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Role of CYP2E1 in the Hepatotoxicity of Acetaminophen*

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CYP2E1, a cytochrome P-450 that is well conserved across mammalian species, metabolizes ethanol and many low molecular weight toxins and cancer suspect agents. The *cyp2e1* gene was isolated, and a mouse line that lacks expression of CYP2E1 was generated by homologous recombination in embryonic stem cells. Animals deficient in expression of the enzyme were fertile, developed normally, and exhibited no obvious phenotypic abnormalities, thus indicating that CYP2E1 has no critical role in mammalian development and physiology in the absence of external stimuli. When *cyp2e1* knockout mice were challenged with the common analgesic acetaminophen, they were found to be considerably less sensitive to its hepatotoxic effects than wild-type animals, indicating that this P-450 is the principal enzyme responsible for the metabolic conversion of the drug to its active hepatotoxic metabolite.

Cytochromes P-450 (P-450)[1] are a superfamily of hemoproteins that carry out oxidative metabolism of many endogenous and foreign chemicals (1). In mammals, P-450s can be functionally segregated into two groups, those that participate in biochemical pathways leading to the synthesis of steroid hormones and those that primarily metabolize foreign chemicals or xenobiotics such as drugs. The latter enzymes are included in the CYP1, CYP2, CYP3, and CYP4 families (2). Many of the hepatic xenobiotic-metabolizing P-450s also metabolize endogenous compounds, but the significance of these reactions is questionable. A clue for the lack of a critical role for many of the P-450s, particularly those in family 2, in development, reproduction, and longevity, is the marked species differences in their expression and catalytic activities (3). However, some of the xenobiotic-metabolizing P-450s are well conserved, including those in the CYP1 family and CYP2E1, suggesting that they may perform an important physiological function.

CYP2E1 is the principal P-450 responsible for the metabolism of ethanol and is considered as a major component of the microsomal ethanol-oxidizing system (4, 5). Among xenobiotics metabolized by CYP2E1 are acetaldehyde, acetaminophen, acrylamide, aniline, benzene, butanol, carbon tetrachloride, diethylether, dimethyl sulfoxide, ethyl carbamate, ethylene chloride, halothane, glycerol, ethylene glycol, *N*-nitrosodimethylamine, 4-nitrophenol, pyrazole, pyridine, and vinyl chloride (6). Many of these chemicals are known toxins, established chemical carcinogens, or suspected carcinogens. CYP2E1-mediated oxidation of a variety of substrates is also believed to liberate a substantial amount of reactive oxygen that can lead to membrane lipid peroxidation and cell toxicity (7).

CYP2E1 is also capable of metabolizing endogenous chemicals including acetone and acetol, which are key metabolites in the methylglyoxal and propanediol pathways of gluconeogenesis (8, 9). CYP2E1 can also carry out the metabolism of arachidonic acid, resulting in the production of several hydroxyeicosatetraenoic acids (10), some of which may have physiological and pharmacological properties (11).

CYP2E1 is inducible by ethanol and other low molecular weight substrates (5, 12). This induction is primarily due to a postranscriptional mechanism where presence of the substrate stabilizes the enzyme from degradation (13). However, transcriptional mechanisms have not been ruled out (14). This enzyme is also induced by starvation and in uncontrolled diabetes (15, 16).

P-450s have been implicated in the hepatotoxicity of acetaminophen (also called paracetamol), an over-the-counter analgesic and antipyretic that is commonly used worldwide as a substitute for acetylsalicylic acid (aspirin®) due to its lack of gastric ulceration and general low toxicity when used within the recommended dose range (17–19). Acetaminophen causes hepatotoxicity at a low frequency. It is metabolized to *N*-acetyl-*p*-benzoquinoneimine, a metabolite that is capable of reacting with cellular nucleophiles. The bulk of this metabolite is either reduced back to acetaminophen or conjugated with glutathione. It was postulated that toxicity results from low cellular glutathione leaving an excess of active metabolite that can cause cell toxicity (19–22).

