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# *In Vitro* Transformation of the Human Ah Receptor and Its Binding to a Dioxin Response Element

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**SUMMARY**

Many biological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) are mediated by a soluble intracellular protein, the Ah receptor (AhR). After binding of TCDD to the cytoplasmic AhR there occurs a poorly understood "transformation" step, wherein the TCDD-AhR complex is converted to a form that can bind to DNA with high affinity. The binding of transformed AhR to a specific dioxin-responsive element (DRE) upstream of a given gene stimulates transcriptional activation of that gene. Using a gel retardation assay we examined the interaction of transformed human cytosolic TCDD-AhR complexes with a synthetic DNA oligonucleotide containing a single DRE site. Transformation and DNA binding of human AhR *in vitro* was ligand dependent and specific for DRE-containing DNA. Unlike rodent hepatic AhR, *in vitro* transformation of human AhR was completely temperature dependent. Although at 4° AhR binds ligand, no transformation of human TCDD-AhR complex was observed at 4° even after 24 h; however, rapid transformation as measured by DNA binding was detectable as early as 10 min after warming to 22°, with maximal binding by about 60 min. Calf thymus DNA-Sepharose or DRE-Sepharose column chromatography showed that transformed human cytosolic AhR interacts with DNA as a single species. The absolute temperature dependency of human AhR transformation mimics that observed *in vivo* and provides a useful system to study the mechanism of AhR transformation in detail.

Biological responses to the environmental toxicant TCDD are elicited through specific binding of TCDD to the AhR, a soluble protein that is thought to be a member of the steroid hormone receptor superfamily (2). Recently, Ema *et al.* (3) have reported the cDNA sequence for mouse AhR; although the AhR is functionally similar to the steroid hormone receptor superfamily, the cDNA sequence for the ligand-binding subunit of AhR shows little similarity to any of the steroid hormone receptors. A simple model has been proposed for the most extensively characterized response to TCDD, namely the induction of transcription of the *CYP1A1*[1] gene. After high affinity binding of TCDD to cytoplasmic AhR, the TCDD-AhR complex undergoes a temperature-dependent process termed transformation, wherein the AhR is converted to a DNA-binding complex that subsequently accumulates within the cell nucleus (4-7). Binding of ligand-AhR complexes to a specific consensus sequence, or DRE, is necessary for transcriptional activation of the *CYP1A1* gene (8, 9). Numerous studies have shown that the DRE exhibits the properties of a transcriptional enhancer and will confer TCDD responsiveness upon a heterologous promoter and gene, in an AhR-dependent manner (9, 10).

Utilizing a gel retardation assay to measure specific protein-DNA interactions, it has been possible to demonstrate the interaction of transformed ligand-AhR complexes formed *in vivo* and *in vitro* with DRE-containing oligonucleotides (8, 9, 11). Transformed TCDD-AhR complexes bind to the DRE oligonucleotide specifically and with high affinity ($K_d \approx 1$ nM) (12), and the presence of the receptor in this complex has been demonstrated using radiolabeled TCDD and TCDD agonists (9, 12, 13).

Comparison of the DRE sequence from the 5′-flanking region of the mouse and rat *CYP1A1* genes with the flanking sequences of the human gene has revealed the presence of several putative DRE sequences contained within the human TCDD regulatory domain (reviewed in Ref. 14). The action of human AhR appears to be biochemically similar to that described in rodent

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[1] Nomenclature for the cytochrome P450 gene superfamily is as described by Nebert *et al.* (1)

**ABBREVIATIONS:** TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aromatic hydrocarbon receptor; BSA, bovine serum albumin; DRE, dioxin-responsive element; MC, 3-methylcholanthrene; TCDF, 2,3,7,8-tetrachlorodibenzofuran; HAP, hydroxylapatite; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s).

species (15–18); although human nuclear TCDD-AhR complexes have been reported to bind to a DRE (19, 20), the process of human cytosolic AhR transformation and interaction with DREs has not been examined in any detail.

Here we report a series of experiments designed to characterize specific binding of human cytosolic AhR, transformed *in vitro*, to DRE-containing DNA.

## Materials and Methods

**Animals.** Male Sprague-Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories (Wilmington, DE), and male Hartley guinea pigs (250–400 g) were from the Michigan State Department of Health (Lansing, MI). All animals were exposed to 12 hr of light and 12 hr of darkness daily and were allowed free access to food and water.

