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Genetics

Cytochrome P450 1A1 promoter as a genetic switch for the regulatable and physiological expression of a plasma protein in transgenic mice

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ABSTRACT **Transgenic and gene knockout techniques allow for *in vivo* study of the consequences of adding or subtracting specific genes. However, in some instances, such as the study of lethal mutations or of the physiological consequences of changing gene expression, turning on and off an introduced gene at will would be advantageous. We have used cytochrome p450 1A1 promoter to drive expression of the human apolipoprotein E (apoE) gene in transgenic mice. In six independent lines, robust expression of the transgene depended upon injection of the inducer β-naphthoflavone, whereas the seventh line had high basal expression that was augmented further by the inducer. The low level of basal expression in an inducer-dependent line was confirmed upon breeding the transgene onto the hypercholesterolemic apoE-deficient background. In the basal state transgene expression was physiologically insignificant, as these mice were as hypercholesterolemic as their nontransgenic apoE-deficient littermates. When injected with the inducer, plasma cholesterol levels of the transgenic mice decreased dramatically as apoE expression was induced to yield greater than physiological levels in plasma. The inducer could pass transplacentally from an injected mother to her fetuses with concomitant induction of fetal transgene mRNA. Inducer could also pass via breast milk from an injected mother to her suckling neonatal pups, giving rise to the induction of human apoE in neonate plasma. These finding suggest a strategy to temporarily ameliorate genetic deficiencies that would otherwise lead to fetal or neonatal lethality.**

A powerful tool of modern biology is the ability to create transgenic animals by introducing exogenous DNA. In addition, it is now possible to produce induced mutant and gene-knockout mice via homologous recombination in embryonic stem cells. Another important goal of transgenesis is the capability of introducing genes whose expression could be controlled, allowing study of the physiological consequences of specific transgene expression. In addition, were expression of the transgene controlled fetally or neonatally, rescue of induced mutations in the corresponding gene that are fetal or neonatal homozygous lethals as well as the study of the mutant phenotype in animals after extinguishing transgene expression might be possible.

Several inducible gene expression systems have previously been developed and used in transgenic or *ex vivo* transplanted mice, but these are either very leaky, such as the metallothionein promoter (1); inefficient, such as the *lac* operator (2); or require the presence of an additional regulatory transgene, such as the tetracycline responsive and mutant progesterone receptor systems (3, 4). We describe here a simple, single-gene system, using the human cytochrome p450 1A1 (cyp1a1) promoter, which is highly regulatable by an injected inducer and overcomes the weaknesses of the above-mentioned systems. Cyp1a1 metabolizes and is induced by aryl hydrocarbons and xenobiotics such as dioxin (5). The induction of cyp1a1, related cytochromes, and some other proteins is mediated by the aryl hydrocarbon receptor (AHR), a basic helix–loop–helix transcription factor that is expressed constitutively in many tissues, including the liver (6). The sites on the cyp1a1 promoter that interact with the AHR have been characterized (5). Recently, a mouse deficient in the AHR was created. Although homozygous deficient pups were born at the expected ratio, they suffered ≈50% neonatal lethality, and surviving pups had small and fibrotic livers, indicating a role of the AHR in normal hepatic development (6). By studying wild-type and AHR-deficient mice it has been shown that detectable levels of murine hepatic cyp1a1 expression depend upon treatment with an aryl hydrocarbon inducer and on the AHR gene (6). Fetal rescue of genetic lethality may be possible with the cyp1a1 promoter system because the transplacental induction of endogenous cyp1a1 has been reported in rats (7).

Instead of using a nonphysiological reporter gene to test this regulatable promoter in transgenic mice, we opted to link the cyp1a1 promoter to the human apolipoprotein E (apoE) gene. ApoE is a protein constituent of very low density lipoprotein and some classes of high density lipoprotein and is an important mediator of lipoprotein metabolism and plasma lipid levels as it is a ligand for both the low density lipoprotein receptor and the low density lipoprotein receptor-related protein (8, 9). Via homologous recombination, the mouse apoE gene has been knocked out independently by three groups (10–12). ApoE-deficient mice are hypercholesterolemic and spontaneously develop atherosclerosis throughout the arterial tree on a low fat chow diet (10, 13). The apoE-deficient mouse is therefore an excellent model system in which to study the physiological effects of a regulatable introduced gene, as one can easily measure both the plasma apoE and cholesterol levels in the basal and induced states.

