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Genetics

Cyp1a2(−/−) null mutant mice develop normally but show deficient drug metabolism

(cytochrome P450/embryonic stem cells/arylamine carcinogenesis)

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ABSTRACT **Cytochrome P450 1A2 (CYP1A2) is a predominantly hepatic enzyme known to be important in the metabolism of numerous foreign chemicals of pharmacologic, toxicologic, and carcinogenic significance. CYP1A2 substrates include aflatoxin B_1, acetaminophen, and a variety of environmental arylamines. To define better the developmental and metabolic functions of this enzyme, we developed a CYP1A2-deficient mouse line by homologous recombination in embryonic stem cells. Mice homozygous for the targeted *Cyp1a2* gene, designated *Cyp1a2*(−/−), are completely viable and fertile; histologic examination of 15-day embryos, newborn pups, and 3-week-old mice revealed no abnormalities. No CYP1A2 mRNA was detected by Northern blot analysis. Moreover, mRNA levels of *Cyp1a1*, the other gene in the same subfamily, appear unaffected by loss of the *Cyp1a2* gene. Because the muscle relaxant zoxazolamine is a known substrate for CYP1A2, we studied the *Cyp1a2*(−/−) genotype by using the zoxazolamine paralysis test: the *Cyp1a2*(−/−) mice exhibited dramatically lengthened paralysis times relative to the *Cyp1a2*(+/+) wild-type animals, and the *Cyp1a2*(+/−) heterozygotes showed an intermediate effect. Availability of a viable and fertile CYP1A2-deficient mouse line will provide a valuable tool for researchers wishing to define the precise role of CYP1A2 in numerous metabolic and pharmacokinetic processes.**

Cytochromes P450 represent the major class of phase I drug-metabolizing enzymes (1). Members of this enzyme superfamily are responsible for the metabolism of innumerable foreign chemicals. In addition, because of the metabolism of many endogenous compounds such as steroids, vitamin D_3, fatty acids, prostaglandins, and biogenic amines, cytochromes P450 are believed to be essential for such critical life functions as cell division, differentiation, apoptosis, homeostasis, and neuroendocrine functions (2–4).

As of October 1995, the P450 gene superfamily was composed of more than 480 genes classified into 74 families, 14 of which exist in all mammals (5). Both the murine and human *CYP1A* subfamilies comprise two genes, designated *Cyp1a1* and *Cyp1a2* in mouse, and *CYP1A1* and *CYP1A2* in humans (5, 6). In mice, the *Cyp1a* genes appear to be located within a 100-kb region on chromosome 9 (1, 7). The CYP1A enzymes are of particular interest due to their capacity for metabolizing numerous compounds relevant to the fields of pharmacology, toxicology, and carcinogenesis. In addition, both enzymes are induced by many foreign chemicals, including polycyclic aromatic hydrocarbons (e.g., benzo[*a*]pyrene) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (1, 4). The induction process is regulated by the aromatic hydrocarbon receptor (AHR) (8, 9); the murine *Ahr* gene has been cloned (10, 11) and an *Ahr*(−/−) mouse line has recently been produced (12).

CYP1A2 is highly expressed in liver and is involved in the metabolism of many toxicologically significant compounds, including aflatoxin B_1, acetaminophen, and the food-derived heterocyclic amines (reviewed in ref. 13). In addition, most carcinogenic arylamines are known to be substrates for the human CYP1A2 enzyme (6, 14). To date, no endogenous substrate has been identified for the CYP1A2 enzyme, and there is little evidence for any physiologic role other than protection from chemical insult. On the basis of caffeine metabolism, two laboratories have suggested the presence of a human CYP1A2 genetic polymorphism, having a trimodal distribution of metabolizer phenotype (consistent with high/high, high/low, and low/low genotypes) in several populations (15, 16).

To define more clearly the involvement of the CYP1A2 enzyme in toxicity and carcinogenesis elicited by a variety of environmental chemicals, we have generated an embryonic stem (ES) cell-derived mouse line lacking a functional CYP1A2 enzyme. These null mutant mice exhibit normal development, viability, and fertility. The availability of this healthy *Cyp1a2*-deficient mouse line should provide an invaluable resource for researchers wishing to define the precise role of CYP1A2 in the metabolism of foreign, and perhaps endogenous, chemicals.¶

MATERIALS AND METHODS

Cloning of 129/SV *Cyp1a2* Genomic DNA. Using the mouse 1.5-kb 3′ *Cyp1a2*-specific cDNA probe (17), we isolated a 129/SV mouse genomic DNA clone isogenic to the ES cells used for recombination. DNA cloning and purification were performed as described (18). From the isolated clone, spanning 19 kb, we subcloned an 11-kb *Eco*RI fragment of the *Cyp1a2* gene into pBluescript II SK(+) (Stratagene). The wild-type *Eco*RI fragment includes exons 2–7 and the 3′ end of the *Cyp1a2* gene (Fig. 1 *Upper*).

