Intrinsic Hepatic Phenotype Associated with the Cyp1a2 Gene as Shown by cDNA Expression Microarray Analysis of the Knockout Mouse

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Several forms of cytochrome P450 (CYP) appear to metabolize principally pharmaceutical agents, as well as other dietary and plant chemicals. Other CYP forms have major roles in steroid, sterol, and bile acid metabolism. CYP1A2 expression is constitutively high in mouse liver and is well known for metabolizing several drugs and many procarcinogens to reactive intermediates that can cause toxicity or cancer. CYP1A2 is also known to carry out several endogenous functions such as uroporphyrinogen and melatonin oxidation and the 2- and 4-hydroxylations of estradiol. We have used cDNA microarray analysis of the untreated Cyp1a2−/− knockout mouse to search for changes in gene expression that might indicate important intrinsic roles for this enzyme. For 15 of the up- or downregulated genes, these increases or decreases were corroborated by reverse-transcription real-time polymerase chain reaction. Other than upregulation of the Hprt gene (used in the selection procedure for disrupting the Cyp1a2 gene), we found several upregulated genes that are associated with cell-cycle regulation and lipid metabolism. Besides Cyp1a2, the gene exhibiting the greatest downregulation was Igfbp1 (insulin-like growth factor binding protein-1), showing only 12% expression of that in the Cyp1a2+/+ wild-type liver. Recurrent themes between both up- and downregulated genes include cell-cycle control, insulin action, lipogenesis, and fatty acid and cholesterol biosynthetic pathways. Histologically, the Cyp1a2−/− mouse exhibited an approximately 50% decrease in lipid stored in hepatocytes, and 50% increase in lipid present in interstitial fat-storing cells compared with that in the Cyp1a2+/+ wild-type. These data suggest that the CYP1A2 enzyme might perform additional hepatic endogenous functions heretofore not appreciated.

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During more than 2.5 billion years of evolution, it is likely that cytochrome P450 (CYP) genes first appeared in prokaryotes and then in early eukaryotes to carry out important roles in critical life processes; following the divergence of plants and animals some 1.8 billion years ago and especially after the radiation of innumerable phyla about 543 million years ago, animal CYP enzymes then took on the functions of metabolizing many plant products and other environmental chemicals that were consumed, inhaled, or in contact with the animal’s skin (Nebert 1997; Nebert and Dieter 2000). For some CYP forms, evidence clearly indicates that their principal functions include the oxidative metabolism of endogenous molecules such as steroids, sterols, bile acids, retinoic acid, and many of the >100 eicosanoids (Nebert and Russell 2002). In addition, it is likely there are still many endogenous roles of CYP of which we remain ignorant. For instance, although CYP1A1/A2/B1 induction is well established in the metabolism of pro-carcinogenic polycyclic aromatic hydrocarbons and arylamines, these three enzymes are suggested to have pivotal functions in degrading the putative endogenous ligand(s) of the aryl hydrocarbon (AH) receptor and perhaps participate in apoptosis and cell-cycle regulation (Nebert et al. 2000b).

Among the few abundant CYPs that are expressed constitutively in mammalian liver, CYP1A2 carries out several known endogenous functions such as uroporphyrinogen and melatonin oxidation and the 2- and especially 4-hydroxylations of estradiol (Nebert and Russell 2002). The Cyp1a2−/− knockout mouse, however, has no apparent overt phenotype or problems with viability or fertility (Liang et al. 1996). These findings suggest that, in the absence of a foreign chemical, the expression of CYP1A2 might be redundant. In humans, CYP1A2-mediated activity varies ~60-fold between individuals (Nebert 1997; Eaton et al. 1995; Nebert et al. 1996; Dorne et al. 2001), also with no overt phenotype; most of the variation does not depend on lifestyle or nutrition but is likely to be genetically determined (Le Marchand et al. 1997). It seems unlikely, however, that high constitutive CYP1A2 expression in mammalian liver continues without a particular purpose, whereas CYP1A1 expression occurs only under conditions of ligand-activation of the AH receptor (Nebert et al. 2000b). CYP1A2 is absent in fish but present in birds and mammals, suggesting that between 380 million and 320 million years ago this gene arose from CYP1A1 by way of a duplication event; most likely, the duplicated CYP1A2 gene “drifted” from CYP1A1 until it became involved in one or more critical life functions or in the metabolism of dietary components or environmental chemicals, such that the animal gained a reproductive or survival advantage (Heilman et al. 1988; Nebert et al. 1991).

The development of mice with disruptions in specific genes allows testing of hypotheses that various CYPs might have physiologic roles not yet identified (Nebert and Duffy 1997). Many knockout mouse lines show profoundly altered phenotypes from normal, including lethality during embryogenesis if the gene participates in a nonredundant component of a metabolic pathway. In one of our laboratories we have been particularly interested in the effect of knocking out members of the Cyp1a family. For example, Cyp1a1−/− knockout mice are fertile and healthy and show no apparent changes in the activity or expression of other genes in the [Ah] gene battery, including Cyp1a2, following administration of AH receptor ligands (Dalton et al. 2000a). Similarly, the untreated or inducer-treated Cyp1a2−/− null mouse shows no apparent changes in expression of other genes in the [Ah] gene battery, including Cyp1a1 (Liang et al. 1997). On the other hand, both Cyp1a1−/− and Cyp1a2−/− lines show modified biochemical responses to foreign chemicals (Liang et al. 1996; Dalton et al. 2000a; Liang et al. 1997; Pineau et al. 1995; Buters et al. 1996; Peters et al. 1999).