MATERIALS AND METHODS

cyp1a1–apoE Construction and Transgenic Mice. The cyp1a1 promoter extending from −1604 bp to +294 bp relative to the start of transcription (14, 15) was cloned into the *Hin*dIII site of pGem9k2 [prepared by Neil Shachter (Columbia University, New York) from pGem9Zf(−) (Promega) by replacing the *Not* I and *Sfi* I sites with *Kpn* I sites]. An ≈5-kb *Sac* I fragment extending from a site in the first intron to 1.5 kb beyond the 3′ end of the human apoE gene (16) was cloned into the polylinker of the above vector. The DNA insert for microinjection was isolated after *Kpn* I digestion. Transgenic mice were made as described (17), and founders, progeny, and fetuses carrying the transgene were identified from tail tip or

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Abbreviations: cyp1a1, cytochrome p450 1A1; AHR, aryl hydrocarbon receptor; apoE, apolipoprotein E; Tg*X*, transgenic line *X*; E0, apoE-deficient.

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fetal membrane-derived DNA by a PCR assay for the human apoE gene (18). One of the cyp1a1-driven human apoE transgenic lines (Tg*X*) was bred with apoE-deficient (E0) mice (10), and the transgenic progeny heterozygous for apoE deficiency were bred back to apoE-deficient mice to select transgenic progeny homozygous for apoE deficiency (Tg*X*/E0).

Inducer Treatment and Assays for Transgene mRNA and Protein Expression. The inducer β-naphthoflavone (19) was injected i.p. at a dose of 100 μg/g of body weight as a 14 mg/ml suspension in corn oil. Control mice were injected with an equivalent volume of corn oil vehicle. For the RNase protection assay, RNA was prepared from organs or whole fetuses by the acid/phenol/guanidinium method (20). Ten micrograms of RNA was hybridized to a human apoE RNA probe, digested with RNase T1, and run on a 6M urea/polyacrylamide gel as described (21, 22). For the immunoblot assay, 1 μl of plasma was processed by SDS/10% PAGE and transferred to nitrocellulose. After treatment with a casein blocker (Pierce), blots were stained with goat anti-human apoE antiserum (Atlantic Antibodies, Scarborough, ME) and mouse anti-goat IgG linked to horseradish peroxidase (Pierce), and developed with the enhanced chemiluminescent substrate (Amersham). The level of human apoE in the plasma of the Tg1/E0 mouse was determined by a sandwich ELISA (PerImmune, Rockville, MD) as compared with a dilution series of a known human standard (provided by Petar Alaupovic, Oklahoma Medical Research Foundation).

Cholesterol Determinations. Nonfasted blood was taken from the retroorbital plexus into EDTA-containing tubes. The plasma was separated by centrifugation, and plasma cholesterol levels were determined by enzymatic assay (Boehringer Mannheim). Statistical comparisons of plasma cholesterol levels were done by two-tailed *t* test analyses.

RESULTS

Inducible Expression by cyp1a1-Driven apoE Transgenic Mice. The human cyp1a1 promoter region, first exon, and a portion of the first intron were linked to the *Sac* I site in the first intron of human apoE genomic DNA. After oocyte pronuclear microinjection, seven independent transgenic lines were established. Six of the seven transgenic lines yielded no apparent basal expression of the transgene when examined at the level of liver and intestine RNA or at the level of protein in the plasma. However, on prolonged exposure trace amounts of transgene RNA were detectable in liver and intestine of one of these six lines. The remaining transgenic line 4 (Tg4) displayed high levels of basal transgene RNA expression in the liver. One day after injecting the aryl hydrocarbon inducer β-naphthoflavone (100 μg/g of body weight), robust transgene RNA expression was observed in the lines with low-to-undetectable levels of basal expression, strongly in the liver, weakly or not detectable in the intestine (Fig. 1*A*), and not detectable in the brain (data not shown). Inducer injection into the basal expressing Tg4 line led to an increase in transgene RNA expression in the liver and initiated its expression in the intestine (Fig. 1*A*). We selected one of the transgenic lines (Tg1) that had undetectable levels of basal expression for further study. A single β-naphthoflavone injection resulted in the rapid induction and prolonged expression of the transgene-encoded protein in the plasma of Tg1 mice. Fig. 1*B* shows an example of this dramatic appearance and persistence of human apoE in plasma after inducer injection, diminished but still apparent after 19 days. The prolonged expression of the transgene after a single inducer injection appeared due to its relative insolubility, as the yellow inducer could clearly be observed in the peritoneal cavity at autopsy of mice sacrificed 3 days after injection.