Construction of *Cyp1a2* Gene-Targeting Vectors. The hypoxanthine phosphoribosyltransferase (*hprt*) gene-based vectors used for targeting the *Cyp1a2* gene were derived from the parent vector, pHPRT KO; this vector is derived from pBluescript II SK(+), contains a 2-kb *Kpn* I blunt-ended herpes simplex virus (HSV) thymidine kinase (*tk*) gene cassette, and features a 2.9-kb *Not* I–*Eco*RI blunt-ended *hprt* minigene cassette subcloned into the *Hin*dIII site (19). To generate the two vectors used for targeting, we subcloned a 0.4-kb *Hin*dIII–

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Abbreviations: ES cell, embryonic stem cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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¶This work was presented in abstract form at the Annual Meeting of the Society of Toxicology, March 5–9, 1995, Baltimore (43).

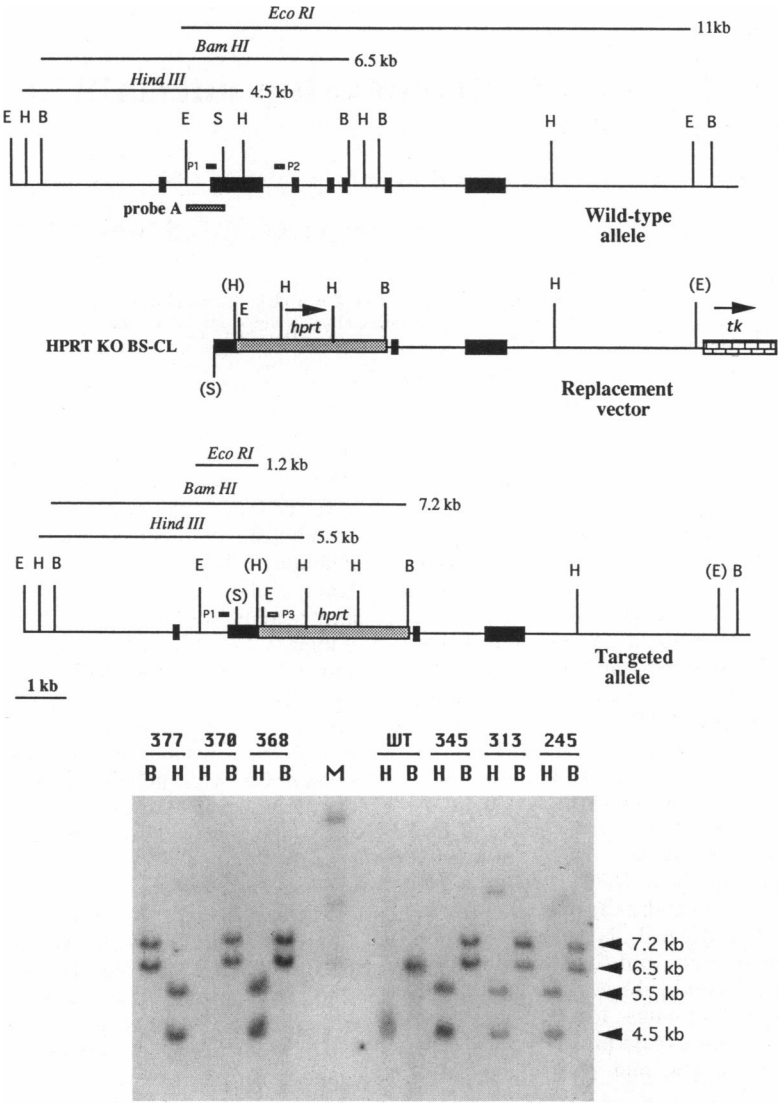
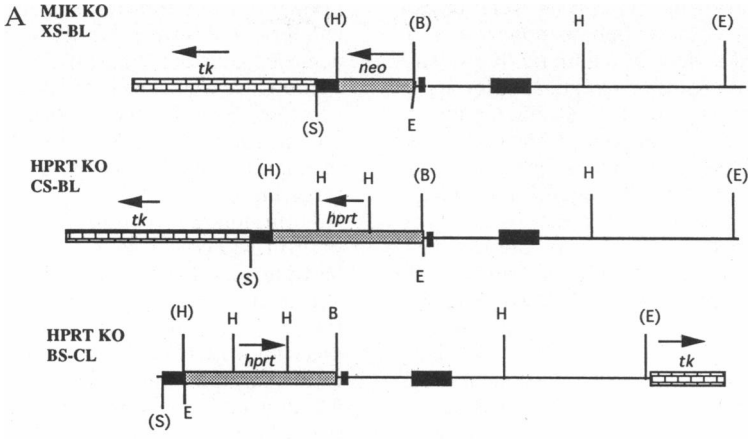


FIG. 1. Targeted modifications of the murine *Cyp1a2* gene. (*Upper*) The wild-type allele with all seven exons (solid boxes) and the targeted allele (containing the *hprt* gene) are shown. Probe A was used for genomic DNA analysis. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Stu* I; sites in parentheses were lost during cloning/ligation. The predicted wild-type, and targeted allele, restriction fragment sizes for *Bam*HI and *Hin*dIII are shown. P1, P2, and P3 represent primers used for PCR analysis. (*Lower*) Diagnostic Southern blots for DNA from wild-type (WT) untargeted ES cells, plus DNA from six targeted ES cell lines designated 377, 370, 368, 345, 313, and 245. *Bam*HI (B) and *Hin*dIII (H) digests were hybridized with the flanking probe A. Heterozygote targeted lines contain the 7.2-kb *Bam*HI and the 5.5-kb *Hin*dIII fragments. M, molecular size markers.