The P-450s responsible for acetaminophen activation have been investigated. Ethanol was reported to increase the toxicity of acetaminophen in mice (20, 23), thus suggesting the involvement of CYP2E1 *in vivo*. *In vitro* studies have also implicated human CYP1A2 in addition to CYP2E1 in acetaminophen metabolism, although the latter P-450 had a lower K_m than CYP1A2 (24, 25).

The conservation across species in expression and catalytic activities of CYP2E1 and its ability to metabolize and be induced by chemicals that are generated endogenously, such as acetone and ethanol, suggests that it has an important physiological role in mammals. To investigate this possibility and to determine if this P-450 is involved in the hepatotoxicities and carcinogenesis potential of many of its substrates, mice lacking CYP2E1 expression were produced and characterized.

MATERIALS AND METHODS

Construction of the Targeting Vector—Genomic clones corresponding to *cyp2e1* were obtained by screening a 129/SV genomic library (Strategene) with a rat CYP2E1 cDNA (26). A clone spanning 14.2 kb and containing all nine exons of the gene was subcloned as a *Sal*I fragment. To disrupt the gene, a 1.9-kb *Hin*dIII fragment containing exon 2 and

[1] The abbreviations used are: P-450, cytochrome P-450; kb, kilobase pair(s); PGK, phosphoglycerate kinase-1; ES, embryonic stem.

A

cyp2e1 GENE

Probe P5

6.3 Spe I
5.9 3.2 Bgl II
12.3 Apa I

TARGETING VECTOR

HSV-TK   pUC9   PGK-NEO

HOMOLOGOUS
RECOMBINANT   PGK-NEO Probe

Probe P5

2.3 7.7 Spe I
5.5 3.2 Bgl II
11.3 Apa I

B

Bgl II   Spe I
AY168 ES   AY168 ES

—7.7
5.9—   —6.3
5.5—

C

Bgl II   Spe I
+/−   +/−
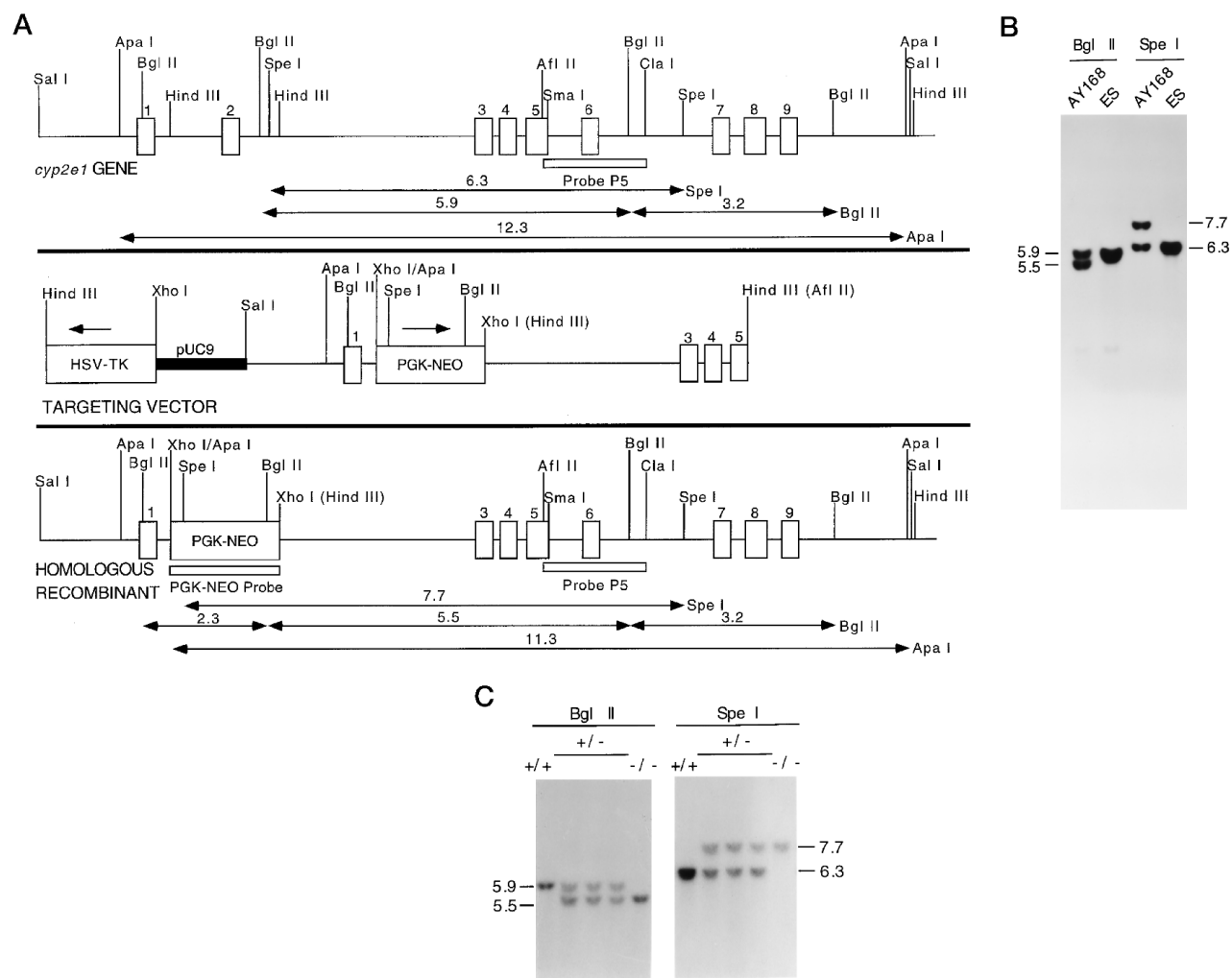+/+   −/−   +/+   −/−

