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COMPARISON OF ARYL HYDROCARBON HYDROXYLASE AND ACETANILIDE 4-HYDROXYLASE INDUCTION BY POLYCYCLIC AROMATIC COMPOUNDS IN HUMAN AND MOUSE CELL LINES*

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Abstract—The human MCF-7 and the mouse Hepa-1 cell culture lines were compared for aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase inducibility by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo[*a*]anthracene (BA) and TCDD- and BA-specific binding in the cytosol and nucleus. The effective concentration of BA in the growth medium required to induce either enzyme to 50% of its maximally inducible activity (EC_{50}) was the same (5–11 μM) in both MCF-7 and Hepa-1 cells. On the other hand, the EC_{50} for TCDD in MCF-7 cells (5–25 nM) was more than 40-fold greater than that in Hepa-1 cells (0.4 to 0.6 nM). P_1-450- and P_3-450-specific mouse cDNA probes were used to quantitate mRNA induction in the Hepa-1 cell line. P_1-450 mRNA was induced markedly by TCDD and benzo[*a*] anthracene, whereas P_3-450 mRNA was induced negligibly. A P_1-450-specific human cDNA probe was used to quantitate P_1-450 mRNA induction in the MCF-7 cell line. Aryl hydrocarbon hydroxylase inducibility by TCDD or BA always paralleled P_1-450 mRNA inducibility in either the mouse or human line. Although the cytosolic Ah receptor in Hepa-1 cells was easily detected by sucrose density gradient centrifugation, gel permeation chromatography, and anion-exchange high-performance liquid chromatography, the cytosolic receptor cannot be detected in MCF-7 cells. Following *in vivo* exposure of cultures to radiolabeled TCDD, the intranuclear concentration of inducer–receptor complex was at least fifty times greater in Hepa-1 than MCF-7 cultures. The complete lack of measurable cytosolic receptor and almost totally absent inducer–receptor complex in the nucleus of MCF-7 cells was, therefore, out of proportion to its capacity for aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase inducibility. This MCF-7 line should provide an interesting model for a better understanding of the mechanisms of drug-metabolizing enzyme induction by polycyclic aromatic compounds, including the Ah receptor-mediated mechanism.

Polycyclic aromatic compounds such as TCDD‡ and BA induce at least two forms of cytochrome P-450 in the laboratory animal (reviewed in Ref. 6). In the mouse hepatoma cell line Hepa-1, inducers bind with high affinity ($K_d \cong 0.5$ nM for TCDD) to a protein receptor, and AHH activity is induced by TCDD at correspondingly low concentrations (reviewed in Ref. 7). Following treatment of C57BL/6N or DBA/2N mice *in vivo* with [^{3}H]TCDD, [^{3}H]TCDD·Ah receptor complexes accumulate in the hepatic nuclear fraction and, within minutes, transcriptional activation of the P_1-450 and P_3-450 genes [9] followed by enhanced levels of P_1-450 and P_3-450 mRNA can be detected [8, 9]. Mutant clones of Hepa-1 have been identified in which defective induction of P_1-450 mRNA is correlated with decreased Ah receptor content or impaired intranuclear accumulation of the TCDD·Ah receptor complex [7]. These data all support the hypothesis that the Ah receptor is essential for AHH induction by polycyclic aromatic compounds.

In this report, we have analyzed AHH and Ac4H induction by TCDD and BA in the human breast carcinoma cell line MCF-7. Although AHH and Ac4H activities were induced by TCDD and BA, the expected Ah receptor could not be detected in MCF-7 cultures. The same was true for several other human breast carcinoma cell lines. The MCF-7 line thus appears to represent an exception to current

* Portions of this work were presented at the Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics, St. Louis, MO, April, 1984[1].

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‡ Abbreviations include: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; [^{3}H]TCDD, [1,6-^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin; BA, benzo[*a*]anthracene; [^{3}H]BA, generally tritiated benzo[*a*]anthracene; AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (EC 1.14.14.1); Ac4H, acetanilide 4-hydroxylase; EC_{50}, effective concentration of chemical required to induce an enzyme activity to 50% of its maximally inducible level; HPLC, high-performance liquid chromatography; 1X SCC, solution of 0.15 M NaCl and 15 mM sodium citrate; 1X Denhardt's solution: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidine, and 0.02% bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate. "P-450" designates any or all forms of the membrane-bound hemoprotein enzyme requiring flavoprotein reductase and reduced pyridine nucleotide(s) for catalytic activity. Mouse "P_1-450" and "P_3-450" proteins are defined as those forms of polycyclic hydrocarbon-induced P-450 in C57BL/6N mouse liver having the highest turnover number for induced aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activity respectively. Mouse P_3-450 was formerly called "P-448" [2]. Mouse P_1-450 and P_3-450 correspond to rat "P-450c" and "P-450d", respectively [3], and to rabbit "form 6" and "form 4", respectively [4, 5].

hypotheses regarding the Ah receptor and its role in the induction of P_1-450.

EXPERIMENTAL PROCEDURES

Materials. All tissue culture solutions and fetal calf serum were purchased from GIBCO (Grand Island, NY). Tissue culture plastic ware was from Falcon Plastics (Oxnard, CA), guanidinium thiocyanate from the Eastman Kodak Co. (Rochester, NY), oligio(dT)-cellulose from Collaborative Research, Inc. (Waltham, MA), and nitrocellulose paper from the Millipore Corp. (Bedford, MA). Nonidet P-40, T1 RNase, yeast RNA, deoxyribonucleoside triphosphates, BA, Ficoll, polyvinylpyrrolidone, bovine serum albumin (fraction V), sperm DNA, NADH, and NADPH were from the Sigma Chemical Co. (St. Louis, MO). The 96-hole dot-blot manifold was from Schleicher & Schuell (Keene, NH), and polynucleotide kinase from Bethesda Research Laboratories (Rockville, MD). [α-^{32}P]dCTP and [γ-^{32}P] ATP were from Amersham Radiochemicals (Arlington Heights, IL); [1,6-^{3}H]TCDD (52 Ci/mmole) was from KOR Isotopes (Cambridge, MA), generally labeled [^{3}H]BA (5 mCi/mmole) from Amersham (Arlington Heights, IL), and ^{14}C-*methyl*-labeled bovine serum albumin (16 μCi/mg) from the New England Nuclear Corp. (Boston, MA), Nonlabeled TCDD was a gift from the Dow Chemical Co. (Midland, MI).

Cell culture. The origin and development of the Hepa-1 line have been detailed previously [10]. MCF-7 and the other human established cell lines were obtained from Marc Lippmann (National Cancer Institute, Bethesda, MD). The origin and development of the MCF-7 line have been described [11, 12]. Cultures were grown as monolayers at 37° in 95% air and 5% CO_2 in α-MEM medium supplemented with 10% fetal calf serum, penicillin (40 units/ml), streptomycin (40 μg/ml) and Mycostatin (25 μg/ml). Cells were plated at a density of $\sim 10^6$ cells per 125-cm^2 T-flask and treated with TCDD and BA during the second half of the logarithmic growth phase. Fetal calf serum was omitted during short-term incubations ($\leqq$3 hr). For experiments lasting more than 24 hr, medium was replaced each day. All experiments were repeated two to four times to ensure reproducibility.

