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Generation of 'humanized' h*CYP1A1_1A2_Cyp1a1/1a2*(−/−) mouse line

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Abstract

Human/rodent CYP1A1 and CYP1A2 orthologs are well known to exhibit species-specific differences in substrate preferences and rates of metabolism. This lab previously characterized a BAC-transgenic mouse carrying the human *CYP1A1_CYP1A2* locus; in this line, human dioxin-inducible CYP1A1 and basal vs dioxin-inducible CYP1A2 have been shown to be expressed normally (with regard to mRNAs, proteins and three enzyme activities) in every one of nine mouse tissues studied. The mouse *Cyp1a1* and *Cyp1a2* genes are oriented head-to-head and share a bidirectional promoter region of 13,954 bp. Using Cre recombinase and *loxP* sites inserted 3′ of the stop codons of both genes, we show here a successful interchromosomal excision of 26,173 bp that ablated both genes on the same allele. The *Cyp1a1/1a2(−)* double-knockout allele was bred with the "humanized" line; the final product is the h*CYP1A1_1A2_Cyp1a1/1a2*(−/−) line on a theoretically >99.8% C57BL/6J genetic background—having both human genes replacing the mouse orthologs. This line will be valuable for human risk assessment studies involving any environmental toxicant or drug that is a substrate for CYP1A1 or CYP1A2.

Keywords: Humanized mouse line; Human *CYP1A1_1A2* locus; Mouse *Cyp1a1_1a2* locus; *loxP*; Cre recombinase; Interchromosomal excision; Benzo[*a*]pyrene; Dioxin

The human and mouse cytochrome P450 *(CYP)* gene superfamilies contain 57 and 102 protein-coding genes, respectively; one of the 18 mammalian *CYP* families is *CYP1*, having three members in both human and mouse—CYP1A1, CYP1A2, and CYP1B1 [1–3]. The *CYP1A* and *CYP1B* subfamily ancestors diverged from one another probably more than 500 million years ago; *CYP1A2* arose as a gene duplication event from *CYP1A1* about 450 million years ago. Thus, land animals (including fowl) carry both *CYP1A1* and *CYP1A2*; sea animals do not have the *CYP1A2* gene [4]. Accordingly, the *CYP1A1* and *CYP1A2* genes are located at human chromosome 15q24.1, in head-to-head orientation, 23,306 bases from one transcription initiation start-site to the other [5]. Of three mammalian genomes studied, estimates are that about 10% of gene duplication pairs share bidirectional promoters [6]. The latest data from the UCSC browser assembled by NCBI and the Mouse Genome Sequencing Consortium puts the *Cyp1a1* and *Cyp1a2* genes on mouse chromosome 9 at cM 31.0, also in a head-to-head orientation, 13,954 bases from one transcription start-site to the other. In contrast, *CYP1B1* is located on human chromosome 2p22.2, and *Cyp1b1* (syntenic with human *CYP1B1*) is located on mouse chromosome 17.

Cyp1a1(−/−) [7], *Cyp1a2*(−/−) [8], and *Cyp1b1*(−/−) [9] knockout mouse lines have previously been generated. Because of location on different chromosomes for the *Cyp1a1_Cyp1a2* locus and the *Cyp1b1* gene, the *Cyp1a1/1b1*(−/−) and *Cyp1a2/1b1*(−/−) double-knockout lines were easy to produce, and have been used in toxicity studies [10]. This lab discovered a recombination "hot spot" in the human *CYP1A1_CYP1A2* spacer region [5]. Therefore, we felt it likely that a "hot spot" would also exist in the mouse *Cyp1a1_Cyp1a2* bidirectional promoter, which

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should aid us in creating the *Cyp1a1/1a2(−)* allele. Despite breeding the *Cyp1a1(+/−)* × *Cyp1a2(+/−)* cross and genotyping several hundred offspring, however, our laboratory was unable to identify any *Cyp1a1/1a2(−)* allele. The present study describes how we were successful in generating the *Cyp1a1/1a2*(−/−) double-knockout mouse.

Human/rodent CYP1A2 orthologs are well known to exhibit species-specific differences in the rates by which various substrates are metabolized [11]. For example, human and mouse CYP1A2 differ by 3- to 7-fold in catalyzing ethoxyresorufin *O*-deethylation [12] and uroporphyrinogen oxidation [13].

Using bacterial artificial chromosomes (BACs), we and others have been able to generate "humanized" h*CYP 1A1_1A2* BAC-transgenic lines [5,14]. Studies have been carried out with the h*CYP1A1_1A2_Cyp1a1*(−/−) line vs the h*CYP1A1_1A2_Cyp1a2*(−/−) line; for example, these lines were used both for theophylline [15] and for the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) [14]. In both cases, human CYP1A2 was shown to be responsible for the "human metabolite profile" in the absence of mouse CYP1A2.

Examining drug or carcinogen metabolism in the rodent, and then extrapolating results to human populations, is therefore prone to error. It would be very much preferred if human metabolism gene(s) could be inserted in place of the mouse orthologous gene(s). Studying the h*CYP1A1_1A2_ Cyp1a1*(−/−) and h*CYP1A1_1A2_Cyp1a2*(−/−) lines separately is cumbersome. It would be much better to have a mouse line carrying the human *CYP1A1* and *CYP1A2* genes in the absence of both mouse orthologs. The present study reports generation of such a mouse line.

Materials and methods

Mice. C57BL/6J (B6) and DBA/2J (D2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Creation of conventional [7] and conditional [16] *Cyp1a1*(−/−) knockout lines has been described. Production of the *Cyp1a1*(−/−) [7], *Cyp1a2*(−/−) [8], and *Cyp1b1*(−/−) [9] knockout mouse lines have been reported. Development of two "humanized" h*CYP1A1_1A2* BAC-transgenic lines have been detailed [5,14]. All experiments involving mice were conducted in accordance with the National Institutes of Health (NIH) standards for the care and use of experimental animals and the University of Cincinnati Institutional Animal Care and Use Committee.