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expression differences between the cDNA microarrays to compare hepatic gene constitutive expression of mediated gene expression and that, in the substrate that modulates AH receptor—that CYP1A1 metabolizes an endogenous (Zaccaro et al. 2001). It has been proposed involve production of a reactive metabolite. be functioning in a mode that does not 1998; Sinclair et al. 2000; Smith et al. 4-aminobiphenyl-induced methemoglobinemia (Shertzer et al. 1998; Sinclair et al. 2000; Smith et al. 2001); in these cases, CYP1A2 appears to be functioning in a mode that does not involve production of a reactive metabolite. Other studies suggest that CYP1A2 might have a role in bilirubin metabolism (Zaccaro et al. 2001). It has been proposed that CYP1A1 metabolizes an endogenous substrate that modulates AH receptor-mediated gene expression and that, in the liver, CYP1A2 can take over this role (Nebert et al. 2000b). Since there is no constitutive expression of Cpaa1 in the liver, it would be interesting to investigate whether nonpathologic changes in hepatic metabolism might occur in the complete absence of constitutive CYP1A2. We have thus used cDNA microarrays to compare hepatic gene expression differences between the untreated Cyp1a2(−/−) knockout and the Cyp1a2(+/+) wild-type mouse.

Materials and Methods

The Cyp1a2(−/−) mouse line. Knockout mice had been generated by removing portions of exons 2 and all of exons 3–5 of the Cyp1a2 gene; insertion of the Hprt mini-gene cassette was used for selection in embryonic stem (ES) cell cultures (Liang et al. 1996). Although the mice were originally generated from a mixture of the C57BL/6J and 129/J inbred strains, the Cyp1a2(−/−) genotype was subsequently backcrossed into the C57BL/6J strain to a theoretical level of >99.8%; if the mouse genome contains 40,000 genes, this would mean that the Cyp1a2(−/−) line in the Nebernt mouse colony should have fewer than about 80 genes that might be expected to be of 129/J origin (Nebert et al. 2000a). For this reason, C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME, USA) were used as the Cyp1a2(+/+) controls in the present experiments. We used untreated males, approximately 10 weeks of age, in the microarray studies; females and males were compared in the histologic studies.

Mouse EST clones and preparation of cDNA. The arrays comprised 4,246 mouse expressed-sequence-tag (EST) clones (2,783 individual Genbank clusters). Two-thirds of the clones were obtained from the I.M.A.G.E. collections held at the MRC Human Gene Mapping Project (http://www.hgmp.mrc.ac.uk). The remaining one-third of the EST clones were obtained from Research Genetics (RG9 set; http://www.resgen.com). All clones described in this article were verified by sequence analysis. cDNA from the EST was obtained via polymerase chain reaction (PCR) amplification using plasmid-specific primers. The PCR products were separated by electrophoresis on agarose gels to ensure that only a single product was obtained for each clone. The reaction products were precipitated and prepared for array, using methods described (DeRisi et al. 1997; Eisen and Brown 1999).

Printing of the arrays. Arrays were printed on poly-l-lysine-coated slides. UV-cross-linked, and blocked prior to use (DeRisi et al. 1997; Eisen and Brown 1999; Turton et al. 2001). The arrays were printed using an arrayer built essentially according to the Stanford designs (cf. http://www.le.ac.uk/cmht/microarray_lab/ Home.htm). The center-to-center distance of the features was 210 µm, and each feature was 90–100 µm in diameter.

Labeling and hybridizations. Total RNA was prepared from mouse liver by sedimentation through CsCl. The RNA of five individual Cyp1a2(+/+) wild-type mice and five individual Cyp1a2(−/−) knockout mice were each separately labeled with both Cy3 dUTP and Cy5 dUTP. RNA labeling was carried out essentially as described (DeRisi et al. 1997; Eisen and Brown 1999; Turton et al. 2001). Priming was achieved with the oligo dT(25), using 4 µg of the oligo with 50 µg of total RNA. After denaturation at 70°C for 8 min, annealing was allowed to occur as the temperature fell to 42°C over 30 min. At this point, dNTPs (Pharmacia/Amersham, Bucks, UK) were added to final concentrations of 0.5 mM, with the exception of dTTP, which was at 0.2 mM. The desired Cy-labeled dUTP (Pharmacia/Amersham) was then added to a final concentration of 0.1 mM. We used the 1X first-strand buffer (Gibco/Invitrogen, Paisley, Scotland). RNAsin (20 U) was added to the reaction. Transcription was initiated by addition of 100 U of Superscript II (Gibco/Invitrogen) and allowed to proceed for 1 hr at 42°C before addition of a second 100 U of Superscript II and another 1-hr incubation at 42°C. RNA was removed from the synthesized cDNA by addition of NaOH/EDTA/sodium dodecyl sulfate (SDS) to final concentrations of 0.195 M/10 mM/0.22%, respectively, and incubated at 70°C for 10 min. The reaction was neutralized by addition of HCl and buffered to pH 7.5 by the addition of Tris–HCl. The reaction products were purified by passage through a Centri-Sep column (Princeton Separations, Inc., Adelphi, NJ, USA), dried, and resuspended in hybridization buffer. Prior to hybridization, the samples were heated to 100°C for 2 min, then at 42°C for at least 30 min.

Hybridizations were conducted in humidified chambers at 42°C for approximately 16 hr. The hybridization buffer consisted of 50% deionized formamide/0.5% SDS/6x SSPE (1 M NaCl/0.33 mM NaHPO4/6.6 mM EDTA, pH 7.4) and 2.5× Denhardt’s/s0.06 µg/µL of polyA800/0.66 µg/µL human Cot 1 DNA/0.27 µg/µL yeast tRNA. The arrays were washed in 1.0x SSC (0.1 M NaCl/0.015 M Na citrate, pH 7.0), 0.03% SDS for 10 min, then washed in 0.2x SSC for 5 min, and a final wash in 0.05x SSC for 5 min at room temperature. The slides were dried by centrifugation at 800 rpm for 5 min. For each pair of Cyp1a2(+/+) and Cyp1a2(−/−) mice, the Cy3-labeled cDNA product was hybridized against the Cy5-labeled cDNA product, and vice versa—giving 10 separate hybridizations from the five pairs of animals being compared.