—7.7
5.9—   —6.3
5.5—

FIG. 1. *Panel A* displays the restriction map of the *cyp2e1* gene, the targeting vector, and the predicted homologous recombinant locus. The *numbers over* the *horizontal double arrows* are the predicted sizes of restriction fragments in kb. *Panel B* shows a Southern blot of the specific ES clone and wild-type ES cells, and *panel C* displays a Southern blot of a typical screen of tail clipping DNA from mice with different genotypes. The sizes of the fragments are in kb.

spanning from intron 1 to intron 2 was deleted and replaced with the bacterial phosphoribosyltransferase II gene, under control of the phosphoglycerate kinase-1 promoter (PGK-NEO), that confers resistance to the neomycin derivative G418 (Life Sciences Inc.). This gene was derived from the plasmid pPNT (27). The PGK-NEO cassette was inserted in the same transcriptional orientation as the *cyp2e1* gene. The herpes simplex virus thymidine kinase gene was inserted at the 3′ end of the *cyp2e1* gene as a negative selection against random integration of the construct (28). The construct used for targeting (see Fig. 1*A*), contained 2.3 kb of 5′ and 3.6 kb of 3′ genomic DNA flanking the PGK-NEO cassette.

The construct was made in six cloning steps (see Fig. 1A). 1) The *Hin*dIII site in the polylinker region of pGEM-3Z (Promega) was destroyed by *Hin*dIII digestion, Klenow polymerase treatment, and religation. 2) An 8-kb *Sal*I-*Sma*I *cyp2e1* genomic fragment was subcloned into the same sites in the modified pGEM-3Z. 3) The plasmid made in step 2 was digested with *Hin*dIII, treated with Klenow polymerase, and ligated with *Xho*I linkers in order to remove the 1.8-kb fragment containing exon 2 and add a restriction site compatible with the PGK-NEO cassette. This 1.9-kb cassette was previously modified by changing the *Bam*HI site at its 3′ end to an *Xho*I site by use of Klenow polymerase and *Xho*I linkers. 4) The *Xho*I fragment containing the PGK-NEO cassette was subcloned into the *cyp2e1* gene at the *Xho*I site. 5) The *cyp2e1* construct, containing the PGK-NEO cassette was digested with *Afl*II, treated with Klenow polymerase, and ligated with *Hin*dIII linkers. 6) The *cyp2e1* gene was released from this construct by digestion with *Sal*I and *Hin*dIII and inserted into the corresponding sites of pMC1TK plasmid (29) containing the herpes simplex virus thymidine kinase gene. The resulting plasmid was used as a targeting vector.

