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Real-Time Polymerase Chain Reaction Analysis of CYP1B1 Gene Expression in Human Liver

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Procarcinogen-activating cytochrome P450 (CYP) enzymes such as CYP1B1, CYP1A1, and CYP1A2 are considered to play an important role in chemical carcinogenesis. However, conflicting data exist with respect to CYP1B1 expression in human liver. In the present study, we measured CYP1B1 mRNA and protein expression in liver samples from 12 individuals (7 nonsmokers, 4 smokers, and 1 ex-smoker) and compared the levels to those of CYP1A1 and CYP1A2. As analyzed by real-time polymerase chain reaction, CYP1B1 mRNA was present in all samples and the inter-individual variability was 16-fold. The group mean level was 5-fold greater in smokers than nonsmokers (121 ± 46 vs. 26 ± 5 molecules/ng double-stranded DNA, $p < 0.05$). By comparison, CYP1A1 mRNA was detectable in samples from 4 of 7 nonsmokers, 3 of 4 smokers, and one ex-smoker, whereas CYP1A2 mRNA was detectable in samples from 5 nonsmokers, 4 smokers, and the ex-smoker. The mean levels of CYP1A1 and CYP1A2 mRNA were 4-fold and 9-fold greater, respectively, in smokers than nonsmokers, but the differences were not statistically significant. The inter-individual variability in CYP1A1 and CYP1A2 mRNA expression was 26-fold and 500-fold, respectively. Immunoblot analysis using several antibodies and with a larger panel ($n = 27$) of liver microsomes showed that CYP1A1 and CYP1B1 proteins were undetectable, whereas CYP1A2 was detectable in all samples and quantifiable in 24 of 27 samples. In summary, our novel finding indicates that CYP1B1 mRNA is expressed in human liver and the levels are increased in smokers, but the protein is undetectable.

Key Words: **cytochrome P450; CYP1A1; CYP1A2; CYP1B1; cigarette smoke; real-time PCR.**

Cytochrome P450 (CYP) enzymes such as CYP1B1, CYP1A1, and CYP1A2 play an important role in the metabolic activation of environmental procarcinogens. Human CYP1B1 catalyzes the oxidation of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene (Shimada *et al.*, 1996) and dibenzo-[*a,l*]pyrene (Luch *et al.*, 1999), and heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, often in a unique stereoselective manner, to yield electrophilic intermediates capable of binding covalently to DNA (Crofts *et al.*, 1997; Shimada *et al.*, 1996), a step believed to be important in the initiation of carcinogenesis. CYP1B1 also metabolizes 17β-estradiol to form the 4-hydroxy metabolite (Hayes *et al.*, 1996), which has been implicated in estrogen-induced carcinogenesis (Yager and Liehr, 1996). The bioactivation of polycyclic aromatic hydrocarbons by CYP1A1 (Whitlock, 1999) and the bioactivation of aryl and heterocyclic amines by CYP1A2 (Guengerich *et al.*, 1999) have been reviewed recently. Due to their involvement in the bioactivation of chemically diverse procarcinogenic compounds to reactive metabolites, the constitutive and inducible expression of CYP1B1, CYP1A1, and CYP1A2 are considered to be important determinants of carcinogenesis, although the exact relationship between CYP1 expression and chemically induced carcinogenesis remains to be established. However, it is thought that high levels of DNA adduct and CYP1A-mediated activity are associated with an increased risk of lung cancer in cigarette smokers (Bartsch *et al.*, 1992; Mollerup *et al.*, 1999).

CYP1B1 protein has been detected in a variety of human tumors, including those of the lung, brain, testis, breast, kidney, and ovary (McFadyen *et al.*, 2001; Murray *et al.*, 2001). Furthermore, it has been suggested that CYP1B1 is the most frequently expressed CYP1 enzyme in breast cancer (McFadyen *et al.*, 1999; McKay *et al.*, 1995; Murray *et al.*, 1997). The issue of whether CYP1B1 is expressed in normal human tissues remains unresolved, but there is evidence that the CYP1B1 protein may be present in at least some human liver samples. In a recent study (Muskhelishvili *et al.*, 2001), it was reported that three of nine human liver samples examined were positive for CYP1B1 protein staining on immunoblots. However, the extent of variability among individuals in human hepatic CYP1B1 expression has not been extensively characterized. Moreover, it is not known if the hepatic expression of this human CYP *in vivo* is amenable to induction by environmental factors. In experimental animals, hepatic CYP1B1 is readily inducible following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polycyclic aromatic hydrocarbons, and other agonists of the aryl hydrocarbon (Ah) receptor (Murray *et al.*, 2001). Induction of CYP1B1 by agonists of the Ah receptor has also been demonstrated in cell culture models such

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TABLE 1
Sample Donor Information

Sample no.	Age (years)	Weight (kg)	Sex	Race	Smoker
1	N/A	N/A	N/A	N/A	No
2	51	70	F	C	No
3	44	82	F	C	No
4	68	61	F	A	No
5	65	98	M	C	No
6	34	90	M	C	No
7	64	98	M	C	No
8	40	69	M	C	Yes, 3 ppd for 25 years
9	45	86	M	C	Yes, 1.5 ppd for 30 years
10	40	76	M	A-A	Yes, 1 ppd
11	56	70	F	C	Yes, 0.5 ppd for 20 years[a]
12	68	69	F	C	Yes, 1.5 ppd for 50 years
13	45	88	M	C	Yes, 1.5 ppd for 25 years
14	55	82	M	A-A	Yes, 1.5 ppd for 5 years
15	44	102	M	C	Yes, 2 ppd for 26 years
16	23	N/A	F	C	Yes
17	40	N/A	M	A-A	Yes
18	52	N/A	M	A-A	Yes
19	34	N/A	F	C	Yes
20	16	N/A	M	C	No
21	25	N/A	M	C	No
22	28	N/A	F	C	No
23	48	N/A	F	C	No
24	36	N/A	F	C	No
25	61	N/A	F	C	No
26	45	N/A	F	C	No
27	2	N/A	F	C	No