Analysis of fluorescence and data processing. The fluorescence of all the features on the slides was measured using the GenePix software (version 3.0.0.85; Axon Instruments, Union City, CA, USA). Feature sizes were determined using the inbuilt automated parameters in the first instance and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined, and the median fluorescence of these pixel measurements was taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default GenePix parameters. The raw feature data for each channel were globally centered by reference to the median fluorescence of the whole feature set for that channel. The changes in gene expression obtained are shown as means ± standard deviations of the ratio between the Cy3 and Cy5 channels for 10 pairs of hybridizations.

Reverse-transcription real-time PCR using SYBR Green. For an alternative estimation of changes in gene expression, the relative mRNA levels of genes of interest were determined by comparison with a
mouse endogenous control gene, β-actin (Actb). The primers used (Table 4) were designed to cross exon-exon boundaries to eliminate the detection of any contaminating genomic DNA.

In the first step, cDNA was synthesized from total RNA. A 1.450-µL master mix was made up as follows: 200 µL PCR buffer, (catalog no. Y02028; Gibco/Invitrogen); 100 µL MgCl2 (50 mM); 20 µL each of dATP, dCTP, dGTP, dTTP (all 100 mM); 19.8 µL random hexamers (catalog no. 27-2166-01; Amersham; 90 OD U/µL); dithiothreitol (Gibco 400147); and 1,030.2 µL water. For cDNA synthesis, a 40-µL reaction contained: 29 µL of the master mix; 8 µL total RNA (400 ng); 40 U RNasin (catalog no. N2118; Promega, Southampton, UK); and 400 U SuperScript II reverse transcriptase (Gibco/Invitrogen). The mixture was heated for 10 min at 23°C, then for 30 min at 42°C, and finally for 10 min at 99°C. In the second step, 1 µL of the product of the cDNA synthesis (from 10 ng RNA), or a non-template control, was incubated with 24 µL SYBR Green PCR Master Mix (part no. 4309155; Applied Biosystems, Warrenton, UK) containing 900 nM forward primer and 300 nM reverse primer in an ABI PRISM 7700 Sequence Detection System. The thermal-cycler protocol was stage one, 50°C for 2 min; stage two, 95°C for 10 min; stage three, 40 cycles at 95°C for 15 sec and 65°C for 1 min. For every sample of Cyp1a2(+/-) and Cyp1a2(–/-) liver, the relative level of each gene examined was compared with that for β-actin. A comparison was then made for the expression of the gene between the wild-type and the knockout animal.

**Histological analysis of liver.** Liver was prepared for routine light- and electron-microscopic histochemistry and morphometry (Dalton et al. 2000b). Twenty-eight Cyp1a2(–/-) and 28 Cyp1a2(+/-) mice were used, with equal numbers of males and females (8–10 weeks of age). Phase-contrast microscopy of toluidine blue-stained 1.5-micron–thick plastic sections was used to quantify the relative amounts of parenchymal and interstitial cells and to determine the volume density of hepatocyte- and of interstitial fat, glycogen pools, and necrosis. A grid of 75 intersections was visualized over the light-microscopic image using a Zeiss Photomik light-microscopy (Carl Zeiss GmbH, Vienna, Austria) and camera lucida, and the number of positive intersections lying over hepatocytes, interstitial cells, lipid, and glycogen was counted. The volume density (Vd) of each parameter was determined by dividing those values by the total number of positive intersections lying over the entire tissue. Statistical analysis of microarray data was performed using the two-tailed paired t-test, taking the reference ratio for the population as 1.0 and comparing this with the ratio of interest. Means and standard-errors-of-the-mean were obtained from morphometric data, using the General Linear Model of SAS 6.1 (SAS Institute, Inc., Cary, NC, USA). A p <0.05 value was regarded as statistically significant.

**Results**

cDNA expression microarray. To investigate potential biochemical phenotypic differences, we compared constitutive hepatic gene expression of Cyp1a2(–/-) mice with that of aged-matched Cyp1a2(+/-) mice, using cDNA microarrays. Labeling of samples with both Cy3 and Cy5 dyes (reverse-labeling) was performed to take into account any methodologic bias. Our information was thus based on 10 separate hybridizations from five mice. Although it is often customary in array work to use an arbitrary cut-off at a 2-fold change in expression, we believe that 2-fold alterations can be important in critical-life-process pathways and therefore chose to list all expressions that had changed significantly as assessed statistically at p <0.05 (Tables 1, 2). Only a relatively few genes were detected that exhibited significant up-or down-regulation. With the particular array used, we found a greater number of downregulated than upregulated genes in the Cyp1a2(–/-) mouse. The >5-fold elevation in Hprt gene expression (Table 1) is likely explained by the Hprt minigene cassette used in the selective elimination to disrupt the Cyp1a2 gene.

| Table 1. Hepatic genes significantly expressed to a greater extent in Cyp1a2(+/-) compared with Cyp1a2(–/-) mice. |
| Description | Symbol | Accession no. | Mean | SD |
| Hypoxanthine guanine phosphoribosyltransferase | Hprt | BF148132 | 5.38 | 1.80 |
| Growth arrest and DNA-damage-inducible 45 gama | Gadd45g | A1227072 | 3.16 | 1.08 |
| CYTOMEGA P450 2a4 | Cyp2a4 | A1118260 | 1.94 | 0.50 |
| Major urinary protein-1 | Mup1 | AI225954 | 1.89 | 1.06 |
| RIKEN cDNA 0610010A22 gene | 0610010A22Rik | AA198519 | 1.85 | 0.52 |
| CYP450 2A4, steroid inducible 3a11 | Cyp3a11 | A0295384 | 1.67 | 0.75 |
| EF4 transcription factor-1 | E4f1 | AA270628 | 1.65 | 0.76 |
| CYP450 7b1 | Cyp7b1 | A0287063 | 1.64 | 0.89 |
| Glutathione S-transferase, pi 2 | Gstp2 | AA165555 | 1.59 | 0.46 |
| Ferritin light chain-1 | Ftl1 | A059749 | 1.61 | 0.70 |
| NADPH-P450 (cytochrome) oxidoreductase | Por | B144049 | 1.53 | 0.38 |