Treatment of the mice. Benzo[*a*]pyrene (BaP), dissolved in corn oil, was given daily in normal rodent chow at a dose calculated to be 125 mg/kg/day [10,17,18]. Mice of five genotypes were sacrificed after 18 days on this diet. In some cases, mice were treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (15 μg/kg i.p. 48 h), vs corn oil for untreated. At least three groups ($N = 3$ each time) were studied to ensure reproducibility.

DNA preparations and polymerase-chain reaction (PCR) analysis. DNA for PCR analysis was isolated from embryonic stem (ES) cells, or from a 5-mm tail biopsy taken from mice on or before postnatal day 21, using previously described methodologies [7].

Cre recombinase/loxP technology. Cre recombinase is an enzyme and *loxP* is a DNA recognition site, present in bacteriophage P1 [19]. A *loxP* site represents 34 bases: inverted repeats of 13 bp, plus an 8-bp spacer sequence that imposes directionality to the recombination event: same orientation results in excision; opposite orientation, inversion [20]. Two *loxP* sites as far apart as 200 kb on the same chromosome have been recognized by Cre recombinase, which then excised all DNA between [21]. Successes at Cre-mediated interchromosomal recombination have also been reported—in which two genes in tandem spanning 40 kb were removed [22], 45 exons spanning 108 kb were excised [23], and non-homologous chromosomes were joined to make novel chimeric chromosomes [24–28].

Generation of the Cyp1a1/1a2(−) allele. To eliminate both *Cyp1a* genes on a single chromosome, we relied on Cre recombinase-mediated interchromosomal recombination between *loxP* sites placed 3′ of the stop codon in both genes (Fig. 1A). Previously, we had deleted the "floxed" (flanked by *loxP* sites) *Cyp1a1(f)* coding sequence using Cre-mediated recombination (lines 2 and 4 of Fig. 1A); this included a floxed *HPRT* minigene in intron 1 and a *loxP* site 187 bp 3′ of the termination codon in exon 7 [16]. Cre-mediated excision gave us the resultant floxed null allele, *Cyp1a1(−)*, containing only exon 1, a portion of intron 1, and one remaining *loxP* site 5′ of the distal 760 bp of the exon 7 untranslated region (UTR) (line 4 of Fig. 1A).

Next, we generated the targeted *Cyp1a2(t)* allele containing a floxed *PGK-NEO* gene [29], placed 350 bp 3′ of the stop codon, using standard gene-targeting techniques; the *loxP* site is located 48 bp 3′ beyond the end of exon 7 (line 3 of Fig. 1A). We prepared a targeting construct with a long arm for recombination encompassing 4429 bp of *Cyp1a2* genomic sequence plus 48 bp of 3′-flanking sequence. This was followed by a floxed *PGK-NEO* gene [22] and then by 1452 bp of 3′-flanking sequence for the short arm. Outside the 3′ *Cyp1a2* homology arm, we placed the *HSK-TK* negative-selection cassette [30]. All targeting vector sequences were assembled in Bluescript SK(−). The targeting vector was linearized by cutting 4 bp downstream of the *HSV-TK* cassette. Following chloroform extraction and ethanol precipitation, this construct was used to electroporate ES cells derived from 129S6/SvEvTac mice [31]. ES cells were selected by using G418 and ganciclovir, and resistant colonies were harvested as described [31]. DNA was prepared from individual colonies and screened by PCR, using two primer sets. One set amplified a fragment of 1617 bp, using primer A in the *PGK-NEO* gene and primer B immediately downstream of the 3′-most *Cyp1a2* sequence that was present in the targeting construct. These primer sequences are:

A: 5′-TCCTCTTGAAAACCACACTGCTCGAC-3′
B: 5′-GATTAGCTGATGGGTGTGTCTATGGG-3′

PCRs included 35 cycles, with initial denaturation at 94 °C for 3 min, followed by cycling at 94 °C for 30 s, 60 °C for 40 s, and then 72 °C for 3 min and 45 s. The second PCR amplified a fragment of 5050 bp, using primer C in the *PGK-NEO* gene and primer D just upstream of the 5′-most *Cyp1a2* sequence that was present in the targeting construct. These primer sequences are:

C: 5′-TGGCTACCCGTGATATTGCTGAAGAG-3′
D: 5′-TAACCCACATCACCGCCAGAGAAATG-3′

PCR conditions were the same as above, except the annealing times were 60 s, and extension times were 6 min.

ES colonies positive for both PCRs were considered "targeted". Several colonies were expanded and used for injecting into B6 blastocysts, as previously described [7,8,20,31]. Coat-color chimeric mice were bred with B6 mice, and agouti offspring were screened by PCR for the targeted *Cyp1a2(t)* allele.

Cyp1a2(+/t) heterozygotes were mated with *Cyp1a1(+/−)* heterozygotes that also carried the universal deleting *CAGGS-CRE* gene [32,33]. *CAGGS-Cre* is a Cre recombinase-expressing transgene driven by a globally expressed *Actb* (β-actin) promoter, which can function developmentally as early as the fertilized ovum [32]. Mice with the genotype *Cyp1a1(+/−)/Cyp1a2(+/t)/CAGGS-CRE(+/−)* were identified using PCR, and these mice were then bred with B6 mice. To detect Cre recombinase-mediated interchromosomal recombination between the *loxP* sites 3′ beyond the stop codons of the *Cyp1a1* and *Cyp1a2* genes (bottom line of Fig. 1A), we used PCR and a primer set in which each primer was 3′