Buffers and solutions. Dulbecco's sodium phosphate-buffered saline (0.85% NaCl, pH 7.2) was used. HEDG buffer consists of 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 1.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (v/v), pH 7.6. HDMG buffer represents 25 mM Hepes, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 10% glycerol (v/v), pH 7.6. HEDGN denotes 25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), and 0.5 M NaCl (pH 7.6). Dextran-charcoal represents 0.5 mg of dextran and 5 mg of charcoal (Norit A) per ml of HEDG buffer (pH 7.6).

Enzyme assays. Following the removal of medium, the cell culture surface was washed three times with 10–20 ml of cold Dulbecco's phosphate-buffered saline without calcium or magnesium. The cells were scraped from the flasks and collected as pellets by centrifugation (800 $g \times 5$ min). In some cases these pellets were stored at −80° for several weeks with no loss in enzyme activity. The fresh or previously frozen pellets were homogenized briefly by sonication in 0.25 M Tris chloride (pH 7.6) at protein concentrations of 5–20 mg/ml. AHH activity [13] and Ac4H activity [14] were assayed by the methods cited; incubation times were 30 min, and final cellular homogenate concentrations ranged between 0.4 and 1.5 mg protein/ml reaction mixture. Both the enzyme activity and protein concentration were determined in duplicate for the homogenate of cells scraped from one T-flask. *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-hydroxybenzo[*a*]pyrene recrystallized standard. *One unit* of Ac4H activity is defined as that amount of enzyme catalyzing in 1 min at 37° the formation of 1 pmole of 4-hydroxyacetanilide. *Specific activity* denotes units per mg of cell homogenate protein. Protein content was assessed by the method of Lowry *et al.* [15] with bovine serum albumin as the standard.

Mouse P-450 mRNA dot hybridization analysis. Mouse P_1-450 and P_3-450 cDNA probes, named clone 46 [16] and p$P_3$450-21 [17], respectively, are 1100-base pair and 1710-base pair inserts in pBR322. These cDNA clones are known to hybridize specifically to P_1-450 (23 S) mRNA and P_3-450 (20 S) mRNA respectively. A human cDNA probe, phP_1-450-5′, is known to represent the 5′ end (1521 base pairs) of P_1-450 cDNA, which includes almost the entire translating region [18].

RNA "dot blots" were performed by the method of White and Bancroft [19]. Cells from one T-flask were pelleted by centrifugation and then resuspended and washed twice in sterile Dulbecco's phosphate-buffered saline. The cells were resuspended in 45 μl of ice-cold 10 mM Tris chloride (pH 7.0) and 1 mM EDTA and lysed by the addition of 5 μl of 5% Nonidet P-40 and 5 min of mixing on ice. Nuclei were pelleted (15,000 $g \times 2.5$ min), and supernatant fraction (40 μl) was transferred to a sterile 1.5-ml tube containing 24 μl of 20X SSC plus 16 μl of 37% (w/w) formaldehyde. Another 10 μl of supernatant fraction was treated with 5 μl of RNase [20 μg/ml in 10 mM Tris chloride (pH 7.6) plus 1 mM EDTA] and incubated at 37° for 1 hr before adding 6 μl of 20X SSC and 4 μl of 37% formadehyde. Following incubation of all mixtures for 15 min at 60°, various aliquots were adjusted to 150 μl in 15X SSC. These solutions (100 μl) were applied with suction to 4-mm diameter spots on nitrocellular paper supported on 3-mm Whatman filter paper holding a 96-hole manifold apparatus. The nitrocellulose sheet was then treated for 90 min at 80° *in vacuo* to fix the cytoplasmic RNA. The filters were next prehybridized for 6 hr at 68° in 10 ml containing 6X SSC, 10X Denhardt's solution, 0.5% $NaDodSO_4$, and denatured salmon sperm DNA (200 μg/ml). The hybridization mixture was the same as the prehybridization mixture. The two probes (clone 46 and p$P_3$450-21) were nick-translated [20] and added ($\sim 1.5 \times 10^8$ cpm; ~ 1 μg DNA) to the hybridization mixture, and the mRNA-DNA hybridization was carred out at 68° overnight. Following hybridization, the filters were washed four times (each time 15 min

at 52°) with 0.1X SSC and 0.05% $NaDodSO_4$ and then three times (each time 20 min at 45°) with 0.1X SSC. The filters were then dried and autoradiographed.

Mouse P_1-450 and P_3-450 mRNA quantitation. Poly(A^+)-enriched RNA from TCDD- or BA-treated cells was purified by the guanidinium thiocyanate method [21], followed by three cycles of oligo(dT)-cellulose chromatography. The RNA was stored at −80°. The inserts in clone 46 and pP_3450-21 corresponding to mouse P_1-450 and P_3-450 DNA, respectively, and the human pP_1450-5′ cDNA clone were electroeluted and fixed to nitrocellulose filters, as previously described [22, 23]. DNA (100 μg) was boiled for 10 min in 1 ml of 0.2 N NaOH, diluted to 15 ml with 2 M NaCl, and filtered through a 0.45-μm nitrocellulose filter by gravity. The filters were washed under light vacuum with 20 ml of 4X SSC, dried at room temperature for 2 hr, and then placed in a vacuum oven at 80° for 16 hr. Circles (10 mm) were cut from the large filter and prehybridized for 2 hr at 45° in 30% (v/v) formamide containing 10 mM Tris chloride (pH 7.2), 0.6 M NaCl, 1 mM EDTA, 0.2% $NaDodSO_4$, and yeast RNA (100 μg/ml). The poly(A^+)-enriched RNA was treated for 15 min at room temperature in kinase buffer composed of 50 mM Tris chloride (pH 9.5), 5 mM glycine, 100 μM spermidine and 10 μM EDTA [23], following which the mRNA was labeled to a specific activity 1×10^7 to 5×10^7 cpm/μg by use of polynucleotide kinase in the presence of [γ-^{32}P]UTP. The DNA fixed on the nitrocellulose filters was then hybridized with 10^6 to 10^7 dpm of [^{32}P]RNA at 45° for 72 hr in 200 μl of buffer containing 0.5 M NaCl, 1 mM EDTA, and 0.5% $NaDodSO_4$; the filters were treated for 30 min more with the same solution without the $NaDodSO_4$. The filters were next incubated with 1 ml of pancreatic RNase (10 μg/ml) and 10 units of T1 RNase for 1 hr at 25° and then washed at room temperature three times with 1X SSC containing 0.5% $NaDodSO_4$ and twice with 10 mM Tris chloride (pH 7.2) containing 1 mM EDTA. Specifically hybridized mRNA was eluted by heating the filters twice at 40° in doubly-distilled water. Controls (background) represented radioactivity eluted from pBR322 DNA-bound filters. The percentage of total mRNA hybridized (experimental minus control) was determined.