**Cell line.** LS180 cells (derived from a human colon adenocarcinoma) were obtained from the American Type Culture Collection (Rockville, MD). LS180 cells were routinely grown in $\alpha$-minimal essential medium containing 10% fetal bovine serum and were passaged at confluency by trypsinization.

**Preparation of cytosol.** Hepatic cytosol was prepared in HEGD buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 10%, v/v, glycerol, 1 mM dithiothreitol), as previously described (21). Cytosol from LS180 cells was prepared in HEGD buffer containing 3 mM EDTA (18). All protein determinations were by the method of Bradford (22), using BSA as the standard.

**Synthetic oligonucleotides.** A complementary pair of synthetic oligonucleotides of the sequences 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′ (containing the 20-bp AhR binding site of DRE3 and designated as the DRE oligonucleotide) and 5′-GATCTGGCTCTTCTCACT-CAACTCCG-3′ and 5′-GATCCGGAGTTGAGTGAGAAGAGCCA-3′ [containing a single nucleotide transversion substitution (underlined) and designated as the mutant DRE oligonucleotide] were synthesized (Dalton Biochemical, Toronto, Canada), purified by high performance liquid chromatography, annealed, and radiolabeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Pharmacia), as previously described (9).

**Gel retardation assay.** Cytosol (16 mg of protein/ml for hepatic cytosol and 10 mg of protein/ml for cell cytosol) was incubated with 20 nM TCDD for 2 hr at 22° before gel retardation analysis. In some instances, as noted in the text, cytosol was incubated with 20 nM MC or 20 nM dexamethasone. The assay was done essentially as previously described (8). Briefly, 80 $\mu$g of cytosolic protein were incubated for 15 min with 225 ng of poly(dI-dC) (Boehringer Mannheim, Montreal, Canada) in HEGD buffer containing 95 mM NaCl, followed by the addition of 100,000 cpm (0.4–0.5 ng of DNA) of [$^{32}$P]DRE oligonucleotide, and the sample was incubated for an additional 15 min (the final incubation volume was 25 $\mu$l). Samples were mixed with Ficoll sample buffer, loaded onto a 4% nondenaturing polyacrylamide gel, and electrophoresed in TAE buffer (6.7 mM Tris, pH 8.0, 3.3 mM sodium acetate, 1 mM EDTA). Variations of the aforementioned procedures are described in the text or in the figure legends. The presence of specific protein-DNA complexes was determined by autoradiography of the dried gel.

In some instances the amount of protein-DNA complex formed was determined by excising the specific radiolabeled band from the dried gel and determining the amount of $^{32}$P radiolabel by liquid scintillation. The amount of specifically bound [$^{32}$P]DRE was estimated by measuring the radioactivity present in the inducible band, identified in a cytosolic sample incubated with TCDD, and subtracting the amount of radioactivity present at the same position in a parallel lane containing cytosol not treated with TCDD. The difference between these two values represents the specific TCDD-inducible binding of transformed TCDD-AhR to [$^{32}$P]DRE and is presented as the amount of TCDD-AhR-DRE complex formed.

**Velocity sedimentation analysis on sucrose gradients.** The procedure used was essentially that of Harper *et al.* (18). Briefly, cytosols to be analyzed for [$^{3}$H]TCDD specific binding were incubated with 20 nM [$^{3}$H]TCDD in the absence or presence of a 100-fold molar excess of the competitor TCDF (the specific time and incubation temperature are indicated in the figures and figure legends). After incubation, unbound or loosely bound radioligand was removed by treatment with dextran-coated charcoal (0.1 mg of dextran-coated charcoal/mg of cytosolic protein) and samples were analyzed on 10–30% sucrose density gradients (18). After centrifugation, each gradient was fractionated and the radioactivity in each fraction was determined by liquid scintillation. [$^{14}$C]Formaldehyde-labeled BSA (4.4 S) and catalase (11.3 S) were included in each gradient as internal sedimentation markers.

