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Discriminating Activation of CYP2B9 Expression in Male C57BL/6 Mouse Liver by β-Estradiol

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The inducible expression of the cytochrome P450 2B subfamily was investigated in male C57BL/6 (B6) and DBA/2 (D2) mice, as well as their hybrids, B6D2F1, at the mRNA level. The expression of hepatic CYP2B mRNAs in B6 was lightly induced by β-estradiol (ES), while that by phenobarbital (PB) or 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) was prominent. Discriminating analysis showed a novelty that ES markedly induced CYP2B9 mRNA expression, whereas PB and DDT increased CYP2B10 more than CYP2B9 expression: albeit both mRNA species responded to all three inducers. Furthermore, the specific induction by ES of CYP2B9 mRNA in B6 male mice, but not D2 male mice, suggests strain dependency in the regulatory pathway of CYP2B9 expression.

Key Words: **estradiol; phenobarbital; CYP2B.**

Cytochrome P450s (P450) represent a large group of monooxygenases considered as the major enzymes in the biotransformation of a myriad of structurally diverse compounds of both endogenous and exogenous origin (1, 2). The expression of these enzymes is regulated by complex mechanisms involving many factors like sex, age, diet composition, and strain (3–7), as well as endocrine sex and glucocorticoid hormones (3, 4, 8–11). The CYP2B subfamily accounts for one of the major P450 species expressed in the liver (10, 12). CYP2B9 and CYP2B10 are major CYP2B isoenzymes constitutively and inducibly expressed in the mouse liver (3, 8, 10, 13) and kidney (14).

Abbreviations used: P450, cytochrome P450; B6, C57BL/6; D2, DBA/2; F1, B6D2F1; ES, β-estradiol benzoate; PB, phenobarbital sodium; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane; PBREM, phenobarbital-responsive enhancer module; RT-PCR, reverse transcriptase–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SSC, standard saline citrate.

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The expression of CYP2B isoenzymes in the murine liver is modulated by various structurally diverse compounds, such as PB (7–16), DDT (14, 17, 18), sex hormones (3, 8–10), and glucocorticoid hormones (3, 5, 6, 8, 10–12). PB induces hepatic CYP2B9 and CYP2B10 mRNA expressions, whereas Dex markedly induces CYP2B10 expression, while simultaneously suppressing CYP2B9 (10). In addition, the PB induction of CYP2B mRNA expression has been reported in both male and female (8, 10). The regulatory mechanism for CYP2B involving PB has been proposed to occur through a key regulatory factor, the liver-enriched orphan nuclear receptor CAR (Constitutive Active Receptor) that interacts with the multiple responsive element PBREM which localize in the 5′-flanking region of the *Cyp2b10* gene (19–22). Along with CYP2B10, we have found that CYP2B9 was induced by PB (8, 10, 14) but, since the induction was weak, a previous paper doubted that CYP2B9 responded to PB (3). CYP2B9 is prominently expressed in the female mouse liver. Since relatively little is known about the regulatory mechanism of CYP2B9 expression, it is worth ascertaining the induction factor(s) involved in the regulatory pathway.

In the present study, we investigated the constitutive and inducible expression of CYP2B9 and CYP2B10 mRNA in the liver of two inbred mouse strains (C57BL/6 and DBA/2), as well as their hybrid, B6D2F1, and determined specific responses to ES. The expression of CYP2B9 mRNA was potently induced by ES in the liver of B6 male mice. In addition, expression of CYP2B9 mRNA was found to be induced by ES in the liver of the F1 hybrid mice, albeit at lower levels than in B6 mice, whereas it was hardly seen at all in D2 male mice. These findings suggest that the expression of CYP2B9 species is under hormonal as well as genetic control. This is the first report of a potent inducer for investigation of the regulatory pathway of CYP2B9 expression.

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MATERIALS AND METHODS

Chemicals. β-Estradiol benzoate, DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane), and phenobarbital sodium were supplied by Nacalai Tech (Tokyo, Japan). The partial cDNA clone of mouse CYP2B10 was a generous gift from Dr. M. Negishi (NIEHS, USA). Restriction endonuclease, the RNA PCR kit, and T4 polynucleotide kinase were obtained from TaKaRa Biomedicals (Shiga, Japan). [α-^{32}P]dCTP (3000 Ci/mol) was an ICN Biomedicals' product (Costa Mesa, CA). Amersham Pharmacia Biotech Co. (Buckingham, England) supplied the [γ-^{32}P]ATP (6000 Ci/mol) and membranes for blotting (Hybond-N). All other laboratory chemicals were of the highest purity and from commercial suppliers.