Stu I fragment from exon 2 of the *Cyp1a2* gene into either the *Bam*HI site or the *Cla* I site of the pHPRT KO plasmid, generating products designated pHPRT KO BS and pHPRT KO CS, respectively. Subsequently, the *Cla* I site of the pHPRT KO BS plasmid and the *Bam*HI site of the pHPRT KO CS plasmid were used to subclone a 6.6-kb *Bam*HI–*Eco*RI fragment of the *Cyp1a2* gene. The resultant plasmids were designated pHPRT KO BS-CL and pHPRT KO CS-BL, respectively (Fig. 2*A*).

The targeting vector used in our earlier experiments was constructed from the multipurpose knockout vector pMJK KO, also derived from pBluescript II SK(+). pMJK KO possesses features similar to pHPRT KO, except that the *Hin*dIII site of pBluescript II SK(+) was used for subcloning a 1.6-kb *Xho* I–*Hin*dIII phophoglycerate kinase promoter–neomycin-resistance gene cassette instead of the *hprt* minigene cassette (20). The 0.4-kb *Hin*dIII–*Stu* I fragment of exon 2 of *Cyp1a2* (Fig. 1 *Upper*) was subcloned into the *Xho* I site of the pMJK KO plasmid. The *Bam*HI site of the pMJK KO plasmid was then used to subclone the 6.6-kb fragment. The resulting construct, designated pMJK KO XS-BL (Fig. 2*A Top*), contains 7.0 kb of target homology and produces a 2.2-kb deletion in the targeted locus. All three resulting plasmids were purified by the CsCl banding technique, linearized at the unique *Not* I site in the pBluescript II SK(+) backbone, and used for electroporation.

ES Cell Cultures. D3 ES cells (21) and E14tg2a ES cells, which are *hprt*$^-$ (22), were maintained at 37°C in a 5% CO_2 atmosphere on feeder layers of murine mitomycin C-treated embryonic fibroblasts in Dulbecco's modified Eagle's medium containing 15% heat-inactivated fetal bovine serum, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, penicillin at 50 units/ml, streptomycin at 50 μg/ml, and leukemia inhibitory factor (LIF; GIBCO) at 1000 units/ml. The fibroblast feeder layer was prepared from 13- to 14-day-old mouse embryos and was grown in Dulbecco's modified Eagle's medium containing glucose at 4.5 mg/ml, penicillin at 50 units/ml, streptomycin at 50 μg/ml, and 1 mM L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum. Confluent fibroblast monolayers were treated with mitomycin C at 10 μg/ml for 90 min at 37°C. The monolayers were then washed four times with phosphate-buffered saline prior to freezing or immediate use



B

Construct	MJK KO XS-BL	HPRT KO CS-BL	HPRT KO BS-CL
ES cells	D3	E14	E14
Screened colonies	>800	200	380
Targeted lines	1	0	11
Chimera generated	13 males 9 females		12 males 1 females
Germline transmission	0		6

FIG. 2. Comparison of the three *Cyp1a2* gene-targeting constructs used in these experiments. (*A*) Diagram of the three constructs. The pMJK KO XS-BL vector carries the neomycin-resistance (*neo*) gene and the *Cyp1a2* gene fragment in opposite orientations. The pHPRT KO BS-CL vector carries the *hprt* gene in the same orientation as the *Cyp1a2* gene fragment, whereas the pHPRT KO CS-BL vector carries the *hprt* gene and the *Cyp1a2* gene fragment in opposite orientations. (*B*) Results of successfully targeted lines, generation of chimeric mice, and germline transmission.

as feeder layers (23). For electroporation, two confluent 100-mm dishes of ES cells were treated with trypsin and resuspended in 3 ml of phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) containing *Not* I-digested targeting vector DNA at 20 μg/ml. Three 1-ml aliquots were electroporated at 900 V and 14 μF in a 0.4-cm-wide cuvette (Gene Zapper; IBI). The cells were then distributed onto 15-mm × 100-mm dishes containing mitomycin C-treated mouse embryo fibroblast feeder cells. Selection in hypoxanthine/aminopterin/thymidine (HAT) supplement (GIBCO/BRL) for E14 cells, or in G418 (150 μg/ml) for D3 cells, was initiated 24 h later. Further selection with 2 μM ganciclovir (Syntex, Palo Alto, CA) was begun at 48 hr following electroporation. Resistant ES colonies were selected 7 days after electroporation, treated with trypsin, and transferred to 24-well plates. After 2 or 3 days in culture, half the cells from each well were frozen, and the other half were transferred to gelatin-coated six-well plates. DNA was then prepared from each colony for PCR and/or Southern blot analysis, in order to identify putative homologous recombination events.

