Biochemical Pharmacology, Vol. 38, No. 9, pp. 1517–1525, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INDUCTION OF CYTOCHROME P450IA1 IN MOUSE HEPATOMA CELLS BY SEVERAL CHEMICALS

PHENOBARBITAL AND TCDD INDUCE THE SAME FORM OF CYTOCHROME P450

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(*Received* 20 *May* 1988; *accepted* 21 *November* 1988)

Abstract—The mouse hepatoma cell line Hepa-1 was studied for aryl hydrocarbon hydroxylase (AHH) inducibility by sixteen compounds known to be inducers of cytochrome P450 of different "classes". Both 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and sodium phenobarbital induced AHH activity. A cytochrome P450IA1-specific (P_1-450) mouse cDNA probe was used to quantitate mRNA induction. There was a good correlation between the amount of cytochrome P450IA1 mRNA induced and AHH activity. Immunoblots with monoclonal antibody 1-7-1, which recognizes rat liver P450IA1 and P450IA2 (P450c and P450d, respectively), showed that both phenobarbital and TCDD increase the amount of a P450 isozyme immunorelated to P450IA1 in this cell line. Hepa-1 mutants with no AHH inducibility (no functional P450IA1 structural gene; no *Ah* receptor; no nuclear translocation of the inducer–receptor complex; and presence of dominant repressor) did not respond to phenobarbital. The cytosolic receptor for TCDD (*Ah* receptor) was characterized to see if phenobarbital induced cytochrome P450IA1 mRNA and the hydroxylase enzyme through the same mechanism as TCDD. 20 mM Phenobarbital almost completely abolished the binding of ^{3}H-TCDD to the cytosolic receptor. These data indicate that phenobarbital can be a weak ligand for the *Ah* receptor and thus induce cytochrome P450IA1 and AHH activity. The observation increases the list of different P450 forms inducible by phenobarbital.

The multiplicity of cytochrome P-450 hemoproteins and their overlapping substrate specificities is well established.§ In rat liver different isozymes of cytochrome P450 are shown to be induced by phenobarbital [2], 3-methylcholanthrene and other polycyclic aromatic compounds, isosafrole [3], isoniazid [4], certain chlorinated biphenyls [5], and synthetic steroids [6]. Totally about 30 different P450 isozymes have been isolated or their genes cloned (for review see Ref. 7).

TCDD induces cytochrome P450IA1 (P1-450) mRNA in mouse liver *in vivo* [8] and in the mouse hepatoma cell line Hepa-1 [9]. The inducer binds with high affinity to a cytosolic receptor, and aryl hydrocarbon hydroxylase (EC 1.14.14.2) activity is induced [10]. *In vivo*, aryl hydrocarbon hydroxylase inducibility by TCDD parallels the induction of cytochrome P450IA1 mRNA [9, 11].

Phenobarbital has been shown to induce several forms of P450 different from polycyclic aromatic hydrocarbon-inducible P450s in animals [2]. The mechanism of induction is largely unknown because of the lack of a suitable cell culture model. Cells in culture tend to loose rapidly the ability to induce P450 by phenobarbital. A receptor for the phenobarbital class of P450 inducers has not been identified. This is also true for other types of inducers except polycyclic aromatic hydrocarbons [12].

In cell cultures derived from fetal rat liver or from rat or mouse hepatoma, aryl hydrocarbon hydroxylase activity is increased by a wide variety of compounds, including phenobarbital, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane, benzo(*a*)-anthracene and 3-methylcholanthrene [13–16]. It has been suggested that the induced enzyme activity is associated with cytochrome P450IA1 [17]. This conclusion was made from experiments with different types of compounds which inhibit aryl hydrocarbon hydroxylase activity *in vitro* and by examining the Soret peak of the reduced hemoprotein–CO-complexes.

The gene for mouse cytochrome P450IA1 has been cloned and sequenced [18]. Also monoclonal antibodies (Mab:s) against various P450 isozymes have become available recently [19, 20]. In this study we used Mab 1-7-1 [19, 21] recognizing rat polycyclic aromatic hydrocarbon-inducible cytochromes P450IA1 (P450c) and P450IA2 (P450d), which are orthologous to mouse cytochromes P1-450 and P3-450, respectively [1]. Using the cDNA and antibody probes we show here that, like TCDD, phenobarbital is capable of inducing the expression of P450IA1 in the mouse hepatoma cell line Hepa-1 by a mechanism involving the formation of the *Ah* receptor–inducer complex.

† Recipient of a Research Fellowship from the Academy of Finland.

§ A common nomenclature for the P450 gene superfamily has been established recently [1]. The P450IA1 and P450IA2 include mouse P1-450 and P3-450, respectively (designations used by D. Nebert's group), and rat P450c and P450d, respectively (designations used by W. Levin's group). These designations will be used interchangeably in the text.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture plastic ware was purchased from Nunc (Denmark). Nitrocellulose filter for dot blot analysis was obtained from the Millipore Corp. (Bedford, MA) and for immunoblot analysis from Schleicher & Schuell (Keene, NH). Nick Translation Kit and α-^{32}P-dCTP were from Amersham (U.K.). Ficoll 400 was purchased from Pharmacia (Uppsala, Sweden), polyvinylpyrrolidone K90, quinine sulphate and $NaDocSO_4$ were from Fluka. Nonidet P-40 was obtained from BDH Chemicals (Poole, Dorset, U.K.). Bovine serum albumin (fraction V), RNase A (type 1-A) and salmon sperm DNA (type III) were from Sigma (St. Louis, MO). NADH, NADPH, formamide and formaldehyde were from Merck (Darmstadt, F.R.G.). 3-OH-Benzo(*a*)pyrene was obtained from NCI Chemical Repository. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was a gift from Dow Chemical Co. (Midland, MI). ^{3}H-2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was a generous gift from Dr. A. Poland (Madison, WI). 3-Methylcholanthrene, aminopyrine and benzo(*a*)anthracene were obtained from Sigma and 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane from Fluka. 1-(2-Isopropylphenyl)imidazole was purchased from DuPont (Wilmington, DE). Sodium phenobarbital (Pharmacopea Nordica, for human use) was from University Pharmacy (Finland) and was tested by NMR spectroscopy to be free from contaminating impurities. Other chemicals used were of the same origin as cited previously [22] and were gifts from Dr. Nebert, NIH. The 96-place microsample filtration manifold was from Schleicher & Schuell.

