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The *CYP2A3* Gene Product Catalyzes Coumarin 7-Hydroxylation in Human Liver Microsomes†

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ABSTRACT: Three cDNAs, designated IIA3, IIA3v, and IIA4, coding for P450s in the *CYP2A* gene subfamily were isolated from a λgt11 library prepared from human hepatic mRNA. Only three nucleotide differences and a single amino acid difference, $Leu_{160} \rightarrow$ His, were found between IIA3 and IIA3v, indicating that they are probably allelic variants. IIA4 displayed 94% amino acid similarity with IIA3 and IIA3v. The three cDNAs were inserted into vaccinia virus, and recombinant viruses were used to infect human hepatoma Hep G2 cells. Only IIA3 was able to produce an enzyme that had a reduced CO-bound spectrum with a λ_{max} at 450 nm. This expressed enzyme was able to carry out coumarin 7-hydroxylation (turnover number of 15 min^{-1}) and ethoxycoumarin O-deethylation. cDNA-expressed IIA3v and IIA4 failed to incorporate heme and were enzymatically inactive. Analysis of IIA proteins in human liver microsomes, using antibody against rat IIA2, revealed two proteins of 49 and 50 kDa, the former of which appeared to correlate with human microsomal coumarin 7-hydroxylase activity. A more striking correlation was found between IIA mRNA and enzyme activity. The rat antibody was able to completely abolish coumarin 7-hydroxylase activity in 12 liver samples. In addition, kinetics of coumarin metabolism in two livers were monophasic over the substrate concentration tested. K_m values obtained from human liver (2.3 μM) were similar to those obtained from lysates of hepatoma cells expressing IIA3 (3.6-7.1 μM). These data establish that the *CYP2A3* gene product is primarily responsible for coumarin 7-hydroxylase activity in human liver. The level of expression of this activity varied up to 40-fold between livers. Levels of IIA mRNA also varied significantly between liver specimens, and three specimens had no detectable mRNA.

Cytochrome P450s[1] are the principal enzymes involved in the metabolism of foreign compounds including drugs, carcinogens, plant metabolites, and environmental contaminants. Nine P450 gene families exist in mammals (Nebert et al., 1989). The *CYP2* family is composed of eight subfamilies, some of which contain a number of P450s. The enzymes within these subfamilies catalyze the oxidation of numerous chemicals. Many of these enzymes are also species-specific. Species specificity can be illustrated by analyzing the metabolism of androgenic steroids. These reactions are not thought to be of physiological importance but may simply reflect the similarities in basic structures of steroids and plant-derived chemicals or stress metabolites, phytoalexins. P450s in rodents oxidize testosterone at numerous positions (Waxman, 1988) whereas human P450s produce primarily the 6β-hydroxytestosterone metabolite (Waxman et al., 1988). On the basis of these and other known species-specific metabolisms, it is becoming more important to characterize human P450s. Indeed, several P450 forms have been purified from human liver specimens (Distlerath & Guengerich, 1987). This direct approach is rather difficult for most laboratories due to the dearth of human liver specimens, the genetic heterogeneity between different livers, and the difficulty in purifying these enzymes, particularly those present at low levels.

Human P450s can also be characterized through cDNA cloning and expression. Antibodies and cDNA probes against rat P450s and P450 mRNAs, respectively, can be used to isolate the cDNAs from human liver libraries. Due to sequence similarities and multiplicity of P450s within gene subfamilies, a single rodent probe can identify multiple human cDNAs.

Previously, we used the rat IIA1 cDNA (Nagata et al., 1987) to isolate a cDNA from a human liver λgt11 library. The complete sequence of IIA3 was recently reported by us (Yamano et al., 1989b) and others (Miles et al., 1989). In the present report, the sequences of two additional variants of IIA3, one of which has an amino acid substitution, designated IIA3v, and a cDNA corresponding to a second gene

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[1] The nomenclature used in this report is that described by Nebert et al. (1989). The proteins and mRNAs are designated IIA3, IIA3v, and IIA4. The IIA3 cDNA, characterized in the present study, appears to be an allelic variant of that cDNA isolated in an earlier report (Yamano et al., 1989b). Since the deduced protein sequences of these two cDNAs are identical, we chose not to designate them with different names in the present report. The IIA3v cDNA displays only three nucleotide differences and one amino acid difference with the IIA3 cDNA characterized in this study; therefore, these cDNAs also are probably allelic variants. However, since IIA3v contains an amino acid difference ($Leu_{160} \rightarrow$ His) and could not be expressed into active P450, we chose to call it a variant (IIA3v variant). The genes encoding IIA3/IIA3v and IIA4 mRNAs are designated *CYP2A3* and *CYP2A4*, respectively.

product and designated IIA4, are presented. We expressed all cDNAs using vaccinia virus and analyzed the proteins and mRNAs encoded by *CYP2A* genes in 12 human liver specimens. Evidence is presented that the *CYP2A3* gene encodes the coumarin 7-hydroxylase activity expressed in human liver with a large degree of interindividual variation.

Materials and Methods

Materials. The sources of human liver specimens used in these studies have been published (Aoyama et al., 1989). Coumarin, 7-hydroxycoumarin, 7-ethoxycoumarin, and benzo[*a*]pyrene were obtained from Sigma. Wild-type vaccinia virus and plasmid pSC11 and the human TK$^-$143 cell line were kindly provided by Dr. Bernard Moss of the National Institutes of Health. Hep G2 cells (ATCC HB 8065) were obtained from the American Type Culture Collection. Radioisotopes used for nick translation ([α-^{32}P]dCTP, 3000 Ci/mmol), 5′-kinasing ([γ-^{32}P]ATP, 6000 Ci/mmol), and sequencing ([α-^{35}S]dATP, 500 Ci/mmol) were purchased from New England Nuclear Corp. Sequenase was obtained from United States Biochemicals.