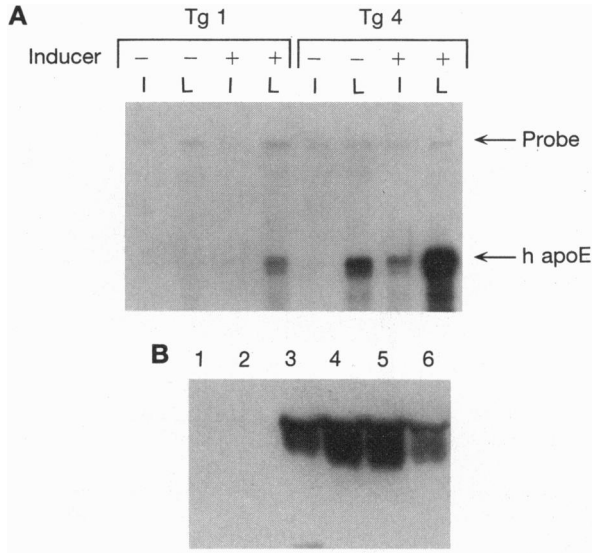

FIG. 1. Inducible expression of human apoE driven by the cyp1a1 promoter in transgenic mice. (*A*) RNase protection assay for human apoE (h apoE) mRNA in intestine (I) and liver (L) of mice, 1 day after injection with vehicle (–) or inducer (+), from the nonbasal apoE-expressing Tg1 line and the basal apoE-expressing Tg4 line. (*B*) Immunoblot for human apoE in plasma of nontransgenic mouse (lane 1), or Tg1 mouse before inducer injection (lane 2) or 3, 6, 11, and 19 days after inducer injection (lanes 3–6, respectively).

Inducible Transgene Expression Effects on Plasma Cholesterol. We bred the inducible Tg1 line onto the hypercholesterolemic E0 background to test for leakiness of the cyp1a1 promoter. Before injection with inducer or vehicle the Tg1/E0 mice had a mean plasma cholesterol level of 463 ± 164 mg/dl (n = 7), not significantly different from the mean cholesterol level of 570 ± 162 mg/dl (n = 9) observed in their nontransgenic E0 littermates. In the E0 mice, plasma cholesterol transiently decreased by 40% (P = 0.02) on the first day after injection of β-naphthoflavone at 100 μg/g, but by the second day cholesterol levels did not differ significantly from pretreatment levels (Fig. 2*A*). This transient decrease in cholesterol was due to the corn oil vehicle and not associated with toxicity of the inducer, as ≈40% decreases in plasma cholesterol levels were also seen in both Tg1/E0 and E0 mice 1 day after injection with the corn oil vehicle (data not shown). In the Tg1/E0 mice, mean plasma cholesterol decreased by 82% (P = 0.0002) to normal mouse levels on the first day after inducer injection and remained at this level for 13 days, after which it gradually increased over the next several weeks (Fig. 2*A*). These cholesterol results support the notion that there were physiologically insignificant levels of transgene expression before induction and reveal the power of this system to express physiologically significant amounts of secreted protein after induction. Human apoE levels in the plasma of Tg1/E0 mice, before and after β-naphthoflavone injection, were measured by an ELISA assay. Basal human apoE levels were undetectable, whereas on the first day after induction plasma apoE ranged from 13 to 38 mg/dl (mean ± SD, 25.0 ± 12.7 mg/dl; n = 4). For comparison, the average apoE level in human plasma is ≈10 mg/dl (23). Onset of the reappearance of hypercholesterolemia (plasma cholesterol >200 mg/dl) varied from 19 days to >50 days in individual inducer-treated Tg1/E0 mice, correlating with variation in the duration of transgene expression at levels of ≈≥0.6 mg/dl. An example of the inverse changes in plasma apoE and cholesterol levels in a single Tg1/E0 mouse before and after induction is shown in Fig. 2*B*. This mouse had a sharp peak of apoE levels on the first day after induction, and hypercholesterolemia returned by the 19th day as apoE levels