*From GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). *Ratio of expression in the Cyp1a2(–/-) compared with that in the Cyp1a2(+/+) mouse. Results are means of five mice per line and significantly different at p <0.05. **This may arise, in part or in total, from the HAT-selective minigene used for the selection of the transfected ES cells. **Also probably recognizing CYP2A5 cDNA and any other mouse CYP2A cDNA.

| Table 2. Hepatic genes significantly expressed to a lower degree in Cyp1a2(+/-) compared with that in Cyp1a2(–/-) mice. |
| Description | Symbol | Accession no. | Mean | SD |
| CYP450 2a, aromatic compound inducible | Cyp2a2 | AA242380 | <0.01 | <0.01 |
| Insulin-like growth factor binding protein-1 | Igfbp1 | W10686 | 0.12 | 0.08 |
| GU51 switch gene-2 | Gbs2 | NM_008059 | 0.29 | 0.09 |
| Fatty acid synthase | Fasn | BF31259 | 0.27 | 0.15 |
| CYP450 4a14 | Cyp4a14 | A163852 | 0.28 | 0.26 |
| Sterol-oxidase-3 A desaturase-1 | Scd1 | AA269438 | 0.33 | 0.17 |
| 3-Hydroxy-3-methylglutaryl–coenzyme A reductase | Hmgcr | W18347 | 0.36 | 0.25 |
| Glucokinase | Gk | AI194797 | 0.38 | 0.18 |
| Fatty acid-binding protein 2, intestinal | Fabp2 | AA56309 | 0.40 | 0.08 |
| Similar to tyrosine aminotransferase | AI225954 | 0.41 | 0.22 |
| CYP450 4a10 | Cyp4a10 | A081842 | 0.42 | 0.15 |
| UDP-glucose ceramide glucosyltransferase | Ugcg | A116263 | 0.45 | 0.28 |
| Protein tyrosine phosphatase, nonreceptor type 16 | Ptpn16 | A1205860 | 0.45 | 0.37 |
| Glutamyl aminopeptidase | Enpep | A170635 | 0.45 | 0.33 |
| Phosphoenolpyruvate carboxykinase-1, cytosolic | Pck1 | A063800 | 0.47 | 0.34 |
| Pre-B-cell leukemia transcription factor 1 | Pbx1 | W05267 | 0.48 | 0.39 |
| Apolipoprotein A-IV | Apoa4 | A058545 | 0.54 | 0.15 |
| Serum amyloid P-component | Sapl | AI157330 | 0.57 | 0.27 |
| Pyruvate dehydrogenase E1alpha subunit | Pdh1 | AW106750 | 0.58 | 0.19 |
| Farnesyl diphosphate synthetase | Fdps | BE988882 | 0.58 | 0.25 |
| Glutathione dehydrogenase | Gud | W03390 | 0.61 | 0.37 |

*From GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). **Ratio of expression in the Cyp1a2(–/-) compared with that in the Cyp1a2(+/+) mouse. Results are means of five mice per line and significantly different at p <0.05.
(Liang et al. 1996), or any other gene (van der Lugt et al. 1991), in ES cells. The Gadd43 gene, the Spr1 gene, and the Cyp2a5 gene(s) were upregulated 3.2- to 2.4-fold, respectively. In addition to the marked decrease in Cyp1a2 gene expression (Table 2), as expected, several other genes were downregulated by 2.5- to 5-fold more. These included the lgalp1, Gbh2, Fasn, Cyp4a14, Sct1, Hmgcr, Gk and Fabp2 genes. Recurrent functional themes among both these up- and downregulated genes include cell-cycle control, insulin action, lipogenesis, and fatty acid and cholesterol biosynthetic pathways.

Besides Cyp1a2, there were at least nine other mouse Cyp genes upregulated (Table 1) and two others downregulated (Table 2). We also documented in our microarray that the expression of at least nine other mouse Cyp genes was not significantly altered (Table 3). Expression of genes encoding the AH receptor and both forms of heme oxygenase was also not significantly changed (Table 3).

### Table 3. Hepatic Cyp and other possibly relevant genes whose expression is not significantly different between the Cyp1a2(+/−) and Cyp1a2(+/+) mice.

| Description | Symbol | Accession no. | Mean | SD |
|-------------|--------|---------------|------|----|
| Cytochrome P450, 17 | Cyp17 | AA081768 | 0.80 | 0.19 |
| Cytochrome P450, 2a12 | Cyp2a12 | A256530 | 1.00 | 0.16 |
| Cytochrome P450, 2b10, phenobarbital-inducible, type b | Cyp2b10 | AI196307 | 1.02 | 0.20 |
| Cytochrome P450, 2c29 | Cyp2c29 | A6529126 | 1.10 | 0.37 |
| Cytochrome P450, 2d9 | Cyp2d9 | A886388 | 0.94 | 0.15 |
| Cytochrome P450, 2a1, ethanol-inducible | Cyp2a1 | A717630 | 0.92 | 0.43 |
| Cytochrome P450, 2t2 | Cyp2t2 | A242462 | 1.17 | 0.31 |
| Cytochrome P450, 2j6 | Cyp2j6 | A329304 | 0.72 | 0.21 |
| Cytochrome P450, 3a25 | Cyp3a25 | A1081713 | 1.15 | 0.37 |
| Aryl-hydrocarbon receptor | Ahr | A7247836 | 1.19 | 0.37 |
| Heme oxygenase (deciing)-1 | Hmox1 | W08692 | 1.30 | 0.16 |
| Heme oxygenase (deciing)-2 | Hmox2 | A1428016 | 1.00 | 0.23 |

*From GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene).