Note. The sample numbers shown in this table correspond to those in Figures 3A–3C, 6A, and 6B. F, female; M, male; C, caucasian; A, Asian; A-A, African-American; ppd, packs of cigarettes smoked per day. N/A, not available.
[a]Stopped 0.5 year before death.

as human epidermal keratinocytes, MCF-7 human breast cancer cells, and human renal adrenocortical cells, which contain functional Ah receptor (Christou *et al.*, 1994; Sutter *et al.*, 1994; Tang *et al.*, 1999). Consistent with these findings, experimental evidence has implicated the Ah receptor as a mediator in CYP1B1 induction (Murray *et al.*, 2001). Therefore, environmental factors such as cigarette smoke, which contains polycyclic aromatic hydrocarbons, may up-regulate hepatic CYP1B1 expression and contribute to the variability in the levels of this CYP among individuals. However, the influence of cigarette smoking on hepatic CYP1B1 expression has not been reported to date.

The purpose of the present study was to conduct a detailed investigation of CYP1B1 protein and mRNA expression in a panel of human liver samples and to compare the levels between smokers and nonsmokers. A real-time, rapid-cycle polymerase chain reaction (PCR) method was employed to quantify CYP1B1 mRNA expression. Immunoblot analysis was performed to measure CYP1B1 protein expression in a larger panel of human liver microsome samples from individuals of known smoking status. For comparison, we also measured CYP1A1 and CYP1A2 mRNA and protein expression in the same samples.

MATERIALS AND METHODS

Chemicals and reagents. TriZol™, dithiothreitol, dNTP mix, oligo(dT)$_{12–16}$ primer, magnesium chloride, deoxyribonuclease I, Superscript II™ reverse transcriptase, and Platinum® *Taq* DNA polymerase were bought from Invitrogen Canada, Inc. (Burlington, Ontario, Canada). SYBR Green-I and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). RiboGreen® RNA Quantitation Kit and PicoGreen® dsDNA Quantitation Kit were purchased from Molecular Probes, Inc. (Eugene, OR). Forward and reverse primers for human CYP1B1, CYP1A1, CYP1A2, and β-actin were synthesized at the University of British Columbia Nucleic Acid and Protein Service Unit (Vancouver, British Columbia, Canada).

Source of human liver samples and preparation of microsomes. Frozen liver tissues from 15 individuals were kindly provided by James R. Olson (Department of Pharmacology and Toxicology, State University of New York, Buffalo, NY) and were stored at –70°C until use. The information on the donors is listed in Table 1. The microsomal fractions of these liver tissues were prepared by differential ultracentrifugation (Lu and Levin, 1972). The microsomal pellet was suspended in 0.25 M sucrose and aliquots of the suspension were stored at –80°C until use. Microsomal protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Ltd., Mississauga, Ontario, Canada) with bovine serum albumin as the standard. In addition, a panel of 9 individual human liver microsomes was purchased from BD GENTEST Corp. (Woburn, MA) and 3 samples were obtained from Human Cell Culture Center, Inc. (Laurel, MD).

Isolation and quantification of total RNA. Of the 15 human liver samples obtained (see above), there was sufficient tissue material to isolate RNA from 12 of them. Total liver RNA was isolated using TriZol™ reagent according to the manufacturer's protocol. The RNA pellet was suspended in diethylpyrocarbonate-treated distilled water and stored at –70°C until subsequent analysis. The purity of each RNA preparation was evaluated by the ratio of the absorbance at 260 nm to that at 280 nm, and the integrity of the preparation was assessed by agarose (1.7%)/formaldehyde (0.66 M) gel electrophoresis. RNA concentration was quantified using the RiboGreen® RNA Quantitation Kit (Molecular Probes, Inc.) according to the manufacturer's protocol (Jones *et al.*, 1998). Calibration curves were constructed with known concentrations of ribosomal RNA standards (16S and 23S rRNA from *E. coli*, supplied in the kit). The fluorescence of the unknown samples and the standards were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (CytoFluor Series 4000 fluorescence microplate reader, Millipore, Bedford, MA).

Reverse transcription and quantification of total cDNA. RNA was transcribed using SuperScript II™ reverse transcriptase as described previously (Chang *et al.*, 2000). The reaction was stopped by heating the mixture at 95°C for 5 min and storing at –20°C until subsequent analysis. The concentration of the synthesized cDNA was determined by the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Inc.) according to the manufacturer's protocol (Singer *et al.*, 1997). Calibration curves were constructed with lambda DNA standards (included in the kit). The fluorescence of the unknown samples and the standards were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Primers for polymerase chain reaction (PCR). Sequences for the forward (5′-CAC-TGC-CAA-CAC-CTC-TGT-CTT-3′) and reverse (5′-CAA-GGA-GCT-CCA-TGG-ACT-CT-3′) primers for CYP1B1 (Huang *et al.*, 1996), forward (5′-TGG-ATG-AGA-ACG-CCA-ATG-TC-3′) and reverse (5′-TGG-GTT-GAC-CCA-TAG-CTT-CT-3′) primers for CYP1A1 (Huang *et al.*, 1996), and forward (5′-AAC-AAG-GGA-CAC-AAC-GCT-GAA-T-3′) and reverse (5′-GGA-AGA-GAA-ACA-AGG-GCT-GAG-T-3′) primers for CYP1A2 (Rodriguez-Antona *et al.*, 2001) were obtained from the cited references.