Preparation of cytosolic and nuclear fractions. Following removal of the growth medium, the cell surface of MCF-7 or Hepa-1 cultures was washed three times with Dulbecco's phosphate-buffered saline, and the cells were removed by scraping and pelleted by centrifugation (800 $g \times 3$ min). The washed pellet was freeze-thawed at −196° and then homogenized with a Teflon–glass homogenizer in 2 vol. of HEDG buffer containing 0.1 M NaCl. After centrifugation at 800 $g \times 10$ min, the supernatant fraction was centrifuged at 105,000 g for 1 hr; this supernatant fraction was regarded as the cytosolic fraction and quickly frozen in 2-ml aliquots (protein concentrations 5–10 mg/ml) and stored at −196°. No losses of TCDD-specific binding capacity were noted during such storage conditions for at least 9 months. In other experiments, cytosolic and nuclear fractions from MCF-7 and Hepa-1 cells were also prepared according to previously described methods [24].

Dextran-charcoal adsorption followed by velocity sedimentation. Either the MCF-7 of Hepa-1 cells were exposed to [^{3}H]TCDD or [^{3}H]BA in culture or the cytosol fractions or nuclear extracts were treated with [^{3}H]TCDD or [^{3}H]BA for 1 hr at 4° *in vitro*. Following exposure of the cultures to radioligand or treatment of the subcellular fraction with radioligand, the fractions were treated with dextran-charcoal (5 mg charcoal/5 mg cellular protein) and incubated at 4° for 15 min. The dextran-charcoal was then removed by centrifugation (4000 $g \times 15$ min). Cytosolic fractions or nuclear extracts (300 μl) were layered onto linear (5–20%) sucrose density gradients prepared in HEDG buffer containing 0.1 M NaCl. The gradients were centrifuged at 2° for 16 hr at 235,000 g in a Beckman SW 60Ti rotor. The gradients were separated in 0.2-ml fractions with an ISCO model 640 gradient fractionater. Radiolabeled bovine serum albumin was routinely used as an internal sedimentation marker.

Gel permeation chromatography. Sephacryl S-300 gels were equilibrated in HEDG buffer containing 0.05 M NaCl [25] or 0.5 M NaCl, and columns with dimensions of 95 × 1.5 cm were prepared. Chromatography of 1 ml of the radioligand-containing cytosolic fraction or nuclear extract was performed at a flow rate of 10 ml/hr. Blue dextran and standard proteins were used as markers to calibrate the columns.

Anion-exchange HPLC. Treatment of MCF-7 or Hepa-1 cytosol with [^{3}H]TCDD alone or [^{3}H]TCDD plus nonlabeled TCDD was carried out as usual for 1 hr at 4° *in vitro*. A Beckman model 344 liquid chromatograph was used for HPLC. The anion-exchange column (Pharmacia, Mono-q HR S/5) was equilibrated in 25 mM Hepes (pH 7.5) containing 150 mM NaCl. The column was cooled on ice. Following injection of 0.5 ml (2–5 mg protein), a linear gradient to 25 mM Hepes containing 500 mM NaCl was developed during 20 min at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and radioactivity was determined.

RESULTS

AHH and Ac4H induction. Maximally induced AHH activity in MCF-7 cells (Table 1) was approximately one-fifth of the maximally induced AHH activity in Hepa-1 cells. We found several interesting differences between MCF-7 and Hepa-1. The EC_{50} for TCDD induction of AHH activity was at least 40-fold higher in MCF-7 cultures than in Hepa-1 cultures (Fig. 1; Table 2). The EC_{50} values for BA induction of AHH activity, on the other hand, were nearly equal in both cell lines and were considerably higher than the EC_{50} values for TCDD induction of AHH activity. Analogous results were obtained for the induction of Ac4H activity.

P_1-450 and P_3-450 mRNA induction. Specific cDNA probes for P_1-450 mRNA and P_3-450 mRNA have been recently isolated and characterized [16, 17]. TCDD and 3-methylcholanthrene have been shown to induce both P_1-450 mRNA and P_3-450 mRNA in mouse liver [8]. We therefore wished to use these probes to detect, by means of RNA dot blot analysis (Fig. 2) and an mRNA

Table 1. AHH and Ac4H activities in four human breast carcinoma cell lines and the mouse Hepa-1 cell line following TCDD or BA exposure*

Cell line	Exposure in culture	Hours exposure to inducer	Specific AHH activity	Specific Ac4H activity
MCF-7	None		2.9 ± 0.13	690 ± 20
	TCDD	6	20.7 ± 0.57	
		24	169 ± 9.7	12,500 ± 120
	BA	6	24.6 ± 0.32	
		24	163 ± 10.4	12,700 ± 430
MCF-7p46	None		2.3 ± 0.09	
	TCDD	24	151 ± 5.7	
MDA-231	None		2.0 ± 0.05	
	TCDD	24	9.2 ± 0.98	
T47-D	None		8.9 ± 0.57	
	TCDD	24	66.4 ± 5.7	
Hepa-1	None		10.2 ± 0.06	760 ± 30
	TCDD	6	381 ± 6.0	
		24	846 ± 17	10,200 ± 520
	BA	6	410 ± 45	
		24	761 ± 16	9,930 ± 350

* All values are means ± S.E. (N = 3) and represent maximally inducible activities found after exposure of cells to a 1000-fold range of TCDD and BA concentrations. These concentrations were 100 nM TCDD and 50 μM BA.

quantitation assay (Fig. 3), the induction of specific P_1-450 mRNA and P_3-450 mRNA in Hepa-1 cells following TCDD or BA treatment. Although P_1-450 mRNA induction has been detected by dot blot hybridization in TCDD-treated Hepa-1 cells [26], P_3-450 mRNA has not been measured previously in this cell line. We were particularly interested in determining the dose–response of mRNA accumulation following TCDD versus BA treatment. Such data would serve to confirm the high EC_{50} values for BA induction of AHH and Ac4H activities.