### Table 4. Real-time PCR changes in expression of some hepatic genes in Cyp1a2(−/−) compared with that in Cyp1a2(+/+) mice.

| Expression | Mean | SD | Forward primer | Reverse primer |
|------------|------|----|----------------|----------------|
| Upregulation |      |    |                |                |
| Gadd43    | 4.26 | 2.57 | GACGCCTGGCGCTCAGCA | GCACGCAAGTTCATGTT |
| Cyp2a4/2a5 | 2.40 | 0.97 | TCAGAGGCGCATCCAA | GACGTGTGGCTAAGTGAAAGG |
| Mup1      | 1.72 | 0.56 | GGGAAACTTCTCATGGTAG | GGATCTTACGTCCTCACAGT |
| Cyp3a11   | 1.36 | 0.55 | TTAAGAATTGGTAGAGAAGATTT | CATCCTAGTATGAGATCTACTTAAAA |
| Cyp7b1    | 1.42 | 0.54 | GCTGTCCGCCCTGTCCTCT | GGSGGCGCAGAAGT |
| Por       | 1.21 | 0.34 | GCTGGAGGCCGCCCTCACTA | CGCACTGTGSCGCAAGGATTT |

| Downregulation |      |    |                |                |
| Igg6p1    | 0.09 | 0.05 | CCATGACCACCTATGACAGCAT | ATTGTTAGATGCCTCATCCTGGCTTCCTCT |
| Gbh2      | 0.13 | 0.03 | AACGCGAAGCAGCAGTCTGA | GTCGGCCAAGGGATCA |
| Fasn      | 0.21 | 0.10 | CATGGTTGGTGGATGGTAG | GACGCCTTGGAATCAGTAA |
| Cyp4a14   | 0.11 | 0.12 | CAGAACCTCCAGCATCTTCC | GCCGCCGAGAACAGCTGAA |
| Sct1      | 0.24 | 0.10 | CCAGAAAGAGCAGTGTTGCTG | GCTGGCGGAGAGATCAGG |
| Hmgcr     | 0.37 | 0.25 | CCTGAGAACAGGTGCTGAGCAT | GTCGACACATGAGTGGTGCTGTTG |
| Gk        | 0.33 | 0.05 | GCACACCTGTGGTGGTGGAS | GCCGTGTGGCTCAGATG |
| Fabp2     | 0.43 | 0.13 | CCTTGCGAGAGACTGAGAACATC | CGCAGGCTTCTGCAGCATCATT |
| Pck1      | 0.53 | 0.27 | TGCTGAGAGAGACTGAGAACATAGA | TGCTGAGTGGAGATACATAG |

*Ratio of expression in the Cyp1a2(−/−) compared with that in the Cyp1a2(+/+) mouse, relative to that of Actb (forward primer, GATTACTGCTGGTGGTGGTGG; reverse primer, GCCACGAGTCACAGAAGT). Results are means ± SD of three to four Cyp1a2(−/−) and three to four Cyp1a2(+/+) mice.

Reverse-transcription real-time PCR. To prove the accuracy of the cDNA microarray, we chose 15 of the genes that had exhibited significant differences in expression between the genotypes (in Tables 1, 2) for further analysis by reverse-transcription real-time PCR, and we used Actb expression as a reference “housekeeping” gene. For the 6 upregulated and the 9 downregulated genes investigated (Table 4), there was good agreement with those changes that had been observed using the cDNA arrays. The data in Table 4 thus confirm the robustness of the cDNA expression microarray approach.

### Discussion

The data in this study strongly suggest that mouse hepatic CYP1A2 is involved in a number of previously unrecognized endogenous functions. These findings are in contrast to popular opinion that CYP1A2 exists solely for the metabolism of pharmaceuticals and other environmental chemicals.

The Vd of hepatic glycogen pools was slightly but not significantly greater in the Cyp1a2(−/−) mouse. The increase in Cyp1a2(−/−) interstitial fat accounted for a small increase in the Vd of interstitium overall, which had a p value of 0.089. Total lipid stores were significantly (p < 0.035) decreased in the Cyp1a2(−/−) liver compared with that in Cyp1a2(+/+) liver. This decrease principally reflected a Vd of hepatocyte-containing lipid droplets in Cyp1a2(−/−) liver that was approximately 55% of that seen in Cyp1a2(+/+) liver. On the other hand, there was a 45% increase in the Vd of lipid found within the Cyp1a2(−/−) interstitial fat-storing cells compared with that in Cyp1a2(+/+) liver. Gender influenced the response, with females showing a greater increase than males in fat stored in interstitial cells; in retrospect, therefore, it might have been worthwhile in the microarray experiments to include comparisons between males and females.

There were no significant differences in hepatocyte necrosis, inflammatory infiltrate, birefringent hepatocytes, or number of apoptotic hepatocytes that could be attributed to the loss of CYP1A2. The mitotic index in Cyp1a2(−/−) liver was slightly elevated but not statistically significant compared with that in Cyp1a2(+/+) liver (p = 0.16).

