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Transfer of PCBs via Lactation Simultaneously Induces the Expression of P450 Isoenzymes and the Protooncogenes c-Ha-ras and c-raf in Neonates

J. T. Borlak,† A. Scott,‡ C. J. Henderson,‡ H. J. Jenke§ and C. R. Wolf‡*

†University of Reading, Department of Biochemistry and Physiology, Reading RG6 2AJ, U.K.; ‡Imperial Cancer Research Fund, Molecular Pharmacology Unit, Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.; and §Gesellschaft für Strahlen- und Umweltforschung, Abteilung für Zellchemie, Postfach 1129, D-85758 Oberschleißheim, Germany

ABSTRACT. At the first day of lactation, maternal rats were injected with a single i.p. dose of 100 or 250 mg/kg body weight of a mixture of polychlorinated biphenyls (Aroclor 1254). This treatment caused significant increases in both material and neonatal hepatic cytochrome P-450, cytochrome b_5, and cytochrome-c-(P-450) reductase. Transfer of PCBs via lactation resulted in significant increases in hepatic enzyme activities catalysed by neonatal CYP1A1, CYP1A2, CYP2B1, CYP3A1, and CYP2E1 using a variety of substrates. In contrast, the metabolism of dimethylnitrosamine and aminopyrine was only marginally (up to 2-fold) increased in maternal animals four days post treatment. Further measurements showed significant increases in maternal and neonatal epoxide hydrolase, glutathione-S-transferase, and UDP-glucuronyl transferase activities, thus suggesting a coordinated response in maternal and neonatal oxidative and post-oxidative drug metabolism. Western blot analysis provided evidence for an induction of CYP1A1, CYP1A2, CYP2A1, CYP2B1, CYP2E1, CYP3A1, and CYP4A1 in both maternal and neonatal liver, albeit at varying intensities. However, PCBs did not modulate the expression of maternal and neonatal CYP2C6, and at the higher dose the expression of neonatal CYP2E1 was significantly reduced. Northern blot analysis provided further evidence for significant increases in maternal and neonatal hepatic CYP1A1, CYP1A2, CYP2B1, and CYP2E1 mRNA, but reduced amounts of CYP2C7 and CYP4A1 mRNA. Additional Northern blot hybridization experiments may suggest an increased expression of the protooncogenes c-Ha-ras and c-raf in the mother and the neonate upon treatment of maternal rats with Aroclor 1254. Lactation itself may result in an increased expression of the latter protooncogenes, but the mRNA of the protooncogenes c-erb A and c-erb B was not detected in any of the tissues examined. BIOCHEM PHARMACOL 51;4:517–529, 1996.

KEY WORDS. PCBs; lactation; neonates; toxicity; CYP450 induction; protooncogenes

The widespread accumulation and distribution of PCBs[‖] is extensively documented [1]. Approximately 31% of the 1.2 million tons of PCBs that have been produced are present in the environment [2]. The majority of PCB oils have been utilised in electrical equipment or other products still in use. Improper disposal has led to the potential of pollution for many years to come [2], and individual PCB isomers and congeners are commonly present in human blood, milk, and other tissue samples, such as biopsy material taken from tumours [3–5].

Individual PCB isomers and congeners, as well as mixtures of PCBs, modulate and disturb normal cellular functions [1, 6, 7]. PCBs elicit a wide range of toxic reactions depending on the experimental conditions, treatment dose, and duration and route of application.

Several excellent reviews are available that provide detailed information on all aspects of the toxic properties of PCBs, including their ability to promote and perhaps to initiate malignant tumour formation. It has been shown that mammalian species differ in their response to PCBs and that developmental and sex differences are important factors to be considered in interpreting their toxicity. The potency of PCBs in inducing certain P450 isoenzymes is well recognised, and the studies by Parkinson *et al.* [8] show how versatile PCBs are in modulating the expression of P450 proteins.

Transfer of PCBs via lactation to the nursing infant has frequently been reported, and there is compelling evidence for *in utero* exposure to PCBs [9, 10]. Based on the evidence available to date, the health risk for the mother and the baby could be significant, but it is notoriously difficult to find universal markers that allow risk assessment with high predictive power.

* Corresponding author: Dr. J. T. Borlak, Solvay Pharma, Department of Drug Metabolism and Preclinical Pharmacokinetics, Hans-Böckler Allee 20, 30173 Hannover, Germany.

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‖ *Abbreviations*: PCB, polychlorinated biphenyl; Aroclor 1254, a complex mixture of individual PCB isomers and congeners; CYP1A1, CYP1A2, CYP2A1, CYP2B1, CYP2C6, CYP2E1, CYP3A1, and CYP4A1 are distinct isoenzymes of the cytochrome P450 gene superfamily that catalyse oxidative reactions; c-Ha-ras, c-raf, c-erb A, and c-erb B are protooncogenes.

Furthermore, as PCBs produce a variety of different toxic reactions at the same time, it is difficult to define a suitable endpoint for a universal interpretation of the toxic properties of PCBs.

In the present study, two markers were chosen as toxicological endpoints: the expression of P450 isoenzymes and protooncogenes measured at the protein and mRNA level. Both endpoints have been implicated in toxic injury and possible tumour development [11]. Therefore, the aim of the present study was to assess early changes in the expression of P450 proteins from families 1 to 4, and to correlate these changes with substrate reactions catalysed by P450 isoenzymes. Furthermore, the concomitant expression of four different protooncogenes was studied to obtain detailed information on the simultaneous expression of the mRNA of P450 genes and protooncogenes. The results, taken collectively, should aid the search by defining suitable markers that predict the risk for the mother and the baby at an early stage.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley (SD) rats with an average body weight of 175 g were supplied by Charles River Ltd., Margate, U.K. Food and water was provided *ad libitum*. The beginning of pregnancy was determined by examination of vaginal plugs.

TREATMENT OF ANIMALS. On the first day *post partum*, maternal rats were injected with a single i.p. dose of either 100 mg or 250 mg/kg body weight Aroclor 1254 dissolved in cornoil, and were killed 4 or 14 days later. The dose selection was based on reports by others and our previous studies where similar doses were used [1, 7, 8].

Chemicals, Reagents, Enzymic Substrates

Unless otherwise stated, all chemicals and reagents were of the highest purity and purchased from Sigma Chemical Co. or from BDH chemicals, Poole, U.K. NADPH-dependent enzyme reactions were assayed by the addition of 12 mM $NADP^+$, 12 mM glucose 6-phosphate, and 60 units of glucose-6-phosphate dehydrogenase in a final volume of 3 mL. Cytochrome P450 catalysed reactions were assayed in a buffer system containing 0.1 M TRIS, pH 7.4, 0.15 M $MgCl_2$, and 0.5 M nicotinamide to prevent the destruction of pyridine nucleotide by tissue nucleosidases.

Preparation of Hepatic Microsomes and Assessment of Microsomal and Cytosolic Enzyme Activity

Hepatic microsomes were prepared as described previously [12]. Cytochrome P-450 and b5 were measured by the methods of Omura and Sato [13]. Protein concentrations were determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard. Ethoxyresorufin-O-de-ethylation (EROD) activity was determined by the method of Burke *et al.* [15], and the N-demethylation of aminopyrine and dimethylnitrosamine were measured essentially as described by Anderson *et al.* [16]. Aldrinepoxidation was measured by the method of Wolff *et al.* [17] with modifications as described previously [18]. The activity of NADPH-cytochrome-c-(P-450) reductase, para-hydroxylation of aniline, O-demethylation of nitroanisole and of glucuronyl-transferase using o-aminophenol as substrate, were assayed as detailed in ref. [19] with modifications described previously [18]. Glutathione-S-transferase activity was measured by the method of Habig *et al.* [20] using 2.4-dinitro-1-chlorobenzene as a substrate. Epoxide hydrolase was assayed using epichlorohydrin as a substrate and an experimental protocol detailed by Guengerich *et al.* [21].

Unless otherwise stated, all enzyme kinetic measurements were found to be linear with respect to protein concentration and incubation time. A detailed description of the above described assays and the validation of linear enzyme kinetics is given by Borlakoglu [18].

Western Immunoblot Analysis of P450 Isoenzymes

At 4 and 14 days following treatment of lactating mothers with Aroclor 1254 at either 100 or 250 mg/kg body weight, hepatic tissue was obtained from maternal and neonatal rats. Hepatic microsomal proteins were isolated as described above and subjected to SDS/polyacrylamide gel electrophoresis (SDS/PAGE) using 9% separating gels [22]. Immunoblotting was performed essentially as described by Towbin *et al.* [23], with modifications according to Lewis *et al.* [24]. After separation, proteins were transferred electrophoretically to nitrocellulose, and probed with various antisera to rat liver cytochrome P-450s. Antibodies to the purified enzymes were isolated as described previously by Wolf *et al.* [25, 26]. These antibodies have previously been used in immunoblotting studies with mouse, rat, and human microsomal samples. In addition, the isoenzyme specificity of these antisera have been demonstrated by immunoblot analysis with expressed human recombinant P-450 proteins [27].

Cytochrome CYP4A1 was isolated according to the method of Tamburini *et al.* [28] and was of high purity, running as a single band on SDS/PAGE. Antibodies used were of CYP1A1, CYP1A2, CYP2A1, CYP2B1, CYP2C6, CYP2E1 (kindly provided by Dr. C. S. Yang), CYP3A1, and CYP4A1 (for nomenclature system, see Nebert *et al.* [29]). A protein loading of 3 mg microsomal protein was applied to enable direct comparisons amongst individual groups. Furthermore, the intensities of coomassie blue staining of SDS/PAGE-separated proteins were compared to ensure a uniform loading (data not shown).