Isolation and Sequencing of cDNAs. Total liver RNA was prepared according to the method of Chirgwin et al. (1979) except cesium trifluoroacetic acid was used instead of CsCl. A λgt11 library was constructed from K19 liver (see below) as described by Watson and Jackson (1985) and screened by using nick-translated human IIA3 cDNA that had previously been isolated (Yamano et al., 1989b). cDNAs were subcloned into pUC9 and then sequenced by using shotgun cloning into M13 (Deininger, 1983) and the dideoxy sequencing strategy (Sanger et al., 1977). Sequence data were aligned by using the Beckman Microgenie program. The cDNAs were inserted into vaccinia virus according to the procedures described in Aoyama et al. (1989).

Analysis of mRNA. Northern blot analysis was carried out using formaldehyde-containing agarose gels (Lehrach et al., 1977) and Nytran membranes (Schleicher & Schuell). Filters were hybridized by using nick-translated IIA3 cDNA insert according to the conditions of Church and Gilbert (1984). In some cases, filters were probed with oligonucleotides that had been synthesized with an Applied Biosystems Model 380B, purified on 15% polyacrylamide gels, and labeled with [γ-^{32}P]ATP and polynucleotide kinase. Hybridization of filters was performed at 50 °C. The filters were washed at 55 °C using a solution containing 1 M NaCl, 0.1 M sodium citrate, and 0.5% SDS.

Protein and Enzyme Analysis. Human liver microsomes were prepared as described in Aoyama et al. (1989). Lysates of vaccinia virus infected cells were generated by brief sonication in 1 × PBS (Biofluids, Inc., Rockville, MD). In some cases, membrane fractions were isolated from cell lysates by centrifugation at 2000*g* for 10 min, followed by centrifugation of the supernatant fraction at 100000*g* for 60 min. The membrane fractions thus obtained probably consist of both microsomes and mitochondria. The pellet was suspended in 0.1 M sodium phosphate, pH 7.4. For spectral analysis, the membrane fraction was solubilized in PBS containing 20% w/v glycerol and 0.3% Emulgen 913. The solubilized material was divided into two cuvettes, and carbon monoxide gas was gently bubbled into the sample cuvette. A few crystals of dithionite were added to sample and reference cuvettes, and difference spectra were measured with an Aminco DW-2000 spectrophotometer.

Coumarin 7-hydroxylase and ethoxycoumarin *O*-deethylase (Greenlee & Poland, 1978) and aryl hydrocarbon hydroxylase (Nebert & Gelboin, 1968) assays were carried out as described in earlier reports. Western immunoblotting was performed as described by Towbin et al. (1979) using SDS-containing 10% polyacrylamide gels (Laemmli, 1970). The blots were developed by using rabbit antibody against rat IIA2 (Matsunaga et al., 1988) and alkaline phosphatase conjugated goat anti-rabbit IgG (KPL Laboratories, Gaithersburg, MD).

Results

Isolation and Sequencing of IIA3, IIA3v, and IIA4 cDNAs. The sequence of a cDNA containing the apparent full amino acid sequence of a P450 encoded by the *CYP2A* subfamily was recently published by us (Yamano et al., 1989b) and others (Miles et al., 1989). This cDNA, designated IIA3, was isolated by using a rat IIA1 cDNA probe, from a human liver λgt11 library (liver sample Pa72). Using IIA3 cDNA as a probe, we screened a Northern blot containing RNAs from 12 livers and found a liver that had high levels of IIA mRNAs (see below). RNA from this liver, designated K19, was used to construct a λgt11 library, and a class of cDNAs were isolated that had deduced amino acid sequences identical with the previously published IIA3 (Yamano et al., 1989b). However, these cDNAs differed from the original IIA3 sequence in the 3′ untranslated region (see below). The cDNA-deduced amino acid sequence of IIA3, isolated from the K19 liver library, displayed 85% and 82% respective similarities with rat IIA3 (Kimura et al., 1989) and a mouse cDNA-deduced P450 protein, designated $P450_{15\alpha}$ (Squires & Negishi, 1988). These high degrees of similarities strongly suggest that the three enzymes are orthologous counterparts.[2] The IIA3 protein displayed only 69% and 64% sequence similarities with rat IIA1 and IIA2, respectively.

Two additional cDNAs were isolated that displayed high nucleotide similarities with IIA3 (Figure 1). A cDNA was found that encoded a protein identical with IIA3 except for a single amino acid change from $Leu_{160} \rightarrow$ His, resulting from a T to A change. Two other differences were observed, a silent G → A change at residue 60 and G → C at residue 1645 in the 3′ untranslated region of the cDNA (Figure 1). The high nucleotide sequence similarity between this cDNA and IIA3 suggests that it is derived from another allele of the *CYP2A3* gene and therefore was designated IIA3v (variant).

A third cDNA, designated IIA4, was also isolated from the K19 liver library. This clone displays 96% and 94% similarity with the IIA3 nucleotide and amino acid sequences, respectively (Figure 1). Several interesting clusters of high dissimilarities were detected in the coding region. For example, 6 out of 12 amino acid differences were found between residues 53 and 64, and 5 out of 7 differences exist between residues 158 and 164 (Figure 1). Several differences were also detected in the 3′ untranslated region between IIA3 and IIA4, including a cluster of 21 base changes between residues 1542 and 1598. These regions of dissimilarity in the coding and noncoding portions of the mRNAs were likely generated from prior gene conversion events. Most interesting is that the complete 3′ untranslated region of IIA4 is identical with the previously characterized IIA3 cDNA isolated from the Pa72 liver library. These data suggest that the original IIA3 cDNA isolated

[2] Based on evolutionary considerations [see Nelson and Strobel (1987)], mammalian P450s should diverge 1% in amino acid sequence every 4 million years. Humans and rodents are believed to have split about 80 million years ago. Therefore, one would expect the same P450 enzyme in both species (orthologue) to have changed 80/4 or 20% during the past 80 million years. The percent similarities of 82–85% between these rat/mouse and human P450s are, in fact, a little more similar in sequence than would be expected.