**HAP adsorption assay.** Specific binding of [$^{3}$H]TCDD to cytosol was measured using the HAP adsorption assay as described previously (21). Aliquots (0.5 ml) of cytosol (0.2 mg/ml) were incubated with 2 nM [$^{3}$H]TCDD in the absence or presence of the competitor TCDF (200 nM). After incubation for 2 hr at 20°, 0.2-ml aliquots of the incubation mixture were added to tubes containing HAP (125 mg of HAP in 250 $\mu$l of HEGD buffer). After a 30-min incubation, the HAP pellets were washed three times with 1 ml of HEGD buffer containing 0.05% (v/v) Tween 80 to remove unbound ligand. After the last wash, radioactivity remaining in the HAP pellet was determined by liquid scintillation. Specific binding of [$^{3}$H]TCDD to the AhR was computed by subtracting the amount of [$^{3}$H]TCDD bound in the presence of TCDF from the amount of [$^{3}$H]TCDD bound in the absence of competitor.

**DNA-Sepharose column chromatography.** Double-stranded concatenated DRE oligonucleotide was prepared as described by Kadonaga and Tjian (23), to give oligomers that ranged in size from about 80 bp to 1500 bp. Ligated DRE oligonucleotide or calf thymus DNA was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer. A 3-ml column of DRE-Sepharose (10 $\mu$g of DRE oligomer) or calf thymus DNA-Sepharose (12 $\mu$g of calf thymus DNA) was equilibrated with HEGD buffer containing 50 mM NaCl. Cytosol (about 18–20 mg of protein), transformed *in vitro* by incubation with 20 nM [$^{3}$H]TCDD at 22° for 2 hr, was applied directly to the column. After sample application, the column was washed with 2 column volumes of HEGD containing 50 mM NaCl and was then eluted with a 50-ml gradient of 50 mM to 1000 mM NaCl in HEGD. Radioactivity and conductivity in each fraction were determined.

## Results

**Formation of the TCDD-AhR-DRE complex.** Incubation of rat hepatic cytosol with 20 nM TCDD for 2 hr at 22° and subsequent analysis by gel retardation in order to measure TCDD-AhR binding to [$^{32}$P]DRE oligomer resulted in the detection of two protein-DNA complexes (Fig. 1, *lanes 1* and 2). The characterization of these two complexes has been previously described by Denison and Yao (12). Complex A, present only in cytosols incubated with TCDD, represents the TCDD-inducible protein-DNA complex, whereas complex B represents a noninducible protein-DNA complex, because it is present in both TCDD-treated and untreated samples. Complex B appears to represent the binding of a protein(s) to single-stranded DRE oligomer remaining after reannealing of the complementary single-stranded oligonucleotides and $^{32}$P-labeling of the DNA (12). The radiolabeled band that migrates the furthest represents free, non-protein-bound, [$^{32}$P]DRE oligomer.

Correspondingly, two distinct protein-DNA complexes were observed using TCDD-treated human cell cytosol. A single TCDD-inducible protein-DNA complex that migrated to the

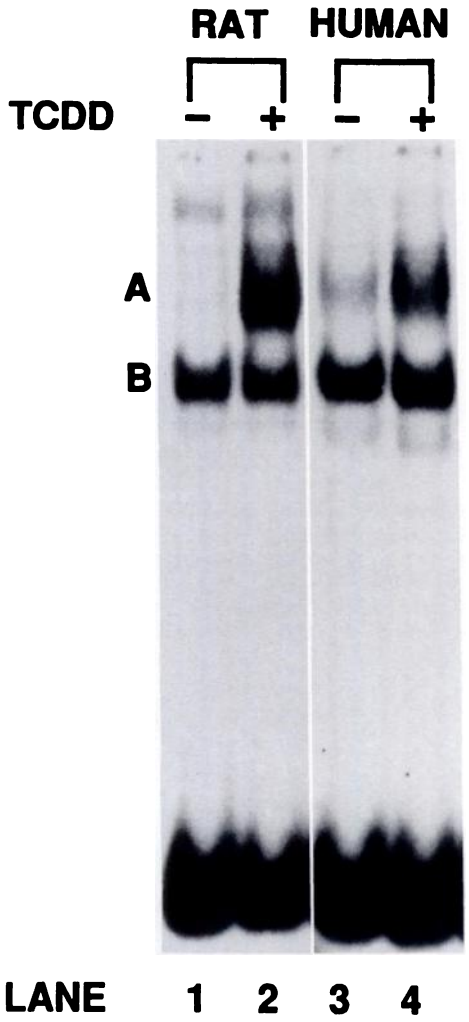


**Fig. 1.** Comparison of DNA-binding complexes from *in vitro* transformed rat hepatic cytosolic AhR and human LS180 cell cytosolic AhR. After 2 hr at 22°, untreated or TCDD-treated (20 nM) rat liver cytosol or human LS180 cell cytosol was mixed with $^{32}$P-labeled DRE oligonucleotide and the formation of protein-DNA complexes was analyzed by gel retardation.

