Biochemical Pharmacology, Vol. 38, No. 14, pp. 2339–2348, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INDUCTION OF ARYL HYDROCARBON HYDROXYLASE AND DEMONSTRATION OF A SPECIFIC NUCLEAR RECEPTOR FOR 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN IN TWO HUMAN HEPATOMA CELL LINES*

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(*Received* 16 *September* 1988; *accepted* 8 *December* 1988)

Abstract—Two established human hepatoma cell lines, Hep3B and HepG2, were examined for aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) induction and for the presence of the murine-equivalent aromatic hydrocarbon (*Ah*) receptor. Both cell lines demonstrated polycyclic aromatic hydrocarbon (PAH)-induced AHH activity; however, assay conditions for induction were different than those established for the control mouse hepatoma cell line, Hepa c1–9. When cytosols from either cell line were exposed to tritiated 2,3,7,8-tetrachlorodibenzo-*p*-dioxin ([^{3}H]TCDD) and analyzed on sucrose gradients with or without prior charcoal treatment, two peaks were observed at positions corresponding to 4–5 S and 8–9 S. The 8–9 S peak was identified as the probable human *Ah* receptor equivalent since, like the mouse *Ah* receptor, this peak: (a) was eliminated only by cytochrome P_1-450 inducers; (b) was sensitive to protease digestion; and (c) was thermolabile. Levels of TCDD specifically bound in the 8–9 S peak for HepG2 and Hep3B were 27 and 34 fmol/mg cytosolic protein respectively. The level of TCDD specifically bound was not affected by charcoal treatment or by the addition of sodium molybdate, which is known to stabilize ligand binding to steroid receptors. Incubation of Hep3B or HepG2 cells with [H]TCDD at 37° for 1 hr effected a redistribution of binding from the cytosol 8–9 S peak to a nuclear 6 S peak. The nuclear peaks from both human cell lines demonstrated similar sedimentation properties, temperature-dependence and inducer-specificity, as for the mouse nuclear *Ah* receptor. Appearance of nuclear 6 S binding is consistent with a temperature-dependent translocation process, supporting the observation that these human hepatoma cell lines contain a binding component which is similar to the mouse *Ah* receptor in structure and function during AHH induction.

The role of the aromatic hydrocarbon (*Ah*) receptor in cytochrome P_1-450 associated aryl hydrocarbon hydroxylase (AHH‡) induction and its link to toxicity and carcinogenesis from polycyclic aromatic hydrocarbons (PAHs), such as 3-methylcholanthrene (MCA), and halogenated aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are well established in experimental rodent systems [1, 2]. Without exception, demonstration of inducible AHH activity has signaled the presence of a functional receptor which specifically binds the xenobiotic compound (i.e. cytosolic receptor) and subsequently interacts with the genome (i.e. nuclear receptor) to stimulate transcription and translation of AHH-related genes [3–5].

While AHH induction is identified primarily with hepatic function, there is evdience that the *Ah* receptor-mediated AHH induction response is also present in many rodent extrahepatic tissues [6–9]. Furthermore, studies in other mammalian [10, 11], non-mammalian [12], and non-human primate systems [13, 14] suggest that, despite structural differences, the basic functional role of the *Ah* receptor in mediating AHH induction is conserved in many species.

Several human tissues and cells in culture have been shown to possess PAH/TCDD inducible AHH activity [15–19]. Although relatively few investigations of the human equivalent *Ah* receptor have been accomplished, a limited number of studies have suggested that a specific binding species similar to rodent *Ah* receptor is present in cytosolic preparations of human tissues and, to a lesser extent, cell lines in culture [14, 20–23]. However, the low variable levels of cytosolic receptor and the apparent absence of nuclear receptor have made it difficult to study the role of the receptor in human AHH induction [17, 24]. Recent reports of high levels of cytosolic receptor and detectable levels of nuclear receptor in human A431 squamous cell carcinoma cells suggest that such studies are now possible [25].

In this report, we have investigated the induction of AHH activity and the role of the *Ah* receptor in two established human hepatoma cell lines, Hep3B and HepG2, and compared the results with those established for the mouse hepatoma cell line, Hepa c1–9 [26]. Our data suggest that the human hepatoma cells contain a receptor specific for TCDD which is similar in nature and function during AHH induction

* This work was supported by a grant from the National Cancer Institute of Canada.

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‡ Abbreviations: AHH, aryl hydrocarbon (benzo[*a*]-pyrene) hydroxylase; BA, benzanthracene; BP, benzo[*a*]-pyrene; DBA, dibenz[*a,h*]anthracene; DCC, dextran-coated charcoal; DMS, dexamethasone; DMSO, dimethyl sulfoxide; DNase, deoxyribonuclease I; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MCA, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; PB, phenobarbital; RNase, ribonuclease A; SDG, sucrose density gradient; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

to the rodent *Ah* receptor.

