Induction of the *Cyp1a-1* dioxin-responsive enhancer in transgenic mice

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ABSTRACT

Cyp1a-1, whose product, aryl hydrocarbon hydroxylase, assists in detoxification of polycyclic aromatic hydrocarbons, is the best characterized of the murine cytochrome P450 genes. The *Cyp1a-1* dioxin-responsive enhancer region has been previously analyzed *in vitro* and found to induce expression of heterologous genes upon exposure of transfected cells to various aromatic hydrocarbons. A 2.58 kbp DNA fragment containing the *Cyp1a-1* enhancer elements and promoter region was coupled to the chloramphenicol acetyltransferase (CAT) reporter gene and used to create transgenic mice. CAT assays were performed on tissues harvested from three different lines of transgenic mice following mock-induction or induction using the aromatic hydrocarbon, 3-methylcholanthrene. Basal levels of expression were detected in the spleen and small bowel of non-induced mice, with little or no expression detected in the liver. Treatment with 3-methylcholanthrene increased hepatic expression levels by as much as 10,000-fold. More modest levels of induction were also recorded in the spleen, small bowel, kidney, and lung. The results indicate that the dioxin-responsive enhancer region functions as a strongly inducible promoter *in vivo*. Differences in the response to induction between male and female mice suggest that *Cyp1a-1* expression may be governed in a gender related manner.

INTRODUCTION

The cytochrome P450-mediated monooxygenases are the most numerous class of drug-metabolizing enzymes. At present, twenty P450 gene families have been identified, ten of which are found in all mammals (1). The membrane-bound enzymes encoded by the P450 gene superfamily act upon a broad range of exogenous and endogenous lipophilic compounds. Interestingly, many of these compounds induce their own metabolism, and hence the formation of mutagenic reactive intermediates, by inducing the expression of certain cytochrome P450 genes (2).

Perhaps the best characterized member of the P450 superfamily is the polycyclic aromatic hydrocarbon-inducible P450A1 (*Cyp1a-1*) gene. This gene is located on mouse chromosome 9

(3) and encodes aryl hydrocarbon (benzo[a]pyrene) hydroxylase (E.C.1.14.14.1). The administration of polycyclic aromatic hydrocarbons, typified by 3-methylcholanthrene (3-MC) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), to certain strains of mice increases aryl hydrocarbon hydroxylase (AHH) activity in a variety of different tissues (4,5). Mouse strains have been divided into 'responsive' and 'non-responsive' strains based upon the degree of induction by aromatic hydrocarbons. Genetic crosses and backcrosses between various inbred strains of mice determined induction of AHH activity by Aromatic hydrocarbons to be a dominant trait encoded by a single autosomal locus (*Ah* locus) (6–8). The *Ah* locus encodes an intracellular receptor which binds saturably and with high affinity to 3-MC, TCDD, and to other polycyclic aromatic hydrocarbons (9,10). There is genetic evidence in mice for the existence of at least three alleles at the *Ah* locus (11) and three forms of the receptor have been distinguished via DNA-Sepharose chromatography in rodent hepatic cytosol (12), but neither the successful purification of the *Ah* receptor nor cloning of the *Ah* gene has been reported.

A direct correlation exists between the presence of an inducer-receptor complex in the nucleus and the synthesis of *Cyp1a-1* mRNA (13–15). Studies performed using *Ah* receptor-defective murine hepatoma cells have determined that activation of transcription requires binding of the inducer ligand to the intracellular *Ah* receptor, a temperature-dependent translocation of the ligand-receptor complex to the nucleus, and binding of the receptor complex to a dioxin-responsive enhancer region located immediately 5' to the *Cyp1a-1* gene (15–21). Gel retardation analysis, exonuclease protection experiments, and DNase footprinting studies reveal that the receptor complex interacts with a specific nucleotide sequence motif present in multiple copies within the *Cyp1a-1* dioxin-responsive enhancer (22–28). Recently, it has been reported that binding of the ligand-receptor to the recognition motifs bends the DNA at or near the site of protein-DNA interaction, suggesting that a conformational change in the enhancer region mediated by the binding of the receptor complex leads to an increase in the rate of *Cyp1a-1* gene transcription (29).