The immunoreactive polypeptides were visualised using horseradish peroxidase-labelled secondary antibody, and the signal was enhanced with ^{125}I-protein A (Amersham International, plc.) and subsequent autoradiography (Kodak X-Omat AR5 X-ray film) at −70°C. Differing exposure times were used to optimise the autoradiographic signal.

Northern Blotting and Determination of P450 mRNA Levels

At four days following treatment of lactating mothers with Aroclor 1254 at 250 mg/kg body weight, hepatic tissue was

obtained from maternal and neonatal rats. RNA was isolated from liver samples of female rats as previously described by Cox [30]. RNA concentration and purity were estimated spectrophotometrically and tested for integrity and equivalence of loading by ethidium bromide staining of a denaturing formaldehyde gel. mRNA was separated electrophoretically, transferred to nitrocellulose, and P450 mRNA content determined using the cDNA probes described below and hybridisation conditions described previously [27]. Blots were washed at 65°C with 0.3 M sodium chloride and 0.03 M trisodium citrate, pH 7.4 cDNA probes were labelled by the random-priming method [31–33]. Human cDNA probes used were CYP1A1, CYP2A6, CYP2B1, CYP2E1 (kind gift from Dr. F. Gonzalez), and CYP3A3/4. The CYP2B1, CYP2C7, and CYP4A1 (kind gift from Dr. G. Gibson [34]) cDNA probes were isolated from rat.

Plasmids and cDNA Probes Used for Northern Blot Hybridization of Protooncogenes

The following cDNA probes were used: v-Ha-ras, 0.62 kb HindIII to BamH1 fragment from the Balb-MuSV-DNA; v-erb A, 0.5 kg PstI fragment of the AEV genome; v-erb B, 0.5 kb BamHI fragment of the AEV genome; v-raf, 1.38 kb XhoI to Bg1II v-raf specific fragment of the 3611 MSV genome. Glyceraldehyde phosphate dehydrogenase (GAPDH) 1.1 kb PstI fragment of the GAPDH gene was cloned in pBR322. Further details about the plasmid and DNA probes have been reported previously [35].

Northern Blot Hybridization of Protooncogenes

The mRNA content of protooncogenes in hepatic tissue was determined following electrophoresis of RNA (10 µg/lane) with glyoxal in 1% agarose gels. Gels were stained with acridine orange prior to the transfer of the RNA onto nitrocellulose membranes. RNA was subsequently fixed to the membranes by heating at 80°C for 2 hr. RNA blots were prehybridized for approximately 3 hr at 42°C in hybridization mixture containing 50% formamide, 5× Denhardts (50× Denhardts is 1% bovine serum albumin, 1% Ficoll, 1% polyvinylpyrollidone), 0.1% sodium dodecylsulphate (SDS), 5× SSPE (20× SSPE in 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.5), and 100 mg denatured salmon sperm DNA, pH 7.5. Hybridizations were carried out in the same buffer containing approximately 2×10^6 cpm/mL of ^{32}P-labelled DNA probes for 1 day at 42°C. The DNA probes were ^{32}P-labelled by the multiprime DNA labelling system (Amersham Buchler GmbH, D-3300 Braunschweig, Germany). After hybridization, the filters were washed at 50°C in 2× SSC (Standard saline citrate), 0.1% SDS, and exposed to Kodak X-Omat S film or Konica X-ray film A2 for various time periods until the autoradiographic signal was optimal. The relative amount of transcript was determined by densitometric analysis of the autoradiographs using an Elscript 400 scanner (Hirschmann, D-8025 Unteraching, Germany). The scanning was done in the linear range of the film. Northern blots were rehybridized to GAPDH to adjust for uneven transfer during the blotting procedure or due to variations in the amount of mRNA applied onto the gel. The expression of this gene served as an internal control for mRNA quantification.

RESULTS

The liver weights, microsomal protein concentrations, and the liver-to-body weight ratios are summarised in Table 1. With maternal and neonatal controls, a time-dependent increase in liver weight, but not microsomal protein concentration, was seen. Treatment of lactating rats with 100 mg/kg Aroclor 1254 led to a significant increase in liver weight after 4 days, although after 14 days the change was no longer significant compared to controls. With the higher dose of Aroclor, liver weight was significantly increased at both time points. In neonates, a significantly higher liver weight was evident with both doses of Aroclor 1254, although only at 14 days post treatment. In these animals, but not the lactating mothers, microsomal protein concentration increased in a time- and dose-dependent fashion. The liver-to-body weight ratio did not differ when control and Aroclor-treated mothers were compared, but a significant dose-dependent increase was observed in neonates 14-days post treatment.

Effect of Aroclor 1254 on Microsomal Monooxygenases

The results of cytochrome P450, cytochrome b5, and NADPH cytochrome c (P450) reductase quantification are summarized in Table 2. In control neonates, a significant 2-fold increase in cytochrome P450 concentration was noted 14 days post partum. Dose- and time-dependent increases in total cytochrome P450 concentration and NADPH cytochrome c (P450) reductase activities, but not cytochrome b5 concentration, were seen in neonates in response to maternal treatment with Aroclor 1254. In contrast, these parameters in lactating mothers either increased by approximately one-third, independent of Aroclor dose (cytochrome P450), increased 2-fold in a dose-dependent fashion (NADPH cytochrome c (P450) reductase), or were significantly reduced (approximately 67%) 4 days post treatment (cytochrome b5 concentration).

Effect of Aroclor 1254 on the Metabolism of Marker Substrates of CYP1A1 and CYP1A2

The catalytic activities of maternal and neonatal cytochrome P450s, with respect to ethoxyresorufin O-dealkylation (EROD), p-nitroanisole O-demethylation, and aniline hydroxylation were assayed, and the results are shown in Table 3. Significant increases in the metabolism of all substrates in both maternal and neonatal samples were recorded in response to maternal treatment with Aroclor 1254. The rate of EROD activity was increased 6-fold at both treatment doses in neonates and lactating mothers at 4 days after treatment. At 14 days, activity in maternal samples fell back, remaining significantly elevated at the higher treatment dose, but virtually at control levels in the 100 mg/kg dose group. In neonates,

TABLE 1. The effects of Aroclor 1254 treatment on maternal and neonatal liver weight, liver to body weight ratio, and hepatic microsomal protein concentration

Treatment group	Liver weight (g)		Microsomal protein (mg/g liver)		Liver/body weight × 100	
	LM	N	LM	N	LM	N
A. control (4 days)	14.6 ± 1.3	1.8 ± 0.2§ (n = 5)	43.5 ± 2.1	22.5 ± 4.2§	4.4 ± 0.4	3.5 ± 0.4§
B. control (14 days)	17.1 ± 0.6	0.9 ± 0.2 (n = 6)	39.0 ± 8.5	28.8 ± 9.7	5.6 ± 0.8	3.1 ± 0.5
C. control (24 days)	17.6 ± 0.7	2.5 ± 1.8 (n = 5)	40.8 ± 7.4	21.0 ± 8.8	5.1 ± 0.9	4.2 ± 0.4
D. 100 mg/kg (4 days)	19.8 ± 1.3* (A,D)	3.1 ± 1.8§ (n = 4)	36.2 ± 2.5* (A,D)	19.1 ± 2.7§	5.9 ± 0.8	3.3 ± 0.3§
E. 100 mg/kg (14 days)	16.4 ± 2.6	3.9 ± 0.5‡ (B,E) (n = 4)	44.2 ± 1.7	62.5 ± 2.1‡ (B,E)	4.8 ± 0.6	4.8 ± 0.4‡ (B,E)
F. 250 mg/kg (4 days)	22.2 ± 3.1* (A,F)	2.1 ± 0.9§ (n = 6)	41.7 ± 3.9	24.0 ± 5.9§	6.2 ± 1.0	3.1 ± 0.3§
G. 250 mg/kg (14 days)	22.5 ± 1.7* (B,G)	2.3 ± 0.6‡ (B,G) (n = 3)	44.7 ± 6.0	78.5 ± 7.8‡ (B,G)	6.1 ± 0.1	6.5 ± 0.2‡ (B,G)

Values are mean ± SD; $n = 3$ animals per treatment group unless otherwise stated.

* $P < 0.05$.

† $P < 0.02$.

‡ $P < 0.01$ significantly different, when compared with the appropriate control (see letters in brackets for a comparison of treatment groups).

§ The entire litter from a single mother was pooled with aliquots taken for individual measurements.

LM, lactating mothers.

N, neonates.

EROD activity continued to rise at the 250 mg/kg Aroclor dose, but appeared to level off at the lower dose. The O-demethylation of p-nitroanisole increased at both treatment doses in neonates after 4 days, although this effect appeared to have subsided by 14 days. No effect on p-nitroanisole O-demethylase activity was seen in lactating mothers, except for a decrease at the lower Aroclor dose 14 days after treatment.

Aniline, which is preferentially metabolised by CYP1A2, although there is evidence for the involvement of CYP2E1, showed a significant dose- and time-dependent increase in hydroxylation in both maternal and neonatal animals at both Aroclor doses and at both time points, although the increase was greater (14-fold) in neonates than in lactating mothers (3-fold).

