Regulation of the mouse liver cytochrome *P*450 2B subfamily by sex hormones and phenobarbital

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The sex-dependent expression and inducibility of the cytochrome *P*450 2B subfamily was studied in DBA/2 and Balb/c mice, and their F_1 recombinants, at the mRNA, protein and activity levels. Analysis of poly(A)$^+$ RNA with specific oligonucleotide probes directed to known mRNAs within the mouse 2B subfamily revealed that the levels of *P*450 2b-10 and 2b-9 mRNAs were co-regulated with two proteins (56 and 53 kDa) detected by a 2B-specific polyclonal antibody. Other mRNAs related to the 2B subfamily were barely or not at all detectable, and did not coincide with protein expression, suggesting that *P*450s 2b-9 and 2b-10 are the major 2B isoenzymes present in mouse liver. Specifically, castration of males increased the expression of 2b-9 and 2b-10 mRNAs and protein up to female levels, and testosterone administration to castrated mice reversed these changes. Ovariectomy of females appears to increase the expression of these *P*450s slightly. 2b-10, but not 2b-9, mRNA and protein were induced by phenobarbital. Based on immunoinhibition studies and the levels of these isoenzymes, *P*4502b-10 appears to be the major catalyst of 7-pentoxyresorufin *O*-dealkylation. Both *P*4502b-9 and *P*4502b-10 contribute up to 30 % of the testosterone 16α-hydroxylation, the balance being catalysed by *P*450s within the 2D subfamily. These experiments show that the female-predominant expression of the two mouse liver isoenzymes *P*4502b-9 and *P*4502b-10 is dependent on sex hormones. The fact that *P*4502b-9 does not respond to phenobarbital, while *P*4502b-10 is regulated by both phenobarbital and sex hormones, demonstrates the complexity of *P*450 expression even within one subfamily.

INTRODUCTION

The enzymes encoded by the cytochrome *P*450 subfamily 2B are generally recognized as being inducible by phenobarbital (PB), and their constitutive expression is similar in both sexes [1–4]. Based on gene analysis, this subfamily is one of the largest within the *P*450 superfamily. For instance, in mouse liver as many as 16 2B-related genes have been identified [5]. However, the number of active genes is not known, and only few mRNAs or proteins have been identified and characterized [5,6]. This suggests that our knowledge of the number and nature of 2B gene products is limited, and thus we have only a scant understanding of the regulation of this subfamily.

We have previously purified and characterized an isoenzyme (*P*450PBI) belonging to the mouse cytochrome *P*450 2B gene family, and showed that this isoenzyme is responsible for microsomal 7-pentoxyresorufin *O*-dealkylase (PROD) activity and is induced by PB and 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) [7]. Some preliminary experiments suggested that, in addition to being PB-inducible, the expression of this isoenzyme may be sex-dependent. First, PROD activity was blocked by 55 % in female liver but only by ~ 20 % in male liver by an anti-*P*450PBI IgG. Second, at least a 3-fold higher level of liver microsomal anti-*P*450PBI-IgG-reactive protein was found in females than in males with a dot-immunobinding assay.

In this paper, we show that the observed sex differences are due to the differential expression of two *P*450 isoenzymes, *P*4502b-9 and *P*4502b-10, the latter form corresponding to

*P*450PBI. Although both isoenzymes are regulated by testosterone, only *P*4502b-10 is responsive to induction by PB, contrary to previous data [8]. The suggested repression of *P*4502b-10 in Balb/c mice [9] does not seem to take place when analysed by protein immunoblotting and probing of mRNA with specific oligonucleotides.

MATERIALS AND METHODS

Chemicals

Oestradiol benzoate, testosterone, testosterone propionate and Tween 80 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Atropine was from Orion (Espoo, Finland). Buprenorphine (Temgesic) was from Reckitt & Colman (Hull, U.K.). Midazolam (Dormicum) was from Roche (Basel, Switzerland), and fentanyl-fluanisone (Hypnorm) was from Janssen Pharmaceutica (Beerse, Belgium). 4-[^{14}C]Testosterone, [γ-^{32}P]ATP, Hyperfilm MP autoradiography films and Hybond N nylon sheets were bought from Amersham International. The sources of other reagents have been described previously [7].