Selection of the Recombinant ES Cells. ES cells were lysed at 65°C for 10 min in 0.5 ml of buffer containing 0.2 M NaCl, 5 mM EDTA, 50 mM Tris·HCl (pH 7.5), 0.2% SDS, and proteinase K at 20 μg/ml. Genomic DNA was precipitated with potassium acetate and ethanol. The DNA was rinsed with 70% ethanol, then rinsed with 95% ethanol, air-dried, and resuspended in H_2O. Mouse tail DNA was prepared similarly, except that lysis buffer containing proteinase K at 15 μg/ml was used, and samples were incubated overnight at 65°C.

For Southern blotting, we digested DNA to completion with an excess of the restriction endonuclease *Eco*RI, *Bam*HI, or *Hin*dIII under reaction conditions recommended by the supplier (GIBCO/BRL). The digested DNA was fractionated through 0.8% agarose gels and transferred to Nytran Plus (Schleicher & Schuell) for further hybridization. Conditions for prehybridization and hybridization were as described (24). A 0.8-kb *Eco*RI–*Stu* I fragment was used as probe A (Fig. 1). Autoradiography was performed with Kodak XAR film and DuPont Lighting Plus intensifying screens.

For PCR screening of the HAT-resistant ES cell colonies, genomic DNA was added to a 20-μl PCR mixture containing 4 μl of 5× reaction buffer, 0.125 μg of each oligonucleotide primer (*Cyp1a2*-5′, CAGCCTGGGATGGAAATCAAGACA; *Cyp1a2*-3′, CGCTGCACACGGCACTCTGAGTAC; and *hprt* 3′, AGCGCCTCCCCTACCCGGTAGAAT), 2.5 units of *Taq* DNA polymerase (GIBCO/BRL), and a mixture of dATP, dCTP, dGTP and dTTP nucleotides at a final concentration of 500 μM for each nucleotide. The 5× reaction buffer contained 250 μM NaCl, 750 μM $MgCl_2$, 100 mM Tris·HCl (pH 8.4), 7.5 mM $MgCl_2$, 0.05% gelatin, and 0.5% Triton X-100. Samples were overlaid with one drop of mineral oil. PCR was performed for 35 cycles of 94°C for 1 min, 62°C for 2 min, and 72°C for 3 min in a thermal cycler (Perkin–Elmer/Cetus). The generated PCR products were 1.2 and 0.9 kb for *Cyp1a2*(+) wild-type and *Cyp1a2*(−) mutant alleles, respectively (Fig. 1). For screening of G418-resistant colonies, the above conditions were used with the following primers: *neo*-3′, ATGGCCGCTTTTCTGGATTCATCGACTTG; *Cyp1a2*-5′, GCGTTCTCCCAGTACATCTCCTTAGCCCCA; and *Cyp1a2*-3′, CTCACCTTGTTGAAGTCTTGGTAGTGCTCC.

Generation of the *Cyp1a2*(−/−) Mouse Line. Chimeric mice were generated by microinjection of targeted ES cells into embryos as described (24). Briefly, 10–15 targeted ES cells derived from the 129/Ola (slate, gray) mouse line were microinjected into the blastocoele cavity of C57BL/6J em-

bryos (nonagouti, black). Surviving blastocysts were transferred to pseudopregnant CD-1 females (albino, nonagouti) by uterine implantation. Identification of chimeric pups was determined by the presence of agouti or slate coat color at 10 days of age, depending on the origin of the ES cells. Chimeric males were bred to CF-1 females (nonagouti, white) or Swiss Black females (nonagouti, black). Germline transmission was determined by the presence of chinchilla-agouti and agouti coat colors in the offspring of the CF-1 and the Swiss Black females, respectively. Germline transmission was confirmed by both PCR and Southern blot analyses, as detailed above.

Northern Hybridization Analysis. Total RNA was isolated from the livers of 7- to 9-week-old mice by the acid guanidinium isothiocyanate extraction method (25). RNA was isolated 36 hr after treatment with a single intraperitoneal dose of either corn oil alone (25 ml/kg of body weight) or β-naphthoflavone (200 mg/kg) in corn oil. Total RNA (30 μg) was loaded onto 1% agarose/formaldehyde gels, transferred to nylon membranes (Nytran Plus; Schleicher & Schuell), and UV-crosslinked. Prehybridization and hybridization were performed in 10% dextran sulfate/1% SDS/6× standard saline citrate. Membranes were probed with a 1.5-kb 3′ fragment of CYP1A2 cDNA or a 1.2-kb 3′ fragment of CYP1A1 cDNA (17). The probe of a 780-bp *Pst* I–*Xba* I fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an RNA-loading control. Hybridization was performed at 60°C, and the blots were washed at 60°C prior to autoradiography.

Zoxazolamine Paralysis Test. Four- to 6-week-old mice were given a single intraperitoneal dose of either β-naphthoflavone (200 mg/kg) in corn oil or corn oil alone. All animals received intraperitoneal zoxazolamine (300 mg/kg) in corn oil 36 hr later. Mice were then placed on their backs, and that time was recorded as time zero. The paralysis time was measured as that period of time until the animal had regained enough consciousness to right itself repeatedly (26).