Animals. Adult C57BL/6, DBA/2, and B6D2F1 male mice weighing 20–25 g were supplied by Charles River (Yokohama, Japan). Three to four mice per group were daily subcutaneously administered with 0.5 mg/kg/day of β-estradiol benzoate in corn oil for 7 days, or intraperitoneally given 100 mg/kg/day of phenobarbital sodium in PBS for 3 days. DDT in corn oil at a dose of 100 mg/kg/day was daily subcutaneously injected for 3 days into adult C57BL/6 male mice. The control group was simply left untreated because the vehicles did not significantly change CYP2B expression. The mice were killed 24 h after the last injection by decapitation.

Preparation of mRNA and hybridization to P450 probes. Livers were quickly excised, and total RNA was immediately prepared as described (8, 10). Northern blotting was performed after the denatured RNA has been size-fractionated on 1.3% agarose gel containing formaldehyde. Hybridization proceeded at 42°C overnight in a mixture containing 50% formamide, 10× Denhardt's solution, 5× SSC, 50 mM sodium phosphate, pH 6.4, salmon testis DNA at 0.25 mg/ml, and a [α-^{32}P]-labeled cDNA probe. The blots were washed twice for 15 min with 2× SSC and 0.1% SDS at 65°C and then for two 15-min periods with 0.2× SSC and 0.1% SDS at 65°C. The membranes were exposed to Fuji X-ray film at −80°C with an intensifying screen (DuPont). Since in both nucleotide sequence and size, the CYP2B9 and CYP2B10 mRNAs are very similar (8, 10, 23, 24), the two cannot be distinguished. Therefore, the mRNA detected on the northern blots is referred to CYP2B.

RT-PCR. Total RNA was reverse-transcribed using random primers and cDNAs for mouse CYP2B9 and CYP2B10 were amplified under the incubation conditions recommended by the supplier (TaKaRa Biomedicals, Shiga, Japan) of RNA PCR kit ver. 2.1. Specific oligonucleotide primers were selected according to Nemoto *et al.* (8) using "Primer Detectives" software (Clontech Laboratories, Palo Alto, CA). The sense primer for both CYP2B9 and CYP2B10 was 5′-CTCTTCCAGTGCATCAC-3′ and the antisense primer was 5′-CAATGTAGTCGAGGAGTTCC-3′. The latter was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The product length was 229 bases, scanning the region from 511 to 739 of the open reading frames. To verify which mRNA species was expressed, the PCR products were digested with a restriction enzyme and then resolved by 10% PAGE. CYP2B9 and CYP2B10 were digestable with *Bgl*II and *Hha*I, respectively. Since the amplification efficiency was equal between CYP2B9 and CYP2B10 cDNAs, we considered that the amounts of RT-PCR products reflect the initial ratio of respective mRNA species. The gel was dried and exposed to an X-ray film. To normalize the RNA quantity applied, portions of the cDNA samples were simultaneously amplified by PCR using primers for GAPDH cDNA. The nucleotide sequence of the sense primer was 5′-TCCACTCACGGCAAATTCAACG-3′ and that of the antisense primer was 5′-TAGACTCCACGACATACTCAGC-3′. The PCR product, 145 bases-in-length, was resolved by 10% PAGE and stained with ethidium bromide (10). The number of PCR cycles was determined within a linear amplification.

