Genetic analysis of the phenobarbital regulation of the cytochrome *P*-450 2b-9 and aldehyde dehydrogenase type 2 mRNAs in mouse liver

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The aim of this study was to investigate the effect of the genetic background on the phenobarbital inducibility of cytochrome *P*-450 2b-9, cytochrome *P*-450 2b-10 and aldehyde dehydrogenase type 2 mRNAs in mice. We analysed the basal expression and the phenobarbital inducibility of both cytochrome *P*-450 mRNAs by semi-quantitative specific reverse transcription-PCR analyses in five inbred mouse strains (A/J, BALB/cByJ, C57BL/6J, DBA/2J and SWR/J). Male mice constitutively expressed cytochrome *P*-450 2b-9 and cytochrome *P*-450 2b-10 mRNAs, but a number of differences in their response to phenobarbital were observed. In all these mouse strains, phenobarbital induced cytochrome *P*-450 2b-10 mRNA whereas it could have either a positive or a negative effect on cytochrome *P*-450 2b-9 expression, depending on the strain and the sex of the mice. Specifically, phenobarbital increased cytochrome *P*-450 2b-9 expression in C57BL/6J males while it decreased it in DBA/2J mice. Interestingly, dexamethasone was able to mimic the phenobarbital effect on both cytochromes *P*-450 in these two strains. Aldehyde dehydrogenase type 2 mRNA was always induced by phenobarbital, except in the C57BL/6J strain. Genetic analysis revealed that the phenobarbital-inducible phenotype was either a semi-dominant or a recessive trait in F1 animals from a C57BL/6J × DBA/2J cross for the cytochrome *P*-450 2b-9 and the aldehyde dehydrogenase type 2 genes, respectively. This study suggests that the genetic basis for phenobarbital induction in mice depends on the target gene, and that more than one regulatory step would be involved in this response pathway.

INTRODUCTION

Phenobarbital (PB) has long been known as a detoxication enzyme inducer in a very extensive set of organisms, including bacteria, yeast, chicken and all mammalian species surveyed [1]. Cytochromes *P*-450 (CYPs) of several families, the cytosolic form of aldehyde dehydrogenase (Ald-2), as well as glutathione S-transferases, UDP-glucuronyl transferases or the epoxide hydrolase, are influenced generally, although not exclusively, in a positive fashion by PB treatment of animals or cells in culture [2]. Presumably, the response to PB involves many more genes than is already recognized, as suggested by sequence representation in cDNA libraries or following differential display PCR [3,4]. In addition to its inductive or suppressing effects on detoxication enzyme-encoding genes, PB induces major modifications of the liver tissue, such as proliferation of the endoplasmic reticulum and weight increase. Moreover, the barbiturate is a well-known tumour promoter, but not a mutagen, in carcinogen-initiated liver cells of experimental animals [5]. Finally, PB is metabolized in the liver by some isoforms of CYP, the expression of which is enhanced by the barbiturate.

Surprisingly, despite the magnitude and the range of the primary responses triggered by PB, these are mostly restricted to the liver, suggesting the involvement of key tissue-specific factors in controlling the majority of the effects of the barbiturate. The existence of a specific receptor for the molecule was postulated many years ago, but the very low potency of PB has prevented any direct identification from any cell compartment [6]. Even

more potent agonists have not resulted in the discovery of such a receptor. In addition, among the myriad of 'PB-like' inducers, no structural similarities have appeared, arguing against the existence of a specific receptor [2].

Most of the attention devoted to the identification of regulatory factors has been focused on looking at the inductive response of CYP genes from family 2, which are strongly transcriptionally activated following PB injection in rats [7]. Recently, upstream sequences of the CYP 2B2 gene have been found to behave as PB-dependent transcriptional enhancers in primary hepatocyte cultures, and to bind nuclear proteins in an activated fashion following PB treatment [8]. Other remote sequences from the upstream region of the CYP 2B2 gene have been shown to be necessary for the establishment of the inducible phenotype in adult transgenic mice [9]. In addition, a PB-responsive enhancer has been described in the chicken CYP 2H1 gene, the orthologue of rat CYP 2B1 [10]. However, the relevant proteins remain to be characterized. A great deal of information on the mechanism by which barbiturates activate transcription of bacterial CYP genes has now been obtained [11]. However, the induction in bacteria is not a saturable phenomenon, unlike that in mammalian species, making it improbable that the primary mechanisms are indeed well conserved [2].