Real-time PCR analysis. Each 20-μl PCR reaction volume contained 1 unit Platinum® *Taq* DNA polymerase in 1X PCR reaction buffer (20 mM Tris–HCl, pH 8.4, and 50 mM KCl), 4 mM magnesium chloride (except for CYP1A2 in which the concentration was 2 mM), 1 ng cDNA (as quantified by the PicoGreen® dsDNA assay, see above), 200 μM dNTP mix, 0.2 μM each of the forward and reverse primers, 0.25 mg/ml bovine serum albumin, and 2 μl of a 3.3X SYBR Green I solution. The conditions for the amplification of CYP1A1 and CYP1B1 were: 94°C for 5 s (denaturation), 65°C for 10 s (annealing), and 72°C for 20 s (extension). To amplify CYP1A2, the conditions for denaturation, annealing, and extension were 95°C for 5 s, 60°C for 10 s, and 72°C for 20 s, respectively. In all cases, the initial denaturation was carried out at 95°C for 5 min. Although little or no primer-dimer formation was detected under these PCR conditions, the real-time DNA thermal cycler (LightCycler™, Roche Diagnostics, Mannheim, Germany) was programmed to take fluorescence readings after each cycle at a temperature several degrees lower than the melting temperature of the amplicon. This step was taken to avoid or minimize any potential contribution of primer-dimers to the overall fluorescence signal. Initial experiments established that an optimal temperature for the fluorescence readings to be taken was 86°C for CYP1B1 and 88°C for CYP1A1 and CYP1A2. All PCR reactions were performed in duplicates. Negative control samples were processed in the same manner, except that the template was omitted. Calibration curve was constructed by plotting the cross point (Ct) against known amounts of purified CYP1A1, CYP1A2, or CYP1B1 amplicon. The Ct is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification.

Purification and sequencing of amplicons. CYP1B1, CYP1A1, and CYP1A2 cDNA amplicons were extracted from gels and purified using the QIAquick Gel Extraction Kit according to the instructions provided by the manufacturer (QIAGEN Inc., Mississauga, Ontario, Canada). Purified amplicons were sequenced on the Applied Biosystems 377 DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) at the Nucleic Acid and Protein Service Unit, University of British Columbia. To determine the identity of the amplicon, the sequence of the amplicon was compared to the known DNA sequence of the gene of interest (BLAST program, www.ncbi.nlm.nih.gov).

Anti-CYP antibodies. Rabbit antihuman CYP1B1 IgG was generated by immunizing three female New Zealand rabbits with a synthetic 16-amino acid peptide corresponding to amino acids 284–299 of the deduced sequence of human CYP1B1 (Sutter *et al.*, 1994) conjugated to keyhole limpet hemocyanin. The procedure for immunization, collection of antisera, and purification of IgG was the same as that used previously for antitrout CYP1A IgG (Lin *et al.*, 1998). Another rabbit antihuman CYP1B1 peptide serum (catalog number A211) was purchased from BD GENTEST Corp. (Woburn, MA). Mouse antirat CYP1A1 monoclonal IgG (a mixture of C1, C7, and C8 antibodies that are specific for CYP1A1), rabbit antihuman CYP1A1 peptide serum, and rabbit antihuman CYP1A2 peptide serum were provided by Paul E. Thomas (Rutgers University, Piscataway, NJ). The antihuman CYP1A1 and CYP1A2 peptide sera are specific for human CYP1A1 and CYP1A2, respectively. They were generated separately against a 5-amino acid peptide corresponding to carboxy-terminus acids of each enzyme. Rabbit antirat CYP1A1 IgG was prepared in our laboratory (Lin *et al.*, 1998). Polyclonal antibody to rat CYP1A2 was raised in a single female New Zealand rabbit immunized with electrophoretically homogeneous CYP1A2 protein, which had been provided by Wayne Levin (Hoffmann-La Roche Inc., Nutley, NJ). The procedure for immunization and collection and preparation of antisera was the same as that used previously (Lin *et al.*, 1998). Initial experiments were performed to verify the specificity of each antibody by immunoblot analysis with a panel of human recombinant CYP enzymes (i.e., CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 from BD GENTEST Corp.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis. Hepatic microsomal proteins were resolved by SDS–PAGE and transferred electrophoretically onto nitrocellulose membranes using a Hoefer transfer unit (Model TE 52; San Francisco, CA) as described previously (Lin *et al.*, 1998). The membranes were incubated with primary antibody at the concentrations given in the figure legends for 2 h at 37^{0}C, followed by a 2-h incubation with alkaline phosphatase-conjugated secondary antibody (1:3000 dilution). Immunoreactive protein bands were detected primarily by reaction of alkaline phosphatase with substrate solution containing 0.01% NBT, 0.05% BCIP, and 0.5 mM $MgCl_2$ in 0.1 M Tris–HCl buffer, pH 9.5. Assay conditions were optimized to ensure that color development did not proceed beyond the linear range of the phosphatase reaction. Enhanced chemiluminescence detection using horseradish peroxidase-conjugated secondary antibody and luminol (SuperSignal® West Pico kit, Pierce, Rockford, IL) was used when protein bands were not detected with the alkaline phosphatase colorimetric substrate. Enhanced chemiluminescence is a more sensitive detection system than colorimetric detection. However, enhanced chemiluminescence, unlike colorimetric detection, does not lend itself readily to reproducible quantification. Thus, CYP1A2 protein levels were quantified by densitometric analysis of immunoblots developed with alkaline phosphatase-labeled antibody and the BCIP/NBT substrate (Lin *et al.*, 1998).