Dot blot analysis (Fig. 2) demonstrated con-

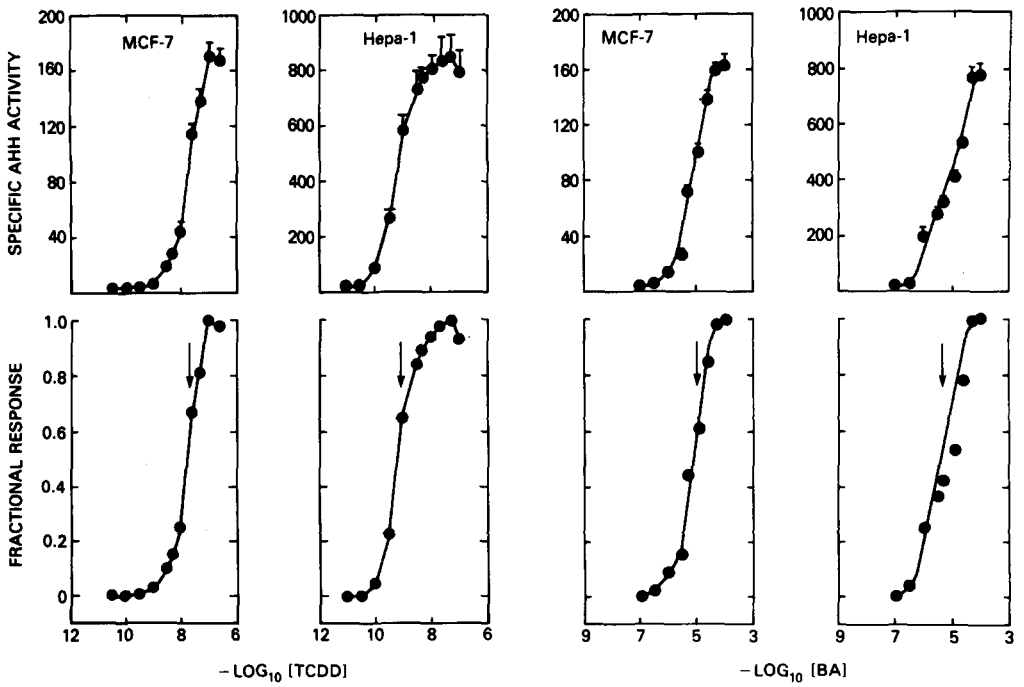

Fig. 1. Potency of TCDD (left) and BA (right) to induce AHH activity in human MCF-7 and mouse Hepa-1 cell lines. Specific AHH activity is shown at top; closed circles and brackets denote means ± S.D. for three experiments done in duplicate. Fractional responses are shown at bottom. Arrows illustrate EC_{50} in each instance. Exposure to TCDD or BA in all experiments shown was 24 hr. Further details are provided under Experimental Procedures.

Table 2. Estimated EC_{50} values for TCDD and BA as inducers of AHH and Ac4H activities in MCF-7 and Hepa-1 cell cultures

Cell line	Inducer	Hours exposure to inducer	EC_{50} AHH	EC_{50} Ac4H
MCF-7	TCDD	6	16 nM	
		24	25 nM	5.9 nM
	BA	6	1.0 μM	
		24	7.9 μM	7.8 μM
Hepa-1	TCDD	6	0.56 nM	
		24	0.63 nM	0.49 nM
	BA	6	0.7 μM	
		24	5.0 μM	11 μM

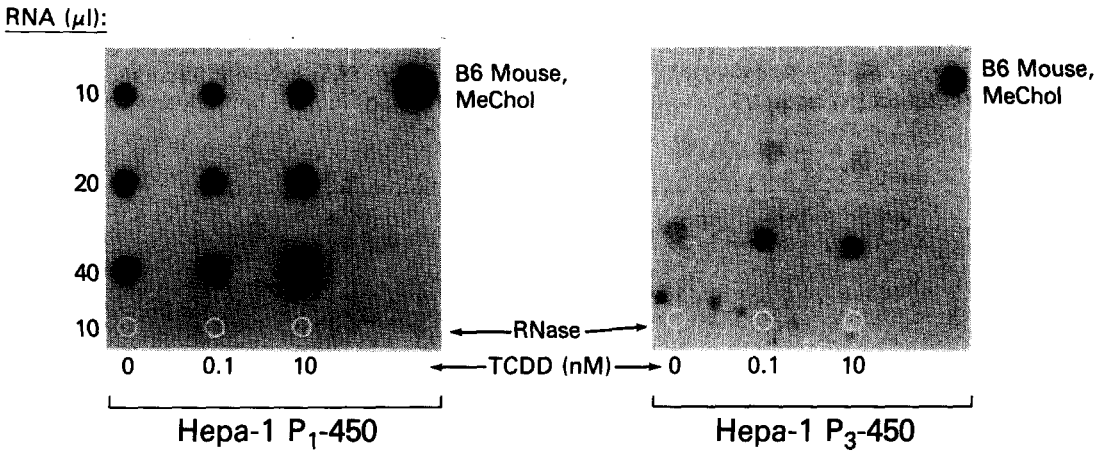

Fig. 2. Cytoplasmic RNA dot hybridization analysis of P_1-450 mRNA and P_3-450 mRNA induction by TCDD in mouse Hepa-1 cultures. The three rows in each case represent three concentrations (10, 20 and 40 μl; approximately 2, 4 and 8 μg) of RNA applied to the spot. At the bottom of the three columns is shown the nanomolar concentration of TCDD in the growth medium during a 24-hr exposure of these cell lines. RNase-treated controls at each TCDD concentration are shown at bottom. The control dot for the mouse P_1-450 and P_3-450 cDNA probes was about 2 μg of liver cytoplasmic RNA from a 3-methylcholanthrene (MeChol)-treated C57BL/6N (B6) mouse. The filters were exposed to X-ray film for 24 hr. Further details are described under Experimental Procedures.

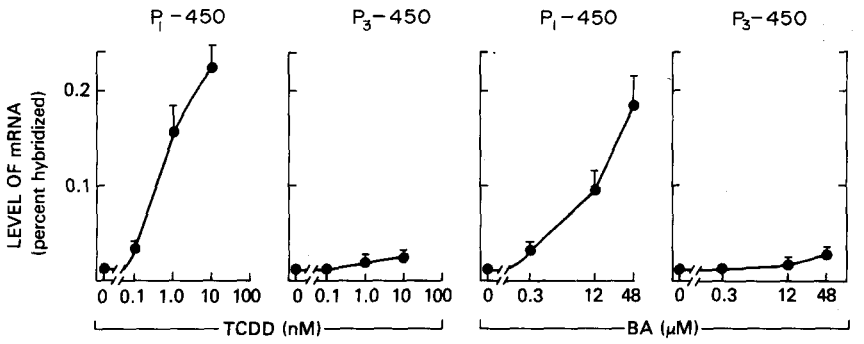

Fig. 3. Quantitation of P_1-450 mRNA and P_3-450 mRNA levels in mouse Hepa-1 cultures as a function of TCDD or BA concentration in the growth medium. Exposure to inducer was 24 hr in each instance. Closed circles and brackets denote means ± S.D. for three duplicate determinations. Following poly(A^+)-enriched RNA isolation and hybridization to nitrocellulose filters containing the appropriate mouse cDNA probe, results are expressed as the percentage of specific hybridized radioactivity per total dpm hybridized. Further details are given under Experimental Procedures.

siderably more hybridization of the P_1-450 messenger than the P_3-450 messenger in TCDD-treated Hepa-1 cells. When analyzed quantitatively (Fig. 3), TCDD and BA treatment of Hepa-1 cells increased dramatically the P_1-450 mRNA and produced only a negligible increase in P_3-450 mRNA. These data demonstrate clearly, however, the induction of P_1-450 mRNA, which we have also found by Northern blot hybridization to be about 2.9 kilobases (kb) (not illustrated). We have not been able to detect increases in the 2.1-kb P_3-450 mRNA with Northern hybridizations. The data in Fig. 3 confirm that TCDD is much more potent than BA as an inducer of P_1-450 mRNA, a finding also true of AHH activity (Fig. 1 and Table 2). Similar findings (not illustrated) were seen with MCF-7 P_1-450 mRNA inducibility with the use of the human phP_1450-5′ cDNA probe. Hence, AHH inducibility by TCDD or BA in either the mouse or human cell line is closely correlated with P_1-450 mRNA inducibility as measured by hybridization to the species-specific P_1-450 cDNA probe.