Discussion
Cell cycle control. The >3-fold upregulation of the Gadd45g gene in Cyp1a2(−/−) mice suggests there is some cellular stress (Fornace et al. 1989), perhaps in genomic stability, or alterations in cell cycle control that might occur in the absence of hepatic CYP1A2 constitutive expression; GADD45y is also associated with apoptosis (Hollander et al. 2001), though this was not reflected in an increase in apoptotic hepatocytes. This marked increase in Gadd45y expression could be related to disruption of the Cyp1a2 gene directly, or to some secondary response caused by the complete absence of CYP1A2 activity. It might also be pertinent that there is a 5-fold downregulation of the G0/G1 switch gene-2 (G0s2) (Table 2) and a 65% increase in the E4f1 transcription factor (E4f1) gene (Table 1), both involved in cell-cycle regulation. Likely reasons for CYP1A2 involvement in oxidative stress, cell cycle control, and apoptosis have been recently reviewed (Nebert et al. 2000b). Histologically, however, the mitotic index in the Cyp1a2(−/−) mice is only slightly elevated (not statistically significant), and the percent binucleated hepatocytes (which reflects hepatocyte hypertrophy and karyokinesis without cytokinesis) in Cyp1a2(−/−) is almost identical to that in the Cyp1a2(+/+) wild type.

Insulin action, lipogenesis, and fatty acid and cholesterol biosynthetic pathways. Other than Cyp1a2, the gene exhibiting apparently the greatest downregulation was Igfbp1 (insulin-like growth factor binding protein-1), which showed only 12% expression of that in the Cyp1a2(+/+) wild-type mouse (Table 2). Again, this may be linked to an overall downregulation of cell proliferation or alterations in lipid metabolism. The Igfbp1 promoter shares common insulin regulatory response elements with the phosphoenolpyruvate carboxykinase (Pck1) gene, which catalyzes the rate-limiting and committed step in gluconeogenesis, although different mechanisms are used by insulin to inhibit expression of these two genes (Yeagley et al. 2001). Pck1 was downregulated more than 2-fold in the Cyp1a2(−/−) mouse (Table 2). Interestingly, glucokinase, another gene associated with insulin action, was also downregulated. In hepatocytes, insulin stimulates a rapid increase in transcription of the gene encoding for sterol response-binding protein-1c, which has been implicated in the expression of lipogenic genes; moreover, cholesterol synthesis genes in hepatocytes include fatty acid synthase (Fasn), sterol-coenzyme A desaturase (Scd1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr), glucokinase (Gki), and farnesyl diphosphate synthase (Fdp5) (Yeagley et al. 2001; Joseph et al. 2002). The expression of these five genes was significantly decreased in Cyp1a2(−/−) mouse liver (Table 2). The 1.6-fold increase in Cyp7b1 (Table 1), and the downregulation of fatty acid-binding protein-2 (Fabp2) and apolipoprotein A-IV (Apoa4) (Table 2), provide further evidence of the possible involvement of CYP1A2 in fatty acid and cholesterol pathways. This possible perturbation of fatty acid and cholesterol pathways might be reflected in the histologic assessment (Table 5) in which the Cyp1a2(−/−) mouse liver has less volume density of lipid within its hepatocytes, concomitant with increases in lipid in the fat-storing cells in its interstitium compared with that in the Cyp1a2(+/+) wild-type liver.

Cyp gene expression. As expected, the signal for Cyp1a2 gene expression was markedly decreased by >99% in the Cyp1a2(−/−) compared with that in the Cyp1a2(+/+) mouse (Table 2). Since the Cyp1a2 gene is constitutively quite highly expressed in wild-type mice, it was of interest to see whether there might be compensatory expression of any other CYP enzymes. The upregulation of Cyp3a11 (Table 1) and the downregulation of Cyp4a10 and Cyp4a14 (Table 2) might be related to alterations in the arachidonic acid cascade or other physiologic homeostasis (Nebert and Dieter 2000; Nebert and Russell 2002) in the absence of CYP1A2. The ESTs for each of the two Cyp4a genes would have detected either cDNA and, thus, these two could not be properly distinguished—although differences in degree of upregulation were seen.

The greatest difference (1.94-fold upregulation) was observed for Cyp2a4 (Table 1). Although this gene encodes steroid 15α-hydroxylase in the synthesis of testosterone and estradiol, both CYP2A4 and CYP2A5 have been shown to be modulated by circadian rhythm (Lavery et al. 1999; Akhtar et al. 2002). CYP2A5 has coumarin 7-hydroxylase activity and metabolically activates many chemicals such as nitrosamines and aflatoxin that are known hepatic carcinogens in mice (Negishi et al. 1989; Camus-Randon et al. 1996). CYP2A5 induction differs from most because it seems to occur by a variety of agents, including not only drugs and chemicals such as pyrazole, phenobarbital and cobalt but also viral and parasitic inflammation and in hepatic neoplasia (Camus-Randon et al. 1996; Wastl et al. 1998). This often occurs under circumstances in which other CYP isoforms are decreased. Thus, it would seem that hepatic Cyp2a5

Figure 1. Histology of mouse liver. Plastic sections (1-µ thick) of tissue were stained with toluidine blue to show lipid droplets. (A) In the untreated Cypyla2(−/−) knockout mouse, the number and size of tiny lipid droplets in hepatocytes (black arrow) are greatly decreased, whereas the amount of large lipid droplets in interstitial fat-storing cells (white arrows) is increased. (B) In the untreated Cypyla2(+/+) wild-type mouse, small osmiophilic lipid droplets (black arrows) are common in the hepatocytes, whereas larger droplets occur occasionally in fat-storing cells of the interstitium (white arrow). Each bar is 25 µm.