Cell culture. A subclone Hepa-1c1c7 [23] of the mouse hepatoma cell line, Hepa-1 [24] and aryl hydrocarbon hydroxylase-deficient mutants derived from it [25–27] were used. One of the mutants had a defect in the cytochrome P450IA1 structural gene, one had no *Ah* receptor and one showed no nuclear translocation of the *Ah* receptor–TCDD complex. One had a defect suggesting the expression of a dominant type of repressor of the hydroxylase system. The defect at the molecular level is unknown for most of the mutants at the present. Cells were grown as monolayers at 37° in 95% air and 5% CO_2 in alpha Minimum Essential Medium lacking ribonucleotides (K.C. Biologicals) supplemented with 5% fetal calf serum (GIBCO, Grand Island, NY) and antibiotic–antimycotic mixture (GIBCO; 100 units of penicillin, 100 μg of streptomycin and 0.25 μg of amphotericin B per ml). Cells were plated at a density of 1.3×10^6 cells per 85-mm dish in 10 ml of medium two days before harvesting. The putative cytochrome P450IA1 mRNA and aryl hydrocarbon hydroxylase inducers were added in the culture medium directly or as acetone, DMSO or ethanol solutions 24 hr prior to the harvesting. The final concentration of solvents was 0.5%. Solvent controls were used. All experiments were repeated two or three times to ensure reproducibility.

Aryl hydrocarbon hydroxylase (AHH) assay. Following removal of the medium, the cell culture surface was washed twice with 5 ml of cold Dulbecco's phosphate-buffered saline without calcium or magnesium (1% NaCl, pH 7.2). Cells were scraped and collected as pellets in Eppendorf Model 5414 Centrifuge (9980 *g*, 40 sec). The pellets were stored at −80°. For the assay the pellets were thawed and sonicated briefly in ice-cold 33 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose (0.8–1 ml of buffer per a confluent dish). AHH activity was assayed by the method cited [28]; the incubation time was 15 min, and final cellular homogenate concentration ranged between 0.05 and 0.2 mg protein/ml reaction mixture. Both the enzyme activity and protein concentration were determined in duplicate from one dish. One unit of AHH activity is defined as that amount of enzyme catalyzing in 1 min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-OH-benzo(*a*)pyrene standard. Specific activity denotes units per mg of total cellular protein. Protein was measured from sonicated cell homogenates with Bio-Rad Reagent by the method of Bradford [29] using bovine serum albumin as the standard.

Analysis of cytochrome P450IA1 mRNA. Cytoplasmic RNA was isolated from the cultured cells by the method of Anderson *et al.* [30] and denatured as described by White and Bancroft [31]. Cells from one 85-mm dish were harvested by scraping in 1 ml of sterile Dulbecco's phosphate buffered saline and pelleted (9980 *g*, 40 sec). The pellet was lysed on ice for 3 min in 0.35 ml of 0.01 M Tris (pH 7.0) containing 0.15 M NaCl, 2 mM $MgCl_2$ and 0.5% Nonidet P-40. Nuclei were pelleted (9980 *g*, 3 min). Supernatant fraction was added to 0.35 ml of 0.01 M Tris (pH 7.6) containing 0.15 M NaCl, 5 mM EDTA and 1% $NaDocSO_4$. The mixture was extracted three times with 0.7 ml of phenol–chloroform–isoamyl alcohol (24:24:1). RNA was precipitated with 2½ vol. of ethanol overnight at −20°. The precipitate was dried *in vacuo* and dissolved in 55 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0). Total RNA was quantitated by measuring A_{260} and part of the sample was treated with RNase (7 μl RNA-solution added to 7 μl TE buffer and 7 μl (0.14 μg) RNase A and incubated 1 hr at 37°). The rest of the sample was denatured by adding 30 μl 20 × SSC plus 20 μl concentrated (37%, w/w) formaldehyde and incubating for 15 min at 60°. These were stored frozen at −80°. For the dot hybridization analysis 1.5–12 μg of total RNA was diluted in 150 μl 15 × SSC. The mixture was applied on a nitrocellulose filter through a filtration manifold. RNA was fixed by baking the filter *in vacuo* at 80° for 2 hr. The filter was prehybridized for about 20 hr at 42° in a mixture (0.2 ml/cm^2 of filter) containing 5 × Denhardt's solution, 6 × SSC, 0.5% $NaDocSO_4$, denatured salmon sperm DNA (100 μg/ml) and 50% formamide. The hybridization mixture contained also 0.01 M EDTA. As a hybridization probe clone 46, a mouse cytochrome P450IA1 (P_1-450) 1100-base pair cDNA derived from the 3′ end of mouse P450IA1 mRNA was used as an insert in pBR322 [18]. The probe was nick-translated to a specific activity of about 2×10^8 cpm/μg DNA and added (about 1×10^7 cpm/ml buffer, 70 μl buffer/cm^2 filter) to the hybridization mixture. The mRNA–DNA hybridization was carried out at 42°. Following hybridization, the filters were washed three times with a large volume (200–300 ml) of 2 × SSC and