Effect of BA metabolism on AHH induction. BA, but not TCDD, is known to be rapidly metabolized by cell cultures having induced AHH activity [27]. Therefore, we measured the EC_{50} for BA induction of AHH activity at shorter exposures (e.g. 6 hr) at which time AHH induction was only about one-fourth of the maximum obtained at 24 hr. The EC_{50} values for TCDD at 6 hr were essentially unchanged from those at 24 hr in either MCF-7 or Hepa-1 cells (Table 2). The EC_{50} values for BA, however, were considerably lower than those at 24 hr. This result confirms that the EC_{50} for BA is dependent on the time of exposure, most likely because BA concentrations in the growth medium (or within the cell) will be affected by induced P_1-450. These data do not, however, account for the apparent insensitivity of MCF-7 cells to AHH or Ac4H induction by TCDD, when compared to Hepa-1 cells.

TCDD binding in vitro *to subcellular fractions of Hepa-1 and MCF-7.* The Ah receptor in Hepa-1 cells has been characterized in several previous studies (reviewed in Ref. 7). In cytosolic fractions, the receptor has an $S_{20,w}$ value of about 6S and an apparent

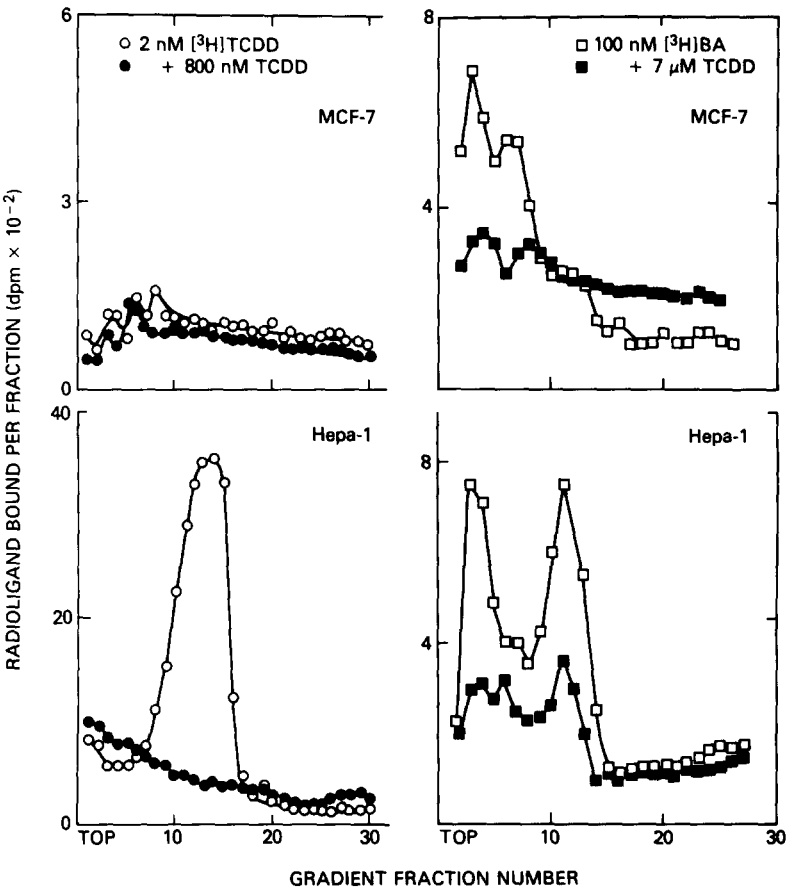

Fig. 4. Velocity sedimentation analysis of TCDD-specific (left) or BA-specific (right) binding moieties of human MCF-7 (top) and mouse Hepa-1 (bottom) cells following *in vitro* treatment of cytosol with the radioligand for 1 hr at 4°. Note the large differences in values on the ordinates and that the specific radioactivity of [^{3}H]BA is only about one-tenth that of [^{3}H]TCDD. Nonspecific [^{3}H]TCDD binding was removed by the addition of 800 nM nonlabeled TCDD (●). Nonspecific [^{3}H]BA binding was removed by the addition of 7 μM nonlabeled TCDD (■). Nonspecific radioligand was then removed with dextran-charcoal, and the cytosol was layered onto 5–20% sucrose density gradients. Further details are provided under Experimental Procedures.

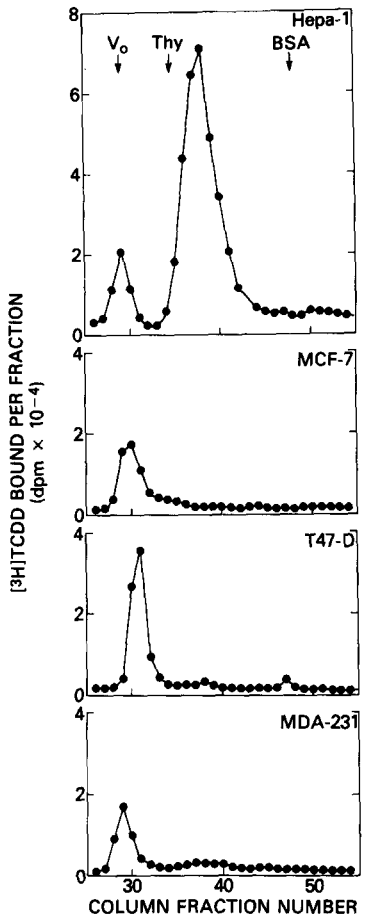

Fig. 5. Gel permeation chromatography of TCDD-specific binding material in mouse Hepa-1 cultures (top) and human MCF-7, T47-D, or MDA-231 cultures. Cytosol from each of these four lines was treated *in vitro* with 2 nM [^{3}H]TCDD for 1 hr at 4°; following dextran-charcoal adsorption the cytosol was applied to Sephacryl S-300 columns [25]. A 400-fold excess of nonlabeled TCDD completely obliterated the Hepa-1 peak centered around fraction 38 but had no effect on the void volume (V_0) peak in any of the four cell lines (data not illustrated). Thyroglobulin (Thy) and bovine serum albumin (BSA) represent standards of 61 Å and 37 Å respectively.