These results, taken collectively, show that individual PCB isomers and congeners differentially modulate the induction of CYP1A1 and CYP1A2. They also elicited a differential response when mothers and neonates were compared. Indeed, the results shown in Table 3 provide strong evidence that neonates are highly responsive to cytochrome P450 induction when milk from Aroclor 1254-treated mothers is consumed. However, this study also shows that lactation itself is an effective means of lowering the maternal body burden of PCBs.

Effect of Aroclor 1254 on the Metabolism of Marker Substrates of the CYP2 Family

The metabolism of marker substrates for the CYP2 family is summarised in Table 4. Treatment of maternal rats with Aroclor 1254 resulted in significant increases in the rates of N-demethylation of both aminopyrine and dimethylnitrosamine in these animals, although this effect was only evident 4 days after treatment at the higher Aroclor dose. Aroclor 1254 had no significant effect on aldrin epoxidation in maternal animals, and a significant difference in this activity was evident in neonates only at the lower dose. Moreover, in neonates, the N-demethylation of dimethylnitrosamine was significantly elevated at both Aroclor doses after 4 days, although in both instances this had diminished by 14 days. When the N-demethylation of aminopyrine was assessed, only the 250 mg/kg Aroclor dose elicited a significant increase in neonates, at both time points.

The N-demethylation of aminopyrine and aldrin epoxidation are catalysed by CYP2B1, and these data provide little evidence for significant increases in enzyme activity. Similarly, the induction of both maternal and neonatal CYP2E1 is only marginal and transient, as judged by the rate of N-demethylation of dimethylnitrosamine. The measurements reported in Table 4 do not mirror the time-dependent profiles of the induction of CYP2B1 and CYP2E1 previously shown [18]. The transient induction of these cytochromes contrasts with the induction of CYP1A1, which remained highly induced. In consequence, the results shown in Tables 3 and 4 point to a complex regulation of the expression of individual P450 isoenzymes that are selectively induced in the mother and neonates by individual PCB isomers and congeners.

Effect of Aroclor 1254 on the Regulation of Postoxidative Drug Metabolising Enzymes

The data from these experiments are summarised in Table 5. With epichlorohydrin as substrate, epoxide hydrolase activity

TABLE 2. The effects of Aroclor 1254 treatment on maternal and neonatal microsomal cytochrome P-450, cytochrome b_5, and cytochrome-c-(P-450) reductase

Treatment group	No. of animals	LM (pmol/mg protein)	LM (nmol/g liver)	N (nmol/g liver)	N (pmol/mg protein)	No. of animals
		Cytochrome P-450 concentration				
A. control (4 days)		350 ± 10	15.3 ± 1.3	3.4 ± 1.2§	150 ± 30§	(n = 5)
B. control (14 days)		500 ± 100	16.1 ± 6.2	6.2 ± 0.1	140 ± 10	(n = 3)
C. 100 mg/kg (4 days)	(n = 3)	500 ± 160	20.6 ± 0.3† (A,C)	6.2 ± 0.1§	320 ± 60‡§	(n = 4)
D. 100 mg/kg (14 days)		450 ± 50	19.6 ± 2.9	14.1 ± 0.4‡	300 ± 30‡	(n = 4)
E. 250 mg/kg (4 days)		500 ± 50	20.9 ± 3.9	6.9 ± 2.2§	280 ± 40‡§	(n = 6)
F. 250 mg/kg (14 days)		650 ± 80	20.8 ± 6.8	23.9 ± 2.2‡	560 ± 90‡	(n = 3)
		Cytochrome b_5 concentration				
A. control (4 days)		250 ± 10	10.9 ± 1.0	2.1 ± 0.6§	80 ± 20§	(n = 3)
B. control (14 days)		200 ± 20	7.7 ± 2.4	2.9 ± 0.6	60 ± 20	(n = 4)
C. 100 mg/kg (4 days)	(n = 3)	190 ± 30	6.3 ± 0.1† (A,C)	2.1 ± 0.2§	110 ± 10§	(n = 4)
D. 100 mg/kg (14 days)		170 ± 40	7.2 ± 1.2	10.4 ± 1.5‡	230 ± 70	(n = 4)‡
E. 250 mg/kg (4 days)		180 ± 10	7.2 ± 1.4	3.6 ± 0.6§	130 ± 30§	(n = 5)
F. 250 mg/kg (14 days)		260 ± 30	13.2 ± 0.5* (B,F)	6.8 ± 1.4*	150 ± 20	(n = 3)§
		NADPH cytochrome-c-(P-450) reductase activities				
		nmol/mg protein/min			nmol/mg protein/min	
A. control (4 days)		45.8 ± 4.0			26.8 ± 4.6§	(n = 4)
B. control (14 days)		51.3 ± 2.7			not determined	
C. 100 mg/kg (4 days)	(n = 3)	69.6 ± 3.4‡			21.9 ± 1.9§	(n = 4)
D. 100 mg/kg (14 days)		50.2 ± 1.8			136.3 ± 16.4	(n = 4)
E. 250 mg/kg (4 days)		73.2 ± 4.1‡			65.2 ± 0.4‡§	(n = 5)
F. 250 mg/kg (14 days)		73.1 ± 11.6*			237.8 ± 6.2	(n = 3)

Values are mean ± SD.

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$ significantly different, when compared with the appropriate control (see letters in brackets for a comparison of treatment groups).

§ The entire litter from a single mother was pooled with aliquots taken for individual measurements.

LM, lactating mothers.

N, neonates.

was elevated in both maternal and neonatal animals. Moreover, if these results are expressed on a liver weight basis (Table 5a), increases in epoxide hydrolase activities were measurable in lactating mothers (3-fold over control) and in neonates (7-fold over control). This suggests that neonates are more responsive to an induction by PCBs for epoxide hydrolase. Compared with neonates, lactating mothers had reduced epoxide hydrolase activities 14 days post treatment, thus suggesting that lactational transfer of PCBs is protective for the mother. In contrast, UDP-glucuronyl transferase using 2-aminophenol as substrate and GST using CDNB as substrate were both significantly raised in maternal and neonatal animals, in a time- and dose-dependent manner (Table 5). The data shown in Table 5 indicate that lactational transfer of PCBs is protective for the mother.

Western Blot Analysis of Cytochrome P450 Proteins

Western immunoblotting experiments were carried out to obtain unequivocal evidence for the expression of individual P450 isoenzymes present in maternal and neonatal hepatic tissue. Furthermore, the expression of immunoreactive proteins were compared with mRNA levels determined by Northern blot analysis (see below).

The expression of individual isoenzymes was determined by comparing immunoreactive bands and their electrophoretic mobility with those estimated for purified isoenzymes (i.e. standards). This comparison permitted an identification of constitutively expressed isoenzymes and the effects of Aroclor 1254 treatment on the induction of P450 proteins. The results of the Western immunoblotting studies are summarised in Fig. 1. Control lactating mothers had a strong immunoreactive band corresponding to CYP2C6 and a weak immunoreactive band corresponding to CYP3A1, but there was little evidence for the expression of CYP1A1, CYP1A2, CYP2B1, CYP2E1, and CYP4A1 at a loading of 3 μg total protein per well. A comparable result was obtained with neonatal controls (Fig. 1), but the expression of neonatal Aroclor CYP3A1 was stronger than in the maternal control. There was also a faint cross reactivity in the neonatal control with the CYP4A1 antibody.

Comparison of control and PCB-treated mothers show an impressive induction of CYP1A1, CYP1A2, CYP2A1, CYP2B1, CYP2C6, CYP2E1, CYP3A1, and CYP4A1, albeit at varying intensities. In the case of CYP1A1, CYP1A2, CYP2B1, and CYP4A1, the induction was dose-dependent. For the other isoenzymes, the lower dose of 100 mg/kg body weight may represent a "saturation" response with respect to P450 induction. The antibody raised to CYP2A1 recognised

TABLE 3. The effects of Aroclor 1254 treatment on monooxygenase activities predominantly catalysed by maternal and neonatal CYP1A1 and CYP1A2

Treatment group	No. of animals	Aniline hydroxylase (pmol/mg protein/min) LM	N	No. of animals
A. control (4 days)		270 ± 10	130 ± 30	(n = 5)
B. control (14 days)		Not determined‖	Not determined‖	
C. 100 mg/kg (4 days)	(n = 3)	360 ± 20‡ (A,C)	260 ± 10‡§ (A,C)	(n = 4)
D. 100 mg/kg (14 days)		410 ± 10	400 ± 130	(n = 4)
E. 250 mg/kg (4 days)		410 ± 30‡ (A,E)	440 ± 70‡§ (A,E)	(n = 6)
F. 250 mg/kg (14 days)		680 ± 80	1830 ± 160	(n = 3)
		p-nitroanisole-O-demethylase (pmol/mg protein/min)		
A. control (4 days)		520 ± 20	480 ± 110§	(n = 5)
B. control (14 days)		Not determined‖	Not determined‖	
C. 100 mg/kg (4 days)	(n = 3)	550 ± 20	1230 ± 170‡§ (A,C)	(n = 4)
D. 100 mg/kg (14 days)		420 ± 10	510 ± 160	(n = 4)
E. 250 mg/kg (4 days)		600 ± 40	2310 ± 160‡§ (A,E)	(n = 6)
F. 250 mg/kg (14 days)		410 ± 60	520 ± 40	(n = 3)
		Ethoxyresorufin-O-deethylase (pmol/mg protein/min)		
A. control (4 days)		56 ± 10	24 ± 10§	(n = 5)
B. control (14 days)		56 ± 10	42 ± 3	(n = 4)
C. 100 mg/kg (4 days)	(n = 3)	340 ± 50‡ (A,C)	161 ± 40‡§ (A,C)	(n = 4)
D. 100 mg/kg (14 days)		93 ± 30	156 ± 10‡ (B,D)	(n = 3)
E. 250 mg/kg (4 days)		206 ± 10‡ (A,E)	131 ± 20‡§ (A,E)	(n = 6)
F. 250 mg/kg (14 days)		156 ± 30* (B,F)	259 ± 20‡ (B,F)	(n = 3)

Values are mean ± SD.