Statistics. The difference between the means of the groups was analyzed by the two-tailed, independent *t*-test. Correlation analysis was performed to calculate the coefficient of determination (r^2). The level of significance was set *a priori* at $p < 0.05$.

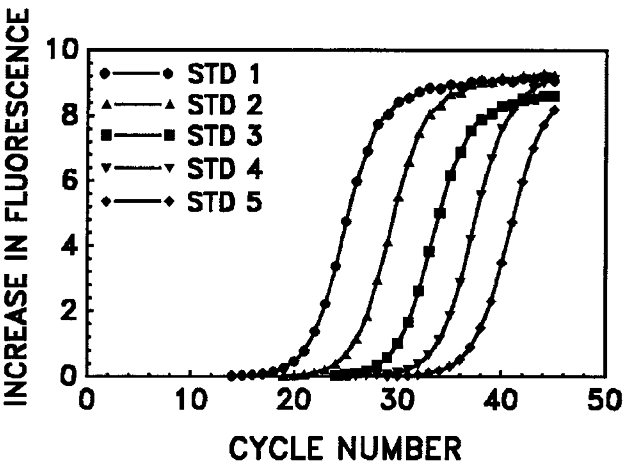

FIG. 1. Representative progress curve for the real-time amplification of CYP1B1 cDNA. Shown is a plot of an increase in fluorescence signal versus PCR cycle number for the amplication of known amounts (see Fig. 2) of purified CYP1B1 amplicon, the quantity of which was determined by the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Inc.).

RESULTS

Real-Time PCR Analysis of CYP1B1, CYP1A1, and CYP1A2 Gene Expression

To quantify *CYP1B1* gene expression in human liver samples, initial experiments were performed to establish the conditions for the real-time PCR assay. These experiments indicated that a suitable magnesium concentration was 4 mM for the amplification of CYP1A1 and CYP1B1, but 2 mM for that of CYP1A2. An appropriate annealing temperature was 65°C, except for CYP1A2, in which a suitable annealing temperature was 60°C. Shown in Figure 1 is a typical progress curve for the amplification of CYP1B1 cDNA. Figure 2 is a representative calibration curve in which the cross point is plotted as a function of known amounts of purified CYP1B1 amplicon. Under our PCR conditions, the fluorescence signal was log-linear ($r^2 > 0.99$) for at least 4 orders of magnitude. The efficiency (E) of our PCR method for the amplification of CYP1B1 cDNA, as calculated by $E = 10^{1/m} - 1$, where m is the slope of the standard curve (Bieche *et al.*, 1999), was typically 80–90%. Real-time PCR methods were also used for the analysis of CYP1A1 and CYP1A2 mRNA expression and similar results were obtained with respect to the dynamic range of the calibration curve and the efficiency of the PCR (data not shown).

Determination of CYP1B1, CYP1A1, and CYP1A2 mRNA Levels by Real-time PCR

Real-time, rapid-cycle PCR analysis of a panel of 12 individual human liver samples (7 nonsmokers, 4 smokers, and 1 ex-smoker) indicated that CYP1B1 mRNA was present in each of the samples analyzed (Fig. 3A) and the inter-individual expression varied by 16-fold. When stratified according to smoking status (Fig. 4), the mean hepatic CYP1B1 mRNA level was 5-fold greater ($p < 0.05$) in smokers (121 ± 46 molecules/ng double-stranded DNA [dsDNA]; mean ± SEM) than nonsmokers (26 ± 5 molecules/ng dsDNA). We also performed real-time PCR analyses of *CYP1A1* (Fig. 3B) and *CYP1A2* (Fig. 3C) gene expression in the same panel of individual human liver samples. CYP1A1 mRNA was detectable in samples from 4 of 7 nonsmokers, 3 of 4 smokers, and 1 ex-smoker, whereas CYP1A2 mRNA was detectable in samples from 5 nonsmokers, 4 smokers, and the ex-smoker. The mean CYP1A1 and CYP1A2 mRNA levels were 4-fold and 9-fold greater, respectively, in smokers than nonsmokers. However, the differences were not statistically significant because of the large inter-individual variability in CYP1A1 (26-fold) and CYP1A2 (500-fold) mRNA expression.

Immunoblot Analysis of CYP1B1, CYP1A1, and CYP1A2 Protein Expression

To determine whether hepatic CYP1B1 protein is expressed in smokers and nonsmokers, we performed immunoblot analysis using enhanced chemiluminescence detection on a panel of 27 individual human liver microsome samples (12 of these were prepared from the same liver samples as those used in the RT-PCR analysis and the rest were obtained commercially). Two antibodies that were monospecific for human CYP1B1 were used; one was prepared in our laboratory and the other was obtained commercially (see Materials and Methods). Both antibody preparations reacted strongly with 0.1 pmol of human recombinant CYP1B1 on immunoblots. As shown in a representative immunoblot (Fig. 5A) probed with the commercial antibody, CYP1B1 protein was not detected in any of the liver microsome samples (lanes 4–10 and 12–17), but human re-