Table 1. Aryl hydrocarbon hydroxylase activities and cytochrome P450IA1 mRNA levels in the mouse Hepta-1 cell line following exposure to various chemicals

Chemical	Final concentration (μM)	Aryl hydrocarbon hydroxylase activity Specific	Normalized	Cytochrome P450IA1 mRNA (relative units)	Ratio
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	0.002	192 ± 7	1	1	1
Beta-naphthoflavone (BNF)	10	331 ± 60	1.72	1.09 ± 0.19	1.58
Alpha-naphthoflavone (ANF)	10	157 ± 45	0.82	1.12 ± 0.09	0.73
3-Methylcholanthrene (MC)	1	133 ± 9	0.69	0.63 ± 0.20	1.10
Benzo(*a*)anthracene (BA)	13	131 ± 11	0.68	0.68 ± 0.16	1.00
Sodium phenobarbital (PB)	2000	93 ± 35	0.48	0.34 ± 0.04	1.42
Benzo(*a*)pyrene (BP)	1	63 ± 4	0.33	0.28 ± 0.01	1.17
2,5-Diphenyloxazole (PPO)	100	46 ± 4	0.24	0.60 ± 0.04	0.40
2,2-Bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT)	20	31 ± 17	0.16	0.26 ± 0.02	0.62
1-(2-Isopropylphenyl)imidazole (IPI)	50	23 ± 7	0.12	0.14 ± 0.02	0.86
Isoproterenol (Isop)	1000	12 ± 1	0.06	0.12 ± 0.05	0.50
2-Methyl-1,2-bis-(3-pyridyl)-1-propanone (MTP)	100	9 ± 5	0.05	0.09 ± 0.03	0.56
Aniline (Anil)	1000	8 ± 1	0.04	0.10 ± 0.03	0.40
Diethylstilbestrol (DES)	10	6 ± 0	0.03	0.10 ± 0.05	0.30
Aminopyrine (AP)	1000	5 ± 0	0.03	0.09 ± 0.00	0.33
2-Allyl-2-isopropylacetamide (AIA)	1000	5 ± 1	0.03	0.10 ± 0.01	0.30
Control (DMSO)		6 ± 1	0.03	0.10 ± 0.02	0.30
Control (Acetone)		3 ± 0	0.02	0.07 ± 0.01	0.29
Control (ethanol)		3	0.02		

The cells were exposed for 24 hr to a medium containing the chemical at optimal concentration. Each value represents the mean of duplicate (or three) dishes of cells ± SE. The values for mRNAs are expressed relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced mRNA which is given a value 1 in each separate assay. Specific aryl hydrocarbon hydroxylase activities (fmol/mg protein/min) have also been normalized relative to TCDD which is given the value 1. The column ratio means the ratio between the normalized AHH activities and the respective mRNA values.

0.1% $NaDocSO_4$ at room temperature, once with 1 × SSC and 0.1% $NaDocSO_4$ (60°, 10–20 min) and once with 0.2 × SSC and 0.1% $NaDocSO_4$ (60°, 5 min). The filter was air-dried and autoradiographed (Kodak X-Omat AR-5 film). The relative amounts of cytochrome P450IA1 mRNA were obtained by densitometric scanning (500 nm, Shimadzu Dual-Wavelength TLC Scanner CS-930) of the X-ray films and by integrating the surface areas of the peaks.

Receptor for 2,3,7,8-*tetrachlorodibenzo*-p-*dioxin.* The receptor analysis was performed according to Legraverend *et al.* [27]. 1 nM ^{3}H-2,3,7,8-tetrachlorodibenzo-*p*-dioxin was used to label the receptor *in vitro*. In the competition assays a 400 mM stock solution of phenobarbital dissolved in dimethyl sulfoxide was used.

Immunoblot analysis. Mab 1-7-1 was raised against purified 3-methylcholanthrene-treated rat liver P450 as described earlier [21]. Sodium dodecyl sulphate-polyacrylamide electrophoresis was run overnight using 7.5% gels with a gradient of 20 mA/gel [32]. Proteins were transferred from the gels to nitrocellulose filters as described by Towbin *et al.* [33]. The molecular masses of the P450s were determined using molecular weight standards (Pharmacia) and Coomassie Blue staining. For specific P450 detection, the filters were first blocked with 3% (w/v) gelatin in TBS (50 mM Tris–200 mM NaCl, pH 7.4) for 1 hr at 37°. The filters were subsequently reacted with (a) Mab 1-7-1 at 1:500 dilution in TBS, (b) biotinylated antimouse IgG (Amersham), and (c) streptavidin–biotinylated horseradish peroxidase complex (Amersham). The filters were developed with 4-chloro-1-naphthol (Bio-Rad) and hydrogen peroxide as substrates.

RESULTS

Induction of aryl hydrocarbon hydroxylase activity in Hepa-1 cell line by various compounds. Hepa-1 cells were exposed to sixteen compounds which are known inducers, substrates or inhibitors of cytochrome P450-mediated monooxygenase activities. A 24-hr induction time was chosen as a convenient time point where the effect of solvent had almost disappeared and the enzymatic activity due to the chemicals showed only minor changes. Optimal concentrations for aryl hydrocarbon hydroxylase induction were used. In the case of alpha- and beta-naphthoflavone ten or five times less chemical, respectively, was used than with fetal rat liver primary cultures [17] because of differing optimal concentrations. For 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane the highest concentration which did not show apparent toxicity was used.

TCDD gave a good induction at the lowest concentration (2 nM) of all the compounds tested (Table 1). Beta-naphthoflavone had a higher maximal inducing capacity than TCDD. It was, however, used at a much higher concentration (10 μM) than TCDD. Other effective inducers included alpha-naphthoflavone and benzo(*a*)anthracene. Phenobarbital induced aryl hydrocarbon hydroxylase about as much as did benzo(*a*)pyrene and 3-methylcholanthrene. 2,2-bis(*p*-Chlorophenyl)-1,1,1-trichloroethane, 2,5-diphenyl-oxazole and 1-(2-isopropylphenyl)-imidazole also gave a notable induction.