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$ significantly different, when compared with the appropriate control (see letters in brackets for a comparison of the treatment groups.

§ The entire litter from a single mother was pooled with aliquots taken for individual measurements.

‖ Statistical analysis was not done, as the 14-day control group was not determined.

LM, lactating mothers; N, neonates.

an additional epitope in microsomal proteins isolated from Aroclor 1254-treated lactating mothers. Similarly, the antibody raised to CYP2B1 recognised an additional epitope, most likely CYP2B2, in microsomal proteins isolated from Aroclor 1254-treated lactating mothers.

Similar results to these obtained with lactating mothers were observed with neonatal samples. There was a dose-dependent expression of CYP1A1, CYP1A2, CYP2B1, and CYP4A1. In contrast, the induction of CYP2A1 and CYP3A1 was independent of the dose given to the mother. Again, the lower dose of 100 mg/kg body weight may represent a "saturation" response with respect to the P450 induction of these isoenzymes.

At the higher treatment dose, a highly significant reduction in the expression of neonatal CYP2E1 was observed, and CYP2C6 did not respond to treatment with Aroclor 1254.

Northern Blot Hybridization Analysis of Cytochrome P450 mRNA

Figure 2 summarises the Northern blot hybridization experiments. The molecular size of the mRNA transcripts are detailed in Table 6. It should be noted that the CYP1A probe hybridised to two mRNA transcripts. The upper band (2.9 kb) was assigned to CYP1A2, whereas CYP1A1 was assigned to the lower band (2.2 kb). The molecular sizes of the individual mRNAs are detailed in Table 6 and are fully consistent with the values reported by others [38]. The Northern blots were scanned within the linear range.

There was little expression of CYP1A1 and CYP1A2 mRNA in control lactating mothers, but treatment with Aroclor 1254 produced approximately 62-fold and 44-fold increases, respectively. In contrast, approximately 2-fold and 14-fold increases in mRNA levels of CYP2A1 and CYP2B1, respectively, were observed, whereas no change was seen in CYP2E1 mRNA levels. Further treatment of lactating mothers with Aroclor 1254 produced a highly significant reduction in CYP2C7 mRNA to 56% of control values, and similar results were obtained when total RNA samples were analysed for CYP4A1 where the mRNA was reduced to 40% of control values. The increase in CYP2E1 protein expression was unexpected in the face of reduced mRNA levels, but could be the result of protein stabilisation against degradation. Such a mechanism of protein induction has been reported for CYP2E1 using pulse labelling with amino acids (reviewed in ref. [38]).

Only CYP2A1, CYP2C7, CYP2E1, and CYP4A1 mRNA transcripts were detected in extracts of hepatic tissue from control neonates, and lactational transfer of PCBs resulted in 22-, 55-, 14-, 49-, and 16-fold increases in CYP1A1, CYP1A2,

TABLE 4. The effects of Aroclor 1254 treatment on monooxygenase activities predominantly catalysed by maternal and neonatal CYP2B1 and CYP2E1

Treatment group	No. of animals	Dimethylnitrosamine N-demethylase (nmol/mg protein/min) LM	N	No. of animals
A. control (4 days)		1.1 ± 0.4	1.4 ± 0.3§	(n = 5)
B. control (14 days)		Not determined‖	Not determined‖	
C. 100 mg/kg (4 days)	(n = 3)	1.8 ± 0.1	4.6 ± 0.9‡§ (A,C)	(n = 4)
D. 100 mg/kg (14 days)		1.6 ± 0.1	1.8 ± 0.5	(n = 4)
E. 250 mg/kg (4 days)		2.1 ± 0.2* (A,E)	5.2 ± 0.3‡§ (B,E)	(n = 6)
F. 250 mg/kg (14 days)		2.0 ± 0.3	2.1 ± 0.2	(n = 3)
		Aminopyrine-N-demethylase (nmol/mg protein/min)		
A. control (4 days)		2.5 ± 0.1	2.9 ± 0.9§	(n = 5)
B. control (14 days)		2.6 ± 0.3	2.3 ± 0.1	(n = 3)
C. 100 mg/kg (4 days)	(n = 3)	2.2 ± 0.2	3.7 ± 0.2§	(n = 4)
D. 100 mg/kg (14 days)		2.5 ± 0.0	1.5 ± 0.3	(n = 4)
E. 250 mg/kg (4 days)		4.9 ± 0.5‡ (A,E)	4.9 ± 0.4†§ (A,E)	(n = 6)
F. 250 mg/kg (14 days)		2.6 ± 0.3	4.3 ± 0.4‡ (B,F)	(n = 3)
		Aldrin epoxidation (nmol/mg protein/min)		
A. control (4 days)		2.2 ± 0.6	2.5 ± 0.5§	(n = 3)
B. control (14 days)		2.9 ± 0.8	2.7 ± 0.2	(n = 6)
C. 100 mg/kg (4 days)	(n = 3)	2.7 ± 0.4	4.8 ± 0.2*§ (A,C)	(n = 3)
D. 100 mg/kg (14 days)		Not determined	8.4 ± 3.6* (B,D)	(n = 3)
E. 250 mg/kg (4 days)		3.3 ± 0.1	3.1 ± 0.9§	(n = 6)
F. 250 mg/kg (14 days)		Not determined	6.3 ± 3.3	(n = 3)

Values are means ± SD.

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$ significantly different, when compared with the appropriate control (see letters in brackets for a comparison of the treatment groups).

§ The entire litter from a single mother was pooled with aliquots taken for individual measurements.

‖ Statistical analysis was not done as the 14-day control group was not determined.

LM, lactating mothers; N, neonates.

CYP2A1, CYP2B1, and CYP2E1 mRNA, respectively. In contrast, exposure to PCBs caused a highly significant reduction in CYP2C7 mRNA to 22% of control values and a 50% reduction in CYP4A1 mRNA. Nevertheless, the Northern blot analysis accords, in part, with the Western blot analysis shown in Fig. 1.

Hybridization analysis of CYP1A1 and CYP1A2 mRNA (Fig. 2) details the presence of two transcripts. Densitometric scanning of Northern blot autoradiographs suggests higher CYP1A1 mRNA levels in response to the lactational transfer of PCBs.

Expression of the Cellular Protooncogenes c-raf, c-Ha-ras, c-erb A, and c-erb B

The results of Northern blot hybridization experiments are summarised in Figs. 3 and 4. An approximate 3-fold increase in the mRNA expression of c-raf was found when control females and lactating mothers were compared. A similar level of c-raf mRNA expression was found when control females and control neonates were compared (Fig. 3).

Compared with female controls, treatment with Aroclor 1254 resulted in 7- and 4-fold increases in lactating mothers and neonates, respectively. The increase in c-raf mRNA expression was only 2-fold when compared with control lactating mothers and control neonates.

The changes in c-Ha-ras expression were less significant. Control neonates expressed c-Ha-ras mRNA at twice the level of control females, whereas little change was seen in control lactating mothers. Treatment of lactating mothers resulted in 4- and 3-fold increases in mothers and neonates, respectively, compared to female controls. The increase in c-Ha-ras mRNA expression was only 2-fold when the treatment group was compared with control lactating mothers. Only marginal changes in the expression of neonatal c-Ha-ras mRNA were observed due to lactational transfer of PCBs.

No c-erb A and c-erb B mRNA transcripts were detected with liver extracts from control and treated animals. In consequence, PCBs did not modulate the expression of these protooncogenes.

DISCUSSION

Aroclor 1254 treatment of lactating rats resulted in significant induction of P450 isoenzymes in both lactating mothers and neonates. This response was not uniform, and depended on the isoenzymes studied. There were differences in the response when maternal rats and their offspring were compared, as the induction of neonatal P450 isoenzymes depended on the transfer of PCBs via lactation.