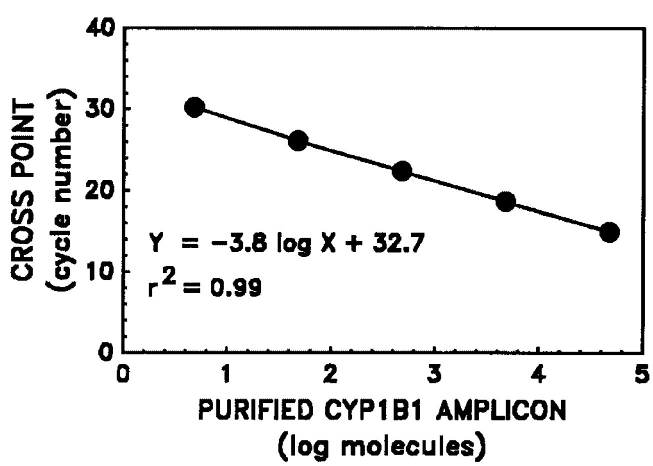

FIG. 2. Calibration curve for the real-time PCR amplification of CYP1B1 cDNA. Shown is a plot of the cross point (Ct) versus known amounts of purified CYP1B1 amplicon. The Ct is the cycle number at which the fluorescence signal is greater than a defined threshold, one in which all the reactions are in the logarithmic phase of amplification.

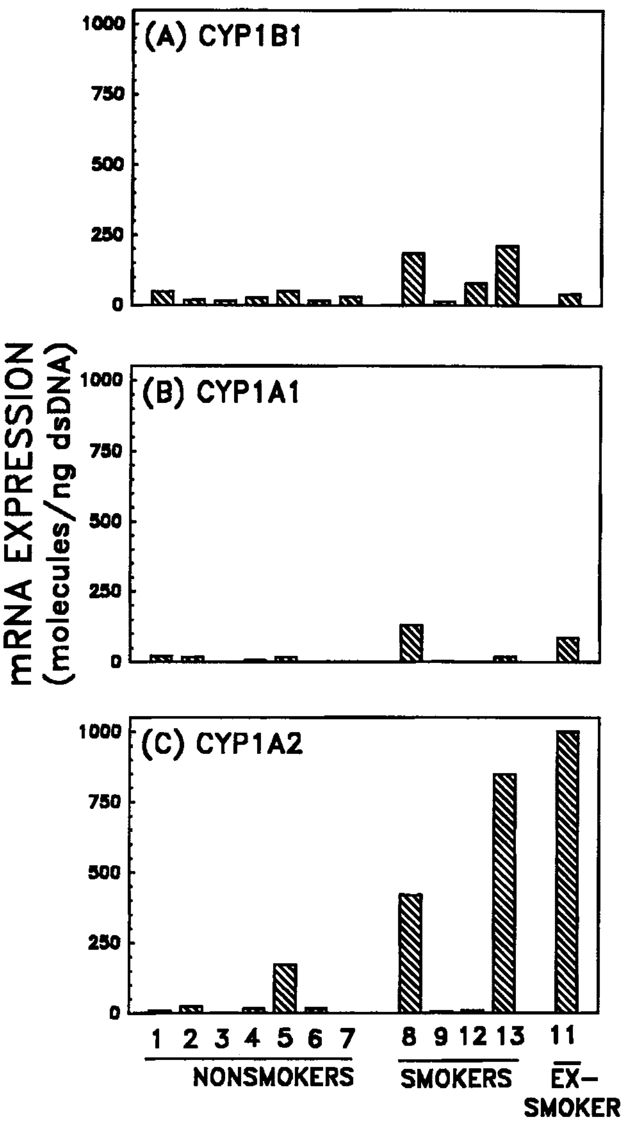

FIG. 3. CYP1B1, CYP1A1, and CYP1A2 mRNA expression in individual human liver samples. Total RNA was isolated from individual liver samples and reverse transcribed. CYP1B1 (A), CYP1A1 (B), and CYP1A2 (C) cDNA samples were amplified by real-time PCR analysis as described in Materials and Methods. Results are expressed as the mean of duplicate determinations for each individual sample. The sample numbers shown in this figure correspond to those in Table 1 and in Figures 6A and 6B.

combinant CYP1B1 protein was detected (lanes 2, 3, 11, 18, and 19). Similar results were obtained with the antibody prepared in our laboratory.

CYP1A1 protein was also not detected in any of the samples (see Fig. 5B for a representative immunoblot), as assessed by immunoblot analyses using enhanced chemiluminescence detection with three different antibody preparations. These were: (1) an antibody prepared to the COOH-terminus of human CYP1A1, which is monospecific for CYP1A1; (2) a mixture of monoclonal antibodies against rat CYP1A1 (C1, C7, and C8), which reacts with human recombinant CYP1A1 but not with human recombinant CYP1A2; and (3) antirat CYP1A2 serum, which reacts with both human CYP1A1 and CYP1A2. A modified discontinuous polyacrylamide gel system that clearly resolved human CYP1A1 and CYP1A2 was used. The antihuman CYP1A serum and the mixture of antibodies against rat CYP1A1 did not react with human liver microsomes, although both easily detected 0.05 pmol of human recombinant CYP1A1 on immunoblots. We estimate that we could detect CYP1A1 protein if it was present at a concentration greater than 0.5 pmol per mg of microsomal protein. With the antihuman CYP1A2 serum, we routinely detected 0.025 pmol of human recombinant CYP1A1 and CYP1A2 proteins by colorimetric detection. This antibody recognized a single band, which had the same electrophoretic mobility as human recombinant CYP1A2, with human liver microsomes. We confirmed this band as CYP1A2, using antibody prepared against a peptide corresponding to the COOH-terminus of human CYP1A2 (provided by P. E. Thomas, Rutgers University, Piscataway, NJ) and which is monospecific for human CYP1A2.