Production of Chimeric Mice—The plasmid DNA used for targeting

was purified by banding twice on cesium chloride. After linearization with *Hin*dIII, 40 µg was electroporated into J1 embryonic stem (ES) cells (30) using conditions described previously (31). ES cell clones resistant to both G418 and ganciclovir (gift of Syntex) were selected and screened for homologous recombination, and clones having the expected Southern blot pattern for a homologous recombinant (see below) were regrown and injected into C57BL/6N blastocysts. The blastocysts were transferred into the uterus of a pseudopregnant recipient NIH Swiss mouse in order to produce an animal exhibiting chimerism (32). Male chimeras presenting greater than 95% 129/SV contribution, as determined by coat color, were bred with C57BL/6N females to determine if the trait was transmitted to the germ line. Southern blot genotyping performed on DNA extracted from tail clips, was used to score for the presence of the mutated *cyp2e1* gene in the progeny. Homozygotes were produced by crossing the F1 generation.

Genotyping of ES Cells and Mice—DNA was isolated from ES cells and mouse tail clips as described previously (33) and digested with either *Bgl*II or *Spe*I. The digested DNAs were subjected to electrophoresis in 0.6% agarose gels and transferred to GeneScreen Plus nylon membranes (DuPont) using 0.4 N NaOH. The conditions for hybridization and washing were described previously (31). A 3′-flanking probe derived from a *Afl*II-*Cla*I genomic fragment (see probe P5, Fig. 1*A*) was labeled with [^{32}P]dCTP using random primers. This probe hybridizes with 5.9- and 3.2-kb *Bgl*II fragments and with a 6.3-kb *Spe*I diagnostic fragment for the wild-type *cyp2e1* allele. The homologous recombinant allele generated fragments of 5.5 and 7.7 kb corresponding to digestions of *Bgl*II and *Spe*I, respectively (see Fig. 1, *A–C*). Mice homozygous for the disrupted *cyp2e1* allele were designated cyp2e1$^{−/−}$.

Analysis of CYP2E1 Expression—Mice were killed by carbon monoxide asphyxiation, and 400 mg of liver tissue was disrupted using a

Teflon-glass homogenizer in 3 ml of a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10% (v/v) glycerol. The homogenate was centrifuged for 20 min at 10,000 × *g*, and the supernatant was centrifuged for 12 min at 500,000 × *g* in a Beckman Optima TL tabletop ultracentrifuge to recover microsomes. All operations were performed at 4 °C. The microsome pellets were resuspended by homogenization in 0.1 M sodium potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol and stored at −80 °C until use. Protein concentrations were determined with the bichinchoninic acid reagent (Pierce) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (34) using 10 μg of microsomal protein. Proteins were electroblotted to nitrocellulose membranes by semi-dry transfer. Immunoblotting was performed according to Towbin *et al.* (35). Rabbit antibodies against CYP1A2 (36), CYP2A1 (37), CYP2B1 (38), and CYP3A1 (39) were produced as described earlier. Rabbit antisera against CYP2C6 was produced by Dr. Kiyoshi Nagata (Tohoku
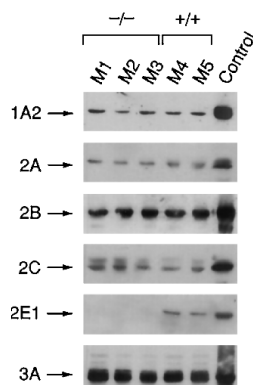
University, Sendai Japan). Antibody to CYP2E1, produced in goat, was obtained from the Gentest Corp. The secondary antibodies, labeled with horseradish peroxidase, were from Amersham Corp.