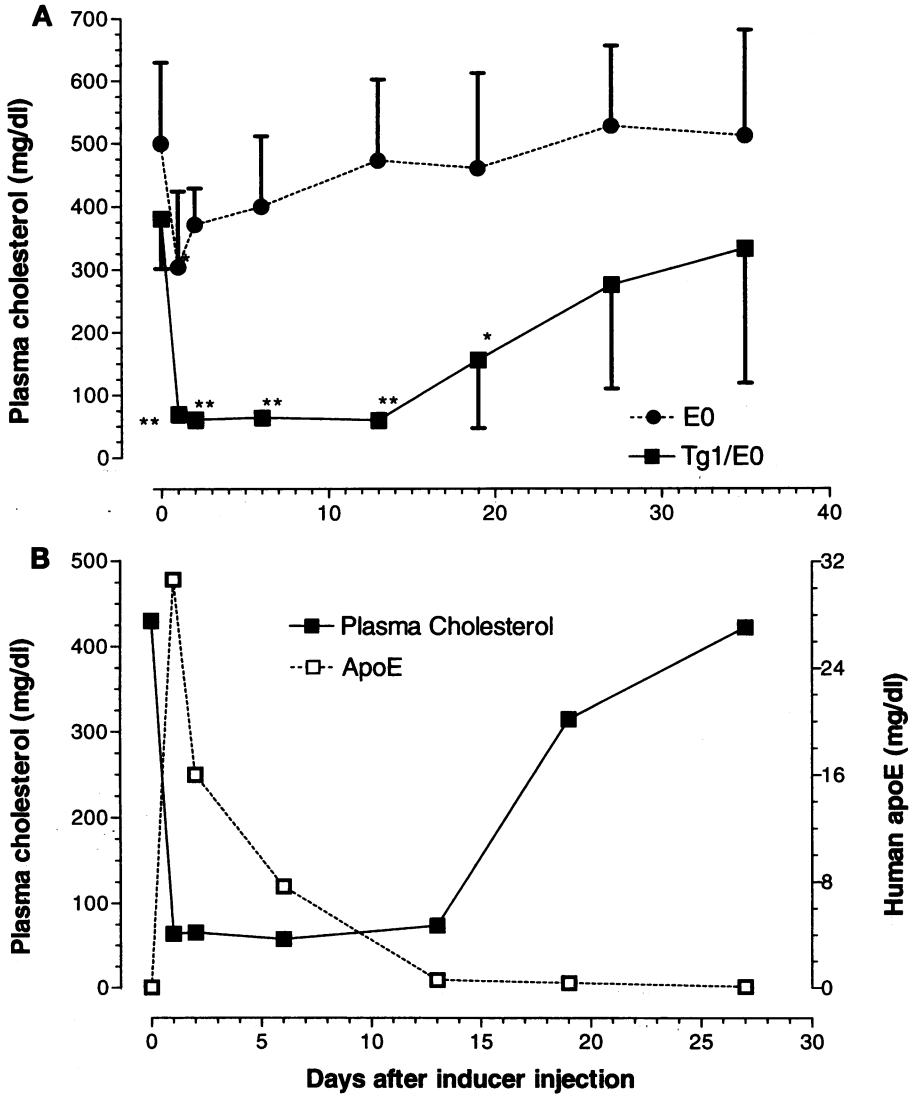

FIG. 2. Effect of inducer on plasma cholesterol and human apoE in Tg1 mice bred onto E0 background. (*A*) Plasma cholesterol levels of Tg1/E0 (■, $n = 4$) and E0 (●, $n = 6$) mice before and after β-naphthoflavone induction (mean ± SD). **, $P = 0.0002$; *, $P = 0.02$, compared to uninduced (day 0) mice. (*B*) Human apoE levels (□) and plasma cholesterol (■) in a Tg1/E0 mouse before and after β-naphthoflavone induction.

decreased to 0.38 mg/dl. Other mice had approximately equivalent apoE levels on the first and second days after induction and had a longer duration of apoE expression at levels >0.6 mg/dl.

Neonatal and Fetal Transgene Induction. To determine whether this promoter system could induce gene expression in neonatal mice via suckling, E0 dams that had been mated to Tg1/E0 males were injected with β-naphthoflavone or vehicle control on the first day after birth, and pups were sacrificed on the following day to obtain plasma for detection of human apoE by immunoblot analysis (Fig. 3*A*). We detected human apoE in the plasma only from Tg1/E0 pups whose mothers had been injected with inducer. Similarly, we injected inducer or vehicle into pregnant E0 mice 14 days postcoitus with Tg1/E0 males and analyzed transgene expression with an RNase protection assay in day 16 fetuses (Fig. 3*B*). Human apoE mRNA was detected in transgenic fetuses from inducer-injected mothers but not in control RNA samples from non-transgenic fetuses or from transgenic fetuses whose mothers were injected with vehicle. These results show the usefulness of the cyp1a1 inducible system in promoting the conditional expression of transgenes during fetal development and during the neonatal period.