Animals

Male and female (6–7-week-old) DBA/2N//Kuo (D2) and Balb/c//Kuo (Balb/c) mice, and their Balb/c × DBA/2N F_1 hybrids (F_1), were obtained from the National Laboratory Animal Center Kuopio, Finland. They were kept in macrolon cages with aspen chips as underlay. The animals had free access to drinking water and standard rodent feed (R3, Ewos, Södertälje

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Sweden). The temperature and relative humidity of the animal rooms was 22 ± 1 °C and 50–60% respectively. The light/dark cycle was 14/10 h.

Atropine-medicated 7–8-week-old female and male mice were anaesthesized with a mixture of Dormicum (6–8 mg/kg, intraperitoneal) and Hypnorm (12–16 mg/kg, intraperitoneal). The males were castrated by removing the testes through incisions made in the abdominal skin and peritoneal muscles. Female DBA/2 mice were ovariectomized through laterodorsal incisions to the abdominal cavity. The veins and ducts were ligated and the organs were carefully removed. In sham-operated animals, the organs were exposed and then returned back to the abdominal cavity. All wounds were sutured with an absorbable thread. Temgesic (0.1 mg/kg, subcutaneous) was given to relieve postoperational pain. Animals were allowed to recover for 2 weeks.

Treatment groups consisted of 4–6 mice. Castrated mice received intraperitoneal injections of either testosterone propionate (20 mg/kg per day) dissolved in olive oil or the vehicle for 5 days. Ovariectomized DBA/2 mice were given either oestradiol benzoate (1 mg/kg per day) dissolved in olive oil or the vehicle for 5 days. Sham-operated mice were given an equal volume of olive oil. Intact, age-matched male and female mice were used as controls. Some of the intact female mice were given a single injection of PB (100 mg/kg) 24 h before the animals were killed. These experiments were conducted with the approval of the local committee on the use and welfare of experimental animals.

Sample preparation

The animals were killed, and livers were removed and divided in two parts. The first portion was frozen quickly in liquid nitrogen and stored at −80 °C for mRNA preparation. From the remainder, microsomes were isolated and stored as described [10]. Ovariectomized mice were dissected to verify the absence of ovaries.

Enzymes, antibodies and oligonucleotide probes

P450PBI, a 56 kDa protein, was purified from PB-treated D2 mice, and a polyclonal antibody was raised in rabbits. This antibody does not recognize mouse P450s from subfamilies 1A, 2A or 2C on Western blots, and does not inhibit testosterone 6β-hydroxylase (3A isoenzymes), ethanol-induced aniline hydroxylase (P4502E1) or male-specific mouse testosterone 16α-hydroxylase (2D isoenzymes) activities ([7]; P. Honkakoski, unpublished work). Specific oligonucleotide probes were synthesized according to Noshiro et al. [6]. The probe 26-cu was GCATAACTAGC-TGAGCTG ($5' \rightarrow 3'$) specific for the mouse 2b-9 (testosterone 16α-hydroxylase A) mRNA. The probe f9 (AACCACAGGAC-AGAGTGGT) recognizes the minor 2b-9-related (16α-hydroxylase B) mRNA [5,6]. The probe 46-i (TCCCACAGACTTTTGA-TCAAAAATTTG) hybridizes with a 2b-10-related (16α-hydroxylase C) mRNA containing an additional 27-mer insert, and the probe pf3/46-u4 (GGAAGGTTGGCTCAACGA) recognizes the 2b-10 (16α-hydroxylase D) mRNA [6]. The probe OP-1 (AAGAATACCATGACTCCAGA) is specific for the mRNA of the C-P45016α (gene ca, 2d-9; [11]). To control the mRNA content on each slot, sheets were reprobed with an oligo(dT)$_{15}$ probe. The oligonucleotides were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Pharmacia) to a specific radioactivity of about 10^8 c.p.m./μg.

Enzyme activities

All enzyme activities were determined from individual microsomal samples. Testosterone hydroxylation [12] was determined with a 100 μM substrated concentration and 100–500 μg of protein. The detection limit was 6 pmol/min per mg of protein.