Messenger RNA was analyzed by Northern blots using liver RNA and the rat CYP2E1 cDNA as a probe. Total RNA was isolated from liver tissue using guanidinium thiocyanate extraction (40) and cesium trifluoroacetic acid centrifugation as described previously (31). Ten μg of total RNA was subjected to electrophoresis on 1% agarose gels containing 2.2 M formaldehyde (41) and blotted to GeneScreen Plus (DuPont) nylon membranes using 3 M NaCl and 0.15 M sodium citrate, pH 7.0. The CYP2E1 cDNA was labeled using random primers and [^{32}P]dCTP. The conditions for prehybridization, hybridization, and
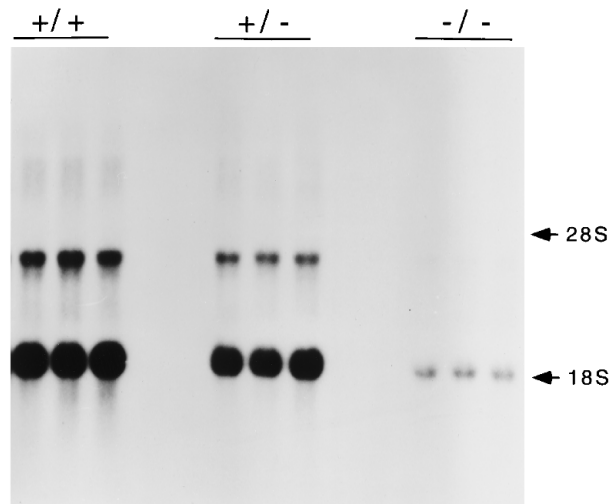

FIG. 2. **Western immunoblots of different P-450s in cyp2e1$^{−/−}$ mice.** Each lane was loaded with 10 μg of microsomal protein from a single mouse.

FIG. 3. **Analysis of RNA in livers of cyp2e1$^{−/−}$ mice.** Each lane was loaded with 10 μg of total liver RNA from a single mouse. The blot was exposed for 24 h with aid of an intensifying screen.
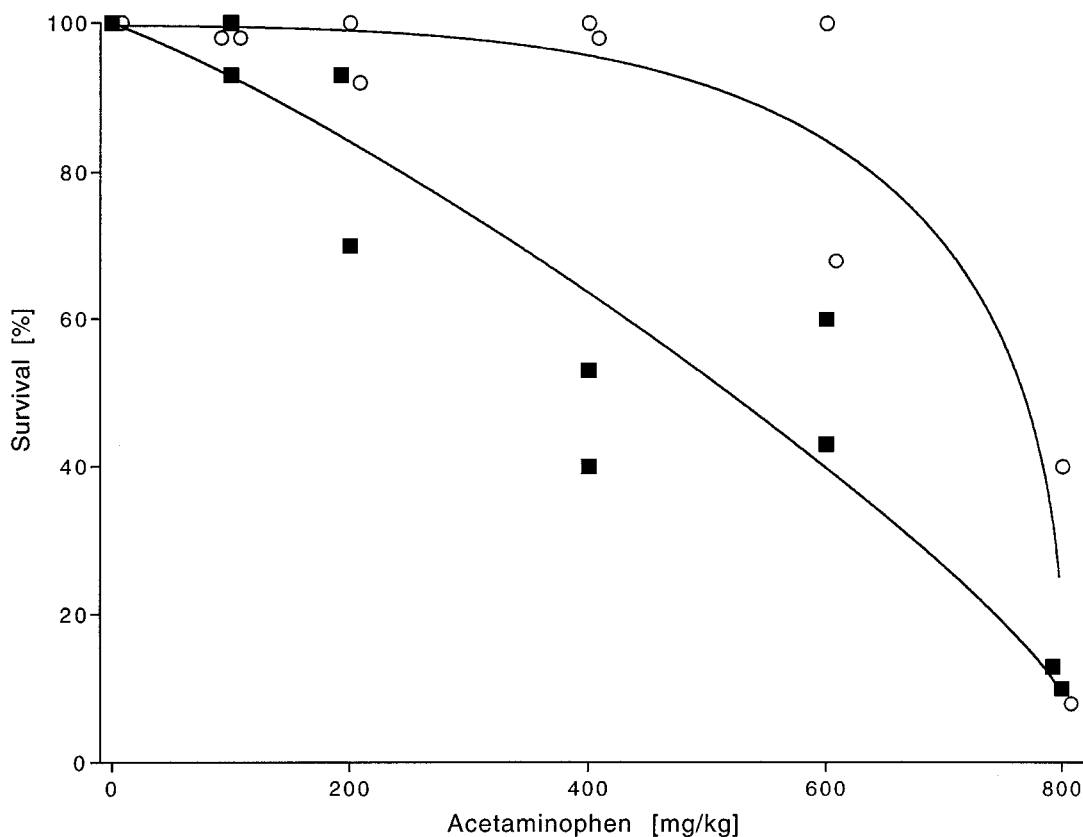