DISCUSSION

We have described the cyp1a1 promoter as a single-gene, highly inducible promoter system that may be useful in transgenic mice under many circumstances to provide regulatable gene expression of either intracellular hepatic or secreted serum proteins. By linking this promoter to a human apoE transgene, we demonstrated physiological levels of expression of human apoE in adult transgenic mice after a single injection with an aryl hydrocarbon inducer. Furthermore, upon breeding this inducible transgene onto the hypercholesterolemic apoE-deficient background we demonstrated the inducer-dependent lowering of plasma cholesterol levels. We also showed the usefulness of the cyp1a1 promoter for inducible gene expression in both fetuses and neonates after maternal injection of the inducer.

We observed variable basal levels of apoE expression in the seven transgenic lines described here and in the one transgenic line in which Shachter *et al.* (14) used this promoter to drive a human apolipoprotein CII transgene. This apoCII-expressing line had basal expression of the transgene in liver and brain, which upon injection with an aryl hydrocarbon inducer was further augmented in liver and also appeared in intestine (14). Altogether six of eight lines had very low-to-

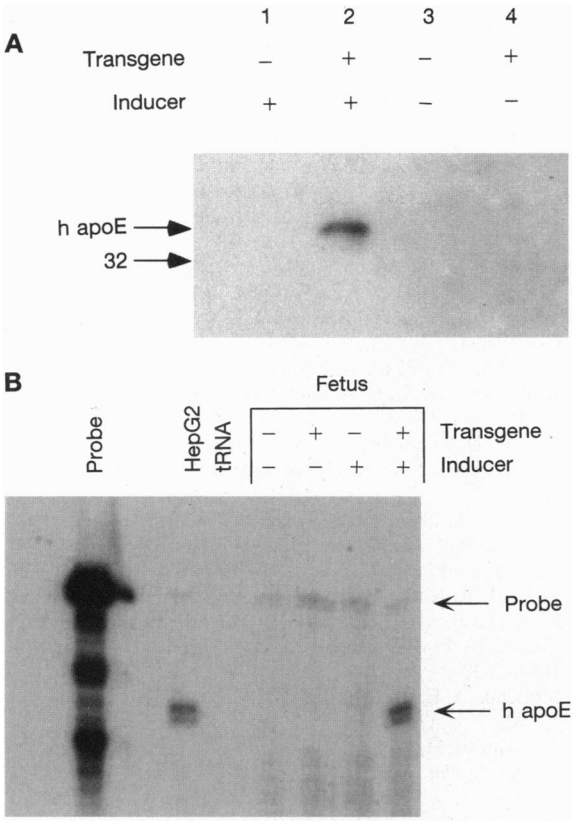

FIG. 3. Human apoE transgene expression induced in neonates and *in utero*. (*A*) Immunoblot analysis for human apoE (h apoE) in plasma of transgenic and control neonates whose mothers were injected with inducer or vehicle. A Tg1/E0 male was mated to two E0 females that were separated after becoming pregnant. On the first day after birth, the dams were injected i.p. with either β-naphthoflavone or vehicle. On the subsequent day pups were bled by decapitation. Pup tissue was genotyped for the transgene by PCR, and human apoE was detected in plasma by immunoblot analysis. 32, Position of 32-kDa marker. (*B*) RNase protection assay for transgene expression in day 16 fetuses. Pregnant E0 mice 14 days postcoitus with Tg1/E0 males were injected i.p. with 0.2 ml of a β-naphthoflavone suspension (14 mg/ml) in corn oil or vehicle alone. Two days later, fetuses were dissected out, and RNA was prepared from the whole fetus for use in the RNase protection assay. Lanes: 1, human apoE RNA probe not digested with RNase; 2–7, 10 μg of RNA hybridized to human apoE RNA probe and digested with RNase T1 before loading. HepG2 and tRNA were used as positive and negative controls, respectively.