Induction of cytochrome P450IA1 *mRNA.* The inducers were used to study cytochrome P-450IA1 mRNA levels to see if in each case the same form of cytochrome P450 was induced. TCDD and benzo(*a*)anthracene were used as model compounds to choose the suitable time point for harvesting mRNA. The mRNA level increased rapidly (30 min) and remained fairly constant for at least 26 hr. This result agrees well with those of Israel and Whitlock [9]. Twenty-four hours was chosen as the harvesting time, thus equalling the harvesting time for hydroxylase activity. RNase treatment was performed for each RNA sample to verify the hybridization of the cytochrome P450IA1 probe specifically to RNA. Different RNA loads were used to ensure the linearity of the response. Several film exposures were made to obtain linear conditions. Figure 1 shows an example of mRNA–DNA hybridization pattern for several chemicals. The results from densitometric scanning of X-ray films are presented in Table 1. TCDD was always included as a reference compound because it has been shown earlier that aryl hydrocarbon hydroxylase activity and cytochrome P450IA1 mRNA have a linear correlation when TCDD is used as an inducer [9]. The amounts of mRNA were calculated relative to TCDD-induced mRNA. The most potent inducers of cytochrome P450IA1 mRNA were TCDD and beta- and alpha-naphthoflavone. Other effective inducers were benzo(*a*)anthracene, 2,3-diphenyloxazole, 3-methylcholanthrene, sodium phenobarbital, benzo(*a*)pyrene and 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane. No induction was observed with 2-allyl-2-isopropylacetamide, aminopyrine, diethylstilbestrol, aniline and 2-methyl-1,2-bis(3-pyridyl)-1-propanone.

Aryl hydrocarbon hydroxylase activity as a function of the cytochrome P450IA1 *mRNA induced by various inducers.* In Fig. 2, the relative increase in P450IA1 mRNA is plotted against AHH specific activity. The shapes of the spots (size of the axes) show the observed variation (range) in enzyme activity or mRNA level. Simple regression was used to draw the "best fit" straight line through the datapoints. This gave a correlation coefficient (R) of 0.91. Also the calculated ratio of specific AHH activity to relative mRNA amount was close to 1 for each chemical (Table 1).

Competition of ^{3}H-*TCDD from the* Ah *receptor by phenobarbital.* An important question was whether the *Ah* receptor was required in the induction of aryl hydrocarbon hydroxylase by phenobarbital in Hepa-1. The cytosolic fraction prepared from Hepa-1 was thus treated *in vitro* by ^{3}H-TCDD and different amounts of phenobarbital (4 mM, 8 mM, 12 mM, 16 mM, 20 mM) were added to the mixture. Sucrose density gradients showed (Fig. 3) that 20 mM phenobarbital almost completely competed TCDD from the receptor site.

Immunoblot experiments. Immunoblot analysis with Mab 1-7-1 was done to see whether also the amount of P450IA1 protein is increased in response to phenobarbital treatment. As shown in Fig. 4, a protein species immunodetectable by Mab 1-7-1 and

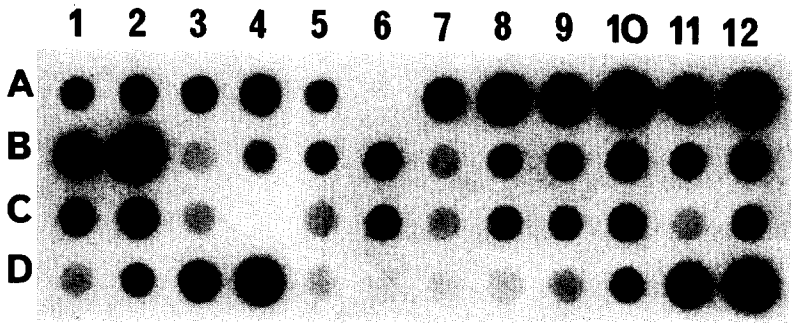

Fig. 1. Induction of cytochrome P450IA1 mRNA by several chemicals. Chemical concentrations used are seen in Table 1. On the left side of each set of spots there is 6 μg of total cytosolic RNA, on the right side 12 μg of total RNA. A 1–2, control (DMSO); A 3–4, benzo(*a*)pyrene; A 9–10, alpha-naphthoflavone; A 11–12, beta-naphthoflavone; B 1–2, benzo(*a*)anthracene; B 3–4, 2-methyl-1,2-bis-(3-pyridyl)-1-propanone; B 5–6, 1-(2-isopropylphenyl)imidazole; B 7–8, 2-allyl-2-isopropylacetamide; B 9–10, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane; B 11–12, 3-methylcholanthrene; C 1–2, sodium phenobarbital; C 3, 6 μg control (acetone); C 5–6, aniline; C 7–8, aminopyrine; C 9–10, isoproterenol; C 11–12, control (DMSO); D 1–2, diethylstilbestrol; D 3–4, 2,5-diphenyloxazole; D 9–10, control; D 11–12, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