PROD was measured using an end-point modification of the previous method [7]. Microsomes (15–150 μg of protein) were preincubated in 25 mM-potassium phosphate, pH 7.4, containing 5 mM-MgCl$_2$ and 2 μM substrate for 3 min, NADPH (0.75 nM final concn.) was added, and the reaction was allowed to proceed at 37 °C for 10 min. Methanol (2.5 ml) was added and the mixture was centrifuged at 3500 g for 5 min. The fluorescence of the supernatants and identically treated resorufin standards were measured with a Shimadzu RF-5000 spectrophotofluorometer. The detection limit of the assay was 0.25 pmol/min per mg of protein. The bicinchonic acid method [13] was used for protein determination.

Immunoinhibition analyses

In preliminary tests we determined the amount of anti-P450PBI antibody which maximally inhibited PROD and testosterone 16α-hydroxylase activities in microsomes from control and PB-induced female mice. This saturating amount of 7 μg of IgG/pmol of P450 was then used in subsequent experiments. Anti-P450PBI or preimmune IgG was incubated with microsomes for 15 min prior to the start of the reaction. The activity difference between the preimmune-IgG-treated and the specific-IgG-treated samples is defined as 2B-dependent activity. In the case of testosterone 16α-hydroxylase, any losses during sample extraction and application could be compensated for by normalizing the result with the formation of 15α-hydroxytestosterone. This metabolite is produced mainly by mouse P4502a-4 [14], and its formation is not affected by anti-P450PBI IgG. Affinity-purified anti-P4502d-9 IgG was kindly donated by Dr. Masahiko Negishi, NIEHS. A concentration of 0.1 μg of IgG/pmol of P450 was used to inhibit P4502d-9-dependent activity [15].

Western blotting

Electrophoresis of individual samples was done on 9% acrylamide gels [16], the proteins were transferred on to nitrocellulose sheets in ice-cold buffer containing 12.5 mM-Tris, 96 mM-glycine and 20% methanol [17]. The sheets were blocked for at least 30 min in 2% powdered milk diluted in Tris-buffered saline (20 mM-Tris/HCl and 500 mM-NaCl, pH 7.6) (TBS) and then incubated in: (i) a 1:500 or 1:1000 dilution of anti-P450PBI in blocking solution for 1 h, and (ii) a 1:1000 dilution of anti-(rabbit IgG)–alkaline phosphatase conjugate in blocking solution for 2 h, with two intervening washes in TBS supplemented with 0.05% Tween 80. The sheets were then processed as before [7]. The bands of the samples and P450PBI standards were quantified with a Shimadzu CS-9000 scanner. The quantification of the 56 and 53 kDa proteins detected (see below) is based on 20 μg protein samples, with the exception of PB-treated samples, for which 5 μg of protein was used. The detection limit was 0.1 pmol/ mg of protein. It is realized that immunological quantification of closely related isoenzymes may be complicated by the fact that distinct proteins might possess differentially reactive epitopes.

mRNA preparation and hybridization to probes

mRNA was isolated from pooled frozen liver samples [18,19], quantified by u.v. absorbance. Slot-blots of mRNA were prepared on to Hybond N nylon membranes. We raised the (pre)hybridization temperatures to more stringent levels than those used by Noshiro et al. [6] to minimize any cross-reactions, although single bands were detected on Northern blots [6]. Hybridization was done in 5 × SSC/5 × Denhardt's/0.1% SDS/ salmon sperm DNA (100 μg/ml) at 46 °C or at 50 °C (probe 46-i). The filters were washed with 2 × SSC/0.1% SDS twice at 25 °C and twice at 38 °C. After washing, Hyperfilm MP was exposed to nylon sheets with intensifying screens at −80 °C. The bands were quantified using the Shimadzu CS-9000 scanner.

Table 1. N-Terminal sequences of purified and cloned mouse P450s within subfamily 2B

The residues different from those of P450PBI are in **bold**. X denotes an unidentified residue. The P450 proteins and clones are named according to the references given. The clone pf26 codes for P4502b-9, and clones pf3/pf46 have been assigned as P4502b-10 [1].