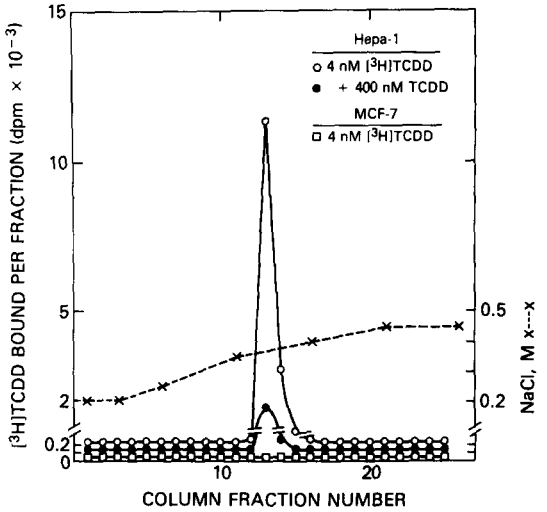

Fig. 6. Anion-exchange HPLC of TCDD-specific binding material in mouse Hepa-1 but not human MCF-7 cells. As was the case in Figs. 4 and 5, cytosol was treated with radioligand, with and without a large excess of nonlabeled TCDD, for 1 hr at 4° *in vitro*, following which the cytosol was ready for HPLC analysis. The salt gradient from 0.2 M to about 0.45 M NaCl was monitored by conductivity. Free [^{3}H]TCDD (i.e. TCDD not associated with macromolecules) is adsorbed to the column resin; hence, charcoal treatment is not required for analysis of TCDD binding by anion-exchange HPLC.

K_d of about 0.5 nM for TCDD. Because the EC_{50} for TCDD induction of AHH activity appeared to be about 20 nM in MCF-7 cells (Fig. 1), it was important to analyze the cytosolic and other subcellular fractions of MCF-7 cultures for TCDD-specific binding moieties. With the use of sucrose density gradient centrifugation (Fig. 4), the Ah receptor was detected in the Hepa-1 cytosolic fraction with either TCDD or BA; however, no comparable saturable [^{3}H]TCDD-binding moiety or [^{3}H]BA-binding moiety was detected in the MCF-7 cytosolic fraction.

In other experiments not illustrated, MCF-7 cytosolic fractions were treated *in vitro* with 20 nM [^{3}H]TCDD, and no saturable TCDD binding was detected on sucrose density gradients. We also examined cytosolic fractions from both cell lines (and other human breast carcinoma cell lines) by gel permeation chromatography (Fig. 5). Although the Ah receptor (R_s about 55 Å) was detected in Hepa-1 cells, no comparable moiety was detected in any of the human lines. Some [^{3}H]TCDD eluted in the void fraction, but this material has been shown in previous studies [24] to represent a nonsaturable binding component in mouse liver cytosolic fractions. Further, this void fraction was not saturable in Hepa-1 or MCF-7 cytosolic fractions (data not included).

Both sucrose density gradient centrifugation and gel permeation chromatography require considerable time (16 and 6 hr respectively). If MCF-7 cells contain an Ah receptor with decreased affinity for [^{3}H]TCDD, then TCDD might dissociate rapidly and be undetectable by these two assays. Because the Ah receptor binds to anion-exchange resins such as DEAE-cellulose, we used anion-exchange HPLC as a rapid assay (about 20 min) to analyze TCDD-binding moieties (Fig. 6). Hepa-1 cytosolic fractions, treated *in vitro* with 4 nM [^{3}H]TCDD, contained a single TCDD-binding peak which was saturable by excess nonlabeled TCDD. MCF-7 cytosolic fractions incubated with the same concentrations of [^{3}H]TCDD did not contain any TCDD-binding peak (Fig. 6).

The short time required for satisfactory resolution by anion-exchange HPLC is comparable to other "rapid" methods (e.g. dextran-charcoal adsorption [28] and hydroxylapatite adsorption [29]) used for detection of [^{3}H]TCDD · Ah receptor complexes. By anion-exchange HPLC we also examined MCF-7 cytosolic and nuclear fractions after treatment *in vitro* with 200 nM [^{3}H]TCDD (Fig. 7). This concentration is several-fold higher than that required for maximal induction of AHH activity in these cells. Although the radioligand was eluted from the

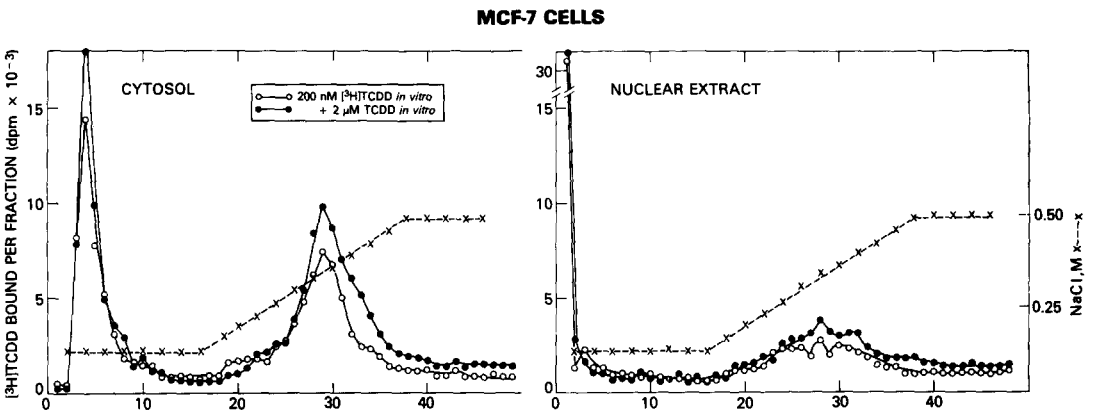

Fig. 7. Attempt to detect TCDD-specific binding by anion-exchange HPLC of MCF-7 subcellular fractions treated *in vitro* with 200 nM [^{3}H]TCDD. Experimental conditions were otherwise the same as those described in the Fig. 6 legend.