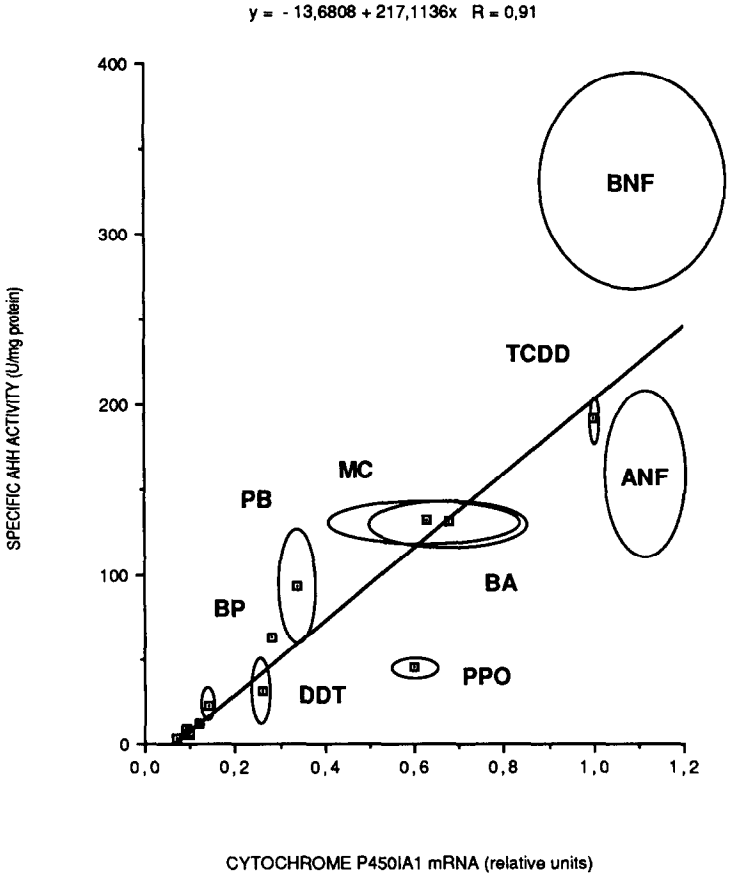

Fig. 2. Aryl hydrocarbon hydroxylase activity as a function of the cytochrome P450IA1 mRNA induced by various inducers. The shapes of the spots indicate the variation observed.

migrating slightly faster (minimum M_r approximately 55 kDa) than rat P450IA1 (P450c, M_r 56 kDa) was strongly increased by TCDD treatment in the Hepa-1 cells. Also phenobarbital treatment clearly increased the amount of this P450, although less than TCDD.

Inducibility by phenobarbital of aryl hydrocarbon hydroxylase in hydroxylase-deficient mutants. Four different types of aryl hydrocarbon hydroxylase-deficient mutants were exposed to phenobarbital. The mutants have defects in cytochrome P450IA1

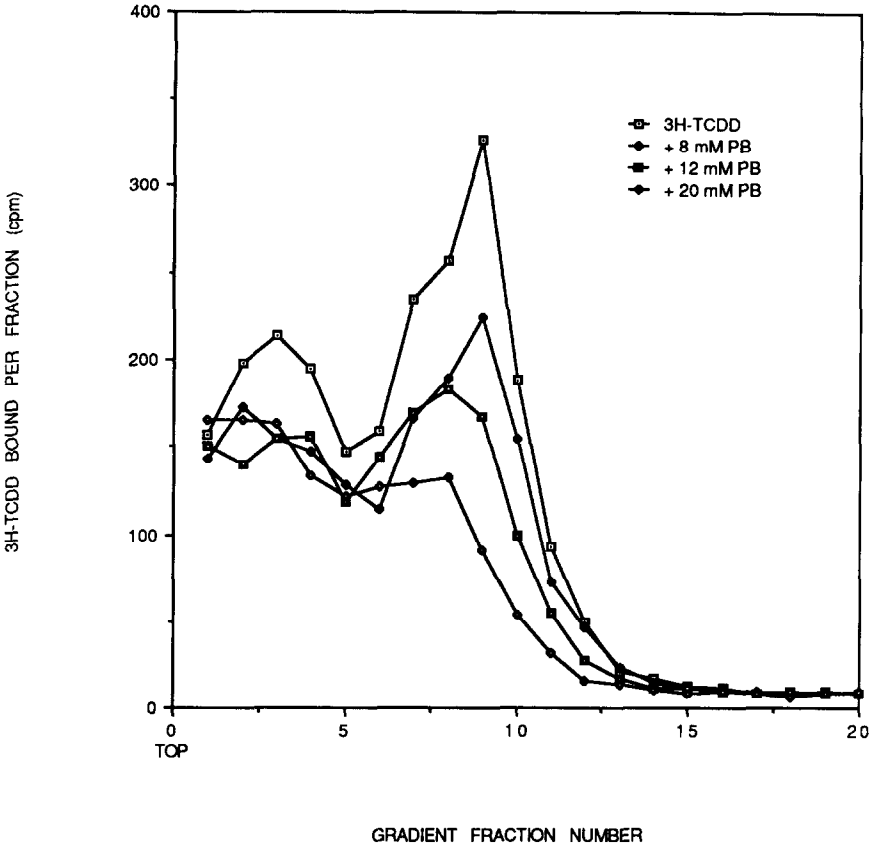

Fig. 3. Competition by phenobarbital for specific high affinity ^{3}H-TCDD binding sites in Hepa-1. Cytosol (3.32 mg protein/ml) from Hepa-1 cells was incubated with 1 nM ^{3}H-TCDD and different amounts of phenobarbital. Each sample containing 0.996 mg of protein was run through a sucrose density gradient. The concentration of phenobarbital displacing 50% of ^{3}H-TCDD was about 12 mM.