Another aspect of the response to PB is its interplay with second messenger pathways triggered by hormones, cyclic AMP and cytokines. Indeed, growth hormone and interleukin 1β have been shown to prevent PB-mediated induction of several CYP genes [12,13], whereas cyclic AMP was able to either prevent or

potentiate PB induction of CYP 2B or Cyp 2a genes in rats and mice, respectively [12,14,15]. Moreover, sex hormones could antagonize the negative effect of growth hormone on the PB induction of rat CYP genes [16]. In addition, it has been suggested that a steroid receptor was involved in induction of CYP 2 genes in rat hepatoma cells, since PB induction was prevented by RU486, a glucocorticoid and progesterone antagonist [17]. Furthermore, dexamethasone (DEX)-induced mouse Cyp 2b genes and the core sequence of the glucocorticoid response element (GRE) has been found at six positions in the 5′-flanking regions of the Cyp 2b-9 gene [18,19]. In addition, transfection experiments have established the presence of a functional GRE between residues 1339 and 1359 upstream of the rat CYP 2B2 gene transcription start site [20].

Despite the large body of evidence currently available concerning the mechanisms by which PB activates gene expression, and in view of the fact that the barbiturate displays strong suppressive effects as well, we decided to develop a strategy, based on a genetic analysis in mice, to get an estimate of the number of genes involved in this pleiotropic control phenomenon. Indeed, several experimental data have demonstrated the existence of a genetic basis for the response of mouse and rat liver functions to PB [21,22]. However, the existence of a unifying mechanism that would account for the pleiotropic effects, namely the inductive or suppressive potential of PB over most if not all of the target genes, is still widely discussed. Large differences in PB and DEX induction factors of Cyp mRNAs from families 2b, 2c and 3a have been found between mouse strains [23]. However, since the probes used could not discriminate between highly similar sequences, it is possible that some of the differences are due to a reduction or a complete block in induction of one or several Cyp mRNAs in some of the strains. In the present study, we have developed a reverse transcription (RT)-PCR-based strategy allowing discrimination between Cyp 2b-9 and Cyp 2b-10 mRNAs and determination of their specific responses to both PB and DEX. In addition, we have looked at the PB response of the Ald-2 mRNA, the cytosolic form of the aldehyde dehydrogenase, in five inbred mouse strains, F1 hybrids, and their F2 derivatives.

EXPERIMENTAL

Animals

Male and female mice from the strains A/J (A), BALB/cByJ (BALB), C57BL/6J (B6), DBA/2J (D2), SWR/J (SWR), C57BL/6J × DBA/2J (B6D2F1) and B6D2F1 × B6D2F1 (B6D2F2) mice were obtained from CERJ (Le Genest St.-Isle,

France). The animals were housed in plastic cages on wood-chip bedding (U.A.R., Villemoisson-sur-Orge, France) and fed AO4 (U.A.R., Villemoisson-sur-Orge, France) and Wayne Breeder Box (Continental Grain Co., Chicago, IL, U.S.A.) food *ad libitum*. At ages ranging between 8 and 12 weeks old, mice were treated with one intraperitoneal injection of drug (80 mg/kg for PB and 200 mg/kg for DEX) 18 h before use. PB and DEX were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dissolved to a final concentration of 10 mg/ml (in 0.9 % NaCl and trioctanoin for PB and DEX, respectively). Control animals received the vehicle alone. Animals were killed by cervical dislocation. Liver and spleen samples were rapidly removed and frozen in liquid nitrogen. All experiments involving animals were conducted according to French regulations.