METHODS

Chemicals and reagents. [^{3}H]TCDD (ring-labeled, 50 Ci/mmol) and unlabeled TCDD were purchased from KOR isotopes (Cambridge, MA). Other stocks of [^{3}H]TCDD (ring-labeled, 31 and 33.8 Ci/mmol) were a gift from Dr A. B. Okey (Division of Clinical Pharmacology, Hospital for Sick Children, Toronto, Ontario, Candada) and Dr S. Safe (Texas A&M University). TCDD is an extremely toxic substance, and its use necessitates special handling and safety disposal precautions as described by Poland *et al.* [2]. ^{14}C-Labeled methylated bovine serum albumin (16 μCi/mg) and Formula 947-prepared water accepting LSC mixture were from New England Nuclear (Boston, MA). Dibenz[*a,h*]anthracene (DBA) and 3-methylcholanthrene (MCA) were from the Eastman Kodak Co. (Rochester, NY). Dithiothreitol (DTT), dextran, dexamethasone (DMS), benzo-[*a*]pyrene (BP), albumin (bovine serum, Fraction V) (BSA), trypsin, DNase I, RNase A, glycerol, NADPH, NADH, and Tris–HCl were from the Sigma Chemical Co. (St Louis, MO). Acetone and *n*-hexane were from Sargent Welch Scientific (Weston, Ontario). Hepes was from the Calbiochem-Boehring Corp. (La Jolla, CA). Sucrose (density gradient grade) was purchased from Beckman Instruments Inc. (Toronto, Ontario). Molybdate (sodium salt) and phenobarbital (PB, sodium salt) were from British Drug House (BDH, Toronto, Ontario). Dimethyl sulfoxide (DMSO), charcoal (Norit A), NaOH, and EDTA were from the Fisher Scientific Co. (Toronto, Ontario). Gentamicin sulfate, alpha-MEM, and fetal calf serum were from Gibco Laboratories (Burlington, Ontario). The Bio-Rad protein assay kit was from Bio-Rad Laboratories (Mississauga, Ontario). All other chemicals were of reagent grade or better and were purchased from the Fisher Scientific Co. Glycerol phosphate buffer (GPO_4) consisted of 0.25 M K_2HPO_4 and 67 mM KH_2PO_4 in 30% glycerol (v/v). Standard homogenization buffer HEDG consisted of 25 mM Hepes, 1.5 mM EDTA, 1 mM DTT, and 10% glycerol by volume, pH 7.6. HEDGM buffer consisted of HEDG plus 20 mM sodium molybdate, pH 7.6, and HEDGK buffer was HEDG with the addition of 0.5 M KCl, pH 8.5.

Cell cultures. The human cell lines, supplied by Dr B. Knowles and Dr D. Aden (Wistar Institute of Anatomy and Biology, Philadelphia, PA), were originally derived and established from liver biopsies of two children with primary hepatoblastoma (HepG2) and hepatocellular carcinoma (Hep3B) [27, 28]. Hepa c1–9 is a highly PAH-inducible clone established in this laboratory from Hepa 1c1, a cell line originally derived from a transplanted hepatoma BW7756 produced originally in the C57L/J (B6) mouse. Hepa c1–9 was used as a non-human control cell line. The AHH activity and *Ah* receptor characteristics of Hepa c1–9 have been described previously [26]. All three cell lines were routinely maintained in alpha-MEM supplemented with 10% fetal calf serum (v/v) for both human cell lines and 5% fetal calf serum for Hepa c1–9. Gentamicin was added to the medium at a concentration of 50 μg/ml of medium. Cell cultures were maintained at 37° in an atmosphere of 5% CO_2–95% air.

Aryl hydrocarbon hydroxylase assay. AHH activity was determined using the standard fluorometric assay developed for mammalian liver cell cultures [29] with minor modifications [26]. One unit of AHH activity is defined as that amount of enzyme catalyzing per minute at 37° the formation of hydroxylated product causing fluorescence equivalent to 1 picomole of hydroxybenzo[*a*]pyrene recrystallized standard [30]. Specific AHH activity was expressed in units per milligram of protein.

Ah *Receptor assay.* The procedures for measuring *Ah* receptor for [^{3}H]TCDD binding in culture and *in vitro* using sucrose density gradients have been described previously [7, 31]. Procedures were modified in particular experiments as indicated in the legends to figures and tables.

Approximate sedimentation coefficient values for radioactive peaks were determined by the method of Martin and Ames [32] relative to ^{14}C-labeled methylated BSA (4.6 S), which was added as an internal sedimentation marker. Specific [^{3}H]TCDD binding peaks are those eliminated by the presence of excess unlabeled competitor. When required, peak area disintegrations per minute were converted to femtomoles [^{3}H]TCDD specifically bound per milligram of protein or per 10^6 cells as previously described [26]. In this manuscript all sucrose density gradient (SDG) profiles are representative of at least three independent experiments.

Protein. Protein determinations were obtained by the method of Bradford [33].

RESULTS

Induction of AHH activity using conditions established for Hepa c1–9. In mouse and rat tissue and cell culture, TCDD is one of the most potent inducers of AHH activity [1, 8, 13, 26, 31]. The results for the two human hepatoma cell lines presented in Table 1 are consistent with this observation. When HepG2 and Hep3B were assayed for AHH activity using inducer concentrations and induction times known to generate maximal levels of AHH activity in Hepa C1–9 [26], the levels of induced activity, in units/mg protein, in both human cell lines were consistently lower than that found in Hepa c1–9. The relative potencies of the inducers, however, were similar in all three cell lines with TCDD inducing the maximum induction response. Moreover, while the absolute values of induced AHH activity varied from experiment to experiment, the relative values remained the same.

Effect of varying AHH assay conditions on expression of induced activity. Since the data in Table 1 were obtained using assay conditions established for murine tissues and cells [29, 31] the effect of changing several important AHH assay conditions on levels of induced activity in the two human cell lines was examined. The results of varying inducer concentration, length of induction period, and sample protein concentration are presented in Fig. 1 (panels A through D).