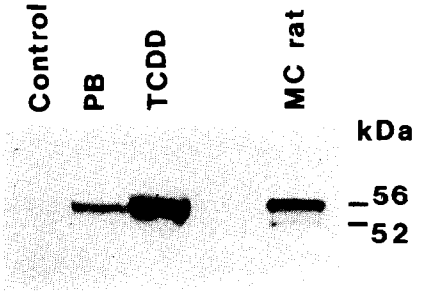

Fig. 4. Immunoblots of Hepa-1 cells with Mab 1-7-1. Protein samples were resolved by electrophoresis in a 7.5% SDS–polyacrylamide gel and transferred electrophoretically to nitrocellulose. The lanes contained 100 μg of protein from control (DMSO treated), phenobarbital (PB)-treated, and TCDD-treated Hepa-1 cells. The cytochromes P450IA1 (56 kDa) and P450IA2 (52 kDa) were derived from a Wistar rat treated with 3-methylcholanthrene (MC rat).

structural gene or *Ah* receptor system [25–27]. Mutant c1 has a single mutation leading to premature termination of P450IA1 protein [34]. Mutant c2 is receptorless [27] and mutant c4 has no nuclear translocation of the *Ah* receptor–TCDD complex [27]. Mutant c3 expresses a dominant repressor [26–27]. All these mutants have almost totally lost the ability to induce aryl hydrocarbon hydroxylase activity when exposed to benzo(*a*)anthracene or TCDD [27]. In our experiments none of these mutants showed a marked (more than 5% of the induction in Hepa-1 parent cells) induction of aryl hydrocarbon hydroxylase activity by phenobarbital.

DISCUSSION

Aryl hydrocarbon hydroxylase activities and cytochrome P450IA1 mRNA levels were compared in a mouse hepatoma cell line, Hepa-1, after exposure to sixteen structurally differing chemicals. The relative ability of the chemicals to induce aryl hydrocarbon hydroxylase in this study differed somewhat from that in fetal rat liver primary culture [17], and also from an earlier study in Hepa-1 [16]. Several compounds which are inducers in cultured rat cells did not induce aryl hydrocarbon hydroxylase in mouse hepatoma cells: 2-methyl-1,2-bis-(3-pyridyl)-1-propanone, 2-allyl-2-isopropylacetamide, aniline, aminopyrine and diethylstilbestrol. The greatest differences were seen in the response to 2-methyl-1,2-bis-(3-pyridyl)-1-propanone and diethylstilbestrol

which did not act as inducers in our study. 2,5-Diphenyloxazole induces higher levels of aryl hydrocarbon hydroxylase in rat cells than in mouse hepatoma cells.

Cytochrome P450IA1 mRNA levels correlated well with the respective aryl hydrocarbon hydroxylase activities assayed under same conditions. A correlation between induced aryl hydrocarbon hydroxylase activity and P450IA1 mRNA has been observed earlier in Hepa-1 for TCDD [9, 11]. The results also support the suggestion of Owens and Nebert [17] that the induced aryl hydrocarbon hydroxylase activities are always associated with cytochrome P450IA1.

Phenobarbital induced aryl hydrocarbon hydroxylase and P450IA1 mRNA in Hepa-1 cells to a similar level as did 3-methylcholanthrene and benzo(*a*)pyrene. This finding is different from several previous ones. In the rat liver *in vivo*, phenobarbital does not induce cytochrome P450IA1 [35]. In rat mammary epithelial cells grown in primary culture sodium phenobarbital has no effect on aryl hydrocarbon hydroxylase [36]. In fetal rat liver 3-methylcholanthrene induces "phenobarbital"-type cytochrome P450 and maternal liver "cytochrome P448"-type cytochrome P450 [17, 37]. In chick embryo liver 2-allyl-2-isopropylacetamide and phenobarbital induce the same form of cytochrome P450, which differs from that induced by 3-methylcholanthrene and beta-naphthoflavone [38]. In mouse liver *in vivo* phenobarbital does not cause any measurable increase in cytochrome P450IA1 mRNA, yet it increases aryl hydrocarbon hydroxylase activity 2-fold [39].

Immunoblot experiments with Mab 1-7-1 demonstrated that a P450 isozyme immunorelated to rat P450IA1 (P450c) is inducible by phenobarbital in the Hepa-1 cells. Several lines of evidence imply that the protein species detected by Mab 1-7-1 in the present work is indeed P450IA1: (1) Mab 1-7-1 is well characterized and known to detect exclusively polycyclic aromatic hydrocarbon-inducible cytochrome P450IA1 and P450IA2 in the rat [19, 21]; (2) several studies have shown that the mouse P_1-450 is orthologous to the rat P450c (see e.g. Ref. 1); (3) the minimum M_rs of the P450s detected by Mab 1-7-1 in our conditions (56 kDa for rat P450c, 52 kDa for P450d, and approximately 55 kDa for mouse P_1-450) agree well with the ones reported in other laboratories (see Ref. 20).

The present experiments using a cDNA probe and monoclonal antibody specific for P450IA1 thus clearly demonstrate that phenobarbital can, in certain conditions, induce P450 isozymes of the polycyclic aromatic hydrocarbon-inducible subfamily (P450I). The results also suggest that only one form of P450 is formed in response to the inducer in the Hepa-1 cells. It has been shown earlier that 3-methylcholanthrene and phenobarbital cause increases in aryl hydrocarbon hydroxylase activity and that the effect is additive when these inducers are administered simultaneously in the culture medium of fetal rat primary hepatocytes [13] or of Hepa-1 and H-4-II-E cells [16]. Also McManus *et al.* [40] demonstrated with a specific cDNA probe that there was at least a 10-fold increase in P450IA1 transcript in a phenobarbital-treated rat hepatoma cell line.

It was therefore of interest to examine if mutants defective in different stages of aryl hydrocarbon hydroxylase induction process—especially in the *Ah* receptor function—would induce aryl hydrocarbon hydroxylase in response to phenobarbital. Practically no induction was detectable in any mutant examined: a mutant in cytochrome P450IA1 structural gene, a mutant with no *Ah* receptor, a mutant with no nuclear translocation of the *Ah* receptor–TCDD complex, and a mutant expressing a dominant type of repressor. The induction studies with these mutants rules out the possibility that the regulatory portion of Hepa-1 P450IA1 structural gene is altered so that phenobarbital is able to bind directly to a *cis*-acting gene regulatory element and thus induce the synthesis of P450IA1 protein without an involvement of the *Ah* receptor. It also appears that a complete, functional aryl hydrocarbon hydroxylase induction system is required for the induction by phenobarbital to occur in Hepa-1.