Total RNA extraction and hybridization

Total liver RNA was extracted by the one-step guanidinium thiocyanate–phenol–chloroform extraction method according to the method of Chomezynski and Sacchi [24]. About 10 μg aliquots of RNA samples were then subjected to electrophoresis under denaturing conditions [6 % (v/v) formaldehyde, 1.2 % (w/v) agarose], transferred on to Hybond-N nylon filters (Amersham, Arlington Heights, IL, U.S.A.) and fixed with a UV cross-linker (Amersham, Arlington Heights, IL, U.S.A.). Prehybridization, hybridization and washes were performed according to the method of Church and Gilbert [25]. cDNA probes were ³²P-labelled by random priming using a T7 DNA polymerase random priming kit (Amersham, Arlington Heights, IL, U.S.A.). Filters were then exposed to autoradiography at −80 °C. Probes used in this study were full-length rat cDNA for PB-inducible rat aldehyde dehydrogenase (ALDH-PB) [22], 1250 bp rat cDNA from the 5′ end of the coding region for CYP 2B1 and a full-length rat CYP 2B1 cDNA (a gift from M. Adesnik, NYU Medical Center, New York, NY, U.S.A.) and chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) full-length cDNA.

RT-PCR analyses

Semi-quantitative PCR

Oligonucleotide primers (20 bp) derived from the cDNA sequences of mouse Cyp 2b-9, Cyp 2b-10 [26] and β-actin were synthesized (Table 1). Base sequences of the Cyps 2b reverse primers were chosen to be highly divergent (65 %) in order to obtain specific annealing with Cyp 2b-9 and Cyp 2b-10 mRNAs prior to RT. Specific cDNAs for mouse Cyp 2b-9 and Cyp 2b-10 were obtained from 1–2 μg of total RNA (treated with DNase I

Table 1   Parameters of the PCR analyses

For both Cyps, the forward and reverse primers were from nt 1240 to 1259 and nt 1550 to 1531, respectively.

	Cyp 2b-9	Cyp 2b-10	β-actin	D19Mit13	D19Mit28
Primers Forward Reverse	5'-TGAAGCTTTTCTGCCCTTCT-3' 5'-GTGTGAGCAGCTACCAATGG-3'	5'-TGAAGCTTTTCTGCCCTTCT-3' 5'-TGGAGACATGCAATAGGAGG-3'	5'-GCCCAGAGCAAGAGAGGTAT-3' 5'-GGCCATCTCTTGCTCGAAGT-3'	5'-TCTGGCACAAAGAGTTCGTG-3' 5'-CTTTTGCAGGAGCAGGTAGG-3'	5'-TCTTCATGCCCAAAAGAGCT-3' 5'-GCATCCTGAATCTCCTGCG-3'
Length	311 bp	311 bp	513 bp	246 bp (B6) 262 bp (D2)	148 bp (B6) 154 bp (D2)
PCR parameters	30 cycles 30 sec at 94°C 30 sec at 53°C 2 min at 72°C		23 cycles 30 sec at 94°C 30 sec at 55°C 2 min at 72°C	35 cycles 40 sec at 94°C 40 sec at 55°C 1 min at 72°C	

to remove any contaminating genomic DNA) under incubation conditions recommended by the supplier of the cDNA synthesis kit (Clontech Laboratories, San Palo, CA, U.S.A.). Subsequently, PCR was performed in 100 μl of PCR buffer: 20 mM Tris/HCl, 50 mM KCl, 300 nM of each primer, 1 mM MgCl$_2$, 125 μM dNTPs and 0.5 unit *Taq* DNA polymerase (Eurogentec, Seraing, Belgium). Annealing temperature and the MgCl$_2$ concentration were determined to obtain the best purity of PCR products (Table 1). Aliquots (20 μl) of the PCR reactions were subjected to electrophoresis (2 % agarose gel) and products were visualized by ethidium bromide staining. Gels were blotted on to Hybond-N$^+$ filters (Amersham, Arlington Heights, IL, U.S.A.) and subjected to hybridization with rat β-actin or CYP 2B1 cDNA probes. After autoradiography, signals were quantified by densitometry using imaging software (Bioprobe systems, Montreuil, France). Digestion of Cyp PCR products with *Bgl*II restriction enzyme led to two bands of 185 and 126 bp, as expected from the known structural features of the Cyp 2b-9 and Cyp 2b-10 genes, respectively. Cyp PCR products were sequenced and found to be identical with the published mouse cDNAs (results not shown).