Histology. Histologic specimens were prepared from 15-day embryos, newborn pups, and 3-week-old mice for analysis of any pathologic changes associated with the *Cyp1a2*-deficient genotype. The 15-day embryos and newborn pups were fixed whole in Bouin's fixative, whereas virtually all organs were dissected from 3-week-old mice and fixed in 10% formaldehyde. Paraffin-embedded sections (5 μm) were visualized by staining with hematoxylin and eosin.

RESULTS

Gene Targeting. Targeted disruption of the *Cyp1a2* gene was successfully achieved by insertion of the *hprt* gene in place of part of exon 2 and all of exons 3–5 (Fig. 1). Our design of the three gene-targeting vectors (Fig. 2*A*) was based on important structural features in the murine *Cyp1a2* gene, including a highly conserved cysteine-containing peptide in the N-terminus of the protein encoded by exon 2 and the so-called cytochrome P450 "conserved tridecapeptide" (27), encoded by exon 5 in the case of both of the *CYP1A* genes. Based on these structural details, homologous recombination constructs were designed in which the regions encoding the N-terminal cysteine-containing fragment and the conserved tridecapeptide would be replaced with either the *neo* or *hprt* positive selection markers. Targeted deletion of these essential gene components would be predicted to generate a null mutation.

Interestingly, pHPRT KO BS-CL (having the *hprt* promoter in the same orientation as the *Cyp1a2* gene) was the only successful construct of three used in our experiments (Fig. 2*B*). Whereas a high targeting frequency was achieved with pHPRT KO BS-CL in E14 ES cells, no homologous recombinant clones were generated from pHPRT KO CS-BL. In our earlier work using the *neo* gene in D3 ES cells, it is noteworthy that only a single homologous recombinant clone (of 800 screened) was obtained with the pMJK KO XS-BL construct, from which 13 chimeric males were produced, yet none of these was able to achieve germline transmission (Fig. 2*B*). The most widely accepted reason for the transmission of coat color but not the targeted gene is that a locus has been lost which allows colonization of the ES cells in the germline; this comes about through continued passage of ES cells both before and after targeting.

Homologous recombinant clones generated with the pHPRT KO BS-CL construct were confirmed by both genomic blotting and PCR analysis. Genomic blot analysis demonstrated that heterozygote clones were obtained, as indicated by the presence of extra 5.5-kb and 7.2-kb fragments upon digestion with *Hin*dIII and *Bam*HI, respectively (Fig. 1 *Lower*). PCR analysis of heterozygote clones showed the presence of a 0.9-kb band, corresponding to the mutant allele, in addition to the wild-type 1.2-kb band (Fig. 3). Two homologous recombinant clones generated with the pHPRT KO BS-CL construct were injected into the blastocoele cavity of C57BL/6J embryos; we subsequently generated 12 male chimeras, of which 6 gave germline transmission. Germline mice were derived from two independent ES clones, lines 368 and 377.

Viability and Fertility. The heterozygous *Cyp1a2*(+/−) mice displayed normal viability and fertility and were then used to generate homozygous mutants. Breeding of the heterozygotes produced offspring in the expected Mendelian distribution of one *Cyp1a2*(+/+) to two *Cyp1a2*(+/−) to one *Cyp1a2*(−/−), indicating no *in utero* lethality due to loss of both functional alleles of the *Cyp1a2* gene.

Homozygous *Cyp1a2*(−/−) null mutants were identified by the presence of only the 0.9-kb band upon PCR analysis (Fig. 3). Genotype was confirmed by Southern blotting. We found the homozygous *Cyp1a2*(−/−) mouse to be completely viable, fertile, and indistinguishable from its *Cyp1a2*(+/+) or *Cyp1a2*(+/−) littermates by appearance, mortality rate, reproductive capacity, and histologic examination of many organs and tissues; this has remained true—currently beyond 15 months of age. The organs and tissues examined histologically included: liver, lung, kidney, stomach, duodenum, small and large intestine, spleen, thymus, lymph nodes, heart, and brain.

CYP1A2 mRNA Analysis. The absence of CYP1A2 mRNA in *Cyp1a2*(−/−) mice was confirmed by Northern blot analysis of liver RNA from both control and β-naphthoflavone-treated animals. Fig. 4 *Left* shows a gene-dose effect: constitutive CYP1A2 mRNA levels in the heterozygote were intermediate between the null mutant, in which no mRNA was detectable even with 21-day exposures of the filter to x-ray film, and the wild type, which showed abundant mRNA. Induction by β-naphthoflavone treatment was found to increase the

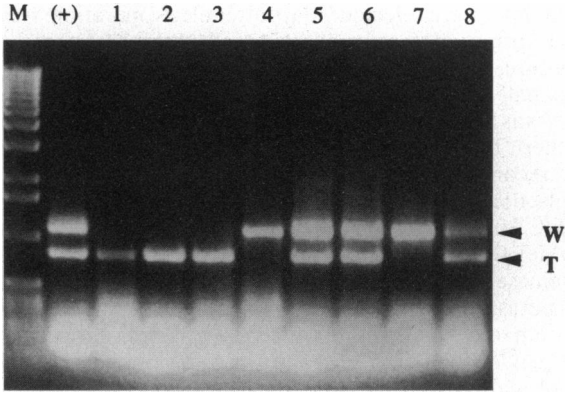


FIG. 3. PCR analysis of tail DNA from eight pups in the same litter generated from a *Cyp1a2*(+/−) × *Cyp1a2*(+/−) intercross. PCR products are 1.2 and 0.9 kb for the wild-type (W) and targeted (T) alleles, respectively. M, 1-kb nucleic acid markers. (+), targeted ES cell DNA.