Inducer concentration. From the data in Fig. 1A, the concentration of TCDD that induced half-maxi-

Table 1. AHH activity of Hepa c1–9, Hep 3B and HepG2 using assay conditions established for Hepa c1–9

	AHH activity* (units/mg protein)			
	Induced with			
Cell lines	1 μM BA	1 μM BP	1 μM MCA	1 nM TCDD
Hepa c1–9	13.6 ± 2.1	7.5 ± 1.7	20.6 ± 2.1	35.2 ± 2.7
Hep3B	0.9 ± 0.2	0.7 ± 0.2	1.3 ± 0.3	3.1 ± 0.7
HepG2	1.3 ± 0.3	0.8 ± 0.2	2.5 ± 0.8	7.6 ± 1.3

* Cells in logarithmic growth were incubated at 37° in medium containing one of the inducers listed for 18 hr and assayed for AHH activity. Induced specific activity was calculated by subtracting the activity for solvent-treated cells from that for cells treated with solvent and inducing agent. One unit of AHH activity is defined as that amount of enzyme catalyzing, per minute at 37°, the formation of hydroxylated products causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[*a*]pyrene recrystallized standard. Abbreviations: BA, benzanthracene; BP, benzo[*a*]pyrene; MCA, 3-methylcholanthrene; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Values are means ± SD, N = 6.

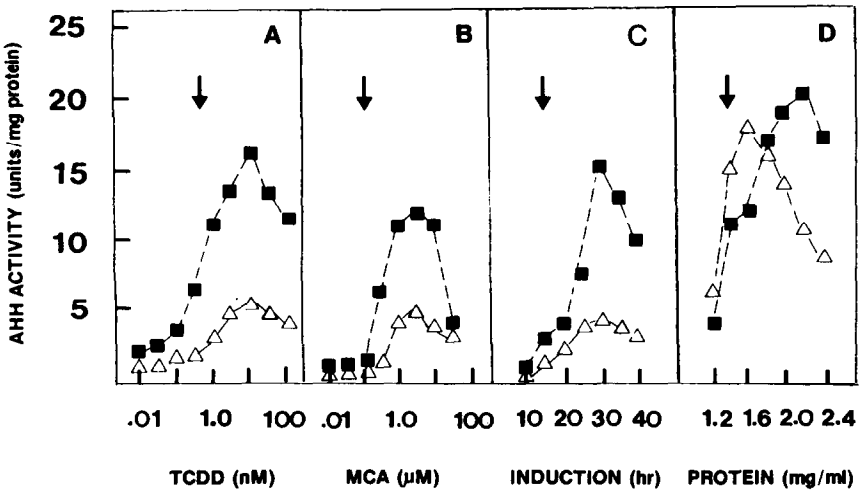

Fig. 1. Effect of varying (A) TCDD concentration, (B) MCA concentration, (C) induction period, and (D) sample protein concentration on the expression of induced AHH activity in Hep3B (△) and HepG2 (■). For each variable the point of highest induced AHH activity for the control mouse hepatoma cell line, Hepa c1–9, is indicated by a vertical arrow. Cells were treated with medium containing TCDD for 18 hr, unless otherwise indicated, at 37° and then assayed.

mal activity (ED_{50}) was calculated to be 0.7×10^{-9} M for HepG2 and Hep3B compared with 0.8×10^{-11} M, a value previously reported for Hepa c1–9 [26]. From similar experiments (Fig. 1B) the ED_{50} for MCA was calculated to be $0.5–0.6 \times 10^{-6}$ M for HepG2 and Hep3B compared with 0.5×10^{-7} M for Hepa c1–9.

Induction period. With respect to length of induction period, the results presented in Fig. 1C demonstrated that the highest levels of induced AHH activity occurred in Hep3B and HepG2 after a 30-hr exposure of cultured cells to TCDD. Maximum levels of activity in Hepa c1–9 cells, on the other hand, were obtained after an 18-hr exposure of cells to TCDD. These levels remained constant with induction periods up to 48 hr. In HepG2, a decrease in activity was observed with induction periods greater than 35 hr. However, our observations indicate that this was due to cell death.

Sample protein concentration. Under the microscope, Hep3B appears larger than either HepG2 or Hepa c1–9. This is reflected in the saturation densities for the three cell lines: 0.6×10^5 cells/cm^2 for Hep3B compared with 1.3×10^5 cells/cm^2 and 1.4×10^5 cells/cm^2 for Hepa c1–9 and HepG2 respectively. Hep3B also contains more total protein per cell. From our data, the number of picograms of protein per cell for Hep3B, HepG2 and Hepa c1–9 was 187, 110 and 58 respectively. The possibility that the human cells, especially Hep3B, require more total protein to produce an Hepa c1–9 equivalent amount of AHH-associated protein was examined. The results presented in Fig. 1D are consistent with this possibility.

In addition to the three assay conditions described above, the effect of substrate concentration on levels

Table 2. AHH activities of Hep3B and HepG2 induced with different inducers

Cell line	Inducer*	AHH activity† (units/mg protein)	AHH activity† (units/10^{12} cells)‡	Relative AHH Activity§
Hep3B	TCDD	6.7 ± 0.7‖	1.2 ± 0.1	1.0
	MCA	2.4 ± 0.6	0.5 ± 0.1	0.4
	DBA	1.1 ± 0.2	0.2 ± 0.1	0.2
	BA	1.8 ± 0.2	0.4 ± 0.1	0.3
	BP	1.8 ± 0.2	0.4 ± 0.1	0.3
	PB	ND¶	ND	
	DMS	ND	ND	
HepG2	TCDD	13.2 ± 2.3	1.5 ± 0.3	1.0
	MCA	4.2 ± 0.8	0.5 ± 0.1	0.3
	DBA	1.2 ± 0.2	0.2 ± 0.1	0.1
	BA	2.7 ± 0.5	0.3 ± 0.1	0.2
	BP	1.4 ± 0.3	0.2 ± 0.1	0.1
	PB	ND	ND	
	DMS	ND	ND	

* TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; MCA, 3-methylcholanthrene; DBA, 1,2,3,4-dibenz[*a*,*h*]anthracene; BA, benzanthracene; BP, benzo[*a*]pyrene; PB, phenobarbital; and DMS, dexamethasone.