Lactational transfer of PCBs resulted in highly significant

TABLE 5. The effects of Aroclor 1254 treatment on the activities of epoxide hydrolase, glutathione-S-transferase, and UDP-glucuronyl transferase

Treatment group	No. of animals	Epoxide hydrolase (pmol/mg protein/min) LM	N	No. of animals
A. control (4 days)		130 ± 10	170 ± 50§	(*n* = 5)
B. control (14 days)		Not determined‖	Not determined‖	
C. 100 mg/kg (4 days)	(*n* = 3)	410 ± 40‡ (A,C)	320 ± 70*§ (A,C)	(*n* = 4)
D. 100 mg/kg (14 days)		250 ± 10	370 ± 100	(*n* = 3)
E. 250 mg/kg (4 days)		440 ± 50† (A,E)	380 ± 150‡§ (A,E)	(*n* = 6)
F. 250 mg/kg (14 days)		340 ± 60	300 ± 0.04	(*n* = 3)
		Glutathione-S-transferase (nmol/mg protein/min)		
A. control (4 days)		138.0 ± 24.0	85.0 ± 18.0§	(*n* = 5)
B. control (14 days)		174.0 ± 39.0	85.0 ± 10.0	(*n* = 6)
C. 100 mg/kg (4 days)	(*n* = 3)	202.0 ± 15	84.0 ± 8.0§	(*n* = 4)
D. 100 mg/kg (14 days)		491.0 ± 39† (B,D)	353.0 ± 98.0† (B,D)	(*n* = 3)
E. 250 mg/kg (4 days)		399.0 ± 34.0‡ (A,E)	123.0 ± 33.0§	(*n* = 4)
F. 250 mg/kg (14 days)		657.0 ± 114.0† (B,F)	905.0 ± 68.0‡ (B,F)	(*n* = 3)
		UDP-glucuronyl transferase (nmol/mg protein/min)		
A. control (4 days)		2.3 ± 0.1	2.6 ± 0.4§	(*n* = 5)
B. control (14 days)		2.8 ± 0.3	2.2 ± 0.3	(*n* = 6)
C. 100 mg/kg (4 days)		3.6 ± 0.4† (A,C)	2.6 ± 0.3§	(*n* = 4)
D. 100 mg/kg (14 days)	(*n* = 3)	4.5 ± 0.2‡ (B,D)	5.2 ± 0.7‡ (B,D)	(*n* = 3)
E. 250 mg/kg (4 days)		3.7 ± 0.1‡ (A,E)	3.0 ± 0.1§	(*n* = 6)
F. 250 mg/kg (14 days)		6.4 ± 0.3‡ (B,F)	6.7 ± 0.7‡ (B,F)	(*n* = 3)

Values are means ± SD.

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$ significantly different, when compared with the appropriate control (see letters in brackets for a comparison of the treatment groups).

§ The entire litter from a single mother was pooled with aliquots taken for individual measurements.

‖ Statistical analysis was not done as the 14-day control group was not determined.

LM, lactating mothers; N, neonates.

changes in CYP1A1 and CYP1A2 protein, mRNA, and associated enzyme activity (Figs. 1 and 2, Table 3). The present study extends the earlier findings by several authors [36], [37], who did not measure the induction of P450 isoenzymes at the protein and mRNA levels. An intriguing finding of the present study was the effectiveness of lactational transfer of PCBs to neonates and their potency in inducing neonatal P450 proteins. The results shown in Table 3 provide evidence for a dose- and time-dependent increase in activities, which became maximal at the higher PCB dose 14 days after maternal treatment. Neonatal CYP1A2 was more responsive to lactational transfer of PCBs as judged by the larger amount of mRNA that hybridised with the CYP1A2 cDNA probe and the higher activities of aniline hydroxylase. This contrasts with the results obtained in lactating mothers. It is noteworthy that aniline is also metabolised by CYP2E1 but, as Western blot analysis did not reveal enhanced CYP2E1 protein expression (see Fig. 1), the results reported in Table 3 are approximate values for the catalytic activities of neonatal CYP1A2.

Parkinson and co-workers [37] reported comparable results

TABLE 5A. The effects of Aroclor 1254 treatment on the activities of epoxide hydrolase

Treatment group	No. of animals	Epoxide hydrolase (nmol/g liver/min) LM	N	No. of animals
A. control (4 days)		377.2 ± 15.3	358.7 ± 20.1§	(*n* = 5)
B. control (14 days)		Not determined‖	Not determined‖	
C. 100 mg/kg (4 days)	(*n* = 3)	977.1 ± 49.8‡ (A,C)	394.5 ± 35.8§	(*n* = 4)
D. 100 mg/kg (14 days)		732.8 ± 53.7	2413.8 ± 320.1	(*n* = 3)
E. 250 mg/kg (4 days)		1221.3 ± 108.5	558.9 ± 18.3‡§ (A,E)	(*n* = 6)
F. 250 mg/kg (14 days)		1012.5 ± 30.5	1578.1 ± 62.0	(*n* = 3)

Values are means ± SD.

‡ $P < 0.01$ significantly different, when compared with the appropriate control (see letters in brackets for a comparison of the treatment group).

§ The entire litter from a single mother was pooled with aliquots taken for individual measurements.

‖ Statistical analysis was not done as the 14-day control group was not determined.

LM, lactating mothers; N, neonates.

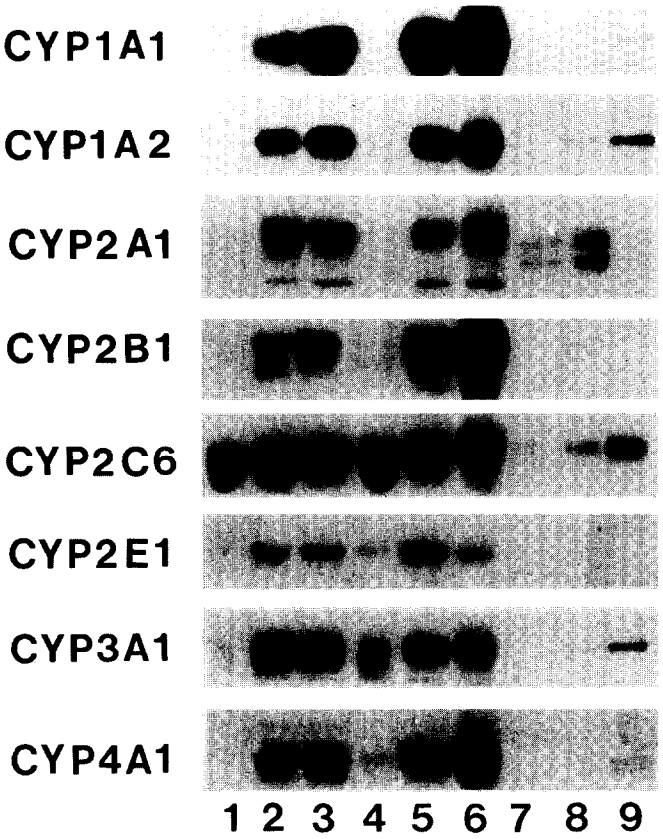

FIG. 1. **Western blot analysis of maternal and neonatal microsomal P450 proteins. Microsomal proteins were separated on SDS/PAGE, transferred to nitrocellulose, and probed with P450 antisera, as detailed in Materials and Methods. Control lactating mother, lane 1; Aroclor 1254-treated mothers, lanes 2 and 3 (100 mg/kg and 250 mg/kg body weight, respectively). Lane 4, control neonate; lanes 5 and 6, exposure of neonates to PCBs via milk in response to a maternal treatment dose of 100 mg/kg and 250 mg/kg body weight Aroclor 1254, respectively. Lanes 7 to 9, standards (0.4, 0.8, and 1.2 pmol purified P450, with the exception of CYP2A1).**

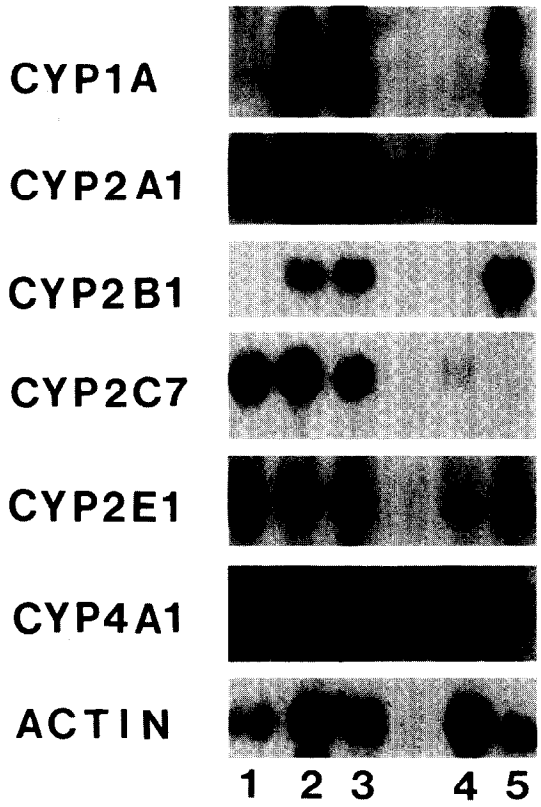

FIG. 2. **Cytochrome P450 mRNA levels in maternal and neonatal liver samples. RNA (10 μg) from maternal and neonatal liver samples was separated on denaturing formaldehyde agarose gels, transferred to Hybond N, and probed with P450 cDNA probes as outlined in Materials and Methods. Lane 1, control mother; lanes 2 and 3, Aroclor 1254-treated mothers (100 mg/kg and 250 mg/kg body weight), respectively; lane 4, control neonate; lane 5, exposure of neonates to PCBs via milk in response to a maternal treatment dose of 250 mg/kg body weight Aroclor 1254.**

with rats exposed to human breast milk that contained a PCB mixture similar to the isomeric and congeneric mixture of PCBs determined in the Osaka Prefecture in Japan. When compared with the commercial PCB mixture Kanechlor 500, the reconstituted human breast milk possessed 7-fold increased potency in inducing aryl hydrocarbon hydroxylase. This finding provides evidence for a selective ability of certain PCBs to modulate the induction of CYP1A proteins, since those PCBs that tend to accumulate are more potent in inducing proteins of the CYP1A family.