Microsatellite analysis

High-molecular-mass DNA was prepared from spleen tissue according to the method of Blin and Stafford [27]. PCR was performed from 20 ng of genomic DNA with D19Mit13 and D19Mit28 [28,29] amplimer set markers (Table 1), in 50 μl of PCR buffer: 20 mM Tris/HCl, 50 mM KCl, 200 nM of each primer, 1.5 mM MgCl$_2$, 125 μM dNTPs and 1 unit of *Taq* DNA polymerase (Eurogentec, Seraing, Belgium). Aliquots (10 μl) of the PCR reactions were subjected to electrophoresis (6 % polyacrylamide gel) and products were visualized by ethidium bromide staining. PCR products showed the expected strain-specific sizes in B6 and D2 mice (Table 1).

RESULTS AND DISCUSSION

PB induces Cyp 2b-10 mRNA, but either induces or suppresses Cyp 2b-9 mRNA, depending upon the strain and the sex of the mice

The use of specific oligonucleotide probes to analyse the PB response of Cyp 2b-9 and Cyp 2b-10 in polyA$^+$-enriched RNA did not show any significant hybridization signal with male RNAs, although these probes could detect female RNAs [26,30]. Based on these observations, as well as on Western blot analyses, the expression of Cyp 2b-9 and Cyp 2b-10 was reported to be female-specific. Moreover, a locus has been described to be involved in repression of the Cyp 2b-9 gene in some male mice [31]. We decided to re-examine this question by using the highly sensitive RT-PCR method [32,33] with liver RNAs from males of five inbred strains. Initial experiments were designed to determine the amount of cDNA (corresponding to an amount of total RNA) over which each target was amplified exponentially (results not shown). Cyp 2b-9 and Cyp 2b-10 cDNAs, corresponding to the major forms of Cyp 2b in mice [26], were synthesized by 3′-specific annealing in order to enrich for these targets from the initial pool of mRNAs.

In all male strains, basal expression of Cyp 2b-10 mRNA was nearly undetectable, even after 30 cycles of amplification, whereas Cyp 2b-9 was expressed to various extents, in several strains. A higher level of Cyp 2b-9 mRNA was detected in A and D2 males than in BALB or B6 mice (Figure 1a). Cyp 2b-10 mRNA was always induced by PB in all these males and in B6 and D2 female animals. These results agree with previous studies which showed
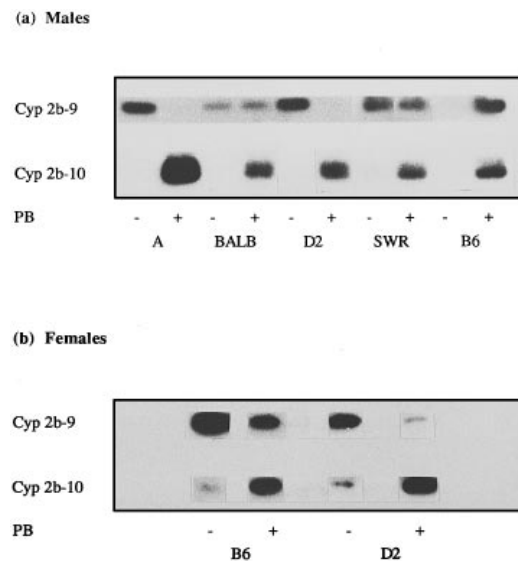

Figure 1   Regulation of Cyp 2b-9 and Cyp 2b-10 mRNAs by PB in male and female mice

(**a**) RT-PCR analyses were conducted from 250 ng (Cyp 2b-9) and 100 ng (Cyp 2b-10) of total RNA for both control and PB-treated animals. PCR products were analysed by Southern blotting and submitted to hybridization with a full-length rat CYP 2B1 cDNA probe. Conditions for RT-PCR experiments are described in the Experimental section. Basal expression of Cyp 2b-10 mRNA, although not apparent on the autoradiogram, can be detected upon longer exposure. Typical results from one of two independent experiments are shown. (**b**) Cyp 2b-9 PCR products were obtained from 50 ng (untreated and PB-treated D2 mice), or 100 ng (untreated and PB-treated B6 mice) of total RNA. Cyp 2b-10 PCR products were obtained from 100 ng of total RNA. PCR products were analysed by Southern blotting and hybridized with a full-length rat CYP 2B1 cDNA probe. One representative experiment of three independent experiments is shown.