† Cells in logarithmic growth were exposed to 10 nM TCDD and a 1.0 μM concentration of the other agents listed for 30 hr, and AHH activity was assayed in duplicate. The values given for specific AHH activity were calculated by subtracting the activity for solvent-treated cells from the activity of cells treated with solvent and one of the inducing agents.

‡ AHH activity was converted from units/mg protein to units/10^{12} cells using the following experimentally determined relationships: 1 mg Hep3B cells corresponded to 5.4×10^{12} cells; 1 mg HepG2 cells corresponded to 9.1×10^{12} cells.

§ Relative AHH activity was determined by dividing the AHH activity induced by MCA, DBA, BA, BP, or PB by that induced by TCDD.

‖ Mean ± SD, N = 4.

¶ ND, no activity detected over solvent controls.

of AHH activity was examined. It was found that the concentration of BP routinely used for Hepa c1–9 was also sufficient in the human cell lines. The highest levels of TCDD-induced AHH activity were routinely observed between 0.07 and 0.11 mM BP for all three cell lines.

Effect of various inducers on levels of AHH activity. Using information established from the above studies, the effects of various other inducers of cytochrome P_1-450, (i.e. MCA, BA, DBA and BP), one inducer of cytochrome P-450 other than cytochrome P_1-450 phenoobarbitol (PB), and a synthetic steroid hormone, dexamethasone (DMS), were examined. In each case the cells were induced for 30 hr. The results are presented in Table 2. To minimize variation, the cells were assayed at the same time and under the same conditions. From these results it can be seen that TCDD, MCA and DBA induced AHH activity, in the same relative order with respect to potency, in both Hep3B and HepG2, whereas PB and DMS did not induce AHH activity in either human hepatoma cell line. This result is consistent with those previously reported for Hepa c1–9 [26].

In general, regardless of the assay condition examined or the type of PAH inducer used, the levels of induced AHH activity—in units/mg protein—were consistently highest in Hepa c1–9 and lowest in Hep3B. However, when the number of cells per milligram of protein was determined and the AHH results corrected for this difference by expressing AHH activity in units/10^{12} cells, the levels of induced AHH activity in Hep3B and HepG2 were similar (Table 2).

Binding of [^{3}H]*TCDD to Hep3B and HepG2 cytosols.* Cytosol preparations from Hepa c1–9, Hep3B and HepG2 were assayed for the *Ah* receptor using the method of sucrose density gradient centrifugation following treatment with dextran-coated charcoal [31]. A representative profile is presented in Fig. 2. Hepa c1–9 cytosol labeled with 1 nM [^{3}H]TCDD resulted in a single peak of binding sedimenting at a region corresponding to 9–10 S (Fig. 2A). Binding of [^{3}H]TCDD at the 9–10 S region appeared specific in that it was competitively inhibited by the presence of a 1000-fold molar excess of unlabeled TCDD. In the human cells, 1 nM [^{3}H]TCDD was not sufficient for receptor detection. However, when Hep3B cytosol was incubated with 5 nM [^{3}H]TCDD, two binding peaks at regions corresponding to 4–5 S and 8–9 S were observed (Fig. 2B). Binding of [^{3}H]TCDD at both peaks was eliminated by the presence of excess unlabeled TCDD in Hep3B cytosol. A similar pattern was obtained with HepG2 cytosolic fractions (Fig. 2C).

Effects of competitors on the [^{3}H]*TCDD binding peaks.* The effects of different chemicals on [^{3}H]TCDD binding in Hep3B and HepG2 cytosols were determined by incubating cytosols in 5 nM [^{3}H]TCDD or 5 nM [^{3}H]TCDD plus excess concentrations of the inducers of cytochrome P_1-450, TCDD, MCA, and DBA; the inducer of other forms