The induction of maternal and neonatal CYP2A1 (see Fig. 1) reported in this study extends the findings of Parkinson *et al.* [8], as treatment of three-week-old Long Evan rats with Aroclor 1254 (500 mg/kg body weight) resulted in a 3–5-fold increase in immunoreactive protein. Treatment of male rats with 3,4,5,3′,4′-penta- or 3,4,5,3′,4′,5′-hexachlorobiphenyl caused an unprecedented high level of induction. A similar result was obtained with 3,4,5,4′-tetra, 3,3′,4,4′-tetra-, and 3,4,5,3′,4′-pentabromobiphenyl. These results document how versatile PCBs are in modulating the expression of CYP2A1, as treatment with eight isomeric and congeneric mono-ortho-substituted halogenated biphenyls produced additional expression of this protein, albeit at varying degrees.

The present report suggests that the lower dose is effective in producing maximal induction of the CYP2A1 protein. In addition, neonates responded with a 14-fold increase in

TABLE 6. Summary of cDNA probes used in northern blotting experiments

cDNA probe	Species	Molecular size of cDNA hybridized mRNA (kb)
CYP1A1	Human	2.2
CYP1A2	Human	2.9
CYP2A1	Human	2.0
CYP2B1	Rat	1.8
CYP2C7	Rat	2.0
CYP2E1	Human	1.8
CYP3A	Human	1.8
CYP4A	Rat	2.1

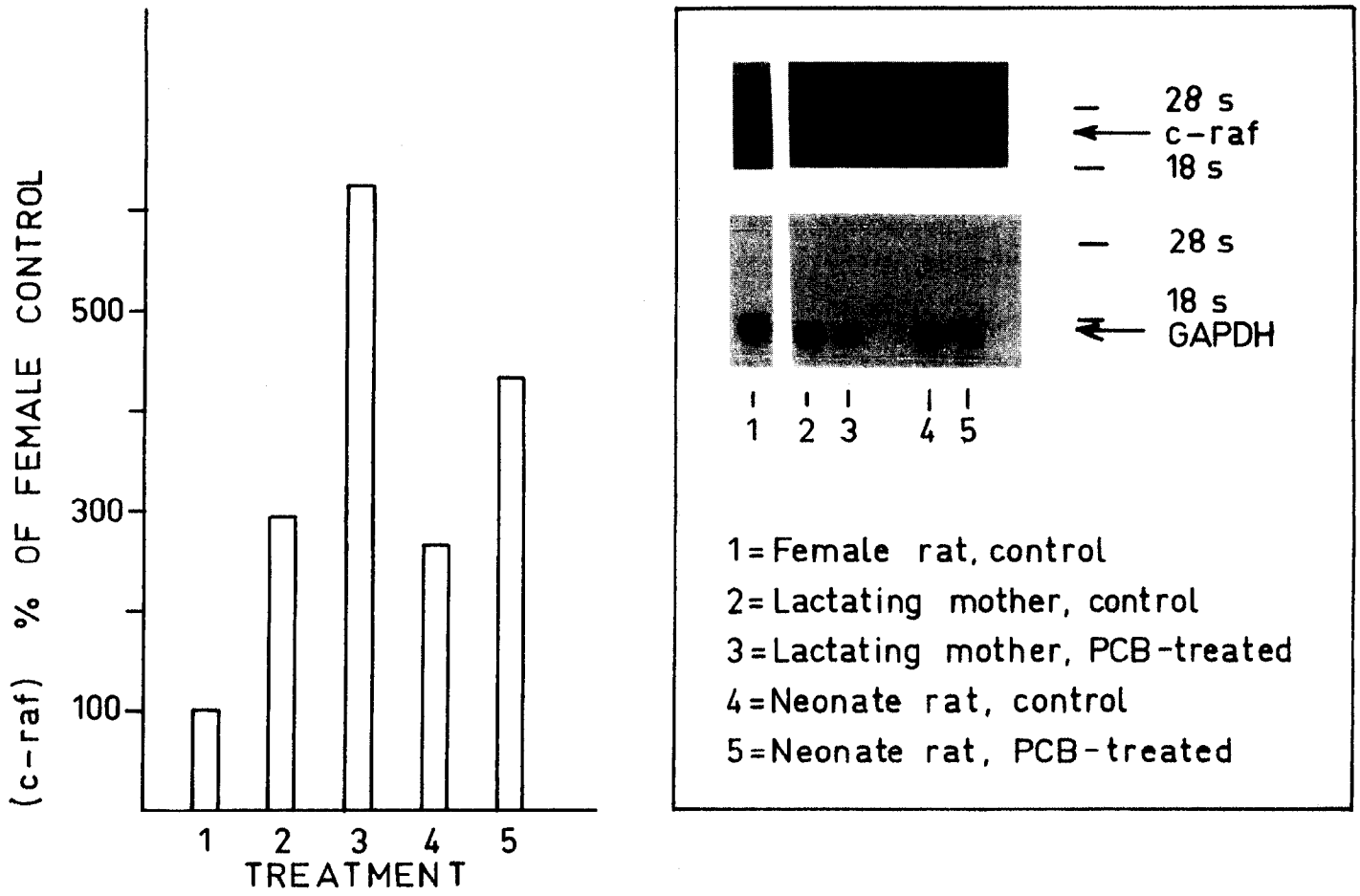

FIG. 3. c-raf mRNA levels in control and Aroclor treated maternal and neonatal rat liver samples. Fourteen days after treating lactating mothers with Aroclor 1254 (250 mg/kg body weight), the livers of 4 control females, 4 untreated, and 4 treated neonatal rats were pooled separately. Then, total RNA was isolated and separated on agarose gels and transferred to nitrocellulose membranes. Ten μg of total RNA was applied and hybridisation with cDNA probes was carried out as outlined in Materials and Methods. The autoradiographic signals were quantified densitometrically and normalized to GAPDH mRNA signals.

CYP2A1 mRNA expression when milk of Aroclor 1254 treated mothers was consumed. This compares with a mere 2-fold increase in maternal CYP2A1 mRNA. Thus, the Northern blot hybridization experiments provide striking evidence for the protective effect of lactation against the inductive effect of PCBs in Aroclor 1254-treated mothers.

Our results are in agreement with reports by others [38], as we were unable to detect CYP2B1 mRNA in control maternal and neonatal hepatic tissue (Fig. 2 and Table 5). Treatment with Aroclor 1254 produced highly significant 14- and 49-fold increases in maternal and neonatal CYP2B1 mRNA (Fig. 2). Again, this shows the protective effect of lactation against the inductive effect of PCBs, but also exemplifies how sensitive neonates are when they consume milk of Aroclor 1254-treated mothers, as neonates had a highly induced CYP2B1 protein compared with the lactating mothers (see Fig. 1 and the enzyme activities shown in Table 4).

As shown in the Western blot analysis (Fig. 1), the CYP2C6 protein was not strongly modulated by PCBs in mothers or neonates. In contrast, a strong induction was seen with CYP3A1 when lactating mothers were treated with Aroclor 1254, but only 2-fold increases in CYP3A1 mRNA levels were measured in maternal and neonatal tissue extracts (results not shown). There was a diminished expression of neonatal CYP3A1 at the higher treatment dose. This suggests a differential response in the expression of CYP3A1 in maternal and neonatal animals to an increasing Aroclor 1254 dose. Again, the results illustrate how versatile PCBs are in modulating the induction of CYP3A1.

Treatment of lactating rats with Aroclor 1254 caused significant CYP2E1 induction in the mother and the suckling offspring, but this response was not uniform and, with the higher dose, a reduced expression of the neonatal CYP2E1 protein was observed. Less CYP2E1 mRNA was detected in the treatment groups compared to the controls. In contrast, the activities towards N-nitrosodimethylamine were increased 4 days post treatment, but were independent of the dose. These results are in agreement with reports by others (reviewed in ref. [38]), as CYP2E1 induction has been shown to occur in rats in response to treatment with acetone, pyrazole, and ethanol without measurable increases in CYP2E1 mRNA. Experimental evidence has been reported [38, 39] that suggests