In contrast to CYP1B1 and CYP1A1, the CYP1A2 protein band was visible on immunoblots with all hepatic microsomal samples analyzed (see Fig. 5C for a representative immunoblot). The levels were below the limit of quantitation for three of these samples (all from nonsmokers; Fig. 6A). The mean ± SEM hepatic CYP1A2 protein content was 5.6 ± 1.7 pmol/mg microsomal protein in smokers and 3.3 ± 0.9 pmol/mg microsomal protein in nonsmokers. However, the difference between the means of the two groups was not statistically significant. This is likely due to the considerable inter-individual differences in hepatic CYP1A2 protein content (Figs. 6A and 6B). The variation in CYP1A2 content among smokers and nonsmokers was 23-fold and 72-fold, respectively.

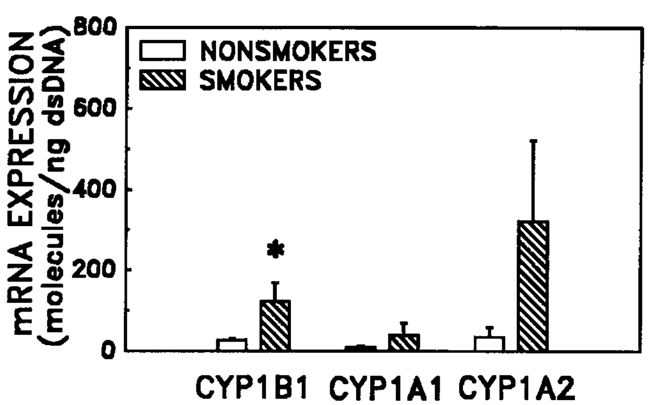

FIG. 4. Group mean CYP1B1, CYP1A1, and CYP1A2 mRNA expression in smokers and nonsmokers. The results from the individual samples (Figs. 3A–3C) were stratified according to smoking status. Shown are the mean (± SEM) for 4 smokers and 7 nonsmokers; *significantly different from the nonsmokers; $p < 0.05$.

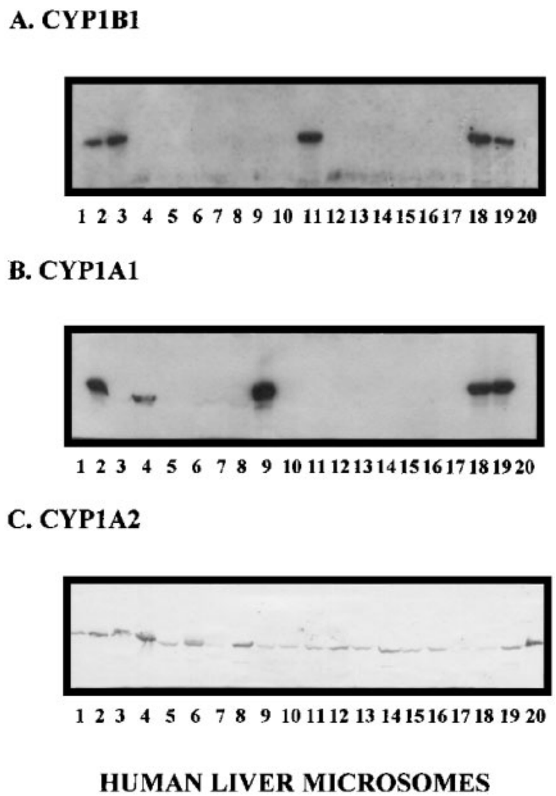

FIG. 5. Immunoblot analysis of CYP1B1, CYP1A1 and CYP1A2 protein expression in liver microsomes. Human liver microsomes were subjected to SDS–PAGE (one individual sample per lane) and immunoblots were probed with antihuman CYP1B1 peptide serum (1:500 dilution, A), antirat CYP1A1 monoclonal IgG (2 μg/ml, B), or antirat CYP1A2 serum (1:250 dilution, C). (A) Lanes 1 and 20, sample dilution buffer; lanes 2 and 19, 0.3 pmol of human recombinant CYP1B1; lanes 3, 11, and 18, 0.6 pmol of CYP1B1; lanes 4–10 and lanes 12–17, liver microsomes from smokers. (B) Lanes 1 and 20, sample dilution buffer; lanes 2 and 19, 0.2 pmol of human recombinant CYP1A1; lane 9, 0.4 pmol of CYP1A1; lanes 3–8 and 10–13, liver microsomes from smokers; lanes 14–16, liver microsomes from nonsmokers; lane 4, a mixture of CYP1A1 and liver microsomes from a smoker; lane 17, 0.4 pmol of human recombinant CYP1A2; and lane 18, a mixture of CYP1A1 and CYP1A2, each at 0.2 pmol. (C) Lanes 1–4, 0.025, 005, 0.1, and 0.2 pmol of human recombinant CYP1A2, respectively; lanes 5–12, liver microsomes from smokers; lanes 13–19, liver microsomes from nonsmokers; and lane 20, 0.1 pmol CYP1A2. Liver microsomes were applied to the gels at 20 and/or 40 μg per lane in A and B and at 10 μg per lane in C.