There is evidence that the aryl hydrocarbon hydroxylase induction process by polycyclic aromatic hydrocarbons is mediated by the *Ah* receptor [10]. There are also reports on aryl hydrocarbon hydroxylase induction by TCDD in cells where no *Ah* receptor has been detected [11]. There is no evidence of a receptor-mediated induction of aryl hydrocarbon hydroxylase or any other form of cytochrome P450 by phenobarbital. Phenobarbital as a 10 μM concentration does not measurably compete TCDD from the *Ah* receptor in mouse liver cytosol [41]. In this report we show that the *Ah* receptor in Hepa-1 permits phenobarbital to form a complex with the receptor site and thus elicit cytochrome P450IA1 protein synthesis. The reason why the binding of phenobarbital has not been observed earlier might be the much lower amount used in the competition assays [41]. The observations made in the present study may explain some of the cases *in vivo* [39] where phenobarbital has been shown to induce aryl hydrocarbon hydroxylase activity. The detailed structure of the *Ah* receptor may determine the accessibility of phenobarbital to the receptor in different animals or cells.

Acknowledgements—We thank Dr. Daniel W. Nebert (NICHHD, NIH, Bethesda, MD, U.S.A.) for helpful discussions and for providing us with clone 46 and several inducers. We are indebted to Drs. S. S. Park and H. V. Gelboin (NCI, NIH) for providing us with the Mab 1-7-1. Ms Raisa Malmivuori is thanked for technical help. We also thank Dr. Simo Lötjönen (University of Kuopio) for performing the NMR analysis of phenobarbital and Professor Olavi Pelkonen (University of Oulu) for criticism during the preparation of the manuscript. This research was supported by Research Grant 07/026 from the Academy of Finland.

REFERENCES

1. Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Waterman MR, The P450 gene superfamily: recommended nomenclature. *DNA* **6**: 1–11, 1987.

2. Ryan DE, Thomas PE, Korzeniowski D and Levin W, Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J Biol Chem* **254**: 1365–1374, 1979.
3. Haniu M, Ryan DE, Levin W and Shively JE, Structures of cysteine-containing peptides in isosafrole-inducible rat hepatic microsomal cytochrome P-450d: Sequence homology with 3-methylcholanthrene-induced cytochrome P-450c. *Proc Natl Acad Sci USA* **81**: 4298–4301, 1984.
4. Ryan DE, Ramanathan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS and Levin W, Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J Biol Chem* **260**: 6385–6393, 1985.
5. Ryan DE, Thomas PE and Levin W, Purification and characterization of a minor form of hepatic microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls. *Arch Biochem Biophys* **216**: 272–288, 1982.
6. Elshourbagy NA and Guzelian PS, Separation, purification, and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile. *J Biol Chem* **255**: 1279–1285, 1980.
7. Adesnik M and Atchison M, Genes for cytochrome P-450 and their regulation. *CRC Crit Rev Biochem* **19**: 247–305, 1986.
8. Gonzalez FJ, Tukey RH and Nebert DW, Structural gene products of the *Ah* locus. Transcriptional regulation of cytochrome P_1-450 and P_3-450 mRNA levels by 3-methylcholanthrene. *Mol Pharmacol* **26**: 117–121, 1984.
9. Israel DI and Whitlock JP Jr, Induction of mRNA specific for cytochrome P_1-450 in wild type and variant mouse hepatoma cells. *J Biol Chem* **258**: 10390–10394, 1983.
10. Nebert DW, Eisen HJ and Hankinson O, The *Ah* receptor: binding specificity only for foreign chemicals? *Biochem Pharmacol* **33**: 917–924, 1984.
11. Jaiswal AK, Nebert DW and Eisen HJ, Comparison of aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase induction by polycyclic aromatic compounds in human and mouse cell lines. *Biochem Pharmacol* **34**: 2721–2731, 1985.
12. Whitlock JP Jr, The regulation of cytochrome P-450 gene expression. *Annu Rev Pharmacol Toxicol* **26**: 333–369, 1986.
13. Gielen JE and Nebert DW, Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. I. Stimulation of enzyme activity in nonhepatic cells and in hepatic cells by phenobarbital, polycyclic hydrocarbons, and 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane. *J Biol Chem* **246**: 5189–5198, 1971.
14. Nebert DW and Gielen JE, Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. II. Effects of Actinomycin D and cycloheximide on induction processes by phenobarbital or polycyclic hydrocarbons. *J Biol Chem* **246**: 5199–5206, 1971.
15. Gielen JE and Nebert DW, Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture. III. Effects of various sera, hormones, biogenic amines, and other endogenous compounds on the enzyme activity. *J Biol Chem* **247**: 7591–7602, 1972.
16. Owens IS and Nebert DW, Aryl hydrocarbon hydroxylase induction in mammalian liver-derived cell cultures. Effects of various metabolic inhibitors on the enzyme activity in hepatoma cells. *Biochem Pharmacol* **25**: 805–813, 1976.
17. Owens IS and Nebert DW, Aryl hydrocarbon hydroxylase induction in mammalian liver-derived cell cultures. Stimulation of "cytochrome P_1-450-associated" enzyme activity by many inducing compounds. *Mol Pharmacol* **11**: 94–104, 1975.
18. Negishi M, Swan DC, Enqvist LW and Nebert DW, Isolation and characterization of a cloned DNA sequence associated with the murine *Ah* locus and a 3-methylcholanthrene-induced form of cytochrome P-450. *Proc Natl Acad Sci USA* **78**: 800–804, 1981.
19. Park SS, Fujino T, West D, Guengerich FP and Gelboin HV, Monoclonal antibodies that inhibit enzyme activity of 3-methylcholanthrene-induced cytochrome P450. *Cancer Res* **42**: 1798–1808, 1982.
20. Thomas PE, Reik LM, Ryan DE, Baldiera S and Levin W, Polyclonal and monoclonal antibody probes of the structure and function of hepatic cytochrome P450 isozymes. *Biochem Pharmacol* **34**: Suppl 1. 131–139, 1985.
21. Gelboin HV and Friedman FK, Monoclonal antibodies for studies on xenobiotic and endobiotic metabolism. Cytochromes P-450 as a paradigm. *Biochem Pharmacol* **34**: 2225–2234, 1985.
22. Goujon FM, Nebert DW and Gielen JE, Genetic expression of aryl hydrocarbon hydroxylase induction. IV. Interaction of various compounds with different forms of cytochrome P-450 and the effects of benzo[*a*]pyrene metabolism *in vitro*. *Mol Pharmacol* **8**: 667–680, 1972.
23. Hankinson O, Single-step selection of clones of a mouse hepatoma line deficient in aryl hydrocarbon hydroxylase. *Proc Natl Acad Sci USA* **76**: 373–376, 1979.
24. Bernhard HP, Darlington GJ and Ruddle FH, Expression of liver phenotypes in cultured mouse hepatoma cells: Synthesis and secretion of serum albumin. *Dev Biol* **35**: 83–96, 1973.
25. Hankinson O, Evidence that the benzo(*a*)pyrene-resistant, aryl hydrocarbon hydroxylase-deficient variants of the mouse hepatoma line, Hepa-1, are mutational in origin. *Somat Cell Genet* **7**: 373–388, 1981.
26. Hankinson O, Dominant and recessive aryl hydrocarbon hydroxylase-deficient mutants of mouse hepatoma line, Hepa-1, and assignment of recessive mutants to three complementation groups. *Somat Cell Genet* **9**: 497–514, 1983.
27. Legraverend C, Hannah RR, Eisen HJ, Owens IS, Nebert DW and Hankinson O, Regulatory gene product of the *Ah* locus. Characterization of receptor mutants among mouse hepatoma clones. *J Biol Chem* **257**: 6402–6407, 1982.
28. Nebert DW and Gelboin HV, Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J Biol Chem* **243**: 6242–6249, 1968.
29. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
30. Anderson CW, Lewis JB, Atkins JF, Gesteland RF, Cell-free synthesis of adenovirus 2 proteins programmed by fractionated messenger RNA: A comparison of polypeptide products and messenger RNA lengths. *Proc Natl Acad Sci USA* **71**: 2756–2760, 1974.
31. White BA and Bancroft FC, Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. *J Biol Chem* **257**: 8569–8572, 1982.
32. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227**: 680–685, 1970.
33. Towbin H, Staehelin T and Godan J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: procedure and some applications. *Proc Natl Acad Sci USA* **79**: 4350–4354, 1979.
34. Kimura S, Smith HH, Hankinson O and Nebert DW, Analysis of two benzo[*a*]pyrene-resistant mutants of the mouse hepatoma Hepa-1 $P_1$450 gene via cDNA