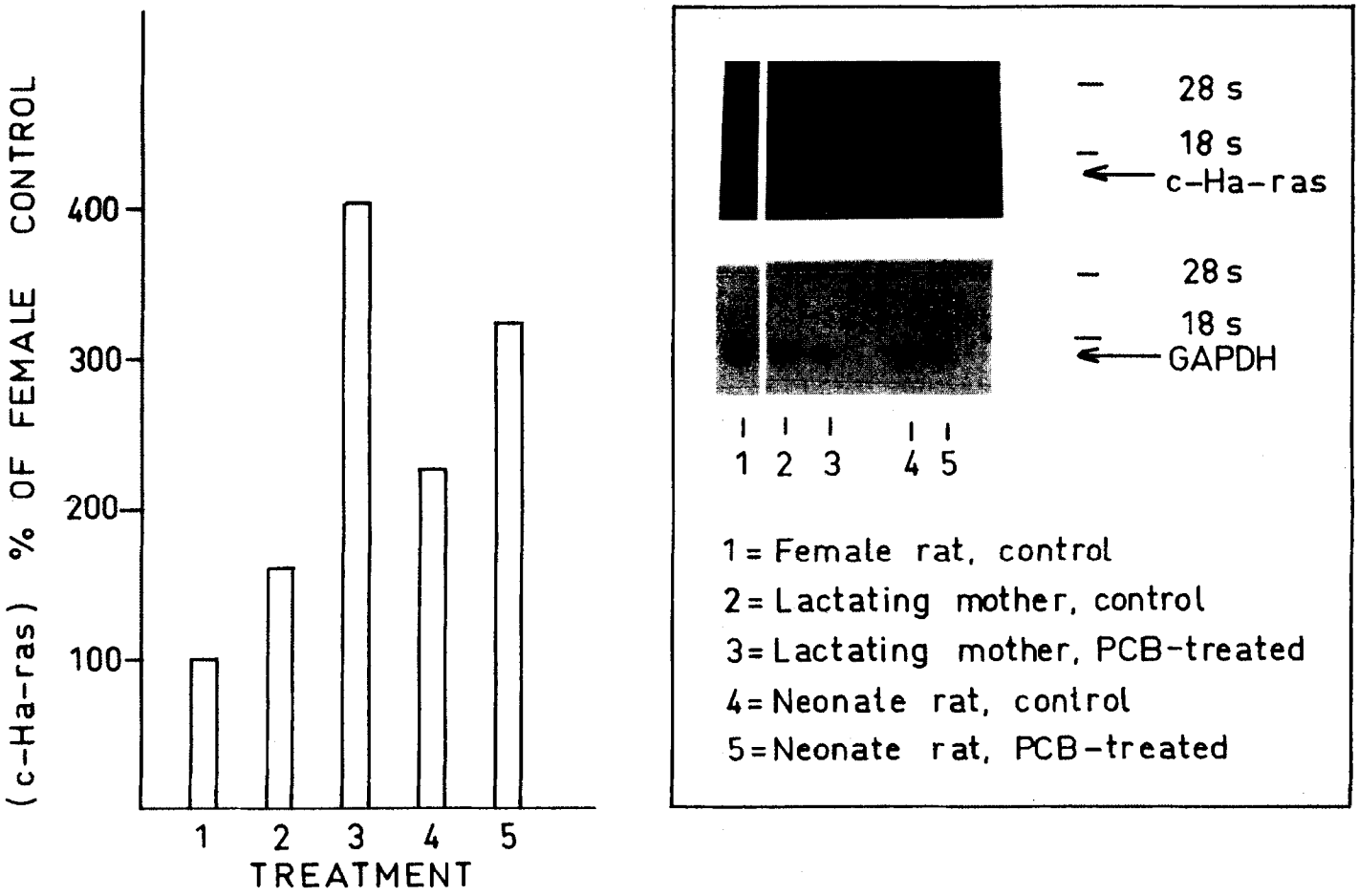

FIG. 4. c-Ha-ras mRNA levels in control and Aroclor-treated maternal and neonatal rat liver samples. Fourteen days after treating lactating mothers with Aroclor 1254 (250 mg/kg body weight), the livers of 4 control females, 4 untreated, and 4 treated neonatal rats were pooled separately. Then, total RNA was isolated and separated on agarose gels and transferred to nitrocellulose membranes. Ten μg of total RNA was applied and hybridisation with cDNA probes was carried out as outlined in Materials and Methods. The autoradiographic signals were quantified densitometrically and normally to GAPDH mRNA signals.

high affinity binding of certain inducers to CYP2E1 to stabilise the protein against degradation.

A similar binding of certain PCB isomers and congeners to CYP2E1 may stabilise the protein against degradation. For comparison, the maternal and neonatal CYP2C7 mRNA transcripts are shown in Fig. 2, and reductions were found in response to Aroclor 1254 treatment. This may indicate enhanced degradation or reduced synthesis of CYP2C7 mRNA. The physiological implications of diminished CYP2C7 mRNA in maternal and neonatal liver in response to PCBs remain uncertain.

In the present study, a dose-dependent increase in the induction of CYP4A1 was observed. This induction was not paralleled by increases in maternal or neonatal CYP4A1 mRNA. This may indicate post-transcriptional regulation of CYP4A proteins against degradation. Treatment of rats with a dose of 600 μmol/kg body weight Aroclor 1254 resulted in the specific induction of 12-hydroxylation of lauric acid [40]. Furthermore, treatment of maternal rats with a low dose of 5 mg/kg body weight of either 2,2′,4,4′,5,5′-hexachlorobiphenyl or 3,3′,4,4′-tetrachlorobiphenyl resulted in an increase in immunoreactive CYP4A protein measured in the neonate [41]. This suggests that the induction of CYP4A may not require the rigid molecular substitution pattern of PCBs, as reported for the induction of other P450 isoenzymes such as CYP1A, CYP2A, and CYP2B proteins (see discussion above and ref. [8]). There is additional evidence to suggest a synergistic mode of action when both PCBs are given in combination, as further increases in immunoreactive protein could be measured [41]. A recent report (reviewed in ref. [38]) suggests a mechanism of induction that involves binding of hypolipidemic drugs to a steroid hormone receptor, but it remains uncertain whether PCBs modulate this receptor.

The expression of protooncogenes in rat liver induced by a diet supplemented with a mixture of PCBs was reported [35]. Of the ten protooncogenes investigated, c-Ha-ras, c-erb A, c-raf, and c-yes were increased at the mRNA level in adult rat liver. Similar results were obtained for weaning rat liver mRNA extracts, but c-erb B was additionally expressed in comparable experiments. This points to developmental differences in the expression of protooncogenes in adult and adolescent rats. In the present study, evidence is presented to

suggest some hormonal control, at least in part, of the protooncogenes c-Ha-ras and c-raf. This hormonal control may explain the increased mRNA expression of the above named protooncogenes in untreated mothers and neonates, but PCBs additionally enhanced their expression to a similar extent in mothers and neonates.

DNA sequence analysis of the 5′-upstream flanking region of the rat c-raf protooncogene resulted in the discovery of the dioxin responsive element (DRE) (Jenke *et al.*, unpublished data). This undoubtedly will increase our understanding of how PCBs modulate the expression of the c-raf protooncogene, as certain PCBs bind with high affinity to the dioxin (Ah) receptor, leading to a cascade of cellular events including binding to the DRE [42]. It is of considerable interest that an estrogen responsive element (ERE) was also discovered in the promoter region of the c-raf protooncogene (Jenke *et al.*, unpublished results), in view of the fact that certain PCBs and their metabolites bind to the estrogen receptor [43, 44]. The latter finding is likely to aid in the interpretation of our recent finding that female rats were found to have higher expression of the c-raf protooncogene in pre-neoplastic lesions and isolated nodules when compared with male rats [45]. Taken collectively, PCBs are likely to modulate protooncogene expression via interaction with the ERE and DRE. The expression of protooncogenes is considered to be deleterious. There is compelling evidence implicating c-Ha-ras in the development of neoplasms in colon, lung, and pancreas [46], and c-raf in the onset of hepatocarcinogenesis [47, 48]. In the present study, differences in the mRNA expression of CYP1A1 and c-raf following treatment with Aroclor 1254 were found. It will be of interest to investigate further the difference in sensitivity to Aroclor 1254 exposure, as both genes are modulated by the DRE.

As discussed above, the doses selected for this study were based on those studied by others. Transplacental transfer of PCBs has recently been reported in which similar doses were investigated [49]. Therefore, the dose selection in this study permitted direct comparisons to the findings reported by others. However, it must be pointed out that the doses used in this study are only comparable to those observed in Japan and Taiwan, where over 1000 people were poisoned by consuming bran oil accidentally contaminated with PCBs. Nevertheless, the findings of the present study do not necessarily permit an extrapolation to lower doses. Future studies should establish dose-response relationships at lower doses.

In conclusion, this study shows how effectively the maternal body burden of PCBs can be lowered during lactation. In other words, the transfer of PCBs into milk protected lactating rats to some extent against the inductive effects of PCBs. The toxicological endpoints studied in this report were detoxification and tumour markers, and these endpoints are valuable in predicting the toxicity of PCBs at early stages of cellular alterations. For instance, the induction of CYP1A1 is deleterious due to its role in the bioactivation of pro-carcinogens such as benzo(a)pyrene. Other factors, such as displacement of physiological ligands from receptors by PCBs and their metabolites, are likely to alter significantly the normal physiological functioning of cells. This is exemplified in the activation of P450 genes and protooncogenes, which are pivotal signals for the onset of certain neoplasms [11, 47, 48].

The studies by Nims and co-workers [11] suggest a positive correlation between the induction of alkoxyresorufin O-dealkalases, epoxide hydrolase, and liver weight gain with the tumour-promoting potential of a series of barbiturates. Comparable changes were observed in the present study within 14 days post treatment. Nevertheless, this study also shows the necessity of assessing several if not all markers, as PCBs produce a variety of cellular alterations that cannot be predicted from the study of a single endpoint.