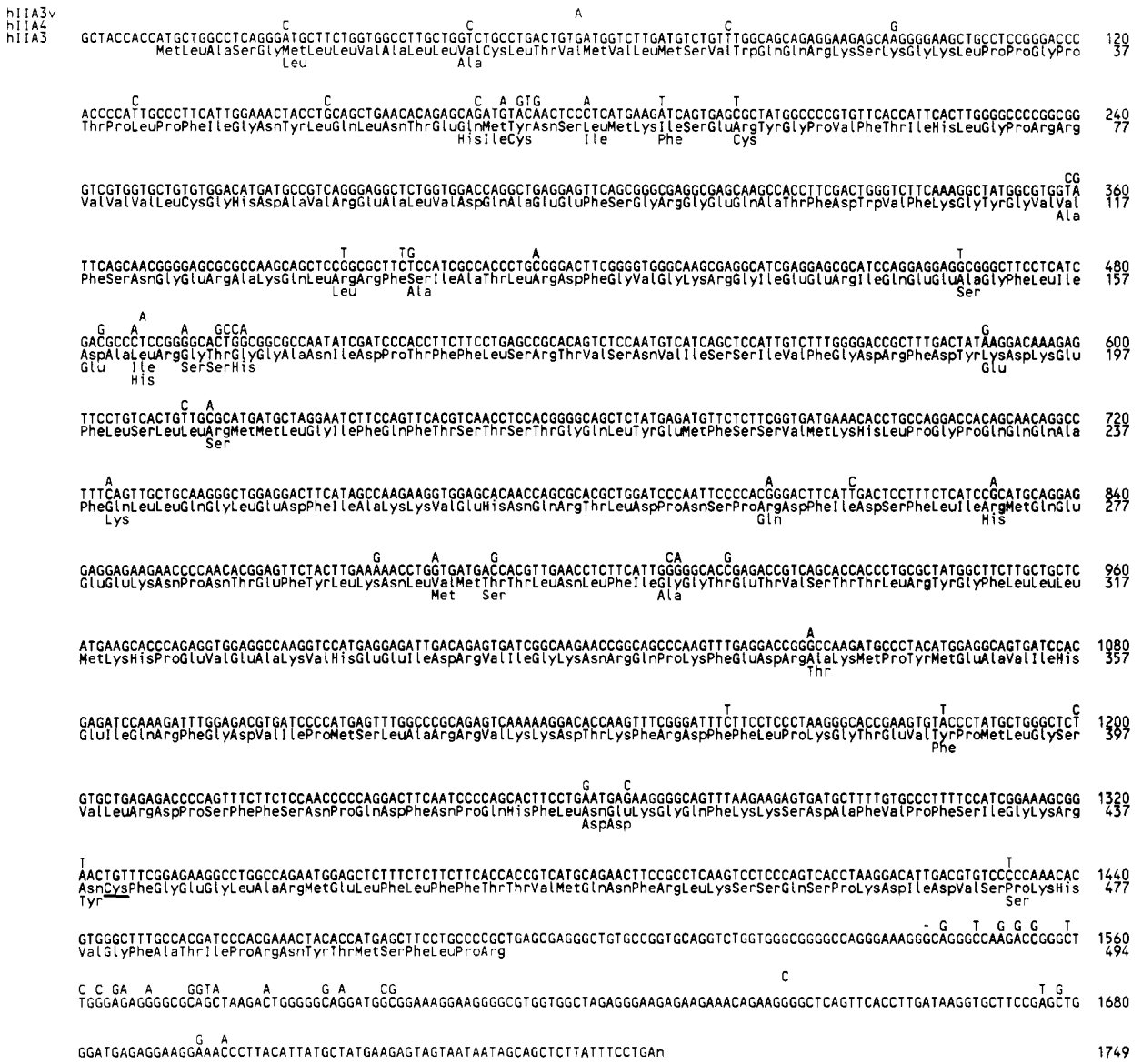

FIGURE 1: Sequence of IIA3, IIA3v, and IIA4 cDNAs. The complete nucleotide and deduced protein sequence of IIA3 is displayed. The IIA4 cDNA nucleotide and amino acid sequences are displayed above and below those of IIA3, respectively. Only those nucleotide and amino acid residues of IIA4 that differ from IIA3 are shown. The IIA3v cDNA nucleotide and amino acid sequences are identical with those of IIA3 except for the T → A at position 488, resulting in the Ile → His at amino acid 160, the G → A at position 60, and the G → C at position 1645. The conserved Cys residue, that serves as the thiolate ligand to the heme iron at the P450 active site, is underlined.

earlier (Yamano et al., 1989b) is from an allelic variant that was generated via a gene conversion between the *CYP2A3* and *CYP2A4* genes. In fact, on the basis of the sequence of the rat *CYP2A3* gene,[3] it appears that the 3′ untranslated region of the *CYP2A* genes is encoded by their ninth exons. A gene conversion encompassing all or part of the ninth exon could yield the Pa72 liver allelic variant.