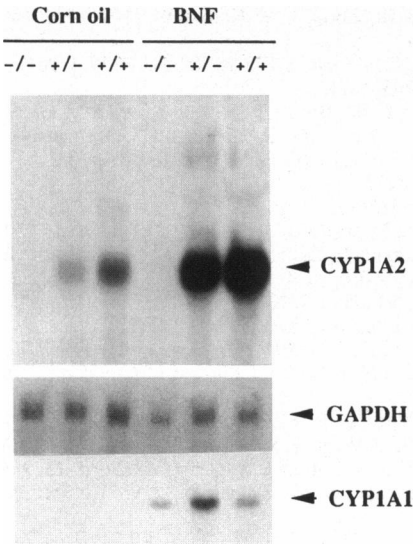


FIG. 4. Northern hybridization analysis of liver RNA from *Cyp1a2*(−/−), *Cyp1a2*(+/−), and *Cyp1a2*(+/+) mice. Probes include the CYP1A2-specific cDNA, the CYP1A1-specific cDNA as a positive control, and GAPDH cDNA to assess RNA loading in each lane. Sizes of the CYP1A2 and CYP1A1 mRNAs are 2.1 and 2.8 kb, respectively (17). Animals were treated with corn oil alone (controls) or β-naphthoflavone (BNF) 36 hr prior to sacrifice. When standardized for GAPDH, CYP1A1 mRNA levels are not significantly different in the three BNF lanes.

CYP1A2 mRNA 4- to 6-fold in the heterozygote and wild-type mouse (Fig. 4 *Right*), whereas no CYP1A2 mRNA was detected in the β-naphthoflavone-treated *Cyp1a2*(−/−) mouse.

CYP1A1 mRNA levels, detectable in liver only after β-naphthoflavone induction, were virtually identical in the *Cyp1a2*(+/+), *Cyp1a2*(+/−), and *Cyp1a2*(−/−) mice (Fig. 4 *Bottom*). These data indicate that expression of this other member of the mouse *Cyp1a* subfamily does not appear to compensate, or to be altered, by absence of the *Cyp1a2* gene.

Zoxazolamine Paralysis Test. The muscle relaxant zoxazolamine is a known CYP1A2 substrate, as well as a CYP2E1 substrate (28), and has been used for more than two decades to phenotype individual recombinant inbred or congenic mice having had CYP1A2 induced by β-naphthoflavone, benzo[*a*]pyrene, or 2,3,7,8-tetrachloro-*p*-dioxin (26, 29). It was expected that mice lacking any CYP1A2 enzyme would metabolize zoxazolamine more slowly and therefore remain paralyzed for a longer period of time and that β-naphthoflavone treatment—while inducing CYP1A2 in *Cyp1a2*(+/+) and *Cyp1a2*(+/−) mice—would have no effect on the *Cyp1a2*(−/−) mouse. The results of the zoxazolamine paralysis test (Table 1) show these expectations to be correct. Interestingly, the data are also correlated with the gene-dose mRNA data of Fig. 4 in that *Cyp1a2*(+/−) animals exhibited an intermediate paralysis time, when compared with the *Cyp1a2*(+/+) wild type and the *Cyp1a2*(−/−) null mutant. The CYP1A2-deficient mouse was paralyzed at least 9 times longer than the wild-type mouse. These results clearly illustrate the major role of CYP1A2 in zoxazolamine metabolism.

Table 1. Results of the zoxazolamine paralysis test in mice of the three genotypes

Genotype	Time paralyzed, min	
	Control	β-Naphthoflavone pretreatment
Cyp1a2(−/−)	>960	>960
Cyp1a2(+/−)	534 ± 225	309 ± 69
Cyp1a2(+/+)	498 ± 121	103 ± 59

The zoxazolamine paralysis test was performed as described (26). Zoxazolamine (chlorzoxazone) was given to all mice 36 hr after a single intraperitoneal dose of β-naphthoflavone (in corn oil); controls received corn oil alone. Paralysis times were assessed as the time taken for mice to right themselves three times. Values (mean ± SEM) are for four mice for each condition.