Table 5. Intrahepatocyte versus interstitial fat in the liver of Cyp1a2(−/−) and Cyp1a2(+/+) mice.

|                  | Cyp1a2(−/−) | Cyp1a2(+/+) | p-value |
|------------------|------------|------------|---------|
| Volume density (%) |            |            |         |
| Interstitium     | 11.0 ± 0.54| 9.52 ± 0.47| 0.089   |
| Glycogen in hepatocytes | 6.59 ± 1.0 | 5.37 ± 0.85| NS      |
| Lipid in the whole liver | 7.84 ± 1.08| 12.1 ± 1.63| 0.035   |
| Hepatocytic lipid (in hepatocytes only) | 6.38 ± 1.09| 11.7 ± 1.72| 0.012   |
| Hepatocytic lipid in whole liver | 5.72 ± 0.93| 10.7 ± 1.58| 0.009   |
| Interstitial lipid (in interstitium cells only) | 25.6 ± 2.29| 17.6 ± 2.09| 0.02    |
| Interstitial lipid in whole liver | 2.19 ± 0.23| 1.35 ± 0.15| 0.004   |

NS, not significant. The morphometric data from 28 Cyp1a2(−/−) and 28 Cyp1a2(+/+) mice, studied in our laboratory for more than 12 months from a number of different experiments, were combined to assess the intrahepatocyte versus interstitial lipid stores.
induction might be associated with a subtle form of liver injury, although nothing was seen histologically. Moreover, mouse Cyp2a5 has been found to possibly be involved in perturbation of the cell cycle and apoptosis (Pelkonen 2002). There was no significant change in the expression of heme oxygenase-1 (inducible form) (Table 3), however, which is often associated with CYP heme turnover and oxidative stress. In addition, there was no detectable elevation in the transcription of Alasl, coding for 5-aminolevulinate synthase, which can also occur in situations of heme insufficiency (unpublished data). The expression of nine other CYPs was detected but was not significantly different between Cyp1a2(+/-) and Cyp1a2(+/+) mice (Table 3). Interestingly, this includes the Cyp2c1 gene, encoding an enzyme that is constitutively quite highly expressed in mouse liver and known to be induced by ethanol and involved in small-molecule intermediary metabolism, diabetes mellitus and ketosis, as well as in the metabolism of drugs and dietary components.

**Hprt gene expression.** The unexpected observation that the Hprt gene is elevated more than 5-fold (Table 1) can most likely be explained by use of the Hprt minigene cassette in the selective elimination of the Cyp1a2 gene in ES cells (Liang et al. 1996). In all probability, Hprt expression is being driven by the nearby Cyp1a2 5′-flanking regulatory region and promoter. We are unable to conclude with certainty that none of the changes observed (Tables 1, 2) are the downstream consequence of this striking elevation in Hprt expression.

**Conclusions**

In summary, absence of the mouse Cyp1a2 gene appears to lead to changes in expression of some genes related to cell growth as well as to a downregulation of genes in energy production mediated by insulin. These endogenous functions would appear to be separate from any activity involving the metabolism of environmental chemicals. It is possible that this Cyp1a2 gene knockout reflects a general disturbed metabolic activity and an intrinsic function (Nebert and Dieter 2000) of CYP1A2 in liver and energy metabolism, which results in the decreased ability of lipid to accumulate within the hepatocyte cytoplasm. It is also not possible at this stage to deduce if there is any phenotypic consequence of Hprt overexpression resulting from the use of this gene in developing the knockout mouse line. It will be easy enough in the near future, however, to disrupt this Hprt selection gene and re-examine the Cyp1a2(+/−) knockout mouse in the absence of Hprt.

**References**

Akhatar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, et al. 2002. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol 12:540–550.

Buters JT, Tang BK, Pineau T, Gelboin HV, Kimura S, Gonzalez FJ. 1996. Role of CYP1A2 in caffeine pharmacokinetics and metabolism: studies using mice deficient in CYP1A2. Pharmacogenetics 6:291–296.

Camus-Randon AM, Raffalli F, Bereziat JC, McGregor D, Konstantid M, Lang MA. 1996. Liver injury and expression of cytochromes P450: evidence that regulation of CYP2A5 is different from that of other major xenobiotic-metabolizing CYP enzymes. Toxicol Appl Pharmacol 138:140–148.

Dalton TP, Dieter MZ, Matulis RS, Childs NL, Shertzer HG, Genter MB, et al. 2000a. Targeted knockout of Cyp1a1 gene does not alter hepatic constitutive expression of other genes in the mouse [Ala] battery. Biochem Biophys Res Commun 267:184–189.

Dalton TP, Miller ML, Wu X, Menon A, Cianciolo E, McKinnon RA, et al. 2000b. Refining the mouse chromosomal location of Cdm, the major gene associated with susceptibility to cadmium-induced testicular necrosis. Pharmacogenetics 10:141–151.

DeRisi JL, Iyer VR, Brown PO. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680–686.

Dorne JL, Walton K, Renwick AG. 2001. Uncertainty factors for chemical risk assessment. Human variability in the pharmacokinetics of CYP1A2 probe substrates. Food Chem Toxicol 39:681–696.

Eaton DL, Gallagher EP, Bammler TK, Kunze KL. 1995. Role of cytochrome P450 1A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity. Pharmacogenetics 5:259–274.

Eisen MB, Brown PO. 1999. DNA arrays for analysis of gene expression. Meth Enzymol 303:179–205.

Farnace AJ Jr, Nebert DW, Hollander MC, Luthy JD, Papathanasiou M, Fargnoli J, et al. 1989. Mammalian genes are constitutively regulated by growth arrest signals and DNA-damaging agents. Mol Cell Biol 9:4196–4203.

Heilmann LJ, Sheen YY, Bigelow SW, Nebert DW. 1988. The trout P450 IA1: cDNA and deduced protein sequence, expression in liver, and evolutionary significance. DNA 7:378–387.

Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC, et al. 2001. Dimethylbenzanthracene carcinogenesis in Gadd45α-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Res 61:2497–2491.

Joseph SB, Laffitte BA, Patel PH, Watson MA, Matsukuma KE, Walczak R, et al. 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. J Biol Chem 277:10119–10125.

Kimura S, Kawabe M, Ward JM, Morishima H, Kudlibar FF, Hammons SJ, et al. 1999. CYP1A2 is not the primary enzyme responsible for 4-aminobiphenyl-induced hepatocarcinogenesis in mice. Carcinogenesis 20:1825–1830.