We isolated and completely sequenced four IIA3 and three IIA4 cDNAs, all of which were isolated from the K19 liver library. Two other IIA4 cDNAs were isolated that had a G at residue 516 resulting in Glu instead of the Asp, a G → A at position 1695, and a A → C at position 1698 of the sequence in Figure 1. We believe these are allelic variants of IIA4. In spite of finding multiple IIA3 and IIA3v cDNAs, we were only able to isolate a single full-length IIA3v cDNA. A second partial cDNA was isolated that was identical with this cDNA from its 5′-most base at position 645 (Figure 1) through to the polyadenylation site. We have been unable to isolate human cDNAs that correspond to rat IIA1 and IIA2.

Expression of IIA3, IIA3v, and IIA4 cDNAs Using Vaccinia Virus. The IIA3, IIA3v, and IIA4 cDNAs, whose sequences are shown in Figure 1, were inserted into vaccinia virus, and the recombinant viruses were designated vIIA3, vIIA3v, and vIIA4, respectively. Cells infected with vIIA3 and vIIA3v produced proteins that were detectable with antibody against rat IIA2 (Figure 2). The IIA3 protein migrated slightly faster than IIA3v in SDS–polyacrylamide gels. These proteins could not be resolved when the vIIA3 and vIIA3v cell extracts were mixed and subjected to immunoblot analysis (Figure 2). The antibody-stained proteins, corresponding to IIA3 and IIA3v, comigrated with proteins detected in the K19 liver from which the λgt11 library was made. Only a small amount of immunostained protein band was detected in cells infected with vIIA4 using anti-rat IIA2 antibody.[4] Interestingly, antibody against rat IIA1 only weakly reacted with IIA3 on immunoblots. This is surprising since the primary sequence of rat IIA1 is actually more similar to human IIA3 than is rat IIA2.

[3] T. Ueno and F. J. Gonzalez, unpublished results.

[4] S. Yamano, unpublished results.

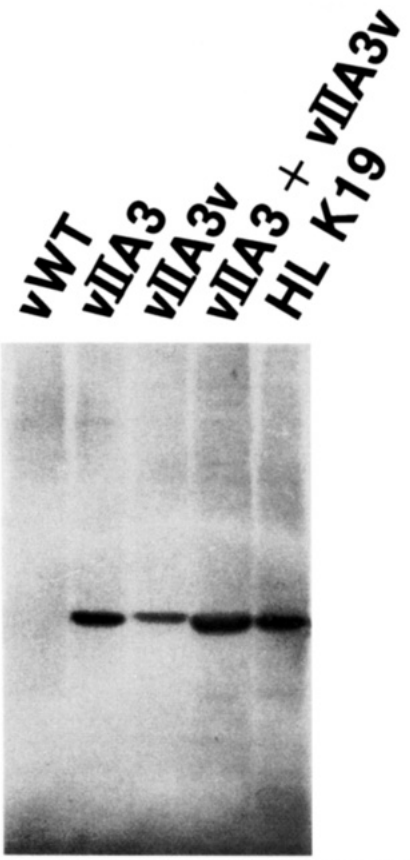

FIGURE 2: Western immunoblot analysis of vaccinia virus expressed IIA proteins. Wild-type vaccinia (vWT) and recombinant vaccinia containing the IIA3 (vIIA3) and IIA3v (vIIA3v) cDNAs were used to infect Hep G2 cells. Twenty four hours after infection, total sonicated cell protein (40 μg) was subjected to electrophoresis on SDS-containing polyacrylamide gels and Western blotting. A sample of microsomal protein (10 μg) from human liver K19 (HL K19) was also examined in parallel. The blots were developed by using rabbit antibody against rat IIA2 and alkaline phosphatase conjugated goat anti-rabbit IgG.

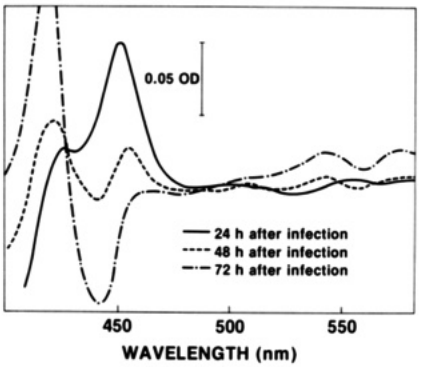

FIGURE 3: Spectral analysis of vaccinia virus expressed IIA3 protein. Human TK$^-$ 143 cells were infected with vIIA3 for various periods of time and then harvested for spectral analysis. Spectra were taken on total cell lysate protein as described under Materials and Methods.

These data suggest that critical immunological determinants are shared between the human protein and rat IIA2.

To determine if the vaccinia-expressed enzymes had a Soret absorption band typical of cytochrome P450s when reduced and complexed with CO, spectral analysis was conducted on cells infected with the recombinant vaccinia. Twenty-four hours after infection with vIIA3, a reduced CO-bound Soret peak that had a λ_{max} at about 450 nm was detected in cell lysates (Figure 3). This peak was markedly decreased at 48 h after infection concomitant with an increase of a Soret band

Table I: Enzyme Activities of Vaccinia-Expressed IIA3[a]

substrate	reaction	activity [pmol min^{-1} (mg of protein)$^{-1}$] vIIA3	vWT
coumarin	7-hydroxylation	172	ND[b]
7-ethoxycoumarin	O-deethylation	29.5	6.75
benzo[a]pyrene	3-hydroxylation	0.84	1.08

[a] Vaccinia-infected cells were harvested at 24 h after infection with vIIA3 or vWT, sonicated, and assayed for various activities. The lysates contained about 14 pmol of P450/mg of cell lysate protein calculated by spectral analysis according to the method of Omura and Sato (1964). Results are averages of duplicate determinations. [b] ND = not detectable.