DISCUSSION

Evolutionary Considerations. We had anticipated that homozygous *Cyp1a2*(−/−) mutant mice would be viable and healthy, yet phenotypically different from wild-type *Cyp1a2*(+/+) and heterozygous *Cyp1a2*(+/−) mice toward CYP1A2-specific substrates such as zoxazolamine, based on several criteria. (*i*) Whereas constitutive expression of the *Cyp1a1* gene and its induction by polycyclic aromatic compounds occur very early during embryogenesis, increases in expression of the *Cyp1a2* gene are not detectable until the neonatal period (1, 30–32). These observations suggest that the *Cyp1a2* gene might not be critical for mouse embryogenesis but more likely is involved in metabolism of dietary and other foreign chemicals encountered after birth. (*ii*) Mammalian *CYP1A2* genes are extinguished in virtually all established and transformed cell lines examined, indicating that absence of CYP1A2 does not affect viability of cells in culture (1, 33, 34). (*iii*) Evolutionary analysis of the *CYP1A* family in trout and mammalian species (5) suggests that, while *CYP1A1* most likely encodes an enzyme critical to life, the *CYP1A2* gene is likely to be the result of a relatively recent gene duplication event in response to dietary selective pressures (35). Whereas trout appears to have only the *CYP1A1* gene, birds and mammals possess both *CYP1A1* and *CYP1A2*. It was therefore proposed that the *CYP1A2* gene originated ≈350 million years ago via a gene duplication event—after the divergence of land animals from sea animals, and before divergence of land animals from birds (5, 35, 36). This duplication event may have been driven by evolutionary pressures caused by animal–plant interactions (35). Thus, it appears more likely that CYP1A2 might play an important role in protecting newborns from the insults of foreign (particularly dietary) chemicals during and after the neonatal period. We therefore had expected that the *Cyp1a2*-deficient mouse would develop normally, be viable, and display normal fertility, and this is what the present study shows.

Comparison of Two CYP1A2-Deficient Mouse Lines. The normal phenotype of the *Cyp1a2*(−/−) mouse line described in our study contrasts sharply with the phenotype of respiratory distress and neonatal lethality observed in another *Cyp1a2*(−/−) mouse line recently described (37). One possibility to explain the differences in phenotype would be differences in genetic background. This is not without precedent: for example, in studies of the insulin-like growth factor (*Igf1*) gene, differences in the phenotype of knockout mouse lines were found to be caused by differences in genetic background (38). In the present study, chimeric males in this laboratory were bred to CF-1 females or Swiss Black females, whereas chimeric males in the other laboratory were bred to C57BL/6J females (37).

Another possible explanation for differences in the phenotype of CYP1A2-deficient mice between the present study and a recent study (37) is a combination of genetic and nongenetic factors—i.e., presence of viral or other respiratory pathogens in a genetically susceptible host. In support of this possibility is that 19 of their 599 *Cyp1a2*(−/−) null mutants did survive to adulthood and are fertile (37).

An additional possible explanation has to do with the gene construct electroporated into the ES cells. It is not without precedent that different genomic approaches to knockouts of the same gene can lead to different phenotypes. For example, several laboratories engineered mutations that created null alleles with no residual expression of the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene (39–41),

whereas a different laboratory created a "leaky" insertional mutation in exon 10 leading to an only mildly affected phenotype (42). It is exciting that both the mild and severe phenotypes of the *Cftr* gene knockout are proving to be valuable experimental model systems. Pineau *et al.* (37) disrupted the *Cyp1a2* gene by inserting the *neo* selection marker into exon 2, whereas we removed much of exon 2 and all of exons 3–5.

Conclusions. We have produced a *Cyp1a2*(−/−) null mutant mouse that develops normally, and is completely viable and fertile, yet exhibits altered drug metabolism. The generation of the *Cyp1a2*(−/−) mouse line described herein will provide an invaluable tool for researchers seeking to define the precise role of the CYP1A2 enzyme in numerous metabolic processes. Such a model will be particularly useful for further investigation of the CYP1A2 enzyme in terms of drug metabolism and toxicity, as well as cancer caused by environmental arylamines. Extrapolation of studies in this mouse line to human populations should also enable more educated predictions of the risk assessment associated with toxic exposures to chemicals via diet, life style, and occupation.