	Sequenced or deduced residues 1–30					Protein or clone	Ref.
MDPSV	LLLLA	VLLSL	FLLLV	RGHAK	IHGHL	pf26 (I-P45016α)	[6]
MDPSV	LLLLA	VLLSL	FLLLV	RGHAK	IHGHL	pf9	[5,9]
MEPSV	LLLLA	LLVGF	LLLLA	RGHPK	EXGNF	P450PBI	[7]
MEPSV	LLLLA	LLVGF	LLLLA	RGHPK	SRGNF	pf46	[6]
MEPSV	LLLLA	LLVGF	LLLLA	RGHPK	SRGNF	pf3	[6]

Statistics

Pearson correlation coefficients between the enzyme activities, protein contents and relative mRNA contents were calculated. For multiple comparisons, the Bonferroni adjusted t test was used {P values less than $P = [1 - (1 - 0.05)^{1/n}]$, where n = number of groups, were considered statistically significant}.

RESULTS

Association of mouse 2B mRNAs with immunologically detected P450s

Comparison of the N-terminal and deduced amino acid sequences of known mouse 2B P450s [5–7] indicated that cDNA clones pf3 and pf46, both assigned as 2b-10 [1], most likely correspond to P450PBI, assuming that strain variation and/or ambiguities in amino acid identification could explain the Gly-26 → Ser change (Table 1). The cDNA clone pf26 coding for P4502b-9 has 83 % sequence identity with clones coding for P4502b-10 [6]. We then synthesized specific probes for the detection of these mRNAs. Out of four known mRNAs possibly present, only 2b-9 and 2b-10 were present in substantial amounts (Fig. 1), while mRNAs corresponding to cDNA clones pf46 and pf9 were not or barely detectable (results not shown). The expression of the 2b-9 and 2b-10 mRNAs in various treatment groups correlated excellently with expression of the 53 kDa and 56 kDa proteins respectively in D2 mice (Table 2) and other strains (results not shown). The observed female specificity and de-repression of P4502b-9 by castration [6] matched the expression of the 53 kDa protein. Therefore we tentatively concluded that the proteins detected corresponded to P4502b-9 and P4502b-10.
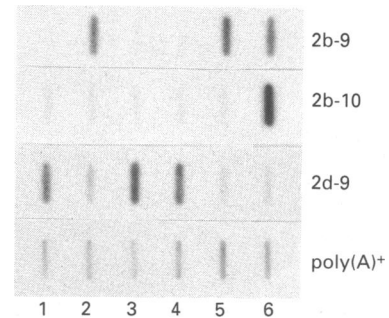

Fig. 1. Regulation of 2b-9, 2b-10 and 2d-9 mRNAs by sex hormones and PB in livers of D2 mice

Serial dilutions of mRNA isolated from livers of sham-operated male (lane 1), castrated male (lane 2), castrated and testosterone-propionate-treated male (lane 3), intact male (lane 4), intact female (lane 5) and PB-treated female (lane 6) mice were probed with oligonucleotides specific for the indicated mRNAs. Poly(A)$^+$ denotes a sheet re-probed with oligo(dT)$_{15}$ probe to control for equal mRNA amounts in each slot. Similar results were obtained with F_1 and Balb/c mice (not shown). For clarity, only the 2 μg loading of mRNA is shown.

Inducibility of mouse 2B P450s by phenobarbital

From earlier studies [7], we predicted that PB would induce P4502b-10. We injected a single dose of PB into female mice 24 h before the isolation of microsomes. Clearly, P4502b-10 was increased while P4502b-9 remained uninduced (Fig. 2). This was also reflected in the corresponding mRNA levels (Fig. 1). When

Table 2. Total and 2B-dependent PROD and testosterone 16α-hydroxylase activities, specific contents of P4502b-10 and P4502b-9 proteins, and relative levels of 2b-10 and 2b-9 mRNAs in the livers of DBA/2 mice