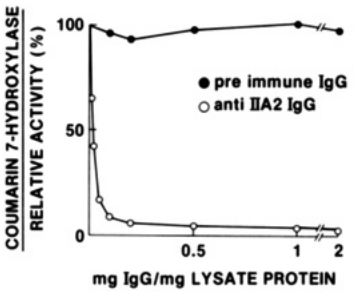

FIGURE 4: Immunoinhibition of coumarin 7-hydroxylase activity in vIIA3-infected cell lysates. Various amounts of preimmune or anti-IIA2 IgG, purified on protein A–Sepharose, were preincubated for 15 min at room temperature prior to asssay of coumarin 7-hydroxylase activity. 100% activity is that obtained in the absence of added IgG.

with a λ_{max} of 420 nm. The latter likely represents a denatured form of the cytochrome (Omura & Sato, 1964). The 450-nm peak vanished at 72 h after infection. It appears, therefore, that expression of IIA3 is maximum at 24 h and the enzyme is unstable in the cell after long periods of infection. Surprisingly, cells infected with vIIA3v contained no Soret peak either at 24 h or at 48 h after infection in the presence of sodium dithionite and CO,[4] even though a IIA3v protein was detected on Western blots. These data suggest that a single amino acid change in IIA3v, $Leu_{160} \rightarrow$ His, decreases the accumulation and/or stability of the P450 protein in vIIA3v-infected cells. vIIA4-infected cells also contained on Soret band as expected from the very low level of IIA4 protein seen on immunoblots.

The enzyme activities of expressed IIA3 were then analyzed by using several common P450 substrates. Cells infected with vIIA3 were able to catalyze 7-hydroxylation of coumarin and O-deethylation of 7-ethoxycoumarin (Table I). No aryl hydrocarbon hydroxylase activity was detected above the level seen in Hep G2 cells. We were unable to detect any of these activities in cells infected with vIIA3v and vIIA4. The turnover number of IIA3 for coumarin, calculated from the spectral analysis, was 15 nmol min^{-1} (nmol of P450)$^{-1}$. These data indicate that IIA3 is quite active at coumarin 7-hydroxylation.

Coumarin 7-Hydroxylation in Human Liver. On the basis of the results of IIA3 expression, we analyzed coumarin 7-hydroxylase activity in human microsomes. First we tested whether the antibody against rat IIA2 was able to inhibit the vaccinia-expressed activity. Indeed, as little as 0.1 mg of IgG/mg of cell lysate protein was able to almost abolish coumarin 7-hydroxylase activity in vIIA3-infected cells (Figure 4). These data demonstrate that this antibody can be used to probe for the coumarin 7-hydroxylase activity and IIA3 protein in human liver specimens.

To determine if human hepatic coumarin 7-hydroxylase activities are exclusively due to the IIA3 P450, microsomes

Table II: Inhibition of Coumarin 7-Hydroxylase and 7-Ethoxycoumarin *O*-Deethylase Activities by Anti-Rat IIA2 IgG in Human Liver Microsomes

substrate	mg of IgG:mg of microsomal protein ratio	percent inhibition											
		K10	K12	K14	K16	K18	K20	K21	M1	K15	K19	M2	KDL12
coumarin	0.1	47	40	26	55	55	39	39	34	60	37	86	62
	2.0	98	97	96	99	99	99	98	98	99	97	97	98
7-ethoxycoumarin	0.1	30	17	13	18	10	14	11	18	3	21	9	
	2.0	52	49	61	54	14	38	42	51	8	60	12	

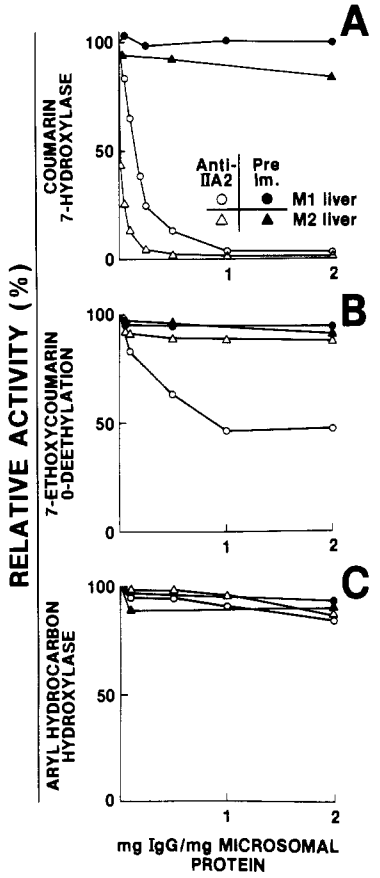

FIGURE 5: Immunoinhibition of coumarin 7-hydroxylase activity (panel A), 7-ethoxycoumarin *O*-deethylase activity (panel B), and aryl hydrocarbon hydroxylase activity (panel C) in two human liver microsomal preparations. Activities were titrated with preimmune IgG and anti-IIA2 IgG.

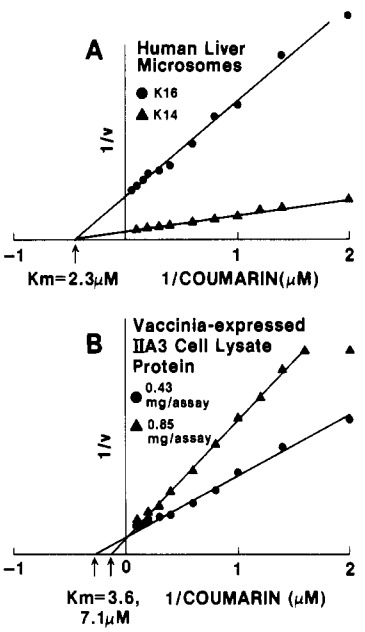

FIGURE 6: Kinetic analysis of coumarin 7-hydroxylase activity in human liver microsomes and in cells infected with vIIA3. Coumarin 7-hydroxylase activities were measured in human liver microsomes (panel A) and sonicated vIIA3-infected cell lysates (panel B) as a function of coumarin concentration.