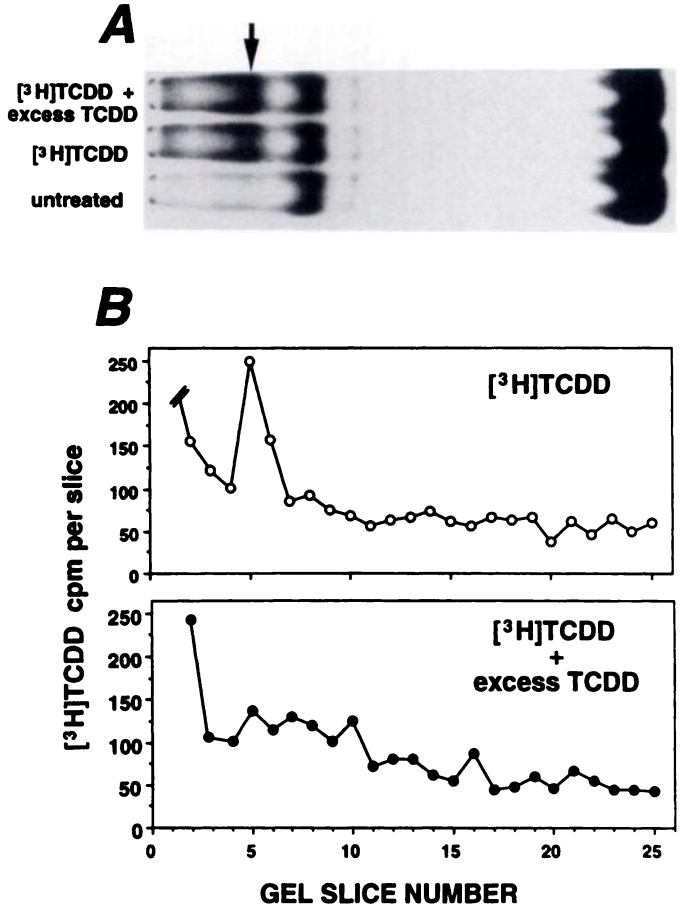


**Fig. 2.** Co-migration of [$^3$H]TCDD with the inducible protein-DNA complex. A, Human LS180 cell cytosol was incubated with dimethylsulfoxide (untreated), [$^3$H]TCDD, or [$^3$H]TCDD and a 10-fold molar excess of unlabeled TCDD, for 2 hr at 22°; protein-[$^{32}$P]DRE complexes were resolved by gel retardation. B, Cytosol was incubated with [$^3$H]TCDD or [$^3$H]TCDD plus a 10-fold molar excess of TCDD for 2 hr at 22°, followed by incubation with unlabeled DRE oligomer, and samples were resolved by gel retardation. Each lane of the gel was sliced into 5-mm sections, and the amount of [$^3$H]TCDD in each slice was determined by liquid scintillation. The *arrow* indicates the position of the TCDD-inducible protein-DNA complex.

same position in the acrylamide gel as that of rat complex A was detected in TCDD-treated cytosol, and a noninducible protein-DNA complex was detected in both TCDD-treated and untreated rat and human cytosols (Fig. 1, *lanes 3* and *4*). Occasionally, a small amount of complex A was detected in untreated human cytosol (Fig. 1, *lane 3*); it could result from the binding to DRE oligomer of AhR transformed by some endogenous ligand present in the cytosol in the absence of TCDD and/or it may represent a noninducible protein-DNA complex that migrates to a position similar to that of complex A.

Similar TCDD-inducible protein-DNA complexes were also obtained using cytosols prepared from Hepa-1 cells (mouse hepatoma), A431 cells (human squamous cell carcinoma), and MCF7 cells (human breast carcinoma). In all instances *in vitro* transformation of AhR with TCDD resulted in the formation of a single TCDD-inducible protein-DNA complex that migrated to the same position as did rat hepatic TCDD-AhR-DRE complexes (data not shown).

