Kempber, B., Levin, W., Phillips, I. R., Sato, R., & Waterman, M. R. (1989) *DNA 8*, 1–13.

Pearson, W. R., & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2444–2448.

Rampersaud, A., & Walz, F. G., Jr. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6542–6546.

Rampersaud, A., & Walz, F. G., Jr. (1987) *Biochem. Genet. 25*, 527–534.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Skoda, R. C., Gonzalez, F. J., Demierre, A., & Meyer, U. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5240–5243.

Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.

Towbin, H., Staehlin, T., & Gordin, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4355.

Watson, C. J., & Jackson, F. J. (1985) *DNA Cloning* (Glover, D. M., Ed.) Vol. 1, pp 79–88, IRL, Washington, DC.

Wong, G. G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay, R. M., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A., & Clark, S. C. (1985) *Science 228*, 810–815.

Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.

Young, R. A., & Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194–1198.

Zanger, U. M., Hauri, H.-P., Loeper, J., Homberg, J.-C., & Meyer, U. A. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8256–8260.

Zanger, U. M., Vilbois, F., Hardwick, J. P., & Meyer, U. A. (1988b) *Biochemistry 27*, 5447–5454.