that PB induced Cyp 2b-10 expression in mice [26,30,34]. Strikingly, PB regulated Cyp 2b-9 expression differently in the same panel of male mice: in D2 and A strains there was a large decrease of Cyp 2b-9 mRNA, while no apparent change was observed for BALB and SWR animals and a strong induction occurred in the B6 mice. This Cyp 2b-9 induction by PB seemed to be male-specific since PB decreased the Cyp 2b-9 mRNA in B6 female mice (Figure 1b). Until now, the Cyp 2b-9 regulation by PB has been widely discussed but studied in only a few strains [30,34,35]. This study reveals that PB can have both positive and negative effects on Cyp 2b-9 expression, depending upon the genetic background of the mice, and that the effect of PB depends on sex-specific constraints. Inhibition of gene expression by PB has been described already. For example, Lechner et al. [3] have shown, by screening induced and uninduced rat liver cDNA libraries, that PB acted also by repressing a significant number of mRNA species. More recently, Früh et al. [4] observed, by mRNA differential display analysis, that over 10 genes could be down-regulated after PB treatment in chicken embryo liver. To our knowledge, our data provide the first demonstration that PB can suppress the expression of a Cyp mRNA species from family 2b in mice.

In a previous study [23], we failed to identify such a variability for the regulation of Cyp 2b by PB in mice using an ortho-logous CYP 2B1 rat probe which was unable to discriminate between several related mRNA species. Indeed, mouse Cyp 2b-10 and rat CYP 2B1 display 95 % similarity, while Cyp 2b-9 exhibits 82 % and 85 % identity in amino acid sequences with CYP 2B1 and Cyp 2b-10, respectively [26]. At this point, it must be emphasized that investigation of the PB regulation of Cyp 2b
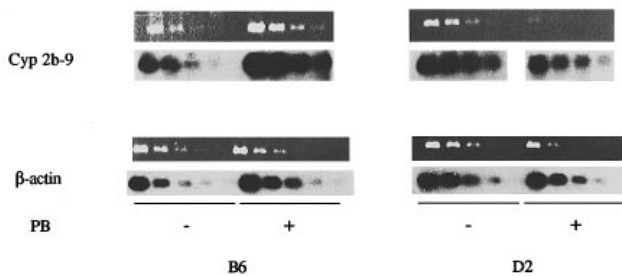
Figure 2   Quantification of the induction and the suppression of Cyp 2b-9 mRNA by PB in B6 and D2 male mice
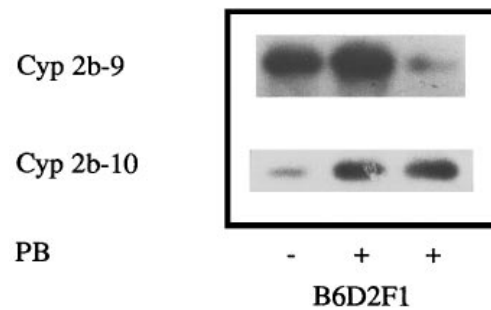
Serial 1:2 dilutions beginning with 500 ng of total RNA for control B6 and PB-treated D2 samples and with 50 ng of total RNA for PB-treated B6 and control D2 samples were prepared and subjected to 30 cycles of amplification with the Cyp 2b-9 amplimer set. Five 1:2 dilutions, beginning with 100 ng of total RNA of the same PB-treated and untreated samples, were subjected to 23 cycles of amplification with the β-actin amplimer set. Ethidium bromide staining of the gel is shown above the hybridization signals. One representative experiment of eight independent experiments is shown.