The presence of TCDD in complex A was demonstrated using [$^3$H]TCDD and nonradiolabeled DRE oligomer (Fig. 2). Cytosolic LS180 AhR was transformed by incubation for 2 hr at 22° with 20 nM [$^3$H]TCDD, in the absence or presence of a 10-fold molar excess of nonradiolabeled TCDD. The treated cytosol was then analyzed by gel retardation using [$^{32}$P]DRE oligomer, protein-DNA complexes were identified by autoradiography (Fig. 2A) or by gel retardation analysis using nonradiolabeled DRE oligomer, and the distribution of [$^3$H]TCDD throughout the lane was quantitated by liquid scintillation counting of gel slices (Fig. 2B). A single peak of [$^3$H]TCDD, corresponding to the position of complex A (Fig. 2A), was detected in samples incubated with nonradiolabeled DRE oligomer (Fig. 2B) and was not detected in samples where the specific binding of [$^3$H]TCDD was competed away by excess nonradiolabeled TCDD. These results are comparable to those previously described using radiolabeled TCDD (13) or TCDD agonist (9, 12) and confirm the presence of the AhR in the human TCDD-inducible protein-DNA complex.

**Ligand specificity and nucleotide specificity of AhR-DNA binding.** Formation of the AhR-TCDD-DRE complex was dependent upon AhR occupancy by AhR agonists (Fig. 3), because incubation of cytosol with 20 nM TCDD or MC resulted

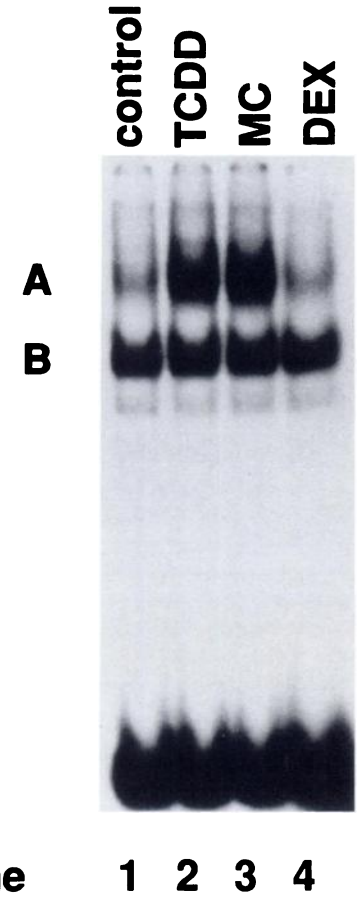


Fig. 3. Ligand specificity of the inducible signal. After 2 hr at 22°, untreated cytosol or cytosols incubated with 20 nM TCDD, 20 nM MC, or 20 nM dexamethasone (*DEX*) were mixed with the $^{32}$P-labeled DRE oligonucleotide and protein-DNA complexes were then analyzed by gel retardation. *A*, Position of the TCDD-inducible protein-DNA complex; *B*, Position of the noninducible protein-DNA complex.

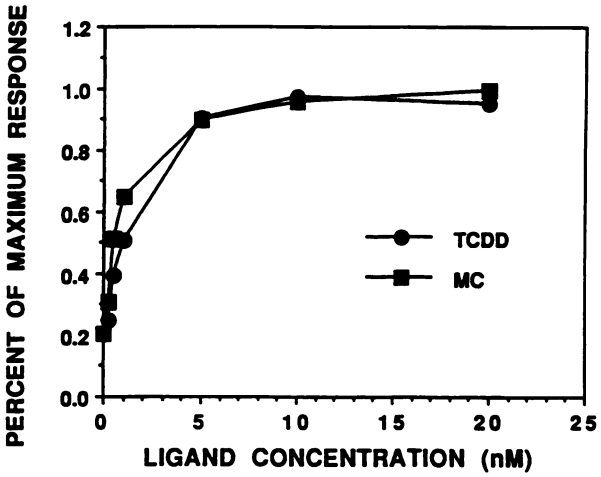


Fig. 4. Dose dependence of the ligand-inducible signal. After 2 hr at 22°, cytosols treated with various concentrations of TCDD or MC (0–20 nM) were mixed with the $^{32}$P-labeled DRE oligonucleotide, and then protein-DNA complexes were analyzed by gel retardation. The ligand-AhR-DRE complexes were excised from the dried gel and the amount of radioactivity in each ligand-AhR-DRE complex was determined by liquid scintillation.