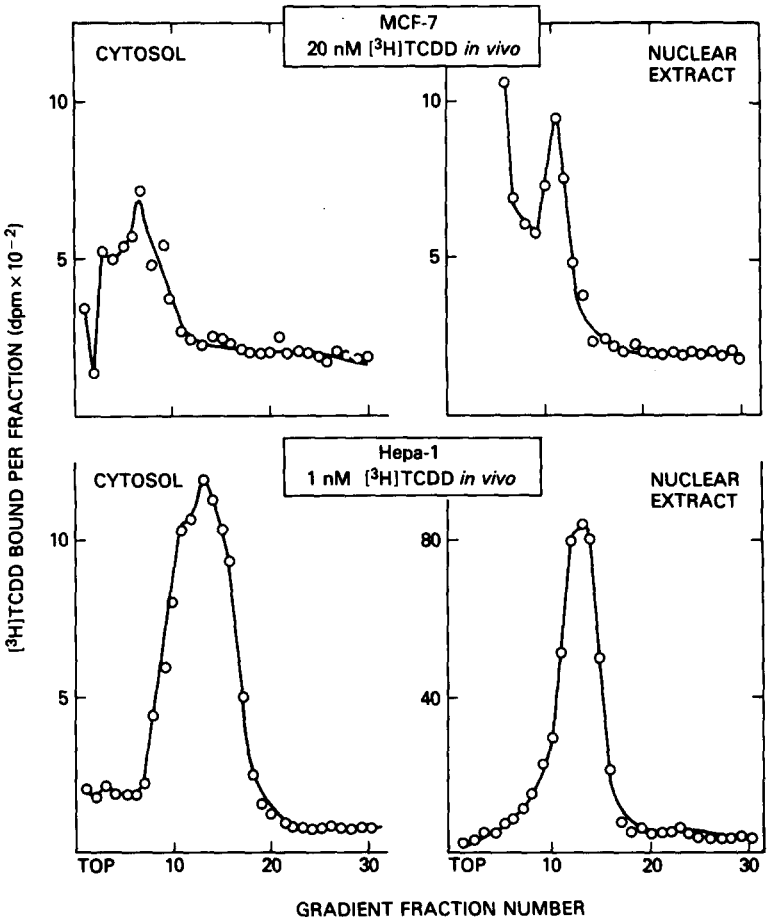

Fig. 8. Velocity sedimentation analysis of cytosolic fractions (left) and nuclear extracts (right) of human MCF-7 cells (top) and mouse Hepa-1 cells (bottom) following *in vivo* exposure to [^{3}H]TCDD in the growth medium for 1.5 hr. After exposure of MCF-7 cells to 20 nM [^{3}H]TCDD and Hepa-1 cells to 1 nM [^{3}H]TCDD, cytosolic fractions and nuclear extracts were prepared [24]. Dextran-charcoal adsorption and sucrose density gradient centrifugation were then carried out, as described in Fig. 4 and under Experimental Procedures.

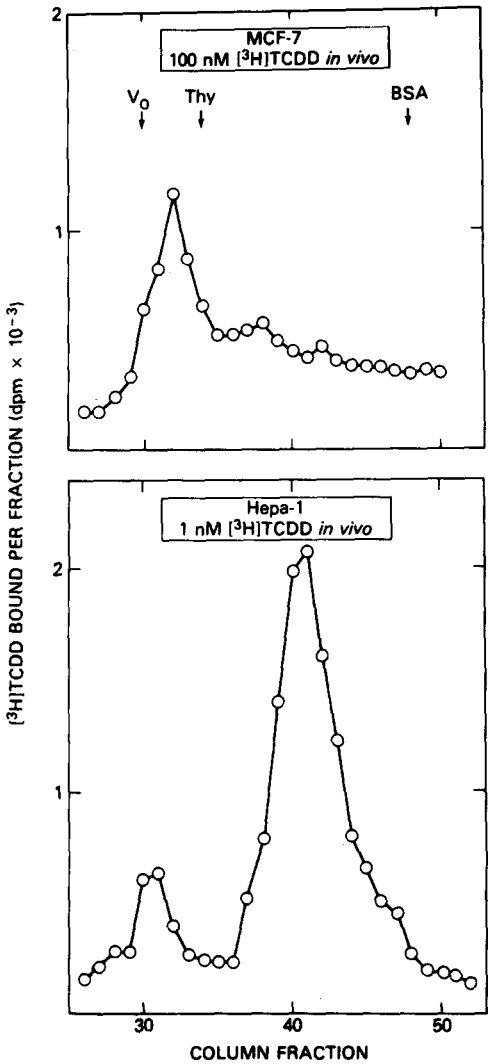

Fig. 9. Gel permeation chromatography of cytosolic fractions of human MCF-7 cells (top) and mouse Hepa-1 cells (bottom) following *in vivo* exposure to [^{3}H]TCDD in the growth medium for 90 min. The experiments were carried out in identical fashion to those in Fig. 8, and further details are described in Fig. 5 and under Experimental Procedures. Nuclear extracts with high salt could not be examined by Sephacryl S-300 in either cell line for reasons not readily apparent (although examination of nuclear extracts of *mouse liver* by Sephacryl S-300 has been done [9, 25]). Abbreviations: V_0, void volume; Thy, throglobulin; and BSA, bovine serum albumin.

column in the initial wash fractions and in a broad peak during NaCl gradient elution, binding of [^{3}H] TCDD to these peaks was not inhibited by excess nonlabeled TCDD.

We also considered the possibility that MCF-7 cells contain factors (e.g. proteases) which degrade the Ah receptor. When [^{3}H]TCDD-bound Hepa-1 cytosolic fractions were mixed with MCF-7 cytosolic fractions, however, no degradation of the Hepa-1 inducer–receptor complex was detected. [^{3}H] TCDD-treated Hepa-1 cells were also mixed with MCF-7 cells, and the usual cytosolic fraction was isolated following homogenization of the intact cells and centrifugation; no degradation of the Hepa-1 Ah receptor was found under these conditions (data not illustrated).

Subcellular distribution of TCDD following in vitro *exposure of Hepa-1 and MCF-7 cells.* After *in vivo* exposure of Hepa-1 cells to [^{3}H]TCDD, the inducer–receptor complex is known [30] to accumulate in the cell nucleus in a temperature-dependent fashion. Figure 8 shows evidence of the inducer–receptor complex in Hepa-1 cytosol and nuclear extracts. The nuclear inducer–receptor complex required extraction with 0.3 M NaCl and displayed a discrete 6S peak at high salt concentrations on sucrose density gradients (Fig. 8).

In contrast, MCF-7 cells that had been exposed to [^{3}H]TCDD *in vivo* exhibited no measurable cytosolic receptor (Figs. 8 and 9). Very small quantities of TCDD-specific binding were reproducibly detected in MCF-7 nuclear extracts (Fig. 8), but the quantity was too low (<2% of that found in Hepa-1 nuclear extracts during comparable experiments) for detailed characterization.

DISCUSSION

A large body of experimental evidence supports the concept that the Ah receptor is required for the induction of AHH activity and P_1-450 by polycyclic aromatic compounds. In the human MCF-7 cell line, however, no Ah receptor is detected—yet AHH activity is highly inducible in these cells. In addition to using procedures such as sucrose density gradient centrifugation and gel permeation chromatography, we have developed anion-exchange HPLC as a method for analyzing [^{3}H]TCDD binding to cellular macromolecules. Even with the use of the rapid (20-min) HPLC method, we are unable to detect specific [^{3}H]TCDD binding *in vitro* to cytosolic or nuclear fractions of MCF-7 cells. This breast carcinoma cell line thus should provide a critical "testing ground" for the current model of P_1-450 induction.