Figure 3   PB regulation of Cyp 2b-9 and Cyp 2b-10 mRNAs in B6D2F1 male mice

Results from two B6D2F1 males treated with PB are shown. Cyp 2b-9 PCR products were obtained from 1 μg of total RNA. Cyp 2b-10 PCR products were obtained from 250 ng (untreated mice) and 62.5 ng (PB-treated mice) of total RNA. PCR products were analysed by Southern blotting and submitted to hybridization with a full-length rat CYP 2B1 cDNA probe. One representative experiment of three independent experiments is shown.

genes has been made possible only by using the exquisitely sensitive and discriminative RT-PCR methodology. Nemoto and Sakurai [36] also distinguished between Cyp 2b-9 and Cyp 2b-10 mRNAs by the RT-PCR method, and reported that Cyp 2b-9 expression was not usually detected in untreated male liver of the B6 strain. These results agree with ours, as we detected only very low levels of this mRNA in males of this strain. By contrast, Cyp 2b-10 mRNA was expressed more than Cyp 2b-9 in B6 males, which differs from our observations. However, our means of detecting mRNA species differs from that of Nemoto and Sakurai [36], who amplified both Cyp 2b-9 and Cyp 2b-10 mRNAs during the PCR reaction, and distinguished between these mRNAs by digestion of the PCR product with specific restriction enzymes. Upon co-amplification of these two mRNAs, and since the relative abundance of each mRNA in the initial pool has a great influence on the kinetics and, consequently, on the overall efficiency of the PCR reaction, expression of one of these mRNAs could have been either underestimated or overestimated. By contrast, our PCR reactions were conducted separately in order to take into account the relative abundance of each mRNA. Furthermore, the amplimer sets used by Nemoto and Sakurai [36] and ourselves were different, since their oligonucleotides do not correspond to a divergent region, making it possible that they could reveal expression of several related Cyp 2b mRNAs. Indeed, it can be expected that the Cyp 2b subfamily could contain at least 16 members (genes and pseudogenes) [19].

Finally, we analysed the effect of DEX on Cyp 2b-9 mRNA in B6 and D2 males with the RT-PCR method (results not shown). Interestingly, the effect of DEX on Cyp 2b-9 mRNA in these mouse strains mimicked the response obtained with PB. Thus, DEX decreased Cyp 2b-9 expression in D2 males and induced this mRNA in B6 males. These observations and our previous report [23] support the idea of a related or common regulatory pathway for PB and DEX in mice.

PB increases Cyp 2b-9 mRNA in B6 males by at least 20-fold, and decreases this mRNA in D2 males by at least 30-fold

We have estimated the levels of the induction and decrease of Cyp 2b-9 mRNA observed in B6 and D2 strains, respectively, by semi-quantitative RT-PCR analyses. In these experiments, in order to take into account variations due to RNA purity and degradation or sampling and measurement errors, we amplified

β-actin mRNA in parallel with Cyp 2b-9 mRNA (Figure 2). As anticipated, there was no significant variation of β-actin PCR product between control and PB-treated samples in D2 and B6 males. After standardization of the amount of Cyp 2b-9 PCR product with the corresponding amount of β-actin, we have estimated, from data obtained from eight independent experiments, that PB increased Cyp 2b-9 mRNA by at least 20-fold in B6 males. Although the quantification of the decrease was much more difficult to obtain because the Cyp 2b-9 PCR product was hardly detectable in PB-treated D2 animals, we have estimated, from data obtained from eight independent experiments, that PB treatment decreased this mRNA by at least 30-fold in D2 males.

The PB induction of Cyp 2b-9 observed in B6 males is suppressed in B6D2F1 male mice

To get information about the genetic basis of the PB induction process of Cyp 2b-9 mRNA, we performed RT-PCR analyses with RNA of B6D2F1 male mice (Figure 3). As expected, in these mice, Cyp 2b-10 mRNA was increased by PB treatment as in the parental B6 and D2 strains. This induction reflected that there was no degradation of our RNA samples and Cyp 2b-10 PCR product was then used as a qualitative control in these experiments. We did not observe any increase or reproducible decrease in Cyp 2b-9 PCR product in male PB-treated mice (Figure 3). In B6D2F1 females, PB never induced this mRNA (results not shown). At this point, we believe that PB induction of the Cyp 2b-9 mRNA behaves as a semi-dominant trait in B6D2F1 males. It is, therefore, likely that the genetic basis for the differential responsiveness of Cyp 2b-9 to PB is due to a difference in the region within or surrounding the gene locus, accounting for the observed co-dominance.