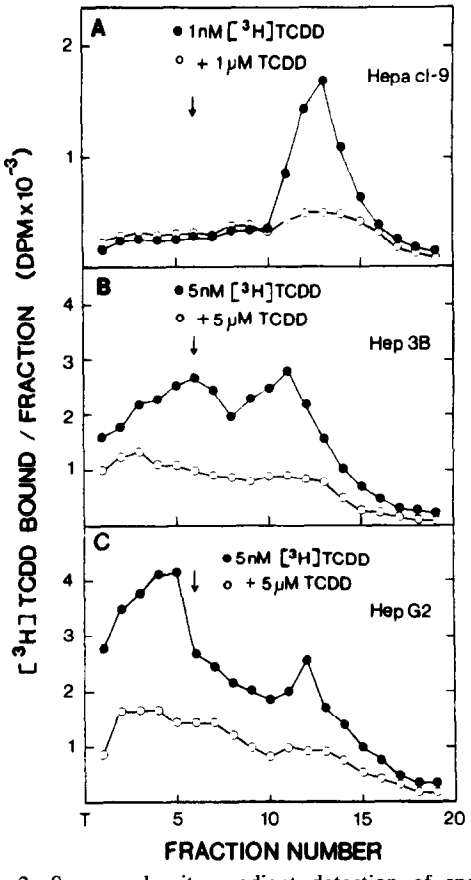

Fig. 2. Sucrose density gradient detection of specific [³H]TCDD binding in cytosolic extracts. Hepa C1–9 (A), Hep3B (B) or HepG2 (C) cytosols were incubated with (A) 1 nM [³H]TCDD (●) or 1 nM [³H]TCDD plus 1 μM nonlabelled TCDD (○) and (B, C) 5 nM [³H]TCDD (●) or 5 nM [³H]TCDD plus 5 μM nonlabeled TCDD (○) for 1 hr at 0–4°. The treated extracts were incubated with DCC, and then specific binding was determined by sucrose density gradient analysis. The position of [¹⁴C]BSA, which is included in the SDG as an internal sedimentation marker (4.6 S), is indicated by the vertical arrow in this and in subsequent figures.

Table 3. Effects of competitors on specific [³H]TCDD binding to Hep3B and HepG2 cytosol extracts

Cell line	Competitor	Fold Concn*	% Elimination (relative to TCDD†) 4–5 S Region	8–9 S Region
Hep3B	TCDD	1000	90	100
	MCA	1000	11	91
	MCA	2000	10	97
	DBA	1000	4	47
	DBA	2000	4	60
	PB	1000	2	0
	DMS	1000	0	0
HepG2	TCDD	1000	93	100
	MCA	1000	12	91
	MCA	2000	9	94
	DBA	1000	0	52
	DBA	2000	0	71
	PB	1000	0	0
	DMS	1000	0	0

* For each cell line, cytosol was prepared from cells not previously treated with [³H]TCDD. The cytosol extracts were subsequently labeled with 5 nM [³H]TCDD ± 1000- or 2000-fold excess of TCDD, MCA, DBA, PB or DMS.

† The amount of [³H]TCDD bound at each peak was 32.3 and 25.4 fmol/mg protein for the 8–9 S peak in Hep3B and HepG2 respectively; and 5.5 and 59.4 fmol/mg protein for the 4–5 S peak in Hep3B and HepG2 respectively.

of cytochrome P-450, PB; and the ligand for steroid receptor, DMS (Table 3). The results reproducibly demonstrate that the 8–9 S peak region in these cells was substantially reduced by an excess of all three known cytochrome P_1-450 inducers. Moreover, increasing the concentration of MCA or DBA resulted in a further reduction of [³H]TCDD dpm in the 8–9 S region (Table 3). Further increases in inducer concentration were not examined. Phenobarbital and DMS had no effect on either the 4–5 S or the 8–9 S peak. It is relevant to note that similar results were obtained for cytosols prepared from cells incubated in culture with 5 nM [³H]TCDD, or 5 nM [³H]TCDD plus excess competitor, for 1 hr at 0°.

Molecular properties of the 8–9 S specific binding peak. The proteinaceous and thermolabile nature of the murine *Ah* receptor is well documented [7, 26, 31]. Comparable analyses in the human hepatoma cell lines demonstrated that, for either Hep3B (Fig. 3A) or HepG2 (Fig. 3B), the specific 8–9 S [³H]TCDD binding peak was effectively eliminated by the serine protease, trypsin, but not by the nucleases, DNase and RNase (data not shown). In addition, for either Hep3B (Fig. 3C) or HepG2 (Fig. 3D), treatment of cytosol at 45° for 10 min eliminated the majority of [³H]TCDD 8–9 S binding. The 4–5 S binding region was not affected by trypsin treatment or by incubation at 45°.

Effects of DCC and molybdate on the 8–9 S binding peak. The values for specific binding content of the 8–9 S peak in Hep3B and HepG2 were calculated to be 34 ± 3.2 and 27 ± 1.5 fmol/mg cytosol protein, respectively. These levels represented approximately 30–40% of levels calculated from the Hepa cl–9 data (Fig. 2A). When expressed in fmol/10^6 cells to correct for the different cell/protein ratios for each of the three cell lines, the mean levels of TCDD-receptor binding in percent were 31% for HepG2 and 66% for Hep3B relative to Hepa cl–9 (=100%). Recently, Manchester *et al.* [23] reported that the level of *Ah* receptor detected in human placental cytosols increases substantially as the ratio of DCC to cytosolic protein decreases and when tissue is initially homogenized in a buffer containing sodium molybdate (10–20 mM). These modifications of the murine assay procedure were examined in the two human hepatoma cell lines. In short, while DCC had some effect on the SDG profile, especially in the 4–

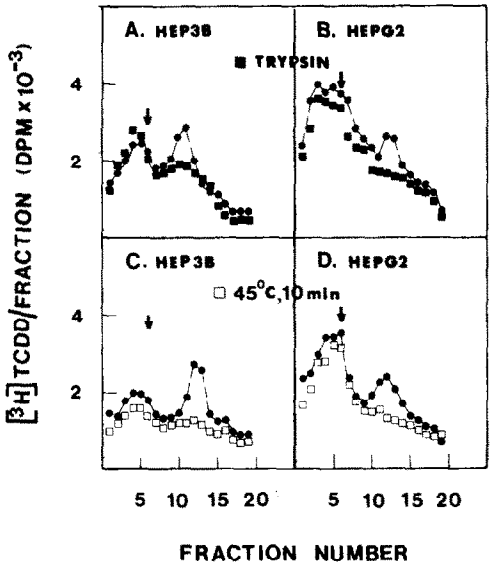

Fig. 3. Effect of trypsin and elevated temperature upon the sucrose density gradient detection of specific [^{3}H]TCDD binding in cytosolic extracts of Hep3B and HepG2. For each cell line, extracts were labeled with 5 nM [^{3}H]TCDD for 1 hr at 0–4° without further treatment (●) or labeled under the same incubation conditions with 100 μg trypsin for an additional hour at 0–4° (panels A and B ■); or incubated for an additional 10 min at 45° (panels C and D □) and then analyzed for specific binding on sucrose density gradients.