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1. Nebert, D. W. & Gonzalez, F. J. (1987) *Annu. Rev. Biochem.* **56,** 945–993.
2. Nebert, D. W. (1990) *Nature (London)* **347,** 709–710.
3. Nebert, D. W. (1991) *Mol. Endocrinol.* **5,** 1203–1214.
4. Nebert, D. W. (1994) *Biochem. Pharmacol.* **47,** 25–37.
5. Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., Waterman, M. R., Gotoh, O., Coon, M. J., Estabrook, R. W., Gunsalus, I. C. & Nebert, D. W. (1996) *Pharmacogenetics* **6,** in press.
6. Nebert, D. W. & McKinnon, R. A. (1994) *Prog. Liver Dis.* **14,** 63–97.
7. Hildebrand, C. E., Gonzalez, F. J., Kozak, C. A. & Nebert, D. W. (1985) *Biochem. Biophys. Res. Commun.* **130,** 396–406.
8. Cobb, R. R., Stoming, T. A. & Whitney, J. B., III (1987) *Biochem. Genet.* **25,** 401–412.
9. Poland, A. P., Glover, E. & Taylor, B. A. (1988) *Mol. Pharmacol.* **32,** 471–478.
10. Burbach, K. M., Poland, A. & Bradfield, C. A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8185–8189.
11. Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsushita, N., Gotoh, O., Funae, Y. & Fujii-Kuriyama, Y. (1992) *Biochem. Biophys. Res. Commun.* **184,** 246–253.
12. Fernandez-Salguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S. T., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M. & Gonzalez, F. J. (1995) *Science* **268,** 722–726.
13. Schweikl, H., Taylor, J. A., Kitareewan, S., Linko, P., Nagorney, D. & Goldstein, J. A. (1993) *Pharmacogenetics* **3,** 239–249.
14. Guengerich, F. P., Shimada, T., Raney, K. D., Yun, C.-H., Meyer, D. J., Ketterer, B., Harris, T. M., Groopman, J. D. & Kadlubar, F. F. (1992) *Environ. Health Perspect.* **98,** 75–80.
15. Butler, M. A., Lang, N. P., Young, J. F., Caporaso, N. E., Vineis, P., Hayes, R. B., Teitel, C. H., Massengill, J. P., Lawsen, M. F. & Kadlubar, F. F. (1992) *Pharmacogenetics* **2,** 116–127.
16. Tang, B. K., Zhou, Y. & Kalow, W. (1994) *Pharmacogenetics* **4,** 117–124.
17. Kimura, S., Gonzalez, F. J. & Nebert, D. W. (1984) *J. Biol. Chem.* **259,** 10705–10713.
18. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (1991) *Current Protocols in Molecular Biology* (Greene/Wiley-Interscience, New York).
19. Li, H., Zeitler, P. S., Valerius, M. T., Small, K. & Potter, S. S. (1996) *EMBO J.*, in press.
20. Small, K. M. & Potter, S. S. (1993) *Genes Dev.* **7,** 2318–2328.
21. Doetschman, T., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. (1985) *J. Embryol. Exp. Morphol.* **87,** 27–45.
22. Hooper, M., Handy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326,** 292–295.
23. Doetschman, T. (1994) in *Transgenic Animal Technology: A Laboratory Handbook*, ed. Pinkert, C. (Academic, New York), pp. 115–146.
24. Li, H., Witte, D. P., Brandford, W. W., Aronow, B. J., Weinstein, M., Kaur, S., Wert, S., Singh, G., Schreiner, C. M., Whitsett, J. A., Scott, W. J. & Potter, S. S. (1994) *EMBO J.* **13,** 2876–2885.
25. Chomczynski, O. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
26. Robinson, J. R. & Nebert, D. W. (1974) *Mol. Pharmacol.* **10,** 484–493.
27. Ozols, J., Heinemann, F. S. & Johnson, E. F. (1981) *J. Biol. Chem.* **256,** 11405–11408.
28. Ono, S., Hatanaka, T., Hotta, H., Tsutsui, M., Satoh, T. & Gonzalez, F. J. (1995) *Pharmacogenetics* **5,** 143–150.
29. Nebert, D. W. (1980) *J. Natl. Cancer Inst.* **64,** 1279–1290.
30. Tuteja, N., Gonzalez, F. J. & Nebert, D. W. (1985) *Dev. Biol.* **112,** 177–184.
31. Kimura, S., Donovan, J. C. & Nebert, D. W. (1987) *J. Exp. Pathol.* **3,** 61–74.
32. Dey, A., Westphal, H. & Nebert, D. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7446–7450.
33. Pasco, D. S., Boyum, K. W., Merchant, S. N., Chalberg, S. C. & Fagan, J. B. (1988) *J. Biol. Chem.* **263,** 8671–8676.
34. Silver, G. J. & Krauter, K. S. (1988) *J. Biol. Chem.* **263,** 11802–11807.
35. Gonzalez, F. J. & Nebert, D. W. (1990) *Trends Genet.* **6,** 182–186.
36. Heilmann, L. J., Sheen, Y. Y., Bigelow, S. W. & Nebert, D. W. (1988) *DNA* **7,** 379–387.
37. Pineau, T., Fernandez-Salguero, P., Lee, S. T. T., McPhail, T., Ward, J. M. & Gonzalez, F. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 5134–5138.
38. Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. & Efstratiadis, A. (1993) *Cell* **75,** 59–72.
39. Ratcliff, R., Evans, M. J., Cuthbert, A. W., MacVinish, L. J., Foster, D., Anderson, J. R. & Colledge, W. H. (1993) *Nat. Genet.* **4,** 35–41.
40. O'Neal, W. K., Hasty, P., McCray, Jr., J. B., Casey, B., Rivera-Perez, J., Welsh, P. J., Beaudet, A. L. & Bradley, A. (1993) *Hum. Mol. Genet.* **2,** 1561–1569.
41. Hyde, S. C., Gill, D. R., Higgins, C. F., Trezise, A. E., MacVinish, L. J., Cuthbert, A. W., Ratcliff, R., Evans, M. J. & Colledge, W. H. (1993) *Nature (London)* **362,** 250–255.
42. Dorin, J. R., Dickinson, P., Alton, E. W., Smith, S. N., Geddes, D. M., Stevenson, B. J., Kimber, W. L., Fleming, S., Clark, A. R., Hooper, M. L., Anderson, L., Beddington, R. S. P. & Porteous, D. J. (1993) *Nature (London)* **359,** 211–215.
43. Liang, H. C., Li, H., Potter, S. S., Duffy, J. J., Puga, A., McKinnon, R. A. & Nebert, D. W. (1995) *Toxicologist* **15,** 59 (abstr.).