Lavery DJ, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquet F, et al. 1999. Circadian expression of the steroid 15α-hydroxylase (Cyp2s) and coumarin 7-hydroxylase (Cyp2s9) genes in mouse liver is regulated by the PAR leucine-zipper transcription factor DBP. Mol Cell Biol 19:6488–6499.

Le Marchand L, Franke AA, Custer L, Wilkens LR, Cooney RV. 1997. Lifestyle and nutritional correlates of cytochrome CYP1A2 activity: inverse associations with plasma lutein and γ-toco-pherol. Pharmacogenetics 7:11–19.

Liang HC, Li, McKinnon RA, Duffy JJ, Potter SS, Puga A, et al. 1996. Cyp1a2(−/−) null mutant mice develop normally but show deficient drug metabolism. Proc Natl Acad Sci USA 93:1671–1676.

Liang HC, McKinnon RA, Nebert DW. 1997. Sensitivity of CYP1A1 mRNA inducibility by dioxin is the same in Cyp1a2(+/−) wild-type and Cyp1a2a(−/−) null mutant mice. Biochem Pharmacol 54:1127–1131.

Nebert DW. 1997. Polymorphisms in drug-metabolizing enzymes: what is their clinical significance and why do they exist? Am J Hum Genet 60:265–271.

Nebert DW, Dalton TP, Stuart GW, Carvan MJ, III. 2000a. “Gene-swap knock-in” cassette in mice to study allelic differences in human genes. Ann NY Acad Sci 919:146–170.

Nebert DW, Dieter MZ. 2000. The evolution of drug metabolism. Pharmacology 61:124–135.

Nebert DW, Duffy JJ. 1999. The use of knockout mouse lines will be used to study the role of drug-metabolizing enzymes and their receptors during reproduction and development, and in environmental toxicity, cancer, and oxidative stress. Biochem Pharmacol 52:249–254.

Nebert DW, McKinnon RA, Puga A. 1996. Human drug-metabolizing enzyme polymorphisms: effects on risk of toxicity and cancer. DNA Cell Biol 15:273–280.

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Fyererisen R, Fuji-Kuriyama Y, et al. 1991. The P450 superfamily: update on new sequences, gene mapping, and recomended nomenclature. DNA Cell Biol 10:1–14.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP. 2000b. Role of the aromatic hydrocarbon receptor and [Ala] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem Pharmacol 59:65–85.

Nebert DW, Russell DW. 2002. Clinical importance of the cytochromes P450. Lancet 360:1155–1162.

Negishi M, Lindberg R, Burkhart B, Ichikawa T, Honakoski P, Lang M. 1989. Mouse steroid 15α-hydroxylase gene family: identification of type II P450(51x) as coumarin 7-hydroxylase. Biochemistry 28:4169–4172.

Pelkonen O. 2002. Cross-talk between signal transduction systems and cytochrome P450 regulation. Pharmacologist 44(2) (suppl 1):A142.

Peters JM, Morishima H, Ward JM, Coakley CJ, Kimura S, Gonzalez FJ. 1999. Role of CYP1A2 in the toxicity of long-term phenacetin feeding in mice. Toxicol Sci 50:82–89.

Pineau T, Fernandez-Salguero P, Lee SS, McPhail T, Ward JM, Gonzalez FJ. 1995. Neonatal lethality associated with respiratory distress in mice lacking cytochrome P450 1A2. Proc Natl Acad Sci USA 92:5134–5139.

Shertzer HG, Dalton TP, Talaska G, Nebert DW. 2002. Decrease in 4-aminobiphenyl-induced methemoglobinemia in Cyp1a2(−/−) knockout mice. Toxicol Appl Pharmacol 181:32–37.

Sinclair PR, Gurnan N, Dalton T, Walton HS, Bement WJ, Sinclair JF, et al. 1998. Uroporphyria produced in mice by iron and 5-aminolevulonic acid does not occur in Cyp1a2(−/−) null mutant mice. Biochem J 330:149–153.
Sinclair PR, Gorman N, Walton HS, Bement WJ, Dalton TP, Sinclair JF, et al. 2000. CYP1A2 is essential in murine uroporphyria caused by hexachlorobenzene and iron. Toxicol Appl Pharmacol 162:60–67.

Smith AG, Clothier B, Carthew P, Childs NL, Sinclair PR, Nebert DW, et al. 2001. Protection of the $\text{Cyp1a2}^{--}$ null mouse against uroporphyria and hepatic injury following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 173:89–98.

Turton NJ, Judah DJ, Riley J, Davies R, Lipson D, Styles JA, et al. 2001. Gene expression and amplification in breast carcinoma cells with intrinsic and acquired doxorubicin resistance. Oncogene 20:1300–1306.

van der Lugt N, Maandag ER, te Riele H, Laird PW, Berns A. 1991. A $\text{pgk::hprt}$ fusion as a selectable marker for targeting of genes in mouse embryonic stem cells: disruption of the T-cell receptor $\delta$-chain-encoding gene. Gene 105:263–267.

Wastl UM, Rossmannith W, Lang MA, Camus-Randon AM, Grasl-Kraupp B, Bursch W, et al. 1998. Expression of cytochrome P450 2A5 in preneoplastic and neoplastic mouse liver lesions. Mol Carcinog 22:229–234.

Yeagley D, Guo S, Unterman T, Quinn PG. 2001. Gene- and activation-specific mechanisms for insulin inhibition of basal and glucocorticoid-induced insulin-like growth factor binding protein-1 and phosphoenolpyruvate carboxykinase transcription. Roles of forkhead and insulin response sequences. J Biol Chem 276:33705–33710.

Zaccaro C, Sweitzer S, Pipino S, Gorman N, Sinclair PR, Sinclair JF, et al. 2001. Role of cytochrome P450 1A2 in bilirubin degradation. studies in $\text{Cyp1a2}^{--}$ mutant mice. Biochem Pharmacol 61:843–849.