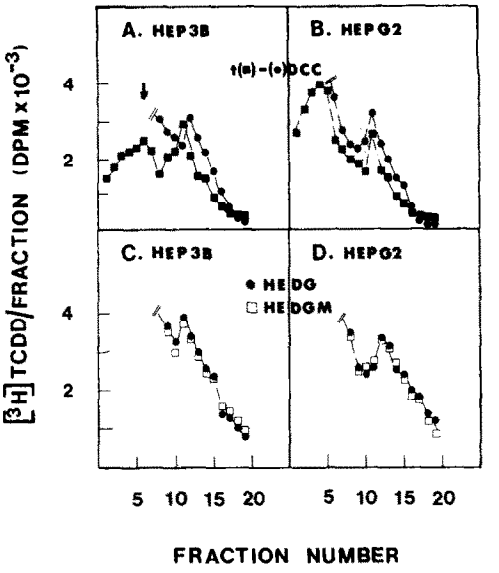

Fig. 4. Effect of dextran coated charcoal and sodium molybdate on the sucrose gradient detection of specific [^{3}H]TCDD binding in Hep3B and HepG2. For either Hep3B (panels A and C) or HepG2 (panels B and D), cytosols were prepared and labeled with 5 nM [^{3}H]TCDD for 1 hr at 0–4°. For experiments presented in panels A and B, cytosols were prepared in HEDG buffer and the labeled cytosols were treated with (■) and without (●) DCC. For experiments presented in panels C and D, cytosols were prepared in HEDG buffer with (□) and without (●) 20 mM sodium molybdate. For all fractions, specific binding was determined on sucrose density gradients.

5 S region, the level of specific binding in the 8–9 S region in either Hep3B (Fig. 4A) or HepG2 (Fig. 4B) measured without DCC was not significantly different from the level of specific [^{3}H]TCDD binding present in Hep3B and HepG2 cytosols treated with DCC prior to SDG analysis. Similarly, the inclusion of 20 mM sodium molybdate in the homogenization buffer had no apparent effect upon the sedimentation properties or the levels of specific [^{3}H]TCDD binding at the 8–9 S region in either Hep3B (Fig. 4C) or HepG2 (Fig. 4D). While the 8–9 S peaks in HepG2 and Hep3B appear small in relation to the background, they are greater than previously published [14] and were reproducible.

Detection of specific binding in nucleus of Hep3B and HepG2. Previous receptor studies with mouse hepatoma cell lines have shown that in culture labeling with [^{3}H]TCDD at 37°, followed by sucrose-gradient analysis of cytosol and nuclear fractions, results in a time- and temperature-dependent appearance of *Ah* receptor:[^{3}H]TCDD complexes in nuclear extracts. These nuclear binding peaks sediment at a position corresponding to 5–6 S [24, 29]. In human tissues/cells, studies of nuclear [^{3}H]TCDD binding are limited to its recent detection [25]. Therefore, specific binding of [^{3}H]TCDD in nuclear extracts prepared from cells treated in culture for 1 hr at 0° or 37° was examined (Fig. 5). In Hep3B, no specific [^{3}H]TCDD binding was detected in the nuclear preparations of cells labeled for 1 hr at 0° (Fig. 5C). However, specific binding of [^{3}H]TCDD was detected in the cytosolic fraction (Fig. 5A). This

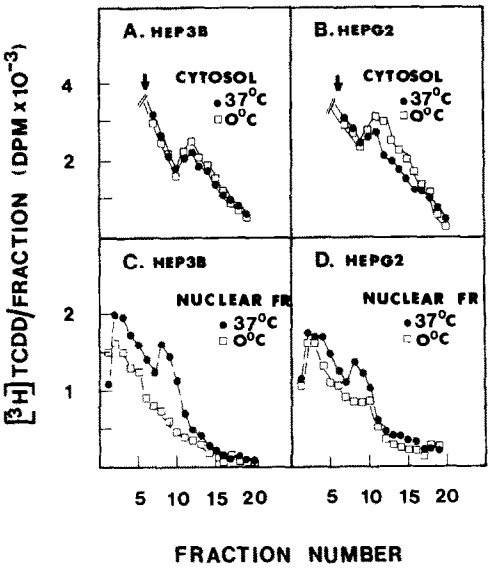

Fig. 5. Sucrose density gradient detection of [^{3}H]TCDD binding in cytosol and nuclear extracts of Hep3B and HepG2 cells. Cells growing in culture were incubated with 5 nM [^{3}H]TCDD for either 1 hr at 37° (●) or 1 hr at 0° (□). Cytosol (panels A and B) and nuclear (panels C and D) fractions were obtained, and specific binding was determined on sucrose density gradients.

Table 4. Effects of competitors on specific TCDD binding to Hep3B and HepG2 nuclear extracts

Cell line	Competitor* (5 μM)	% Elimination (relative to TCDD†)
Hep3B	TCDD	100
	MCA	78
	DBA	37
	PB	0
	DMS	0
HepG2	TCDD	100
	MCA	67
	DBA	29
	PB	0
	DMS	0

* For each cell line, cells growing in culture were incubated with 5 nM [^{3}H]TCDD, or with 5 nM [^{3}H]TCDD plus a 1000-fold excess of TCDD, MCA, DBA, PB, and DMS, for 1 hr at 37°. Nuclear extracts were then prepared and analyzed in SDG.