FIG. 4. **Survival rate of cyp2e1−/− (○) and wild-type (■) mice as a function of the dose of acetaminophen administered.** Groups of 10 mice were injected intraperitoneally with acetaminophen in alkaline saline and survival scored after 48 h. Two complete and independent experiments were performed. The curves were manually fit to the data points.

washing were described previously (31).

Acetaminophen Toxicity—The protocol for dosing mice with acetaminophen was approved by the National Cancer Institute's Animal Care and Use Committee (Protocol LMCE-023). Male cyp2e1$^{-/-}$ and wild-type strains, from 2 to 4 months of age, were administered acetaminophen by intraperitoneal injection at doses ranging from 0 to 800 mg/kg in alkaline saline solution. Each dose group consisted of 10 mice. To score toxicities, the number of surviving animals at 48 h were quantified. Two complete and independent experiments were conducted over the same dose range. From the remaining mice, blood was collected and serum was used to determine the levels of bilirubin, creatinine, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. These measurements were performed by the Diagnostic Services and Clinical Pathology Laboratory of the Uniformed Services University of the Health Sciences Clinical Chemistry Department using a Kodac Ektachem 250 automated plasma analyzer.

RESULTS AND DISCUSSION

Production and Characterization of the cyp2e1$^{-/-}$ Mice—The *cyp2e1* gene was isolated from a 129/SV mouse genomic library. The genomic clone spanned 14.2 kb and contained the complete coding region (Fig. 1*A*). The gene was disrupted by the replacement of exon 2 with the PGK-NEO cassette. A diagnostic probe, designated probe P5 and shown in Fig. 1*A*, was generated that detects homologous integrations of the targeting construct into the gene. Mice having the wild-type allele are expected to yield a 5.9-kb *Bgl*II and a 6.3-kb *Spe*I fragments. A typical autoradiography of a Southern blot of DNA from the ES cell clone AY168 and control ES cells hybridized with the probe P5 is shown in Fig. 1*B*. Upon longer exposure of the blot, an expected 3.2-kb *Bgl*II fragment was also detected. Specific recombinants had diagnostic 5.5- and 7.7-kb fragments from *Bgl*II and *Spe*I, respectively. Screening of mice generated by breeding for heterozygotes for the disrupted *cyp2e1* allele is shown in Fig. 1*C*. Heterozygous mice have the diagnostic fragments corresponding to the wild-type and disrupted alleles, whereas mice that have two copies of the disrupted allele yielded the 5.5- and 7.7-kb fragments after digestion with *Bgl*II and *Spe*I, respectively. Hybridization with the PGK-NEO gene as a probe revealed only a single hybridizing fragments of 2.3, 7.7, and 11.3 kb for the *Bgl*II-, *Spe*I-, and *Apa*I-digested DNA (data not shown), demonstrating that this clone did not contain any additional random integration of the targeting construct.