Unlike Cyp 2b-9, Ald-2 mRNA is down-regulated by PB only in B6 mice

Dunn et al. [22] reported that administration of PB to rats of two selected genotypes could differently affect the expression of ALDH-PB (orthologous to mouse Ald-2). Since we were interested in such a genetic basis for the PB regulation, we looked at Ald-2 mRNA levels in PB-treated mice. To this end, we performed Northern blot analysis of liver RNAs from five male inbred mouse strains. The filter was probed with rat cDNA for
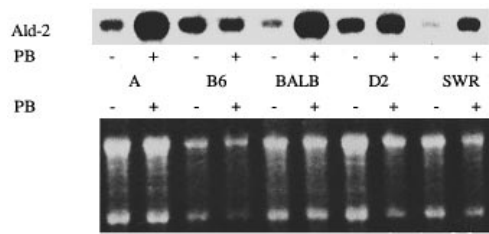
**Figure 4   Regulation of Ald-2 mRNA by PB in five inbred male mice**

Top part, hybridization of total RNA with the rat ALDH-PB cDNA probe. Bottom part, ethidium bromide staining of the RNA gel. One representative experiment of two independent experiments is shown.

ALDH-PB (Figure 4). Ethidium bromide staining of the gel was used to reflect variation in the quality and amount of RNAs between samples. Ald-2 expression increased in all PB-treated animals with the noticeable exception of the B6 strain in which this mRNA seemed to be slightly depressed by PB. Unlike Cyp 2b-9, the same effects were obtained for either sex of PB-treated mice (female results not shown). Interestingly, we observed opposite responses of the Cyp 2b-9 and Ald-2 mRNAs to PB in both the B6 and the D2 strains.

The inducible phenotype of the Ald-2 gene is a recessive trait which possibly involves one major regulatory gene

The PB-inducible phenotype was analysed in B6D2F1 offspring by Northern blot analyses of liver RNA. In animals of either sex, we never observed induction of the Ald-2 mRNA (Figure 5), but rather a slight decrease, similar to the response of B6 mice. Therefore, we believe that the inducible phenotype, observed in the D2 parental strain, is recessive.

To look for the involvement of the chromosomal region containing the Ald-2 gene on chromosome 19 in controlling the PB-inducible phenotype, we genotyped F2 animals from a B6D2F1 intercross with microsatellite markers from both sides of the locus (Figure 6a). We obtained a random distribution of B6 or D2 alleles at these loci, suggesting the lack of a link between the Ald-2 structural gene locus and the locus (loci) controlling the PB-inducible phenotype. In addition, these F2 mice were phenotyped in order to get an estimate of the number of genes involved in regulation of the Ald-2 gene by PB (Figure 6b). We analysed 12 males and 16 females and found that, in both sexes, about three-quarters of these animals were not induced whereas about
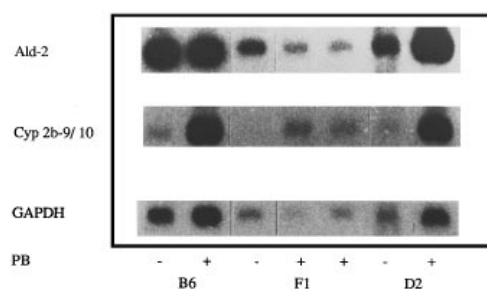
Figure 5   PB regulation of Ald-2 mRNA in B6D2F1 male mice

RNA filters were probed with ALDH-PB, CYP 2B1 and GAPDH cDNA probes. One representative experiment of four independent experiments is shown.
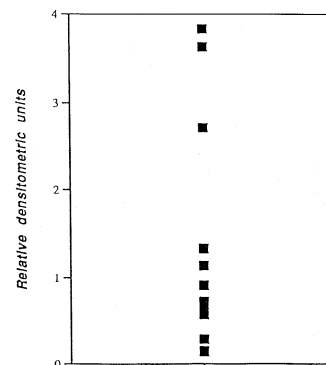
(a)
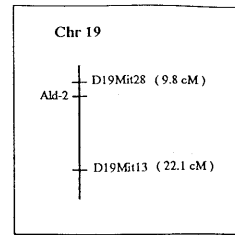
F2 male mice	6	8	12	18	20	22	24	30	32	34	42	44
D19Mit13	BB	DD	BB	BB	BD	BD	BD	BB	BD	BB	BD	DD
D19Mit28	BB	DD	BB	BB	BD	BD	BD	BB	DD	BD	BD	BD
Phenotype	-	-	-	-	-	-	+	-	+	+	+	-