† The amount of [^{3}H]TCDD bound in the absence of any competitor (i.e. control) was 16.7 and 10.4 fmol/mg protein for Hep3B and HepG2 respectively.

pattern was also observed for HepG2 (Fig. 5B and 5D). When the cells were incubated with [^{3}H]TCDD at 37° for 1 hr, low levels of specific binding could be detected in nuclear extracts from Hep3B (Fig. 5C) and HepG2 (Fig. 5D) at a region corresponding to about 6 S. Comparing the cytosol binding profiles at 0° to those obtained at 37° for each cell line (Fig. 5A and 5B), it is apparent that a decrease in cytosolic binding at 37° occurred. However, this decrease was not complete as 60–70% of the total binding (nuclear and cytosolic) remained in the cytosol of both Hep3B (Fig. 5A) and HepG2 (Fig. 5B).

Specificity of nuclear binding in Hep3B and HepG2. To establish that the [^{3}H]TCDD binding at the 5–6 S was specific for inducers of cytochrome P_1-450, binding in nuclear fractions prepared from Hep3B and HepG2 cells previously labeled in culture for 1 hr at 37° with 5 nM [^{3}H]TCDD and with 5 nM [^{3}H]TCDD plus excess unlabeled TCDD, MCA and DBA was examined. For convenience, the SDG analysis data at the 6–S region is summarized in Table 4. In short, the 6–S nuclear peak was reduced by all *Ah* receptor agonists studied. Although the effectiveness of competition for each PAH was similar to that observed for the 8–9 S peak, the effects of MCA and DBA were less than that observed for cytosolic binding competition studies (see Table 3). Neither PB nor DMS had any effect on the nuclear 6–S binding peak in either human cell line. These results are consistent with those properties established for the murine nuclear receptor [31].

DISCUSSION

In this study, the mechanism for induction of AHH activity in two human hepatoma cell lines, Hep3B and HepG2, was examined and compared to that established for the mouse hepatoma cell line, Hepa c1–9. In general, although the level of induced AHH activity varied as a function of the type of cytochrome P_1-450 inducer in Hep3B and HepG2, the pattern of inducer effectiveness was found to be similar to that previously reported for Hepa c1–9, with TCDD always inducing the highest levels of AHH activity [26, 31, 34, 35]. Initially the human cells were assayed using conditions established for rodent cells. Under these conditions three positive inducers of AHH activity in Hepa c1–9, DBA, BA and BP, did not induce AHH activity in the human cells. This lack of induction was found to reflect a difference in assay techniques rather than a difference in induction potential. Specifically, Hep3B and HepG2 required a longer exposure to a greater concentration of inducer than Hepa c1–9. Since later [^{3}H]TCDD binding studies in the human cells also required higher concentrations of TCDD, the requirement for higher concentrations of inducer may reflect a difference at the level of receptor, for example receptor content, affinity of binding sites and/or activation of nuclear receptor and interaction with the appropriate DNA binding sites [26, 31, 34, 36]. These possibilities, as well as levels of cytochrome *c*(P-450) reductase and cytochrome P_1-450, are being examined. Regardless of the molecular explanation, the results discussed thus far may have important practical significance since the majority of human AHH induction studies reported in the literature make use of induction times from 16 to 24 hr [25]. Within this interval, Hep3B and HepG2 appear poorly inducible for TCDD and MCA and not inducible for DBA, BA and BP.

Diamond *et al.* [37] have demonstrated that cell-free preparations of HepG2 cells metabolically activate the classic cytochrome P_1-450 inducer, BP, to known toxic/carcinogenic intermediates. This same group reported that Hep3B also metabolizes BP. Recently, Dawson *et al.* [38] observed a 20- to 30-fold increase in the O-deethylation of 7-ethoxycoumarin in MCA-treated HepG2 cells. In murine cells, this is a cytochrome P_1-450 associated activity mediated by the *Ah* receptor [2].

When the method of SDG following DCC treatment was used with cytosol preparations from either Hep3B or HepG2 incubated with [^{3}H]TCDD, two binding peaks corresponding to 4–5 S and 8–9 S were observed on respective SDG profiles. In contrast, the mouse Hepa c1–9 cell line demonstrated only one peak at 9–10 S, as previously reported [26]. The apparent two-peak profile observed in either human cell line has been reported for murine tissue samples and cell lines [39] and human A431 squamous cell carcinoma cells [25]. In these experimental systems, the 4–5 S binding peak corresponds to the nonspecific binding component, whereas the specific binding component (*Ah* receptor) sediments in the 8–9 S region. This distinction was not readily apparent in either human cell line, since excess unlabeled TCDD consistently reduced both the 4–5 S and 8–9 S peaks. Further evidence, however, identified the 8–9 S as the major specific [^{3}H]TCDD binding moiety in these human cells. First, only the 8–9 S peak was eliminated by MCA and DBA. Second, increasing the concentrations of MCA and DBA resulted in a further decrease in the 8–9 S peak but had no effect on the level of the 4–5 S peak. Third, the cytosolic [^{3}H]TCDD binding component at the 8–9 S region

in Hep3B and HepG2 was shown to be sensitive to proteolytic degradation; the binding component at the 4–5 S region was resistant. Fourth, the 8–9 S peak in both Hep3B and HepG2 was shown to be thermolabile but the temperature used (45°) had no effect on the 4–5 S region. Despite this evidence, the results do not prove that the 4–5 S peak represents nonspecific binding. It has been suggested that the major *Ah* receptor binding peak in cytosols of murine cells can be separated into several binding components, one of which sediments at the 4–5 S region [40]. Like the 4–5 S peak obtained from the human hepatoma cells, this peak demonstrates some, but not all, of the properties associated with the 8–9 S major receptor peak. The results presented in this manuscript suggest that the 8–9 S binding component in Hep3B and HepG2 is similar to that observed for rat cytosolic *Ah* receptor [41] and is protein in nature.