Many previous groups have employed nucleic acid hybridization or substrate affinity testing to examine the spatial and/or temporal pattern of *Cyp1a-1* (or orthologues of *Cyp1a-1*) gene expression in rodents following induction by aromatic

hydrocarbons. Although these studies have greatly contributed to our understanding of the mechanism and response of the *Cyp1a-1* promoter to induction, the high degree of sequence homology among various P450 genes, and the possibility of shared substrate affinities by the P450 gene products renders it difficult unambiguously to document the *Cyp1a-1* promoter response to induction. In this study, we examine the response of the mouse *Cyp1a-1* promoter region to several classic inducers of aryl hydrocarbon hydroxylase activity in transgenic mice. This approach permits induction of the *Cyp1a-1* promoter region to be distinguished from the complex background of cytochrome P450 gene expression. A 2.58 kbp *Cyp1a-1* promoter region fragment containing the multiple dioxin-responsive enhancer motifs was linked to the chloramphenicol acetyltransferase (CAT) reporter gene and microinjected into fertilized oocytes harvested from an Ah-inducible strain of mice. Three lines of transgenic mice were examined for their tissue pattern of promoter response to induction. The results of this study indicate that the *Cyp1a-1* enhancer region functions as both a constitutive and inducible promoter in a variety of tissues and is a strongly inducible promoter in liver. Differences in the level of induction between male and female mice in hepatic and non-hepatic tissues suggest that hormonal influences may also play a role in regulating *Cyp1a-1* expression.

MATERIALS AND METHODS

Creation of transgenic mice

Plasmid pHAVcat contains the CAT reporter gene linked to SV40 large T antigen splice donor/acceptor sites and polyadenylation signals placed under transcriptional control of a 2.58 kbp *HindIII* fragment containing 2.19 kbp of *Cyp1a-1* 5' flanking sequences and the first 390 bp of coding sequences (30). A 4.9 kbp DNA fragment bearing the entire *Cyp1a-1* promoter region-CAT gene and most of the SV40 3' sequence was isolated from pHAVcat by a *PstI-NdeI* double digest, followed by low melting agarose gel electrophoresis, phenol extraction, and ethanol precipitation.

Microinjection of oocytes followed the protocol described by Brinster et al. (31). Founder Fo mice bearing the *Cyp1a-1*-CAT gene were identified by Southern blot analysis of DNA extracted via established methods (32) from approximately two centimeters of tail biopsy at two weeks of age. Southern blots of genomic DNA digested with *EcoRI* were performed using standard techniques (33). An *EcoRI-BamHI* fragment of the CAT gene was isolated from pSV2CAT (34) by low melting agarose gel electrophoresis, phenol extraction, and ethanol precipitation, radiolabeled by extension of random primers (35), and used as a probe in the Southern blots to detect the presence of transgene DNA. Hybridizations were performed as previously described (36).

Induction of transgenic mice

Two-month old F1-generation male sibling mice from the Fo # 2 transgenic line were mock-induced or induced with a single interperitoneal injection of 3-methylcholanthrene (3-MC), β -naphthoflavone (NF), or phenobarbital (PB). Seventy-five mg per kilogram bodyweight of 3-MC or 200 mg per kilogram bodyweight of NF were administered in a minimal volume of Mazola-brand corn oil. Fifty mg per kilogram bodyweight of PB was given in a 0.9% saline solution. Control mice (mock-induced) were injected with corn oil equal in volume to the amount used to administer the 3-MC. Matched male and female F2-generation siblings of all three transgenic lines were induced with 3-MC

via a single interperitoneal injection of 75 mg per kilogram bodyweight in a minimal volume of corn oil. Control mice were mock-induced with corn oil as described above. All mice were anesthetized and sacrificed 24 hours post-induction. No physiologic effect was noticed following injection of 3-MC, NF, or corn oil alone. Some minor impairment of motor coordination lasting approximately 1–2 hours after injection was noticed in the PB-induced mice.