Activities are expressed as pmol/min per mg of protein, and specific contents as pmol/mg of protein (means ± S.D.). Relative levels of mRNA are expressed as the ratio of the hybridization signal to that in the intact female group. Statistically significant difference to intact males (*$P < 0.010$) and to intact females (†$P < 0.013$) are indicated. Abbreviations: TP, testosterone propionate-treated; PB, phenobarbital-treated; T16αOH, testosterone 16α-hydroxylase. The results obtained with Balb/c and F_1 hybrid mice were very similar to those with DBA/2 mice. No qualitative and only insignificant quantitative differences in the activities, amounts of proteins and levels of mRNAs were found (results not shown).

| Mice	Activity (pmol/min per mg)				Specific contents (pmol/mg)		Relative mRNA levels	
	PROD		T16αOH					
	Total	2B-dependent	Total	2B-dependent	P4502b-10	P4502b-9	2b-10	2b-9
Intact	8.8 ± 0.1	1.4 ± 0.2	284 ± 11	< 6	2.1 ± 0.3	< 0.1	0.44	< 0.01
Sham male	7.9 ± 0.7	1.9 ± 0.5	288 ± 15	< 6	2.8 ± 0.7	< 0.1	0.53	< 0.01
Castrated	8.6 ± 0.5†	3.3 ± 0.5*	232 ± 28	26 ± 7*	8.8 ± 2.6*	1.8 ± 0.4*	0.64	0.39
Castrated + TP	8.0 ± 0.5	1.6 ± 0.3	287 ± 12	16 ± 2	5.8 ± 1.3*	0.2 ± 0.05	0.73	0.02
Intact female	10.8 ± 0.1*	4.5 ± 0.6*	230 ± 1*	44 ± 7*	8.7 ± 0.4*	2.4 ± 0.1*	1.00	1.00
Female + PB	168 ± 10†	153 ± 5†	352 ± 32†	78 ± 5†	127 ± 12†	2.6 ± 0.6	16.6	0.71
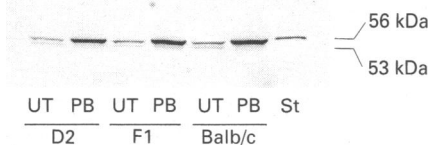
Fig. 2. Inducibility of 2B-immunoreactive 56 and 53 kDa proteins by PB in livers of inbred mice

Liver microsomal protein (20 μg) from D2, F_1 and Balb/c untreated (UT) and PB-treated (PB) female mice, and 250 fmol of P4502b-10 (St), was analysed by immunoblotting (antibody dilution 1:1000)
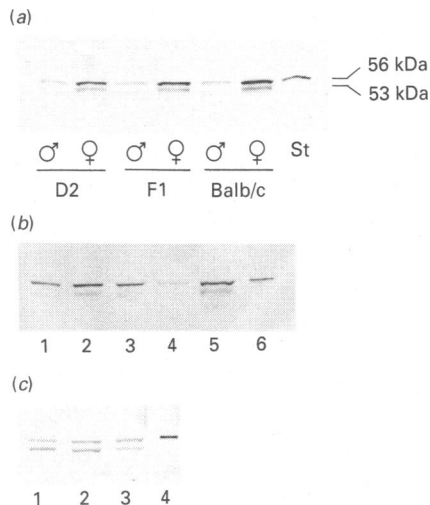

Fig. 3. Sex- and hormone-dependent expression of 2B-immunoreactive 56 and 53 kDa proteins in livers of inbred mice

(a) Liver microsomal protein (20 μg) from D2, F_1 and Balb/c male (♂) and female (♀) mice, and 100 fmol of P4502b-10 (St), was analysed by immunoblotting (antibody dilution 1:500). (b) Liver microsomal protein (20 μg) from sham-operated (lane 1), castrated (lane 2), and castrated + testosterone propionate-treated (lane 3) male D2 mice, intact D2 males (lane 4) and females (lane 5), and 100 fmol of P4502b-10 (St) was analysed by immunoblotting (antibody dilution 1:500). (c) Liver microsomal protein (20 μg) from sham-operated (lane 1), ovariectomized (lane 2) and ovariectomized + oestradiol-benzoate-treated (lane 3) female D2 mice, and 100 fmol of P4502b-10 (St), was analysed by immunoblotting (antibody dilution 1:500).