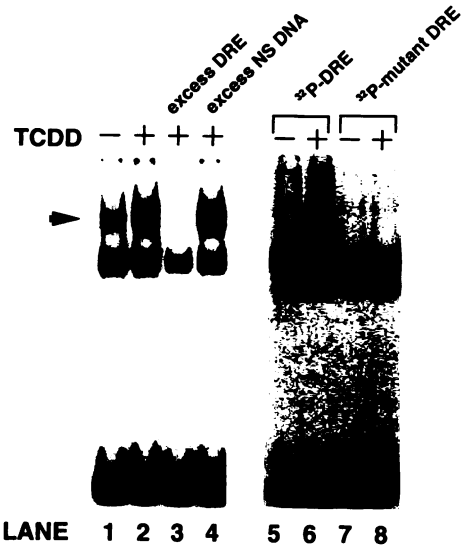


Fig. 5. DNA specificity for the binding of transformed TCDD-AhR complex. *Lanes 1–4*, after 2 hr at 22°, untreated or TCDD-treated cytosol was mixed with the $^{32}$P-labeled DRE oligonucleotide, followed by the addition of HEGD buffer (*lanes 1* and *2*), 100-fold excess nonradiolabeled DRE (*lane 3*), or 100-fold excess nonspecific DNA (*lane 4*), and was analyzed by gel retardation. *Lanes 5–8*, after 2 hr at 22°, untreated or TCDD-treated cytosol was mixed with the $^{32}$P-labeled DRE (*lanes 5* and *6*) or $^{32}$P-labeled mutant DRE oligonucleotide (*lanes 7* and *8*) and then analyzed by gel retardation. The *arrow* indicates the position of the TCDD-inducible protein-DNA complex.

in formation of an inducible protein-DNA complex; incubation with dexamethasone (a non-AhR agonist) did not produce an inducible protein-DNA complex. Additionally, incubation of cytosol with increasing concentrations of TCDD or MC ranging from 0.1 to 20 nM resulted in a concentration-dependent increase in specific protein-DNA complex formation (Fig. 4), with maximum DNA binding being induced by 5 nM TCDD or MC and an $EC_{50}$ of $\approx$1 nM ligand.

The DNA-binding specificity of transformed human TCDD-AhR complex is shown in Fig. 5. Formation of complex A was inhibited by the addition of excess nonradiolabeled DRE oligomer to the incubation mixture (Fig. 5, compare *lanes 2* and *3*), but not by an excess of nonspecific DNA that lacked a DRE sequence (Fig. 5, compare *lanes 2* and *4*). The nonspecific DNA was a 66-bp *Eco*R1-*Hin*dIII DNA fragment isolated from the plasmid pGEM3.79; it spans the region from base −1248 to −1302 upstream of the mouse cytochrome *cyp1a1* transcription start site (24) and contains 12 bp of pGEM3 polylinker. Thus, the transformed human AhR binds specifically to DRE-containing DNA. Sequence specificity of DNA binding was also examined in experiments using a mutant DRE oligonucleotide (Fig. 5, *lanes 5–8*) that contains a single nucleotide transversion substitution within the DRE core consensus sequence (... TTGCGTG ...mutated to ... TTGAGTG ...). We previously observed that guinea pig hepatic AhR binds to this mutant DRE with a 2000-fold lower affinity than to the wild-type DRE.[2] Unlike that found with $^{32}$P-labeled wild-type DRE oligomer, no TCDD-inducible protein-DNA complex was observed using the $^{32}$P-radiolabeled mutant DRE (Fig. 5, compare *lanes 6* and *8*), with human cytosol. Thus, a single nucleotide substitution within the core DRE consensus sequence significantly reduced the binding of transformed human AhR, suggesting that human AhR recognition of DNA is similar to

[2] E. F. Yao and M. S. Denison, unpublished observations.

rodent AhR recognition of DNA. In a more extensive mutational analysis of DRE sequence-specific binding, we have observed no difference between the DNA sequence specificity of human AhR and rodent AhR (25).