Assay of CAT activity

Tissues harvested from induced and mock-induced mice were polytron homogenized on ice in 0.25 M Tris-HCl (pH 7.7) and subjected to three rapid freeze-thaw cycles. Cell debris was removed via centrifugation at 12,000 rpm. for 15 minutes at 4°C in an Eppendorf microfuge. Supernatant was recovered, heated at 65°C for 5 minutes to inactivate endogenous de-acetylases, rapidly cooled, and assayed for protein concentration. CAT assays were performed as described by Gorman et al. (34) with the following modifications: The reaction mix contained 250 mM Tris-HCl (pH 7.7), 0.2 μ Ci of 14 C-chloramphenicol (60.0 mCi/mmol), 2 mM acetyl-CoA, and 100 mg of protein extract in a final volume of 100 μ l. The mixture was incubated at 37°C for 30 minutes, and the reaction terminated by extracting with 1 ml of cold ethyl acetate. The ethyl acetate was evaporated, the pellets redissolved in 10 μ l of ethyl acetate, and the reaction products analyzed by silica gel TLC. Following autoradiography, the reaction products were quantitated by scintillography of the silica gel sections. The reaction kinetics for this assay were found to be linear up to 50% conversion of substrate to products, thus samples yielding conversion values greater than 50% were re-assayed using less extract or for shorter periods of time and the results adjusted accordingly.

RESULTS AND DISCUSSION

Creation of transgenic mice

The FVB inbred strain of mice is routinely used for creating transgenics in our laboratory and in others due to the distinct morphology of the fertilized oocytes and the rapid proliferation of these mice. The Ah-responsive status of FVB mice was examined to ensure that this was a suitable strain (Ah+) for use in creating transgenics for these experiments. The metabolism of zoxazolamine in aromatic hydrocarbon-treated, genetically responsive mice correlates with an increase in hepatic microsomal AHH activity (37). Therefore, zoxazolamine sleeping time offers a quick and reliable test for determining the Ah-responsive status of a mouse strain (38). Four male C57BL/6 (Ah+), DBA (Ah-), and FVB mice were injected intraperitoneally with zoxazolamine (225 mg/kg of body weight) in corn oil twenty-four hours after

Table 1. Duration of zoxazolamine-induced sleep in inbred strains of mice pretreated with 3-MC. The range of zoxazolamine-induced sleep is given in minutes for each inbred strain of mice. Four male C57BL/6 (Ah+), DBA (Ah-) and FVB mice were either mock pretreated or pretreated with 3-MC 24 hours prior to the administration of zoxazolamine. A maximal sleeping time of 180 minutes was assigned to those mice which failed to recover within this time period.

strain	zoxazolamine	
	control	3-MC
C57BL/6	55–180	0–10
DBA	75–145	100–180
FVB	75–180	10–50

receiving a single dose of either 3-MC in corn oil or corn oil alone (control). All of the mice were asleep within several minutes and were placed on their back. The duration of sleep was recorded as the period of time required for the animal to regain consciousness, right itself, and become ambulatory. The results presented in Table I indicate that pre-induction of the FVB inbred strain with 3-MC results in quicker metabolism of zoxazolamine, similar to results obtained with the Ah-responsive C57BL/6 strain. This strain is therefore phenotypically positive for the Ah locus.

Single cell embryos of FVB mice were microinjected with the *Cyp1a1*-CAT construct and transferred into foster mother mice (ICR strain). This experiment yielded 40 offspring that survived the newborn period, seven of which were found to have incorporated the transgene into their genome. Southern analysis revealed that each of the transgenic mice possessed multiple copies of the transgene which were integrated into the genome in a head-to-tail arrangement at a single site. Three founder mice (Fo #2, #6, and #7) were mated with FVB mice to establish F1 and F2 generations for each line. Southern analysis performed on the offspring indicated that the transgenes were stably inherited in a Mendelian fashion in each founder line (data not shown).