from two livers were titrated with anti-rat IIA2 IgG (Figure 5A). Coumarin 7-hydroxylase activity was substantially inhibited in both specimens. Due to the lower levels of enzyme in liver M2, a lower ratio of IgG to microsomal protein was required to inhibit the activity. Analysis of 10 other samples revealed complete inhibition of coumarin 7-hydroxylase activity by anti-IIA2 antibody (Table II). In contrast, human microsomal 7-ethoxycoumarin *O*-deethylase activity was only partially inhibited in 12 specimens (Figure 5B, Table II). Interestingly, the extent of inhibition appeared to correlate with the level of IIA3 protein in these livers. For example, livers K18, K15, and M2, which had low coumarin 7-hydroxylase activities, were only partially inhibited, whereas K19, M1, and K14, having high IIA3, were inhibited by more than 50% (see Figure 7). These data indicate that human microsomal 7-ethoxycoumarin *O*-deethylase activity is, in part, catalyzed by IIA3 and that this activity is also catalyzed by other P450 forms in addition to IIA3. Indeed, human IA2 was found to metabolize 7-ethoxycoumarin (Yamano et al., 1989a). As expected from the lack of catalysis of aryl hydrocarbon hydroxylase activity by vaccinia-expressed IIA3, this activity was not inhibited by anti-rat IIA2 (Figure 5C).

To further establish the relationship between IIA3 and human microsomal coumarin 7-hydroxylase activity, kinetic studies were performed. Microsomes from two liver specimens having a high V_{max} (K14) and intermediate V_{max} (K16) were analyzed and found to have monophasic kinetics with identical K_ms of 2.3 μM (Figure 6A). Lysates of vIIA3-infected cells also displayed K_ms in this range (3.6–7.1 μM) (Figure 6B). A precise comparison of K_ms between human microsomes and cell lysates is difficult due to the different ratios of IIA3 to total protein and lipid in the two systems, resulting in different partitioning of the substrate into nonspecific cellular materials. This is illustrated in Figure 6B in which the K_m values differed depending on the protein content of the assays. In any case, the K_m values are sufficiently similar between vaccinia-expressed IIA3 and human microsomes to conclude that only a single P450 form, IIA3, catalyzes this activity in liver.

Next, we analyzed coumarin 7-hydroxylase activity in 12 liver microsome specimens. Values ranged from 31 pmol min^{-1} (mg of protein)$^{-1}$ (M2) to 1350 pmol min^{-1} (mg of protein)$^{-1}$ (K14) (Figure 7), a difference of 40-fold. Immunoblotting of these livers using anti-rat IIA2 revealed multiple proteins (Figure 7). In most cases, the intensity of the most prominently stained band with the highest mobility (49 kDa, Figure 7) appeared to correlate with the level of coumarin 7-hydroxylase activity. For example, liver specimen M2 contained only trace amounts of the 49-kDa protein, detectable only after overdevelopment of the immunoblots.[4] This liver also contained very low levels of coumarin 7-hydroxylase ac-

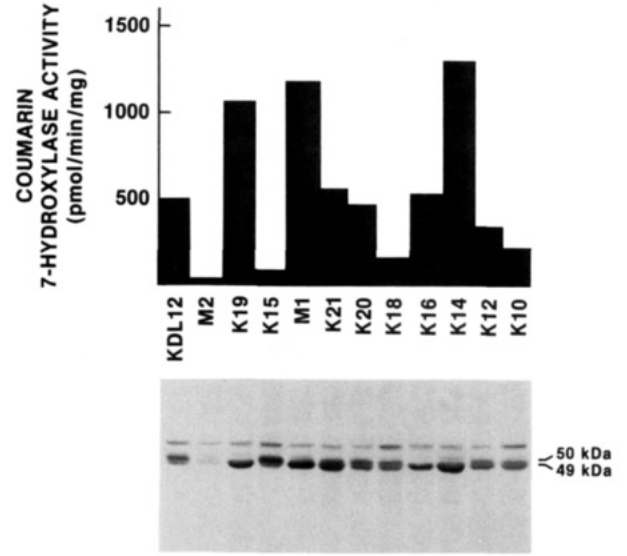

FIGURE 7: Coumarin 7-hydroxylase activities and Western immunoblotting analysis of IIA protein contents in 12 human liver specimens. Each liver microsome sample was subjected to immunoblotting analysis (20 μg/well) and coumarin 7-hydroxylase activity. The arrows denote the 50- and 49-kDa protein described in the text, the latter of which corresponds to IIA3.

tivity. On the other hand, livers K19, M1, and K14 had high activities and prominent 49-kDa proteins (Figure 7). A second 50-kDa protein was also detected in most liver specimens. However, we do not beleive this is IIA3v or IIA4 because even though the vaccinia-expressed protein migrates slighlty slower than IIA3 on SDS–polyacrylamide gels, when the preparations are mixed, they cannot be resolved (Figure 2). This indicates that IIA3v and IIA4 migrate slightly faster than the 50-kDa microsomal protein. Moreover, levels of the 50-kDa protein in human microsomes did not correlate with IIA mRNA (see below). In any case, the nature of this protein is currently unknown, but we believe it may be another P450 in the P450II family. These data indicate that a large degree of interindividual variability in expression of IIA3 and coumarin 7-hydroxylase exists in man.