undetectable levels of transgene hepatic mRNA expression in the basal state. We speculate that the site of integration of the transgene may play a role in its expression in the two transgenic lines that exhibited basal expression. It may therefore be necessary to derive several transgenic lines to obtain one lacking basal expression. After inducer injection levels of the transgene-encoded apoE were greater than the human physiological level. Our inability to detect apoE in the basal state in the Tg1 line could be, in part, due to assay sensitivity. Nevertheless, strong evidence for the low level of basal expression of human apoE transgene is displayed by the lack of any significant effect on cholesterol levels when bred onto the E0 background. Heterozygous apoE-deficient mice (50% normal apoE levels), as well as apoE-deficient mice with apoE introduced via bone marrow transplantation (12.5% normal apoE levels), have normal plasma cholesterol levels, showing that even moderate amounts of apoE affect plasma cholesterol markedly (10–12, 24). Our study shows that human apoE at ≈0.6 mg/dl is sufficient to ameliorate the hypercholesterolemia in E0 mice. In situations where even very low levels of basal expression are detrimental, the cyp1a1 promoter-driven basal expression may be further decreased by feeding a special low-protein minimal cytochrome p450-inducing diet (25). The degree of regulation achieved in our study can be termed binary because the transgene is, in effect, either on or off.

The transgene expression described here can be induced in neonates via breast milk from their inducer-treated mother and also *in utero* via transplacental transfer of inducer from mother to fetus. The cyp1a1 promoter may, therefore, prove useful for fetal and/or neonatal rescue of lethal homozygous genetic disruptions of hepatic or secreted plasma proteins. Recently fetal gene transfer and expression, although highly variable in efficiency and expression, were observed after injection of DNA lipopolyamine complexes into pregnant mice, and this strategy, the authors speculate, could prove useful for rescuing developmental lethal mutations and gene disruptions (26). We speculate that the cyp1a1 promoter-mediated fetal rescue method proposed here may offer more consistency because the transgene is uniformly present in each transgenic fetus. The cyp1a1 promoter system may also be applicable for the inducible expression in adults of transgenes that might be lethal if overexpressed during development.

Of the other inducible promoter systems used in transgenic mice, the one that has been most developed is the tetracycline-responsive promoter, although thus far the binary regulation of this system has been demonstrated only in transgenic mice with nonphysiological reporter genes (3, 27–30). The initial strategy used a fusion of the bacterial tetracycline repressor, which binds the tetracycline operator sequence only in the absence of tetracycline, with the viral VP16 trans-activating domain to create a system in which the presence of tetracycline leads to the repression of genes linked to the tetracycline operator sequence (3, 27). This system has been modified by placing the tetracycline repressor–VP16 fusion gene under the regulation of the tetracycline operator to generate an autoregulatory system with improved levels of derepressed gene expression (28). The tetracycline-mediated repression system has also been modified into a tetracycline-activating system by mutating the tetracycline repressor–VP16 fusion so that it binds DNA only in the presence of tetracycline (29). Although these systems may be useful in extrahepatic tissues in which the cyp1a1 system is inactive, each of these systems requires the presence of two separate transgenes, the repressor/activator transgene and the transgene whose expression is to be regulated by linking to the tetracycline operator. The need to include the repressor/activator transgene requires extra breeding, labor, and expense. For example, if this system is to be used for rescuing a lethal homozygous gene deficiency, both transgenes would have to be bred in two rounds onto the heterozygous knockout background, and these mice, in turn, interbred to get the double transgenic on the homozygous deficient background. The advantage of using the cyp1a1 promoter as a regulatory system is that its transactivator, the AHR, is an endogenous gene; therefore, only one transgene, the one that is to be regulated, must be introduced. In the above example, only one round of breeding would produce transgenic mice on the heterozygous deficient background, which upon interbreeding would generate the desired transgenic mouse on the homozygous deficient background at a 33% greater frequency.

After we completed this study we became aware of a previous study that describes the use of mouse cyp1a1 promoter in transgenic mice to regulate expression of the chloramphenicol acetyltransferase reporter gene (31). Injecting the inducer 3-methylcholanthrene gave rise to up to 10,000-fold regulation of this reporter gene in the liver and, in three transgenic lines, variable induced expression in spleen, intestine, kidney, and lung (31). We have significantly extended this study to show that physiological levels of a plasma protein can be expressed by human cyp1a1 promoter in the induced state, significantly affecting plasma cholesterol levels. We also show that the inducer β-naphthoflavone has a relatively long-lasting

effect on gene expression and that this inducer is effectively transferred from dams to fetuses and neonates. In conclusion, the cyp1a1 inducible promoter provides a single-gene, highly regulatable method to switch on gene expression at will in transgenic mice. The high level of human apoE transgene expression and its effect on plasma cholesterol in the E0 background prove the utility of this system for regulatable expression of either hepatic or hepatic-derived plasma proteins.

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