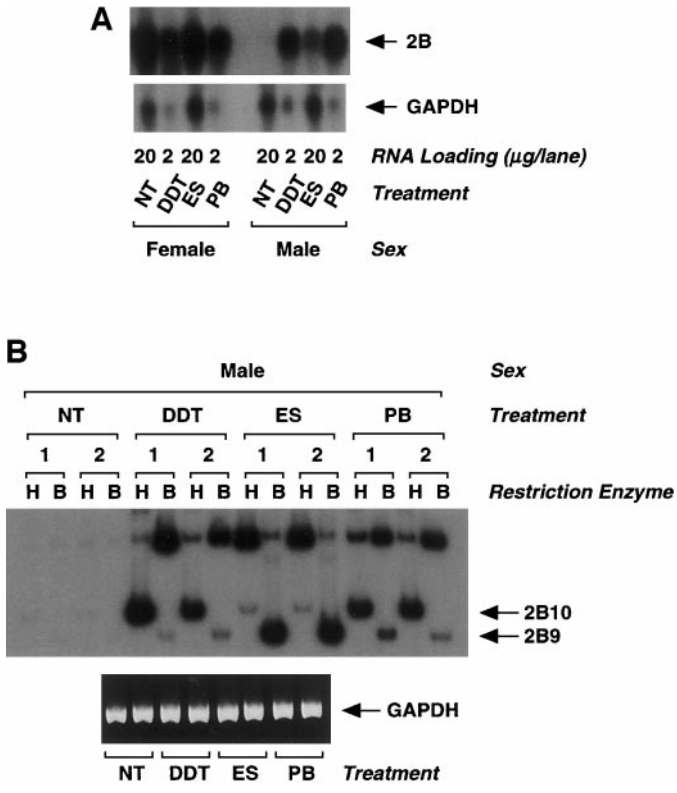

FIG. 1. Induction of hepatic CYP2B9 and CYP2B10 mRNA expression by P450 inducers in C57BL/6 mice. Adult C57BL/6 mice were daily subcutaneously treated with 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) at 100 mg/kg/day for 3 days, or β-estradiol benzoate (ES) at 0.5 mg/kg/day for 7 days, as well as phenobarbital sodium (PB) intraperitoneally at 100 mg/kg/day for 3 days. The animals were killed 24 h after the last injection to prepare total RNA. (A) Twenty micrograms of total RNA from untreated or ES-treated mice and two micrograms from PB- or DDT-treated mice were Northern-blotted using a cDNA probe for CYP2B10. (B) Discrimination of expressed CYP2B9 and CYP2B10 mRNA by RT-PCR. NT, no treatment; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane; ES, β-estradiol benzoate; PB, phenobarbital sodium; H, *Hha*I; B, *Bgl*II; PCR 28 cycles; 1 and 2 indicate individual animals.

RESULTS AND DISCUSSION

Induction of hepatic CYP2B9 and CYP2B10 mRNA expression by P450 inducers in C57BL/6 male mice. The expression of hepatic CYP2B mRNAs was markedly induced by PB and DDT in both sexes of B6 mice, while it was also increased by ES in male, but not prominently in female (Fig. 1A). Since the mRNA detected on the Northern blots is referred to CYP2B as described above, we ascertained whether CYP2B9 or CYP2B10, or both were augmented by these inducers in the male mouse liver. We found that both CYP2B9 and CYP2B10 mRNAs were induced by these inducers (Fig. 1B). Consistent with our previous findings, PB induced the expression of both CYP2B9 and CYP2B10 mRNAs, with CYP2B10 more inducible than CYP2B9 (10, 14). CYP2B10 mRNA was also markedly induced by DDT, while interestingly, ES chiefly increased