Response of the *Cyp1a1* enhancer region to induction

Several known inducers of aryl hydrocarbon hydroxylase (AHH) activity were examined for their ability to induce *Cyp1a1*-driven CAT expression in a variety of tissues. Two-month old male siblings of the Fo #2 transgenic line were either mock-induced or induced with a single interperitoneal injection of methylcholanthrene, naphthoflavone, or phenobarbital. The mice were sacrificed twenty-four hours after injection and liver, spleen, heart, small bowel, kidney, lung, muscle, brain, and testis were harvested and assayed for CAT activity. The results of this experiment (figure 1) indicate that the *Cyp1a1* promoter region functions in the absence of induction in both the spleen and small bowel. Promoter induction of transgene expression occurred in the liver, lung, and kidney when the mice were injected with either 3-MC

or NF. These compounds have been previously documented to induce AHH activity in the liver and lung of inducible strains of mice (39), and our findings agree with an earlier report by Burki et al. which indicated that NF was generally more effective than 3-MC at inducing AHH activity (38).

Figure 1. Response of the *Cyp1a1* Enhancer Region to Induction. F1 generation male offspring of the Fo #2 founder line were mock-induced or induced with 3-MC, NF, or PB as described in 'Experimental Procedures'. Tissues were harvested 24 hours post-induction and assayed for CAT activity. The locations of the acetylated products (1 ac and 3 ac) are given beneath each autoradiograph.

Figure 2. Induction of *Cyp1a1*-CAT Expression in Three Lines of Transgenic Mice. Four male and four female sibling mice from founder lines Fo #2, Fo #6, and Fo #7 were mock-induced or induced with 3-MC as described in Materials and Methods. Tissue extracts were derived from the mice twenty-four hours post-induction and assayed for CAT activity. The results (mean and standard deviation) are expressed as percent conversion of the radiolabelled chloramphenicol substrate to acetylated products per hour per 0.1 mg of extract. Since induction, in some instances, led to differences in the levels of CAT activity which exceeded four orders of magnitude, the ordinates of each graph represent levels of CAT activity on a logarithmic scale.

The administration of PB has been previously reported to yield a modest induction of AHH activity in mouse liver and in rat fetal hepatocyte cultures (40,41). However, this induction was found to occur in liver explants from mice of both inducible and non-inducible strains, suggesting that the mechanism of hydroxylase induction by PB differs somehow from the mechanism utilized by polycyclic aromatic hydrocarbons (42). Subsequent studies have found that PB-induced mouse liver mRNA does not hybridize on Northern blots to *Cyp1a-1* cDNA (43), and polymerase chain reaction-based assays of hepatic mRNA isolated from 3MC-responsive rats fails to detect *Cyp1A1* (P450c) expression following induction by PB (44). These findings indicate that PB-mediated induction of AHH activity does not result from an increase in *Cyp1a-1* synthesis. Our data are consistent with these reports: injection of PB yielded no detectible increase in CAT activity in any tissue. The slight decrease observed in the level of transgene expression in the PB-treated mouse relative to the basal level of *Cyp1a-1*-CAT expression in the control animal may be due to a general decrease in the host's metabolism resulting from the administration of PB.

Induction of *Cyp1a-1*-CAT expression in three lines of transgenic mice

Induction of the *Cyp1a-1* enhancer region by 3-MC was examined in various tissues for the three lines of transgenic mice. Four male and four female siblings from each founder line (F2 generation) were either mock-induced or induced with a single dose of 3-MC. The mice were sacrificed 24 hours post-induction and tissues were harvested and assayed for CAT activity.