DISCUSSION

There are conflicting reports regarding CYP1B1 expression in normal human tissues, especially liver. Immunohistochemical analysis with specific antibodies demonstrated the presence of CYP1B1 protein in various human tumors, including those of the breast, kidney, lung, brain, and testis (McFadyen *et al.*, 2001; Murray *et al.*, 2001), and its absence in normal liver and extrahepatic tissues (McFadyen *et al.*, 1999; Murray *et al.*, 1997). Another study reported that CYP1B1 protein was also not detected by immunoblot analysis in microsomes prepared from human liver or kidney cortex samples collected from several individuals (Baker *et al.*, 2001). In contrast, Kadlubar and coworkers detected CYP1B1 protein by immunoblot and immunohistochemical analyses in several normal tissues including liver, kidney, brain, breast, prostate, ovary, testes, and cervix (Muskhelishvili *et al.*, 2001; Tang *et al.*, 1999), with the lowest level found in liver. Low levels of CYP1B1 mRNA were detected in single and multiple human liver samples by Northern-blot analysis (Shimada *et al.*, 1996; Sutter *et al.*, 1994) and RT-PCR (Finnstrom *et al.*, 2001). Muskhelishvili *et al.* (2001) reported that three of nine human liver samples examined were positive for CYP1B1 protein staining on immunoblots but negative for CYP1B1 mRNA using *in situ* hybridization. In the present study, the expression of CYP1B1 mRNA and protein in a panel of human liver samples was investigated by real-time PCR and by immunoblot analysis. CYP1B1 mRNA but not protein was detectable in all 12 human livers examined. We found approximately a 16-fold variation in CYP1B1 mRNA expression among the human

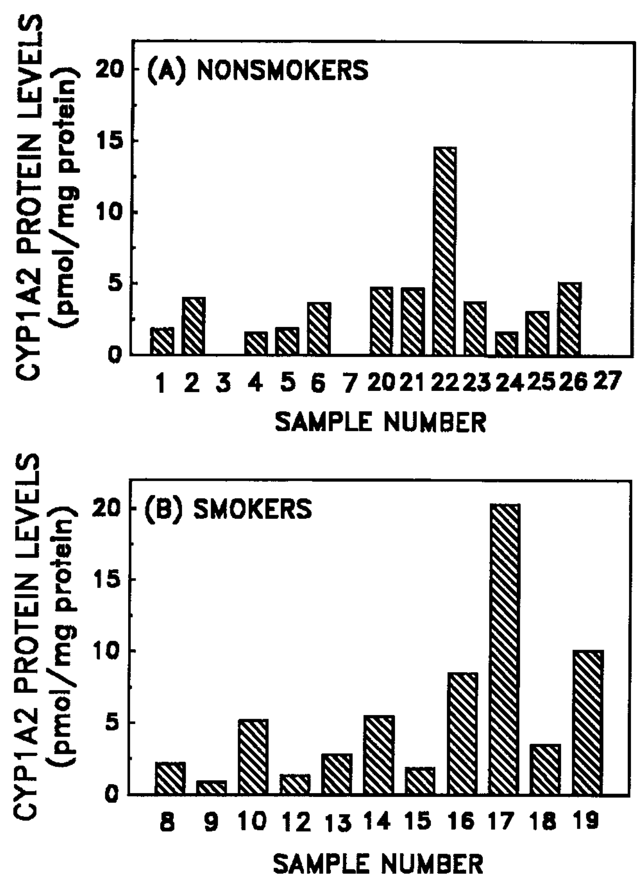

FIG. 6. CYP1A2 protein content in individual human liver samples from smokers and nonsmokers. Shown are the individual CYP1A2 protein content for liver microsome samples from nonsmokers (A, n = 15) and smokers (B, n = 11). CYP1A2 protein was detectable in samples number 3, 7, and 27, but the levels were below the limit of quantitation. The sample numbers shown in this figure correspond to those in Table 1 and Figures 3A–3C. The CYP1A2 protein content in the sample from an ex-smoker (sample number 11, see Table 1) was 1.1 pmol/mg protein.

liver samples. Furthermore, there appears to be an association between smoking and hepatic *CYP1B1* gene expression. The group mean CYP1B1 mRNA level was 5-fold greater in smokers than nonsmokers. An association between smoking and CYP1B1 expression had been proposed, based on the finding that CYP1B1 protein was detected at a greater frequency in bronchoalveolar macrophage samples from smokers than those from nonsmokers (Piipari *et al*., 2000).

Human CYP1A1 expression is considered to be primarily restricted to extrahepatic tissues such as lung and placenta (Wrighton *et al*., 1996). However, it is still controversial as to whether CYP1A1 protein is expressed in human liver. The earlier studies reported the presence of a protein that could be CYP1A1 (Adams *et al.*, 1985; McManus *et al.*, 1988; Schweikl *et al.*, 1993; Wrighton *et al.*, 1986) in human liver, but the data were inconclusive because of the reported or the potential for cross-reactivity of the anti-CYP1A1 antibody preparation, particularly against CYP1A2. In a study that employed an enzyme-specific antibody, CYP1A1 protein was not detected in a panel of individual liver microsome samples ($n = 5$) from renal transplant donors of unknown smoking status (Murray *et al.*, 1993). However, in another study, CYP1A1 protein was reported to be present in 20 individual human liver samples from smokers and nonsmokers, as determined by immunoblot analysis (Drahushuk *et al.*, 1998). In the present study, CYP1A1 protein was undetectable in all human liver samples examined, as determined by immunoblot analyses, using enhanced chemiluminescence detection and several enzyme-specific anti-CYP1A1 antibody preparations. In contrast to CYP1A1 protein, there is general agreement that CYP1A1 mRNA is present in human liver (Hakkola *et al.*, 1994; McKinnon *et al.*, 1991; Omiecinski *et al.*, 1990; Rodriguez-Antona *et al.*, 2001; Schweikl *et al.*, 1993). A novel finding from the present study is that unlike CYP1B1 mRNA, CYP1A1 mRNA was present in some but not all of the liver samples. Moreover, in each of the liver samples analyzed, the level of CYP1A1 mRNA was less than that of CYP1B1 mRNA. The group mean level of CYP1A1 mRNA in our panel of human liver samples was 10-fold less than those of CYP1A2 mRNA. By comparison, a 19-fold difference was reported in the only other study that used real-time PCR analysis (Rodriguez-Antona *et al.*, 2001). In our panel of liver samples, CYP1A1 mRNA levels were not statistically significant between smokers and nonsmokers, but this was because of the substantial inter-individual variability in CYP1A1 mRNA expression, which had been reported in other studies (Rodriguez-Antona *et al.*, 2001; Schweikl *et al.*, 1993).