**Temperature dependency of AhR transformation/DNA binding.** The temperature dependence of *in vitro* AhR transformation was examined by comparing the amount of TCDD-AhR-DRE complex formed at various times and temperatures (Fig. 6). In contrast to the positive signal observed with cytosol incubated for 2 hr at 22°, no TCDD-inducible protein-DNA complex formation was observed when human cytosol was incubated with TCDD at 4° for 2 or 26 hr before gel retardation analysis. Lack of inducible complex formation at 4° was not due to failure of TCDD to bind to the AhR, because we previously observed that incubation of LS180 cytosol with 20 nM [$^3$H]TCDD for 2 hr at 4° resulted in maximal ligand binding to human AhR (18). Prolonged incubation of cytosol at 4° also did not result in any significant decrease in measurable [$^3$H]TCDD specific binding (data not shown). Further, the quantitative similarity in the amount of TCDD-AhR-DRE complex formed in samples incubated for 24 hr at 4° and subsequently warmed to 22° for 2 hr (Fig. 6, *lanes 5* and *6*), compared with samples incubated for only 2 hr at 22° (Fig. 6, *lanes 1* and *2*), indicates that human DRE-binding TCDD-AhR complexes appear to be stable for 24 hr at 4°. In contrast, hepatic cytosolic AhR from guinea pig or rat did not demonstrate this apparent absolute temperature dependency (Table 1). Although the absolute amount of transformation as measured by DNA binding varied between species, inducible protein-DNA complex formation occurred after 2 hr at 4° in all species except human.

We previously observed that liganded AhR binds to the DRE in a 1 to 1 ratio (11); consequently, comparison of the amount of [$^3$H]TCDD specific binding with the amount of DRE bound

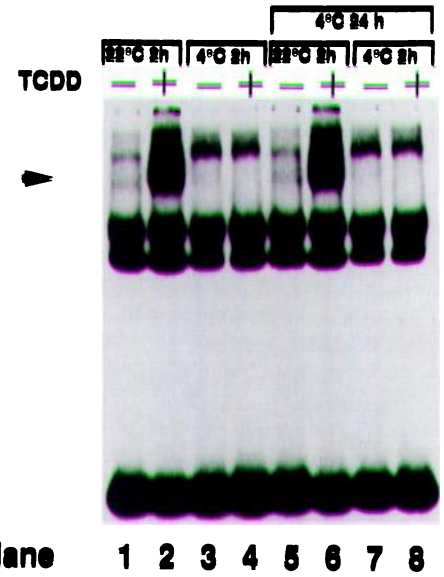


**Fig. 6.** Temperature dependence of *in vitro* transformation of human AhR. *Top*, cytosols (untreated or TCDD-treated) were analyzed by gel retardation. *Lanes 1* and *2*, cytosol was incubated with or without TCDD at 22° for 2 hr; *lanes 3* and *4*, cytosol was incubated with or without TCDD at 4° for 2 hr; *lanes 5–8*, cytosol was incubated with or without TCDD at 4° for 24 hr and then incubated for an additional 2 hr at 22° (*lanes 5* and *6*) or 4° (*lanes 7* and *8*). The *arrow* indicates the position of the TCDD-inducible protein-DNA complex.

TABLE 1

**Effect of temperature on hepatic TCDD-inducible protein-DRE complex formation**

| Species | Amount of specific TCDD-AhR-DRE complex bound[a] | | Relative amount bound[b] |
|---|---|---|---|
| | 22° | 4° | |
| | *cpm* | | % |
| Hartley guinea pig | 12,519 ± 1,703 | 12,922 ± 204 | 103 ± 16 |
| Sprague-Dawley rat | 8,326 ± 955 | 1,970 ± 395[c] | 24 ± 5 |
| Human | 2,147 ± 356 | 55 ± 75[c] | 2.8 ± 7 |

[a] Values indicate the amount (mean ± standard deviation) of specific TCDD-inducible protein-DRE complex formation at the indicated temperatures (three or four experiments).
[b] Ratio of inducible complex formed at 4° compared with 20°.
[c] Values are significantly different from those observed at 22° ($p < 0.01$), as determined by the Student *t* test.

TABLE 2

**Species variation in hepatic AhR transformation and DRE binding**

| Species | AhR[a] | DRE bound | DRE-bound AhR |
|---|---|---|---|
| | *fmol/mg* | *fmol/mg* | % |
| Hartley guinea pig | 43.2 ± 4.5 | 47.6 ± 3.0 | 110.1 ± 6.9 |
| Sprague-Dawley rat | 51.0 ± 2.0 | 25.4 ± 4.9 | 49.8 ± 9.6 |
| Human[b] | 260 ± 30.0 | 11.4 ± 5.1 | 4.4 ± 2.0 |