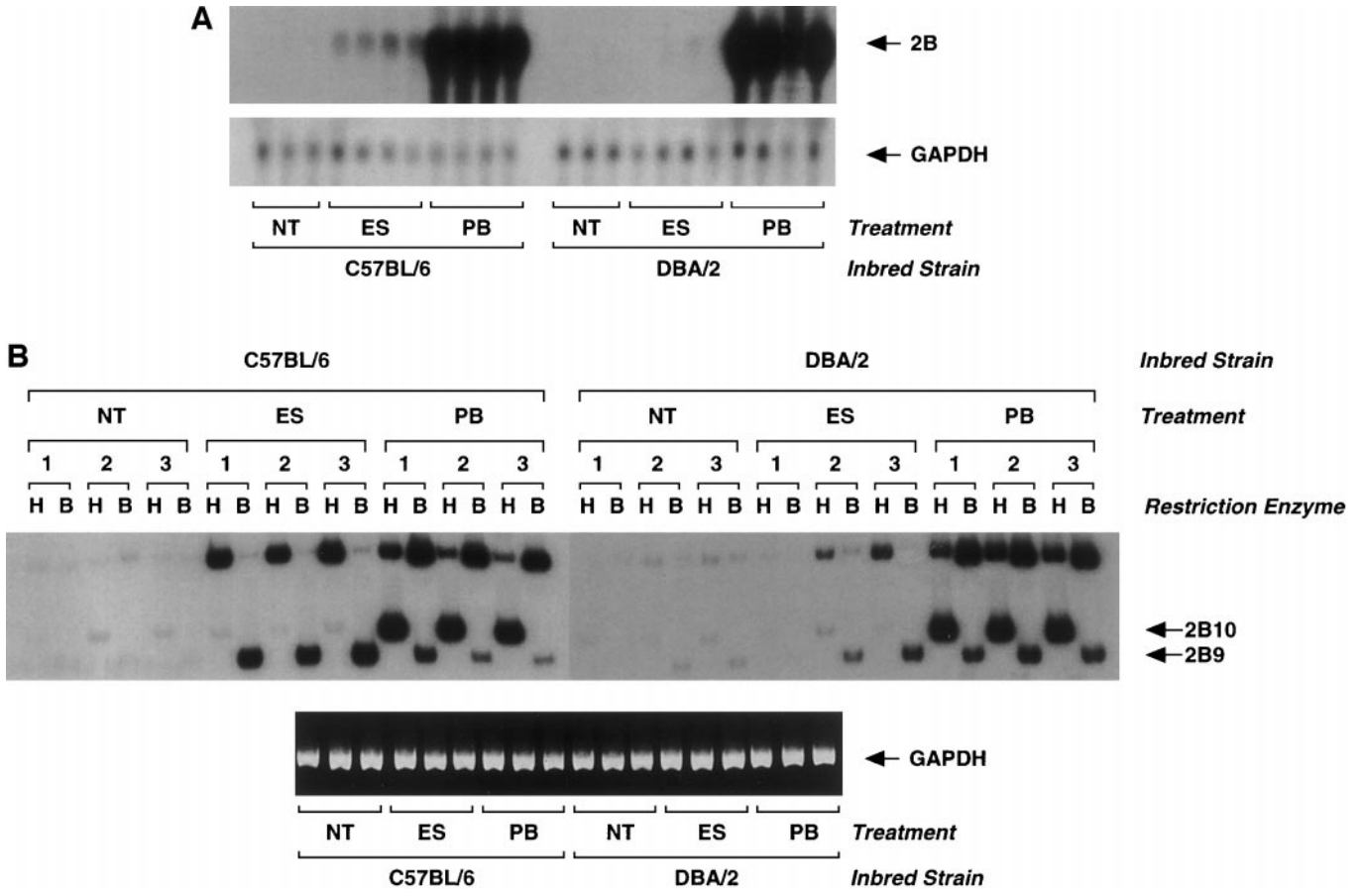

FIG. 2. Induction of CYP2B expression by β-estradiol in male C57BL/6 and DBA/2 mice. Adult male C57BL/6 and DBA/2 mice were daily subcutaneously administered with ES or PB as described in the legend to Fig. 1. The animals were killed 24 h after the last injection to prepare total RNA. (A) Twenty micrograms of total RNA was Northern-blotted using a cDNA probe for CYP2B10. (B) Discrimination of expressed CYP2B9 and CYP2B10 mRNA by RT-PCR. NT, no treatment; ES, β-estradiol benzoate; PB, phenobarbital sodium; H, *Hha*I; B, *Bgl*II; PCR 28 cycles; 1, 2, and 3 indicate individual animals.

CYP2B9. Regarding the discrepancy in the level of CYP2B mRNA expression in the presence of PB and PB-type inducers, DDT, and ES in the Northern blots, it might be that the cDNA probe used in the present experiment is more specific to CYP2B10 than other CYP2B subfamilies, whereas the expression of both

TABLE 1

Inducible Expression of Hepatic CYP2B9 and CYP2B10 mRNAs by Estradiol and Phenobarbital in Male Mice