To determine if levels of mRNA transcribed from the *CYP2A* genes correlated with protein and enzyme activities, Northern blotting analysis was carried out. High levels of a 2-kb mRNA were detected with the IIA3 cDNA probe in livers K14, K16, and K19 (Figure 8). Two larger RNAs of about 2.8 and 4.5 kb were also detected in the K19 liver RNA and, to a lesser extent, in K14 RNA. The origin of these transcripts is unknown, but they may be unprocessed precursor transcripts from the *CYP2A3* gene or a related gene. Both the K16 and K19 livers, having high levels of the 2-kb mRNA, also had high coumarin 7-hydroxylase activities and 49-kDa proteins. Four other livers, M1, K21, K20, and K10, had lower mRNA levels (about one-fifth the levels in K19 and K14). Two specimens, KDL12 and K12, had trace IIA mRNA levels, and three of the specimens, M2, K15, and K18, had little detectable mRNA. Most of these livers, except M1, had low levels of coumarin 7-hydroxylase activities and 49-kDa protein (Figure 7). Only liver M1 did not seem to show a strong correlation between RNA and activity. This may be due to many factors including posttranslational stabilization of IIA3 protein or differences in the distribution of IIA3 expression within areas of the liver. We did not attempt to isolate the RNA and protein from the same piece of disrupted liver.

To control for degradation of RNA in the individual livers, RNAs from these same 12 specimens were also probed for the NADPH–P450 oxidoreductase mRNA (Yamano et al., 1989a). Every liver contained the oxidoreductase mRNA, indicating that the lack of IIA mRNAs is not due to mRNA degradation. Attempts to use oligonucleotides to distinguish the IIA3, IIA3v, and IIA4 mRNAs were unsuccessful due to the low levels of transcripts in most of the liver specimens. We only detected IIA3 and IIA4 mRNAs in RNA isolated from livers K14, K16, and K19.[4] These data indicate a high level of interindividual differences in IIA3 expression in humans.

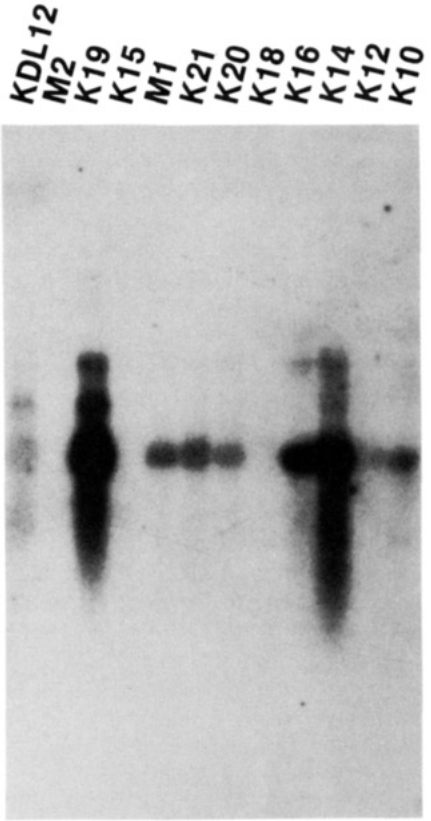

FIGURE 8: Northern blot analysis of human liver RNA. Total RNA was isolated, and 10 μg from each specimen was electrophoresed and transferred to nylon membranes. The membranes were incubated with ^{32}P-labeled IIA3 insert, washed, and exposed to autoradiographic film at −70 °C for 2 days with the aid of Dupont Lightning Plus intensifying screen. In a separate experiment, RNA from liver K19 was electrophoresed in parallel with RNA size standards (RNA ladder; BRL Inc., Bethesda, MD). The two hybridized RNAs were determined to be 2 and 2.8 kb, respectively.

Discussion

In this report, we characterized cDNAs and activities of P450s in the IIA subfamily in man. Two forms, IIA3v and IIA4, were not faithfully synthesized when their cDNAs were expressed using vaccinia virus, as monitored by spectral analysis, Western immunoblots, and catalytic activities. It is unclear, at this time, what causes the lack of accurate production of these enzymes and whether the mRNAs for IIA3v and IIA4 are translated into functional P450 in the intact liver cell. At present, we cannot rule out the possibility that these results are due to some aspect of the vaccinia virus expression system. Indeed, even IIA3 becomes unstable after 2–3 days of infection with vIIA3. The lack of, or low level, expression may be due to faulty translation, incorrect folding and uptake of the critical cofactor heme, or inefficient membrane insertion. Of course, further experimentation must be carried out before we can distinguish among these possibilities. In any case, it is tempting to speculate that humans may produce unproductive IIA P450s, a possibility that is not unprecedented since clearly defective alleles for human IID1 (Gonzalez et al., 1988)

and a P450 in the IIB subfamily (Yamano et al., 1989c) have been found.

Coumarin, a product of certain plants and fungi, is metabolized in man by hydroxylation at the 7 position and subsequent conjugation (Kaighen & Williams, 1961). The activity was first demonstrated in human liver by Kapitulnik et al. (1977) and more recently by Raunio et al. (1988). These latter investigators also demonstrated the immunochemical relationship between the mouse coumarin 7-hydroxylase with the human enzyme. The mouse coumarin 7-hydroxylase cDNA was recently isolated (Lindberg et al., 1989) and expressed using a COS cell cDNA expression system (Lindberg & Negishi, 1989). This work demonstrated that the coumarin 7-hydroxylase was encoded by a member of the P450IIA subfamily. Others also predicted the association of coumarin 7-hydroxylase with this subfamily in man based on chromosome mapping studies in mouse and humans and indirect immunochemical studies using Western blots (Miles et al., 1989). On the basis of our cDNA expression and immunoinhibition results, it appears quite likely that human IIA3 is probably the principal enzyme involved in coumarin metabolism in human liver. It must be noted, however, that the anti-rat IIA2 antibody used in our immunoinhibition studies does bind to two or more human liver proteins on Western immunoblots. Therefore, we cannot completely rule out the participation of other IIA subfamily members or even other family II P450s in coumarin 7-hydroxylation.