Mice homozygous for the disrupted allele, designated cyp2e1$^{-/-}$, were born normally and appeared indistinguishable from their wild-type counterparts. No differences were found between litter size and growth rates for the cyp2e1$^{-/-}$ animals as compared with wild-type littermate controls. The expression of CYP2E1 was determined by immunoblotting with anti-rat CYP2E1 antibody. As expected, a complete absence of protein expression was found in the livers of cyp2e1$^{-/-}$ mice (Fig. 2). The liver is the primary site of expression of this P-450 (16). P-450s in the CYP1A, CYP2A, CYP2B, CYP2C, and CYP3A subfamilies were expressed in the cyp2e1$^{-/-}$ mice at similar levels to those found in control animals, thus indicating that the loss of CYP2E1 was not compensated by an increase in expression of other P-450s, although it remains a possibility that a P-450 not detected with our anti-rat P-450 antibodies is overexpressed.

The expression of CYP2E1 mRNA was also analyzed in the cyp2e1$^{-/-}$ mice. Two transcripts were detected in the liver of normal mice and mice heterozygous for the disrupted allele (Fig. 3). In the cyp2e1$^{-/-}$ mice, neither of these two RNA transcripts were found. Instead, two lower abundance RNAs slightly smaller than the transcripts present in wild-type animals were detected. These may be transcripts from the disrupted allele that should be smaller than a transcript from the normal allele since exon 2 is deleted in the disrupted allele. The
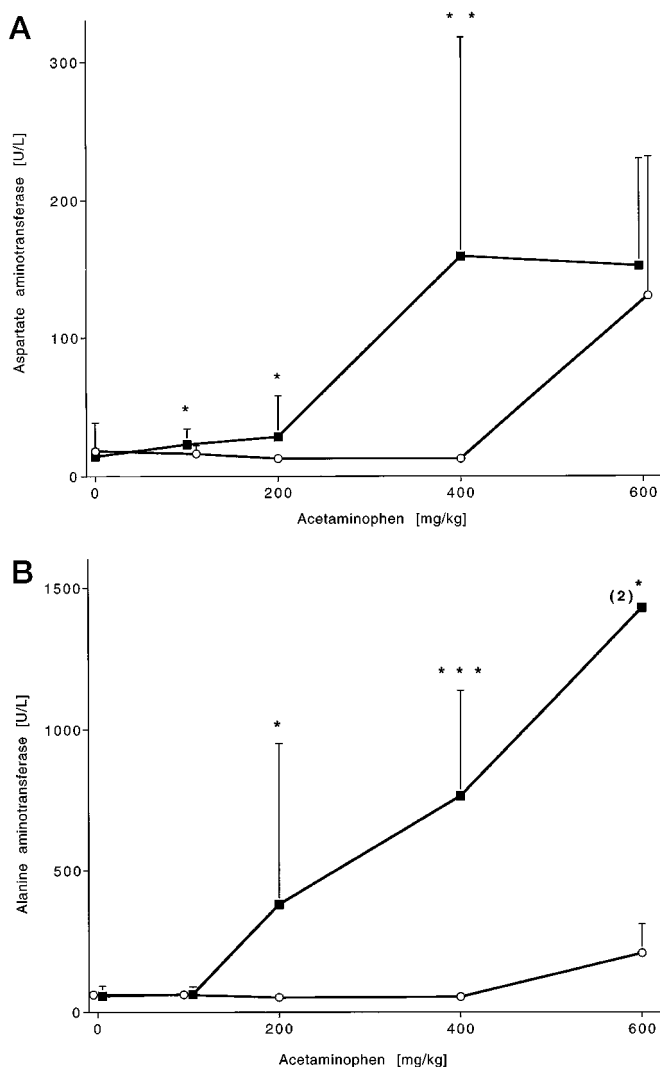