The only other studies reporting the protein nature of a human receptor specific for [^{3}H]TCDD were done with lymphocytes [42] and recently with human thymic epithelial cells [22] using trypsin as the protease. These results are consistent with those determined for the mouse hepatic cytosolic *Ah* receptor [2], rat hepatic cytosolic *Ah* receptor [7, 41], and mouse hepatoma cells [26, 32].

The effects of several *Ah* receptor agonists were also similar in Hep3B, HepG2, and Hepa c1–9. Since greater fold molar concentrations of MCA and DBA were required to compete with [^{3}H]TCDD in all three cell lines, it can be deduced that TCDD has a higher affinity for the specific binding component than does MCA or DBA. This result correlates with the AHH data of the human cells in that TCDD was shown to be the most potent inducer of AHH activity.

Quantitatively, the levels of [^{3}H]TCDD specifically bound at the 8–9 S region obtained for both human cell lines were about 3-fold greater than those reported for the nonhuman primate hepatic *Ah* receptor [13] and about 3- to 4-fold lower than those obtained for the mouse Hepa c1–9 [26] and human A431 squamous cell s[25]. Nevertheless, the levels of specific binding reported for Hep3B and HepG2 were up to 30-fold higher than those reported for cytosol samples of most human tissues and cells in culture.

DCC is routinely used in the receptor assay procedure to remove unbound and/or loosely bound radioligand from [^{3}H]TCDD-treated cytosol samples. However, Manchester *et al.* [23] reported that the level of specific binding detected in human placental tissues increased as the concentration of DCC decreased. A similar inverse correlation was reported recently by Harper *et al.* [25] for human A431 squamous cell carcinoma cells. While the mechanism of this increase is not understood, it was suggested that the affinity of human *Ah* receptor for ligand may be lower than that found in murine tissues/cells and DCC treatment promoted a physical separation of ligand from receptor [23]. Our results demonstrated that, when DCC was omitted, neither the profiles nor the levels of specific binding detected at the 8–9 S region of the gradient varied from the same assay in the presence of DCC extraction.

A striking aspect of the *Ah* receptors is that they share structural and functional properties in many ways similar to the receptors for steroid hormones [43, 44]. The oxyanion molybdate is known to stabilize steroid receptors in a heteromeric 8–9 S complex form that resists dissociation into monomeric 4–6 S DNA binding states [45[. This has prompted others to examine the effect of molybdate on levels of [^{3}H]TCDD specifically bound at the 8–9 S region. Manchester *et al.* [23] have shown that higher levels of *Ah* receptor can be attained if human placental cytosol is initially homogenized in buffer containing molybdate. In our study the inclusion of 20 mM molybdate in the homogenization buffer had no effect on the position or the amount of specific binding detected at the 8–9 S region of the sucrose gradient. It is possible that the concentration of molybdate used was not effective. However, Denison *et al.* [43] also tested molybdate at concentrations ranging from 0 to 30 mM and reported no change in levels of sedimentation positions of *Ah* receptor for either B6 responsive mice or rat hepatic cytosol.

In murine cells, the apparent time- and temperature-dependent translocation of the *Ah* receptor–ligand complex from the cytoplasm to the nucleus has been established as a necessary molecular event in the overall induction of AHH [26, 31]. Without exception, cells which do not have a functional receptor (i.e. cannot form TCDD–receptor complexes or translocate the complexes into the nucleus) do not express inducible AHH activity [46–48]. In contrast to this, nuclear receptor has rarely been detected in any human cell line examined even when these cells have been shown to be highly AHH inducible [17]. This observation has prompted some to imply a mechanism of induction of AHH which is not mediated by a receptor [17]. Results in the present report demonstrated that nuclear binding does occur in Hep3B and HepG2. Moreover, this binding exhibited properties similar to those established for the rodent *Ah* receptor [31]; that is, temperature dependence and specificity for inducers of cytochrome P_1-450. In view of these results, it can be concluded that the 6 S peak detected in nuclear extracts of both human hepatoma cell lines is the equivalent of the murine nuclear *Ah* receptor. Recent studies in this laboratory with human breast cancer cells and human primary lung carcinoma cells and studies with A431 squamous cell carcinoma cells [25] provide additional support for the necessary role of the human *Ah* receptor in the induction of AHH activity.

In summary, Hep3B and HepG2, both known to retain well differentiated functions and the capacity for xenobiotic metabolism, are AHH responsive human cell lines and demonstrate cytosolic and nuclear receptor specific for inducers of cytochrome P_1-450. Therefore, they provide a relevant human model cell system for examining the physiochemical properties of the human Ah receptor and its role in regulation of AHH and other enzyme activities, modulating basic cellular processes such as cell growth and differentiation, and in determining how many PAHs and halogenated aromatic compounds in the environment exert their toxic and carcinogenic effects on humans.

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