It is generally agreed that CYP1A2 is expressed constitutively but variably in human liver. CYP1A2 protein was detected by immunoblot analysis in 20 of 21 human liver samples in one study (Schweikl *et al.*, 1993) and 28 of 28 liver samples in another study (Baker *et al.*, 2001). Both studies noted that hepatic CYP1A2 levels were greater in samples from people with high exposure to cigarette smoke (Baker *et al.*, 2001; Schweikl *et al.*, 1993). Smoking has been associated with greater levels of human hepatic CYP1A2 protein content and CYP1A-mediated enzyme activities in several other studies, but with considerable variation in both variables among smokers and nonsmokers (Pelkonen *et al.*, 1986; Sesardic *et al.*, 1988; Fleischmann *et al.*, 1986; Lucas *et al.*, 1993). Phenacetin *O*-deethylase, caffeine 3-demethylase, and ethoxyresorufin *O*-deethylase activities were shown to vary by more than 50-fold (Butler *et al.*, 1989; Shimada *et al.*, 1994) and CYP1A2 protein content by more than 10-fold (Wrighton *et al.*, 1986) or more than 40-fold (Schweikl *et al.*, 1993) in humans. In the present study, CYP1A2 protein was detectable in all 27 human liver microsome samples examined, and the levels were quantifiable in all but three samples. The lowest value of CYP1A2 content (<0.2 pmol/mg, i.e. below the limit of quantitation) was obtained in a sample from a nonsmoker and the highest value of 20.3 pmol/mg microsomal protein was obtained in a sample from a smoker, representing a difference of at least 100-fold. The group mean (± SEM) hepatic CYP1A2 protein content was 4.3 ± 0.9 pmol/mg microsomal protein. This value is approximately 10 times lower than that reported in another study (Shimada *et al.*, 1994). In agreement with other studies (Hakkola *et al.*, 1994; McKinnon *et al.*, 1991; Rodriguez-Antona *et al.*, 2001; Schweikl *et al.*, 1993), CYP1A2 mRNA expression was detectable in all samples, although it was below the limit of quantitation in two samples. Among the samples in which the levels were quantifiable, the inter-individual variability in CYP1A2 mRNA expression was 500-fold. By comparison, in the only other real-time PCR analysis of CYP1A2 mRNA reported to date, a 582-fold difference was obtained in a panel of 12 individual human liver samples (Rodriguez-Antona *et al.*, 2001). In the present study, CYP1A2 mRNA and protein levels were not significantly correlated ($r^2 = 0.05$, $p = 0.82$) when all 12 human liver samples were analyzed, but a statistically significant correlation ($r^2 = 0.60$, $p = 0.01$) was obtained when samples 2, 6, and 11 were omitted from the analysis. In a previous study, a statistically significant but weak correlation ($r^2 = 0.34$) was obtained between CYP1A2 mRNA and protein levels (Schweikl *et al.*, 1993). Collectively, these data highlight the importance of measuring both mRNA and protein content in studies of CYP enzyme expression.

In agreement with a previous study (Schweikl *et al.*, 1993), a significant correlation ($r^2 = 0.51$) was obtained between CYP1A1 mRNA and CYP1A2 mRNA levels in our panel of human liver samples. A novel finding from the present study is the lack of correlation between levels of CYP1B1 mRNA and CYP1A1 mRNA or between CYP1B1 mRNA and CYP1A2 mRNA. Consistent with these data are reports of differential regulation of CYP1B1 and CYP1A in cultured breast cancer cells by agonists of the Ah receptor (Spink *et al.*, 1998; Coumoul *et al.*, 2001) and differential time-course and dose-response relationships in the induction of hepatic *CYP1B1* and *CYP1A* in rats by TCDD (Santostefano *et al.*, 1997). Together, these data support the notion that while CYP1B1 and CYP1A can be co-expressed, their expression is not subject to the same regulatory control.

Considerable inter-individual variability exists in *CYP1B1*, *CYP1A1*, and *CYP1A2* gene expression in human liver. An explanation for this finding is that the expression of these genes is subject to modulation by environmental factors; for example, CYP1 genes are inducible by polycyclic aromatic hydrocarbons such as those found in cigarette smoke (Murray *et al.*, 2001; Wrighton *et al.*, 1996). However, genetic factors may also play a role. In a recent study, it was reported that a specific set of mutations in the human Ah receptor abolishes *CYP1A1* inducibility (Wong *et al.*, 2001).

In summary, the major findings from the current investigation of *CYP1* expression in human livers are: (1) CYP1B1 mRNA was expressed in all the samples analyzed and the levels were greater in samples from smokers than those from nonsmokers, but CYP1B1 protein was undetectable in any of the samples; (2) CYP1A1 mRNA was detected in some but not all of the samples and CYP1A1 protein was not detected in any of the samples; (3) both CYP1A2 protein and mRNA were expressed in samples from smokers and nonsmokers; (4) considerable inter-individual differences were obtained in CYP1B1, CYP1A1, and CYP1A2 gene expression; and (5) no correlation existed between CYP1B1 and CYP1A mRNA expression, whereas there was a significant positive correlation between CYP1A1 and CYP1A2 mRNA levels.

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