expression in yeast. *EMBO J* **6**: 1929–1933, 1987.
35. Thomas PE, Reik LM, Ryan DE and Levin W, Induction of two immunologically related rat liver cytochrome P-450 isozymes, cytochromes P-450c and P-450d, by structurally diverse xenobiotics. *J Biol Chem* **258**: 4590–4598, 1983.
36. Greiner JW, Malan-Shibley LB and Janss DH, Characteristics of aryl hydrocarbon hydroxylase activity in rat mammary epithelial cells grown in primary culture. *Chem Biol Interact* **27**: 323–334, 1979.
37. Mizokami K, Inoue K, Sunouchi M, Fujimori K, Takanaka A and Omori Y, 3-Methylcholanthrene induces phenobarbital-induced cytochrome P-450 hemoprotein in fetal liver and not cytochrome P-448 hemoprotein induced in maternal liver of rats. *Biochem Biophys Res Commun* **107**: 6–11, 1982.
38. Brooker JD, Srivastava G, Borthwick IA, May BK and Elliott WH, Evidence that 2-allyl-2-isopropylacetamide, phenobarbital and 3,5-diethoxycarbonyl-1,4-dihydrocollidine induce the same cytochrome P_{450} mRNA in chick embryo liver. *Eur J Biochem* **136**: 327–332, 1983.
39. Tukey RH, Negishi M and Nebert DW, Quantitation of hepatic cytochrome P_1-450 mRNA with the use of a cloned DNA probe. Effects of various P-450 inducers in C57BL/6N and DBA/2N mice. *Mol Pharmacol* **22**: 779–786, 1982.
40. McManus ME, Minchin RF, Schwartz DM, Wirth PJ and Huber BE, Induction by phenobarbital in McA-RH7777 rat hepatoma cells of a polycyclic hydrocarbon inducible cytochrome P450. *Biochem Biophys Res Commun* **137**: 120–127, 1986.
41. Okey AB, Bondy GP, Mason ME, Kahl GF, Eisen HJ, Guenthner TM and Nebert DW, Regulatory gene product of the *Ah* locus. Characterization of the cytosolic inducer–receptor complex and evidence for its nuclear translocation. *J Biol Chem* **254**: 11636–11648, 1979.
42. Nebert DW and Gonzalez FJ, P450 genes: structure, evolution, and regulation. *Annu Rev Biochem* **56**: 945–993, 1987.