		Expression[a]		
Strain	Inducer	CYP2B9	CYP2B10	Ratio of CYP2B9/CYP2B10
C57BL/6	No treatment	3.7 ± 0.6 (100)	5.9 ± 2.8 (100)	0.91 ± 0.61 (100)
	Estradiol	190.7 ± 41.1[b,]* (5139)	8.9 ± 2.4 (152)	21.83 ± 2.49[#] (2399)
	Phenobarbital	52.8 ± 29.2[#] (1424)	361.9 ± 63.6[#] (6176)	0.14 ± 0.05* (15)
DBA/2	No treatment	2.1 ± 1.6 (100)	1.8 ± 1.1 (100)	1.48 ± 0.84 (100)
	Estradiol	3.4 ± 2.0 (163)	3.6 ± 2.2 (195)	0.93 ± 0.08 (63)
	Phenobarbital	58.3 ± 15.2[#] (2775)	155.8 ± 49.0[#] (8512)	0.39 ± 0.09* (26)
B6D2F1	No treatment	0.4 ± 0.0 (100)	0.8 ± 0.1 (100)	0.49 ± 0.02 (100)
	Estradiol	20.2 ± 14.6[#] (5305)	2.3 ± 0.7* (292)	8.05 ± 3.71[#] (1642)
	Phenobarbital	13.1 ± 7.3[#] (3439)	285.9 ± 26.6[#] (36190)	0.04 ± 0.02[#] (8)

Note. Results are expressed as the mean ± SD (% Induction) for 3–4 individual mice per group.
[a] Expression level was quantified by BAS2000 Image Analyzer after the gel-exposed imaging plate was scanned by BAS2000 Scanner.
[b] Significantly different from the same strain and P450 isoform of the untreated group by the Student *t* test: $*P < 0.01$, $^{\#}P < 0.001$.

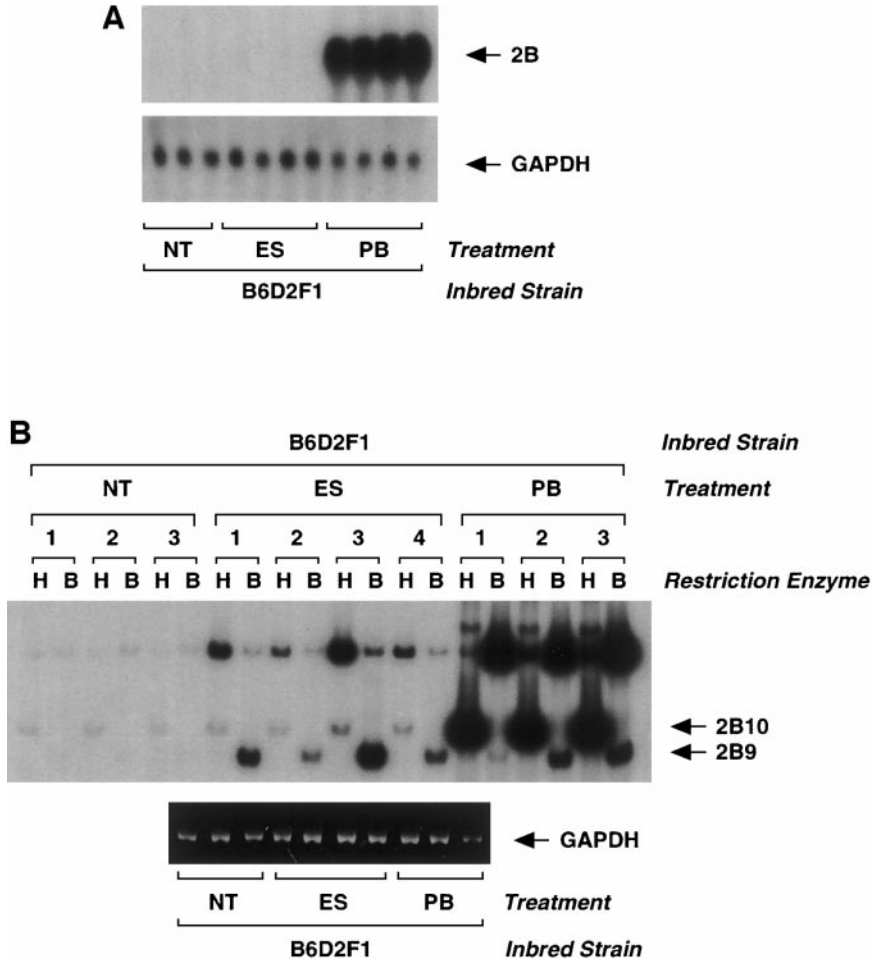

FIG. 3. Induction of CYP2B9 and CYP2B10 mRNA expression by β-estradiol in male B6D2F1 mice. Adult male B6D2F1 mice were daily subcutaneously administered with ES or PB as described in the legend to Fig. 1. The animals were killed 24 h after the last injection to prepare total RNA. (A) Twenty micrograms of total RNA was Northern-blotted using a cDNA probe for CYP2B10. (B) Discrimination of expressed CYP2B9 and CYP2B10 mRNA by RT-PCR. NT, no treatment; ES, estradiol benzoate; PB, phenobarbital sodium; H, *Hha*I; B, *Bgl*II; PCR 28 cycles; 1, 2, 3, and 4 indicate individual animals.