PB was given for 3 days, the P4502b-10 was induced even further but no increase in the 2B-9 protein occurred (results not shown). In male mice, PB was unable to increase P4502b-9 to detectable levels (results not shown). Similar results were obtained with TCPOBOP (results not shown), which is a potent PB-like inducer of P4502b-10 [20,20a].

Sex-dependent regulation of mouse 2B P450s

P4502b-9 was absent in male D2, Balb/c and F_1 hybrid mice, while P4502b-10 was expressed at a 3–4-fold higher level in females than in males of all strains (Fig. 3a). The possible dependence of 2b-9 and 2b-10 expression on sex hormones was studied by gonadectomy and hormone supplementation. Castration of male D2 mice increased both 2b-9 and 2b-10 proteins to female levels, and treatment of the castrated mice with testosterone propionate decreased the level of P4502b-10 and repressed P4502b-9 almost completely (Fig. 3b). These changes took place regardless of the mouse strain (results not shown). In experiments with female D2 mice (Fig. 3c), both proteins were
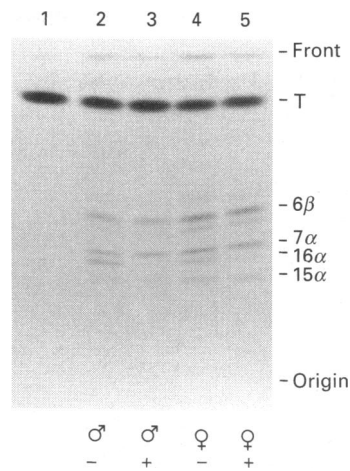

Fig. 4. Effect of affinity-purified anti-P4502d-9 IgG on testosterone 16α-hydroxylation in liver microsomes of male and female D2 mice

Testosterone hydroxylation in the presence of anti-P4502d-9 (+) or an equal amount of preimmune IgG (−) was determined as described in the Materials and methods section. The chromatographed t.l.c. sheet was autoradiographed and spots corresponding to 16α-hydroxytestosterone were scraped and counted for radioactivity. T, testosterone; 6β, 7α, 16α and 15α refer to 6β-, 7α-, 16α- and 15α-hydroxytestosterone respectively. Lane 1 contains a blank reaction without microsomes.

increased about 1.5-fold by ovariectomy. Treatment of operated females with oestradiol decreased P4502b-10 back to normal, and depressed P4502b-9 to ~ 60% of the sham-operated level. The changes in the amounts of the isoenzymes caused by the hormonal manipulations correlated well with the levels of 2b-9 and 2b-10 mRNAs (Table 2 and results not shown), which suggests a pretranslational control by the sex hormones. As a control of the treatments, the androgen-regulated 2b-9 mRNA [9] was expressed at over 5-fold higher levels in livers of male and testosterone propionate-treated castrated male mice than in females or castrated males, as expected.

Association of mouse 2B P450s and mono-oxygenase activities

Two P450-mediated activities were considered relevant in this study. P4502b-10 was purified based on the reconstituted PROD activity, known to be induced by PB [7]. Testosterone 16α-hydroxylase was chosen since it is at least partially catalysed by 2B isoenzyme(s) [8]. 2B-dependent PROD and testosterone 16α-hydroxylase activities were increased over 15-fold and 2–3-fold respectively (Table 2), in accordance with the catalytic specificity of P4502b-10 (catalytic-centre activities: PROD, 4.8 min^{-1} [7]; testosterone 16α-hydroxylase, 8 min^{-1} [20a]. The lower increase in testosterone 16α-hydroxylase is explained by the catalysis of testosterone 16α-hydroxylation also by the non-inducible P4502b-9 (6–8 min^{-1} [8]).

The 2B-dependent PROD activity was clearly higher in females and castrated males than in males and testosterone-treated castrated males. This correlates remarkably well with the microsomal P4502b-10 contents ($r = 0.99$), suggesting that this isoenzyme is the principal catalyst of PROD. Findings that the correlation between P4502b-9 and 2B-dependent PROD activity is weaker, and that anti-P4502b-10 IgG can inhibit PROD in male mice where no P4502b-9 exists, suggest that P4502b-9 does not contribute significantly to PROD activity. The residual PROD activity not affected by anti-P4502b-10 IgG is mediated by P4501a-2 and possibly by another unknown isoenzyme [7]. The P4502b-10 protein did not return completely to basal values

upon testosterone administration, however, 2B-dependent activities were decreased more than protein levels during testosterone propionate treatment (Table 2). This suggests that the 2b-10 protein might be inactivated prior to its degradation, as is rat 2E1 [21], possibly by haem loss [22].