A large degree of interindividual variability exists in human in the expression of IIA3. Up to a 40-fold difference in coumarin 7-hydroxylase activity was detected among human microsome specimens. An even greater difference was seen when mRNA was examined. This difference in IIA mRNA levels was also seen by Miles et al. (1989) using another set of human liver samples. At this time, we do not know if this difference in expression is due to a genetic polymorphism or to a difference in regulation. Indeed, there does not appear to be any relationship between the level of expression of IIA3 or IIA mRNAs and the known drug intake of the liver donors. In most cases, it is difficult to know the precise drug intake history of each donor. However, one liver specimen containing high IIA mRNA (K14) was taken from a kidney donor who had received phenobarbital (Yamano et al., 1989c). In this connection, it is noteworthy that phenobarbital is known to decrease the prothrombinopenic response to coumarin in humans, a phenomenon that is due to the increased clearance and metabolism of the compound (Cucinell et al., 1965). This effect may, in fact, be due to an induction of IIA3 by phenobarbital. It is also noteworthy that livers containing high levels of IIA mRNA (K14 and K19) also expressed high levels of IIB mRNAs (Yamano et al., 1989c), a result that was also found by Miles et al. (1989) in their human liver specimens. These results are especially intriguing since both subfamilies are located in the same region of chromosome 19 within 350 kbp (Miles et al., 1989). Since we detected coumarin 7-hydroxylase activity and the 49-kDa protein in every liver examined, we feel that the difference in expression may not be due to inactive or mutant *CYP2A3* alleles. Perhaps genetic differences in the degree of regulation of different *CYP2A3* alleles may account for low levels of expression. These questions await further analyses of the *CYP2A3* genes.

Finally, it is noteworthy that an abundant P450 expressed in rabbit nasal mucosa, designated P450NMa, appears, based on similar amino-terminal protein sequence, to correspond to human IIA3 or IIA4 (Ding & Coon, 1988). This rabbit enzyme has catalytic activity toward two known nasal carcinogens, hexamethylphosphoramide and *N*-nitrosodiethylamine. In fact, human IIA3[5] does activate a related compound, *N*-nitrosodimethylamine, to toxic and mutagenic metabolites (Davies et al., 1989). The possibility exists, therefore, that high-level expression of IIA3 may be a risk factor for human nasal carcinogenesis.

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Structure and in Vitro Transcription of the Rat *CYP2A1* and *CYP2A2* Genes and Regional Localization of the *CYP2A* Gene Subfamily on Mouse Chromosome 7

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ABSTRACT: The *CYP2A1* and *CYP2A2* genes code for hepatic steroid hydroxylases and differ in their development regulation and expression in male and female rats. In order to explore the mechanism of regulation of these two genes, both genes were isolated and sequenced, their upstream regions compared, and their promoters transcribed in a cell-free system derived from liver. The *CYP2A1* gene was completely sequenced and spanned 12835 bp. The *CYP2A2* gene was sequenced except for 1.5 and 12 kbp in the second and fifth introns, respectively. This gene was about 10 kbp longer than *CYP2A1*. Both genes possess nine exons that displayed overall 93% nucleotide similarity. DNA 4544 and 5529 bp upstream from the *CYP2A1* and *CYP2A2* genes, respectively, was also sequenced, and the transcription start sites were determined. Both genes had typical TATA boxes but did not contain CCAAT boxes within -100 bp of their polymerase start sites. *CYP2A1*, however, contained a reverse CCAAT box between -85 and -90. Search of the Gene Bank revealed a 255 bp region that lies -3 kbp upstream from the transcription start site of *CYP2A1* displaying similarity with retrovirus polymerase. Two regions upstream of *CYP2A2* were also found that displayed 90% sequence similarity with the consensus long interspersed middle repetitive element (LINE). In addition, an unusual 1.6 kbp inserted sequence was detected between -165 and -1779 bp upstream of the *CYP2A2* gene that appears to be a retropseudogene. A nuclear extract derived from adult hepatocytes was used to direct in vitro transcription of the *CYP2A1* and *CYP2A2* gene promoters. Both genes were accurately transcribed in extracts derived from livers of male and female rats. This result is surprising in view of the fact that the *CYP2A1* gene is expressed in adult female rats while the *CYP2A2* gene is expressed exclusively in adult males. The *CYP2A1* promoter was more actively expressed in both extracts than that of *CYP2A2*. By analyzing the segregation pattern of *CYP2A* genes in backcross offspring from an interspecies cross between the laboratory strain NFS/N and the wild mouse *Mus musculus musculus*, the *Cyp2a* subfamily was mapped proximal to the *Gpi-1* locus near the centromere on chromosome 7.

The P450s represent products of a superfamily of genes (Nebert et al., 1989), many of which are under distinct inducer-dependent and developmental control (Gonzalez, 1988). Nine gene families and multiple subfamilies have been described in mammals, and even within closely related members of a single subfamily, P450 genes can be regulated quite differently. For example, the rat *CYP2A* subfamily encodes enzymes having both distinct catalytic activities and regulatory pathways. The most well-studied enzyme in this subfamily is that encoded by the *CYP2A1*[1] gene. This enzyme, designated IIA1, is relatively inactive in the metabolism of various drugs and common P450 substrates but is capable of metabolizing certain steroids such as testosterone (Waxman et al., 1983; Wood et al., 1983) and progesterone (Swinney et al., 1987). IIA1 catalyzes the 7α-hydroxylation of these steroids and also produces low levels of 6α-hydroxysteroid metabolites.

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