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References

1. Safe S, Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, toxicology, and mechanism of action. *CRC Crit Rev Toxicol* **13:** 319:395, 1984.
2. Tanabe S, PCB problems in future: foresight from current knowledge. *Environ Pollut* **50:** 5–28, 1988.
3. Borlakoglu JT and Dils RR, PCBs in human tissues. *Chemistry in Britain* **27:** 815–820, 1990.
4. Wasserman M, Nogneira DP, Tomatis L, Mirra AP, Slubata H, Arie G, Cucos S and Wassermann D, Organochlorine compounds in neoplastic and adjacent apparently normal breast tissue. *Bull Environ Contam Toxicol* **15:** 478–483, 1976.
5. Unger M and Olsen J, Organochlorine compounds in the adipose tissue of decreased people with and without cancer. *Environmental Research* **23:** 257–263, 1980.
6. Safe S, Polychlorinated biphenyls (PCBs): Mutagenicity and carcinogenicity. *Mutation Research* **220:** 31–47, 1989.
7. Borlakoglu JT and Haegele KD, Comparative aspects on the bioaccumulation, metabolism and toxicity with PCBs. *Comp Biochem Physiol* **100C:** 327–338, 1991.
8. Parkinson A, Safe S, Robertson LW, Thomas PE, Ryan DE, Reik LM and Levin W, Immunochemical quantitation of cytochrome P-450 isozymes and epoxide hydrolase in liver microsomes from polychlorinated or polybrominated biphenyl treated rats, a study of structure activity relationships. *J Biol Chem* **258:** 5967–5976, 1983.
9. Gladen BG, Rogan WJ, Hardy P, Thullen J, Tingelstad J and Tully M, Development after exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethane transplacentally and through human milk. *The Journal of Pediatrics* **113:** 991–995, 1988.
10. Lucier GW, Nelson KG, Everson RB, Wong TK, Philpot RM, Tiernam T, Taylor M and Sunahara GI, Placental markers of human exposure to polychlorinated biphenyls and polychlorinated dibenzofurans. *Environ Health Perspectives* **76:** 79–87, 1987.
11. Nims RW, Devor DE, Henneman JR and Lubet RA, Induction of alkoxyresorufin O-dealkalases, epoxide hydrolase and liver weight gain, correlation with liver tumor-promoting potential in a series of barbiturates. *Carcinogenesis* **8:** 67–71, 1987.
12. Borlakoglu JT, Drew MGB, Wilkins JPG and Dils RR, Effects of molecular substitution patterns on the cytochrome P-450 dependent metabolism of 2,2′,3,5,5′,6- and 2,2′,3,4,4′,6-hexachlorobiphenyl by rat liver microsomal monooxygenases. *Biochim Biophys Acta* **1036:** 167–175, 1990.
13. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence of its hemoprotein nature. *J Biol Chem* **239:** 2370–2378, 1964.
14. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein

measurement with the Foulin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
15. Burke MD, Thompsen S, Elcombe CR, Halpert J, Haaparanta T and Meyer R, Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues. A series of substrate to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34:** 3337–3349, 1985.
16. Anderson LM, Van Havere K and Budinger JM, Effect of polychlorinated biphenyls on lung and liver tumours initiated in suckling mice by N-nitrosodimethylamine. *J Natl Cancer Inst* **71:** 157–163, 1983.
17. Wolff T, Denic E and Wanderus H, Aldrin epoxidation: A highly sensitive index specific for cytochrome P-450 dependent monooxygenase activities. *Drug Metab Dispos* **7:** 301–305, 1979.
18. Borlakoglu JT, Comparative studies on the biochemical toxicology of polychlorinated biphenyls (PCBs). PhD Thesis, University of Reading, U.K., 1989.
19. Gibson GG and Skett P, *Introduction to drug metabolism*. Chapman and Hall, Ltd., London, U.K., 1986.
20. Habig HW, Pabst MJ and Jakoby WB, Glutathione-S-transferase. *J Biol Chem* **249:** 7130–7139, 1974.
21. Guengerich FP and Mason PS, Alcoholdehydrogenase coupled spectrophotometric assay of epoxide hydratase activity. *Anal Biochem* **104:** 445–451, 1980.
22. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
23. Towbin H, Staehlin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci* **76:** 4350–4354, 1979.
24. Lewis AD, Hickson ID, Rebson CN, Harris AL, Hayes JD, Griffiths SA, Manson MM, Hall AE, Moss JE and Wolf CR, Amplification and increased expression of alpha class glutathione S-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc Natl Acad Sci* **85:** 8511–8575, 1988.
25. Wolf CR, Moll E, Friedberg T, Oesch F, Buchmann A, Kuhlmann ND and Kurz HN, Characterization, localization and regulation of a novel phenobarbital-inducible form of cytochrome P450, compared with three further P450-isoenzymes, NADPH P450-reductase, glutathione transferases and microsomal epoxide hydrolase. *Carcinogenesis* **5:** 993–1001, 1984.
26. Wolf CR, Seilman S, Oesch F, Mayer RT and Burke MD, Multiple forms of cytochrome P-450 related to forms induced marginally by phenobarbital. *Biochem J* **240:** 27–33, 1986.
27. Forrester LM, Henderson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, Kitteringham NR, Mclaren AW, Miles JS, Skett P and Wolf CR, Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochem J* **281:** 359–368, 1992.
28. Tamburini PP, Mason HA, Bains SK, Makouski RS, Morris B and Gibson GG, Multiple forms of hepatic cytochrome P-450. Purification, characterisation and comparison of a novel clofibrate-induced isozyme with other major forms of cytochrome P-450. *Eur J Biochem* **139:** 235–246, 1984.
29. Nebert DW, Nelso DR, Coon MJ, Estabrook RW, Feyereisen R, Fuji-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR and Waxman DJ, The P450 Superfamily: Update on new sequences, gene mapping, and recommended nomenclature. *DNA and Cell Biology* **10:** 1–14, 1991.
30. Cox RA, The use of guanidinium chloride in the isolation of nucleic acids. *Methods Enzymol* **12:** 120–129, 1968.
31. Rigby PW, Dieckmann M, Rhodes C and Berg PJ, Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Mol Biol* **113:** 237–251, 1977.
32. Feinberg AP and Vogelstein B, A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **136:** 6–13, 1983.
33. Feinberg AP and Vogelstein B, Addendum: A technique for radiolabelling endonuclease fragments to high specific activity. *Anal Biochem* **137:** 266–267, 1984.
34. Earnshaw D, Dale JW, Goldfarb PS and Gibson GG, Differential splicing in the 3′ non-coding region of rat cytochrome P-452 (P450 IVA1) mRNA. *FEBS Lett* **236:** 357–361, 1988.
35. Jenke H-S, Michel S, Hornhardt S and Berndt J, Protooncogene expression in rat liver by polychlorinated biphenyls (PCB). *Xenobiotica* **21:** 945–960, 1991.
36. Alvares AP and Kappas A, Induction of aryl hydrocarbon hydroxylase by polychlorinated biphenyls in the foeto-placental unit and neonatal livers during lactation. *FEBS Lett* **50:** 172–174, 1975.
37. Parkinson A, Robertson LW and Safe S, Reconstituted human breast milk PCBs as potent inducers of aryl hydrocarbon hydroxylase. *Biochem Biophys Res Com* **96:** 882–889, 1980.
38. Gonzalez F, The molecular biology of cytochrome P-450s. *Pharmacological Reviews* **40:** 244–288, 1989.
39. Yang CS, Yoo JSH, Ishizaki H and Hong J, Cytochrome P-4502E1: Roles in nitrosamine metabolism and mechanism of regulation. *Drug Metabolism Reviews* **22:** 147–159, 1990.
40. Borlakoglu JT, Edwards-Webb JD, Dils RR, Wilkins JPG and Robertson LW, Evidence for the induction of cytochrome P-452 in rat liver by Aroclor 1254, a commercial mixture of polychlorinated biphenyls (PCBs). *FEBS Lett* **247:** 327–329, 1989.
41. Borlakoglu JT, Clarke S, SheWen Huang, Dils RR, Haegele KD and Gibson GG, Lactational transfer of 3,3′,4,4′-tetrachloro- and 2,2′,4,4′,5,5′-hexachlorobiphenyl induces cytochrome P450IVA1 in neonates. Evidence for a potential synergistic mechanism. *Biochem Pharmacol* **43:** 153–157, 1992.
42. Denison MS, Fisher JM and Whitlock JP Jr, Inductible, receptor-dependent protein-DNA interactions at a dioxine-responsive transcriptional enhancer. *PNAS* **85:** 2528–2532, 1988.
43. Korach KS, Sarver P, Chae K, McLachlan JA and McKinney JD, Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: Conformationally restricted structural probes. *Molecular Pharmacology* **33:** 120–126, 1988.
44. Romkes M, Piskorska-Pliszczynka J and Safe S, Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin of hepatic and uterine estrogen receptor levels in rats. *Toxicol Appl Pharmacol* **87:** 306–314, 1987.
45. Jenke H-S, Demel E and Oesterle D, c-raf expression in early rat liver tumorogenesis after promotion with polychlorinated biphenyls or phenobarbital. *Xenobiotica*, in press.
46. Bishop JM, Molecular themes in oncogenes. *Cell* **64:** 235–248, 1991.
47. Beer DG, Schwarz M, Sawada N and Pitot HC, Expression of Ha-ras and c-myc protooncogenes in isolated -glutamyltranspeptidase-positive rat hepatocytes and in hepatocellular carcinoma induced by diethylnitrosamine. *Cancer Res* **46:** 2435–2441, 1986.
48. Beer DG, Neveau MJ, Paul Dl, Rapp UR and Pitot HC, Expression of the c-raf protooncogene, -glutamyltranspeptidase, and gap junction protein in rat liver neoplasms. *Cancer Res* **48:** 1610–1617, 1988.