It is of interest that a receptor of the phenobarbital class of P-450 inducers has not yet been identified. Hence, insight gained regarding the mechanism of P_1-450 induction in MCF-7 cells may be applicable to yet unexplained actions of other P-450 inducers.

This laboratory has previously studied AHH inducibility in several cultured cell lines from rodent and primate species [30]. From these data we had concluded that the presence of an Ah receptor was necessary, but not sufficient, for AHH induction. Thus, Hepa-1 and H-4-II-E lines contain similar high-affinity ($K_d < 1$ nM) TCDD-binding proteins in the cytosolic fraction, and TCDD induces AHH activity ($EC_{50} < 1$ nM) in both cell lines. Rat HTC cells contain a high-affinity TCDD-binding moiety but AHH activity is not induced; monkey kidney VERO cells do not contain detectable Ah receptor and AHH is not inducible [30]. Although AHH activity in MCF-7 cells is inducible by TCDD, the induction process was considerably less sensitive to TCDD than that in Hepa-1 cells (Fig. 1): the EC_{50} for TCDD induction of AHH activity was about 40-fold higher in MCF-7 cells. It is thus possible that these cells contain a low-affinity form of Ah receptor

or, alternatively, that AHH induction is mediated in this cell line by a biochemical mechanism that does not involve the Ah receptor.

The human breast carcinoma cell lines we have examined to date indicate varying degrees of impaired AHH inducibility, when compared with the Hepa-1 cell line. How can these cell lines be used to provide evidence for, or against, the presence of a putative low-affinity receptor form (which cannot be detected by current methodology)? We believe that somatic-cell genetics may provide the best approach for further analysis of P_1-450 inducibility in the MCF-7 cells. Study of Hepa-1 variants with defective P_1-450 induction has already given evidence for the involvement of several genes in the induction process (reviewed in Ref. 7). Hence, when Hepa-1 cells are treated with 20 μM benzo[*a*]pyrene, P_1-450 is induced and metabolism of benzo[*a*]-pyrene to toxic metabolites is enhanced. Cells that survive this exposure to benzo[*a*]pyrene have been shown to be defective in AHH induction. Genetic complementation analysis by fusion of different variant clones has been used to identify at least three complementation groups. At least two of these complementation groups represent defects involving the high-affinity Ah receptor [7, 23]: Class B variants have decreased levels of Ah receptor (<10% of that in the wild-type Hepa-1 parent); Class C variants have impaired nuclear uptake of the [^{3}H]TCDD · Ah receptor complex *in vivo*. If similar selection methods can be used to isolate benzo[*a*]pyrene-resistant variants of MCF-7, then fusion of such mutants with Hepa-1 clones may identify the equivalent of Class B variants in MCF-7 cells. Although all of the Hepa-1 Class B variants have low Ah receptor content, they appear to have a normal EC_{50} for TCDD induction of AHH activity. Independently, Miller *et al.* [31] have isolated a Class B variant with a higher EC_{50} for TCDD induction of AHH activity; interestingly, no Ah receptor can be detected in this mutant clone.

We have also used BA as an inducer and as a radioligand. Two problems, however, are associated with the use of BA. First, the compound is metabolized by the AHH activity being induced and, second, the metabolites are toxic. The high EC_{50} for BA induction of AHH activity (about 10 μM) may reflect both metabolism and cytotoxicity. We found that the EC_{50} for BA induction of AHH was considerably lower at earlier times of BA exposure (Table 2). To interpret *in vitro* ligand-binding studies, it would be useful to determine the EC_{50} for BA induction of AHH at the earliest possible time of treatment. Within minutes after addition of TCDD to Hepa-1 cultures, transcription of the P_1-450 gene is enhanced [26] such studies could also be performed with BA.

We examined [^{3}H]BA binding to cytosolic fractions of MCF-7 and Hepa-1 cells (Fig. 4). [^{3}H]BA bound to the Hepa-1 Ah receptor, and no [^{3}H]BA binding was detected in the same gradient fraction of MCF-7 cells. These data confirm the existence of an Ah receptor difference between MCF-7 and Hepa-1 cells with the use of a radioligand other than [^{3}H]TCDD. In both cell lines, we have detected saturable [^{3}H]BA binding in the lower-molecular-weight regions (<4S) of the sucrose density gradients. In this size range, polycyclic-hydrocarbon-binding proteins other than the Ah receptor have been detected in cultured cell lines [24, 25]. Of interest, AHH induction by BA in Hepa-1 cells is impaired in Class B and Class C mutants [24], and we have not found any variants in which the induction response is not the same for both TCDD and BA. These results indicate a close relationship, based on somatic-cell genetics, between TCDD and BA induction of AHH.

In the C57BL/6N mouse, TCDD induces at least two distinct forms of P-450: P_1-450 and P_3-450 [16, 17]. We have used specific cDNA probes to quantitate P_1-450 mRNA and P_3-450 mRNA induction in Hepa-1 by TCDD or BA. We have also measured Ac4H inducibility, in addition to AHH inducibility. It is of interest that TCDD and BA induce proportionately more Ac4H activity in MCF-7 than Hepa-1 cells. Although P_3-450 purified from C57BL/6N mouse liver has the highest turnover number for polycyclic-hydrocarbon-induced Ac4H activity, P_1-450 is also involved in the 4-hydroxylation of acetanilide [2]. P_1-450 mRNA was increased markedly by TCDD and BA (Fig. 3). The difference in the dose–response curve for P_1-450 mRNA induction (Fig. 3) supports the data obtained by measurement of AHH activity (Fig. 1). The minimal increases observed in P_3-450 mRNA induction may represent bona fide induction of P_3-450 mRNA (below the level of detection on Northern blot hybridization) or may represent a low level of cross-hybridization of the P_3-450 cDNA probe with P_1-450 mRNA. Analyses of mRNA transcripts by S_1 nuclease protection, or actual catalytic activities detected following transient expression or transformation by transfected cDNA or genomic clone constructs, will be required to distinguish between these possibilities.

Although several (independently cloned) breast carcinoma cell lines were used in these studies and none was found to have detectable cytosolic Ah receptor, these data cannot be extrapolated as representative of human epithelial cells in general. Several human squamous cell carcinoma lines were surveyed recently for AHH induction by TCDD and Ah receptor content [32]. Two subgroups were found: four lines having an EC_{50} for TCDD of ~1 nM, and one line having an EC_{50} for TCDD of ~0.1 nM. All of these lines appeared to contain low amounts of the cytosolic Ah receptor, as measured by velocity sedimentation analysis. The cell lines used by Hudson *et al.* [32] and in the present study are transformed lines. It is possible that the process of malignant transformation (or other selective pressures of tissue culturing) may alter the normal gene expression of the Ah receptor. It will be important to extend these observations with the use of non-transformed established human cell lines and primary cultures of human epithelial cells. Whatever the aberration in the human MCF-7 line is, these cells will be useful in further characterization and understanding of the Ah receptor.

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