F2 female mice	5	7	9	11	17	19	21	23	29	31	33	35	41	43	45	47
D19Mit13	BB	BD	DD	nd	BB	BD	BB	BB	BD	BB	BB	BB	DD	DD	DD	BB
D19Mit28	BD	BD	DD	BB	BB	DD	BB	BB	DD	BD	BD	BD	DD	DD	DD	BD
Phenotype	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	+

(b)

Figure 6   Genetic analyses of the PB-inducible phenotype of the Ald-2 gene

(**a**) The relative positions of D19Mit28, Ald-2 and D19Mit13 on chromosome 19 are shown. In each of the F2 progeny, the parental B6 (B) and D2 (D) alleles are identified. No correlation was found between the origin of the D19Mit alleles and the PB response phenotypes: ($-$) not inducible by PB; ($+$) inducible by PB. (**b**) Distribution of the Ald-2 mRNA level in PB-treated F2 male mice. For each animal, Ald-2 mRNA levels were determined by standardization to the 18 S RNA hybridization signal.

one-quarter were. These results are consistent with the involvement of a major gene in the control of PB induction of the Ald-2 gene. Studies are in progress in our laboratory to repeat these observations and to map and characterize this PB response locus in mice. In addition, the broad distribution of the low-expressing mice suggests that minor modifier gene(s) could be involved in the PB regulation.

The recessive inheritance of the inducible phenotype of the Ald-2 mRNA is compatible with the idea that a regulatory locus encoding a *trans*-acting factor, which could act either as a repressor in the down-regulated strains, or as an activator in the inducible mice, operates to regulate the PB response. Indeed, if multiple sequence differences were observed in *cis*-acting regions involved in Ald-2 gene expression, one would expect a semi-dominant phenotype in F1 hybrids, which is not the case for this gene, although this was found for the Cyp 2b-9 gene in the same parental cross. In addition, we obtained no evidence for linkage of the chromosomal region containing the Ald-2 structural gene and the PB-inducible phenotype in F2 animals, suggesting the presence of a regulatory locus (loci) elsewhere in the genome. As

a whole, our observations suggest that at least two distinct regulatory steps could be part of the PB response pathway. One possibility could involve a *trans*-dominant locus, active on both Ald-2 alleles, and the other one could be accounted for by *cis*-acting differences between Cyp 2b-9 alleles.

Dunn et al. [22] have established, at both enzyme activity and mRNA levels, that in the Long-Evans rats PB induction of the ALDH-PB gene is genetically controlled and is inherited as an autosomal incompletely dominant trait. Interestingly, these investigators demonstrated that the ALDH-PB, CYP 2B1, CYP 2B2 and GST Ya mRNA levels were not co-ordinately regulated in responsive (*RR*) and non-responsive (*rr*) genotypes. Specifically, the *RR* genotype, although responsive with respect to PB induction of the ALDH-PB, CYP 2B1 and GST Ya mRNAs, responded to PB by suppressing basal expression of the CYP 2B2 mRNA. We also observed a difference between the responses of the Cyp 2b-9 (the orthologue of rat CYP 2B2) and the Ald-2 mRNAs in the D2 strain, since Ald-2, as well as Cyp 2b-10, was induced in this strain, while Cyp 2b-9 expression was strongly down-regulated. In fact, in none of the male mouse strains analysed were all three genes induced by PB. These results obtained in the mouse reinforce the idea that PB regulation of gene expression is gene-specific and may involve more than one regulatory pathway [22].

The mechanisms of the induction or inhibition of gene expression by PB are still essentially unknown even if, recently, several *cis*-acting DNA elements involved in the induction process have been discovered [8,38–40]. However, the regulatory proteins have yet to be characterized. In addition, several studies strongly suggest that hormonal influences could interfere with the PB response of Cyp genes [16,41]. Therefore, the genetic approach used in our study appears as a fruitful alternative method with which to identify the gene(s) involved in the inductive, as well as in the suppressive, aspects of this phenomenon.

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