Basal levels of transgene expression were detected in the spleen, small bowel, and to a lesser extent, in the liver, kidney and lung of mock-induced mice of both sexes from all three founder lines (figure 2). The level of CAT activity in these tissues varied in the different founder lines. The heterogeneity of the results is typical of differences in tissue pattern and levels of transgene expression normally experienced when working with different lines of transgenic mice and is very likely due to differences in the chromosomal site of transgene integration among the three lines of mice.

Non-hepatic levels of *Cyp1a-1* promoter induction ranged from 1.5 - 15 fold (spleen), 14 - 47 fold (small bowel), 5.0 - 14 fold (kidney), and 0.0 - 18 fold (lung) in male mice. Levels of induction were generally lower in female mice in these tissues, with values ranging from 2.2 - 2.8 fold (spleen), 2.5 - 10 fold (small bowel), 2.3 - 51 fold (kidney), and 2.0 - 13 fold (lung). CAT assays performed on liver extracts revealed extraordinarily high levels of *Cyp1a-1* induction in both male and female mice of these three lines, with males exhibiting much greater induction values than females (123 - 10,000 fold vs. 11 - 1,200 fold). Neither basal nor induced transgene expression was detected in the heart, muscle, brain or testis/ovary in the three lines of mice.

Our investigations indicate that the *Cyp1a-1* dioxin-responsive enhancer region functions as an inducible promoter in the spleen, bowel, kidney, and lung of mice, and as a strongly inducible promoter in the liver. These results support an earlier study by Burki et al. which documented a 6- to 7- and 4- to 5-fold elevation, respectively, in total AHH activity in the lung and liver of inducible mice in response to 3-MC (39). Although high levels of basal (non-induced) AHH activity are normally present in these tissues (39), our data suggest that this activity is likely the result of expression of P450 genes other than *Cyp1a-1*. Kawajiri et al. have reported a 50-fold increase in rat *Cyp1A1* (P450c) hepatic mRNA levels following administration of 3-MC (44). Our

findings suggest that the level of hepatic induction of *Cyp1a-1*-encoded AHH activity by 3-MC may be much greater than has been previously reported. However, it should be noted that the 2.58 kbp *Cyp1a-1* promoter fragment used to create the transgene was originally derived from a mouse 'high activity variant' hepatoma cell line, wherein *Cyp1a-1* transcription levels were found to be elevated in response to dioxin relative to levels induced in wildtype hepatoma cells (46). Furthermore, the 2.58 kbp transgene promoter fragment includes a portion of the *Cyp1a-1* coding sequences and first intron (47). Thus, cryptic or altered sequences present in the *Cyp1a-1* promoter-CAT construct may account for the elevated induction levels of *Cyp1a-1*-driven transgene expression.

The underlying basis for the difference between male and female mice in the response of the *Cyp1a-1* enhancer region to 3-MC induction is unclear. In particular, hepatic induction of *Cyp1a-1*-CAT was approximately 10-fold greater in male mice than in female mice in each founder line. The expression of other classes of cytochrome P450 genes are known to be gender regulated (48,49). It is possible that induction of *Cyp1a-1* expression is similarly susceptible to hormonal influences. Further study of the *Cyp1a-1*-CAT induction response in matched male and female sibling should help elucidate the basis of this phenomena.

The tremendous increase in the level of *Cyp1a-1* promoter-driven transgene expression following administration of 3-MC, especially in the liver where expression increases from near zero to very high levels within 24 hours, suggests that the *Cyp1a-1* dioxin-responsive enhancer region is likely to have widespread utility as an inducible regulator of hepatic transgene expression. The ability to promote massive levels of transgene expression in a temporal manner may aid studies aimed at understanding the role of hepatic growth factors in regenerating liver, the effects of antisense transcript production in mice during different stages of development, and facilitate research in liver cancer-causing agents by permitting transgene induction of mutated anti-oncogenes in pre-treated mice. In addition, pharmacologic testing to screen for potential toxicity of drugs which rely upon the cytochrome P450 system for metabolism could be performed utilizing transgenic mice which harbor cytochrome P450 promoter-driven reporter genes.

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