CYP2B9 and CYP2B10 can be determined by RT-PCR analysis. Alternatively, the Northern blots might contain not only CYP2B9 and CYP2B10 mRNA, but other unknown species. Since CYP2B9 mRNA is constitutively expressed at high level in the untreated female mouse liver, we could not observe a clear induction by ES in this experiment.

Although we detected an induction of CYP2B9 species by ES, one report has shown that PB, a classical CYP2B inducer, has a negligible effect on mRNA expression (3) and another the existence of strain dependency in PB-inducible CYP2B9 mRNA expression (5). However, different strains or animal suppliers were used in the present study. We, therefore, further investigated how the mRNA expression of CYP2B9 and CYP2B10 induced by ES in the D2 male mice, classified as a separate PB-responsive strain (13), differs from that induced in B6.

The induction of hepatic CYP2B9 and CYP2B10 mRNA expression by β-estradiol and phenobarbital in C57BL/6 and DBA/2 male mice. ES did not clearly induce the expression of CYP2B mRNA in D2 as in B6 mice, while PB markedly increased the expression in both strains to nearly the same extent (Fig. 2A). After discrimination by RT-PCR, CYP2B10 was constitutively expressed in both B6 and D2 mice, although the level in D2 was very low in the autoradiograph (Fig. 2B). ES dominantly induced CYP2B9 mRNA expression in B6, but not in D2, whereas PB induced both species regardless of the mouse strain, with the expression of CYP2B10 mRNA induced to a greater extent than that of CYP2B9 consistent with our previous studies (10, 14). Our observations did not agree with a previous report (5) that PB reduced CYP2B9 mRNA expression in D2 male mice. However, our procedure for differentiating mRNA species differs from that of

Damon *et al.* (5), who conducted RT-PCR reactions with either CYP2B9 or CYP2B10 separately, and distinguished between these mRNAs. We, however, coamplified the CYP2B9 and CYP2B10 mRNAs during the PCR reaction and discriminated between them by a specific restriction endonuclease digestion as described under Materials and Methods. Moreover, using different animal sources, there may be strain dependency in CYP2B expression.

As evidence of a strain-specific induction by ES of CYP2B9 expression, we have estimated the ratio of CYP2B9 to CYP2B10 (Table 1) and in untreated animals, found it not to differ significantly between these two inbred mouse strains. ES significantly raised the ratio in B6 ($P > 0.001$), but not in D2 mice. PB, although it increased the expression overall, markedly reduced the ratio of CYP2B9/CYP2B10 in both B6 and D2 mice. These observations affirmed the strain-dependency of the increase in CYP2B9 mRNA expression induced by ES in male mice.

The induction of hepatic CYP2B9 and CYP2B10 mRNA expression by β-estradiol and phenobarbital in B6D2F1 male mice. To obtain information on the genetic basis of the ES induction of CYP2B9 mRNA, we carried out an experiment in cross-inbred B6D2F1 male offspring. Since northern-blot analysis did not show a perceptible induction by ES of CYP2B mRNA (Fig. 3A), RT-PCR analysis with RNA of F1 was performed (Fig. 3B). The ratio of CYP2B9 to CYP2B10 mRNA expression in F1 hybrids after treatment with ES was significantly increased (1642% induction) compared to in the untreated group, as it was between B6 and D2 (Table 1). PB also decreased the ratio of CYP2B9/CYP2B10 in F1 hybrid mice. This evidence revealed that the inducibility of CYP2B9 by ES was semidominantly succeeded to F1 hybrid male mice.

The regulatory mechanism behind the expression of CYP2B9 mRNA, a female-specific species inducibly expressed in the mouse liver, has yet to be elucidated. A potent inducer is needed to investigate the regulatory pathway of CYP2B9 expression. This study hints at the existence of sex hormones and of a genetic basis to the regulatory pathway.

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