FIG. 5. **Determinations of aspartate aminotransferase (*panel A*) and alanine aminotransferase (*panel B*) activities in serum of cyp2e1$^{-/-}$ (○) and wild-type (■) mice as a function of the dose of acetaminophen administered.** The mean + standard deviations are shown with $n = 3$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). At the 600 mg/kg dose group for the wild-type mice in *panel B*, two animals were analyzed.

lower abundance of these RNAs, as compared with those from the wild-type allele, is not surprising since mRNAs that do not encode a normal protein are usually not stable. In any case, the protein and RNA establish with certainty that the *cyp2e1* gene is not expressed in the knockout animals. The change in size and abundance of the high molecular weight transcript annealing with the CYP2E1 cDNA in the cyp2e1$^{-/-}$ mice suggest that it is not due to a cross-hybridizing mRNA derived from another gene but is most likely a read-through transcript of the *cyp2e1* gene with an alternate polyadenylation signal.

Acetaminophen Toxicity—P-450s have been implicated in the hepatotoxicity of acetaminophen. To determine whether CYP2E1 influences the toxicity of this compound in mice, the cyp2e1$^{-/-}$ animals were administered the drug and compared with wild-type mice. Survival curves indicated that the cyp2e1$^{-/-}$ mice were more resistant to acetaminophen toxicity than wild-type animals (Fig. 4). Levels of 400 mg of acetaminophen/kg producing toxicity in wild-type mice in this study were similar to those that produced toxicity in other studies (20, 23). Cyp2e1$^{-/-}$ mice survived at doses up to 400 mg/kg, whereas over 50% of wild-type animals died at these doses.

To determine the mechanism of toxicity, levels of enzymes and other serum components, some of which are diagnostic for liver and kidney injury, were measured in serum of treated mice that survived in the experiments described above. At all doses, levels of creatinine, bilirubin, and alkaline phosphatase were within the normal range for mice and were not significantly different between the cyp2e1$^{-/-}$ and wild-type mice. In contrast, liver enzymes aspartate aminotransferase and alanine aminotransferase were elevated at high doses of acetaminophen (Fig. 5). Elevation of these liver enzymes, which are considered a measure of liver cell death, were detected at doses of 200 and 400 mg/kg in wild-type animals but were unchanged at these doses in the cyp2e1$^{-/-}$ mice. These data indicate that liver damage is involved in mediating the toxicity of acetaminophen. This was confirmed by analysis of liver histology of acetaminophen-treated mice (data not shown). At doses higher than 600 mg/kg, a significant level of toxicity was also found in the cyp2e1$^{-/-}$ mice. These data suggest that CYP2E1 mediates the hepatotoxicity of acetaminophen. Other P-450s such as CYP1A2 having a higher K_m for acetaminophen may be responsible for the toxicity in cyp2e1$^{-/-}$ mice at high doses of the drug.

The present study using mice lacking expression of CYP2E1 establish that although this P-450 is highly conserved in mammals, it does not appear to play a significant role in development, reproductive vitality, and physiology. Under conditions of exposure to certain chemicals, CYP2E1 accentuates toxicity. Efforts are underway to use this animal model to determine whether this enzyme is responsible for the carcinogenicity of a number of its chemical substrates including *N*-nitrosodimethylamine and phenacetin.

CYP2E1 may also exert a role in alcoholic liver disease. Lipid peroxidation was found to be associated with alcoholic liver injury in humans and experiment animals (42). This could be the result, in part, of increased oxygen radical production by ethanol-induced CYP2E1 (7). The cyp2e1$^{-/-}$ mice could be used to test this possibility.

During fasting and diabetic ketosis, serum acetone, acetol, and 1,2-propanediol are elevated. CYP2E1 is concommitantly induced due to protein stabilization by acetone (16). Acetone is primarily oxidized to acetol by CYP2E1. Acetol is further metabolized to 1,2-propanediol by the same P-450 in a pathway of gluconeogenesis, suggesting a physiological role for this P-450 during pathophysiological and dietary stress (8). The cyp2e1$^{-/-}$ mice should be of use to determine if CYP2E1 plays an essential role in survival under conditions of starvation.

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