The 2B-dependent testosterone 16α-hydroxylase activity correlated well with *P*4502b-9 levels ($r = 0.91$, excluding PB-treated animals). Furthermore, no inhibition took place in males, where *P*4502b-9 is absent (Table 2). Most of the testosterone 16α-hydroxylation is catalysed by *P*450s not recognized by anti-*P*4502b-10 IgG. This is expected, since several isoenzymes, especially *P*4502d-9, are known to contribute to this reaction [8,15]. The role of *P*4502d-9 is demonstrated well by the ability of the affinity-purified anti-*P*4502d-9 IgG to inhibit at least 90 % of liver testosterone 16α-hydroxylase in males and about 70 % in females (Fig. 4).

DISCUSSION

The sex-specific and growth hormone-dependent expression of *P*450 isoenzymes has been related to subfamilies 2A, 2C, 2E and 3A in the rat, where at least four male-specific forms (2A2, 2C11, 2C13 and 3A1) and four female-predominant forms (2A1, 2C7, 2C12 and 2E1) are known [3,23,24]. No sex differences within the rat 2B family have been detected [3,4], and results on the growth hormone-dependency of rat 2B expression are conflicting [25,26]. In this view, our finding that the female-specific *P*4502b-9 and the female-predominant *P*4502b-10 appear to be repressed by testosterone is intriguing. It would be interesting to see whether growth hormone is responsible for the effects of sex hormones in mice as it is in rats.

The proteins detected by immunoblotting could be assigned as *P*4502b-9 and *P*4502b-10 on the basis of their reported *N*-terminal sequences, and co-regulation with the 2b-9 and 2b-10 mRNAs. Since the complete sequences of the detected proteins are not known, and the cDNAs have not been functionally expressed, the proof of association between the proteins and mRNAs is inconclusive. However, our assumption is supported by the fact that the other two mRNAs were barely detectable, if at all, and poorly correlated with the protein expression.

Surprisingly, and contrary to previous data [8], no increase in 2b-9 protein or mRNA by PB took place, while 2b-10 was readily induced. *P*4502b-9 could not be increased by increasing the exposure time to PB, by administration of the powerful inducer TCPOBOP, or by giving PB to male mice (results not shown), indicating that the *Cyp2b-9* gene is exclusively under hormonal control. All of these treatments induced 2b-10 only. These discrepancies can be explained by the co-detection of *P*4502b-9 and *P*4502b-10 proteins by the anti-I-*P*45016α IgG (a single 54 kDa band was seen) and of respective mRNAs by the cDNA clone [8]. Devore *et al.* [8] also reported that testosterone 16α-hydroxylase activity could be blocked by anti-I-*P*45016α IgG almost totally in PB-treated mice, which clearly disagrees with our modest 30 % inhibition. Our result can be explained by the presence of *P*4502d-9 having at least 10-fold higher turnover for testosterone 16α-hydroxylase than does *P*4502b-9 [8,15]. Indeed, over 70 % of the 16α-hydroxylase activity is mediated by isoenzyme(s) related to 2D family, as judged by immuno-inhibition with anti-*P*4502d-9 IgG.

Finally, our results do not support the suggested lack of

expression of *P*4502b-10 in Balb/c mice [9], since approximately equal levels of both 2b-10 protein and mRNA were detected in D2 and Balb/c mice and their F_1 hybrids. However, the conclusion of Lakso [9] was based indirectly on the number of clones identified by cDNA cloning.

In conclusion, as have identified two mouse *P*450 isoenzymes which are predominantly (*P*4502b-10) or exclusively (*P*4502b-9) expressed in the female liver. *P*4502b-9 is under a strict hormonal control and does not respond to PB, while *P*4502b-10 is regulated both by PB and sex hormones.

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