[a] AhR concentration represents the number of fmol of [$^3$H]TCDD specific binding/mg of cytosolic protein. Values for guinea pig and rat were determined by HAP binding assay and the value for human was determined by sucrose density gradient analysis. Values represent the mean ± standard deviation of at least three determinations.
[b] Cytosol was from the human intestinal epithelial cell line LS180.

in the TCDD-inducible complex should allow quantitative estimation of the relative degree of AhR transformation (Table 2). These data indicate a highly variable degree of overall hepatic AhR transformation among species. A high degree of transformation was observed with guinea pig cytosol, whereas less inducible complex was formed with rat. Comparatively, human cytosolic TCDD-AhR complexes cannot efficiently transform and/or bind to the DRE oligonucleotide *in vitro*.

We previously observed (18) that *in vivo* transformation of human AhR results in its conversion from a form sedimenting at 9 S ($M_r$ 285,000) to a form sedimenting at 6.2 S ($M_r$ 175,000). To examine whether temperature-dependent transformation *in vitro* results in an analogous change in AhR sedimentation, we carried out gradient centrifugation analysis of [$^3$H]TCDD-AhR complexes formed at 4° and 22° (Fig. 7).

Incubation of [$^3$H]TCDD-treated LS180 cytosol for 2 hr at 22° resulted in a significant loss of AhR ([$^3$H]TCDD specific binding), compared with incubation for 2 hr at 4° (Fig. 7, compare A and B). Because unoccupied human AhR is relatively unstable (15), perhaps the decrease in [$^3$H]TCDD specific binding was due to loss of ligand-binding ability of unoccupied AhR during the 22° incubation period. However, preincubation of LS180 cytosol with [$^3$H]TCDD for 2 hr at 4° followed by 2 hr at 22° also resulted in decreased amounts of AhR, similar to that observed with 22° incubation alone (Fig. 7, compare C and B). Thus, AhR sedimentation coefficient(s) for transformed AhR could not be accurately determined under these conditions. The data shown in Fig. 7 are from parallel incubations of aliquots from one cytosol preparation; the amount of 9 S AhR depicted in Fig. 7A is 280 fmol/mg of cytosolic protein, in Fig. 7B is 30 fmol/mg of cytosolic protein, and in Fig. 7C is 75 fmol/mg of cytosolic protein.

Because *in vitro* transformation does not occur at 4°, it was

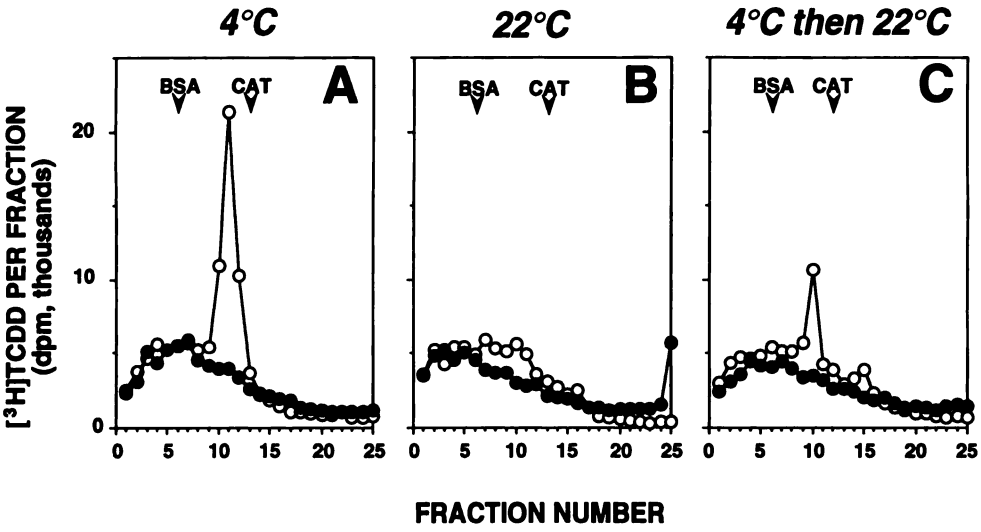


Fig. 7. Sucrose density gradient analysis after incubation at 4° and 22°. Aliquots of the same cytosol were incubated with [$^3$H]TCDD in the absence or presence of a 100-fold excess of nonradiolabeled TCDF at various temperatures and then analyzed by sucrose density gradient centrifugation. *Arrowheads*, positions of [$^{14}$C]BSA (4.4 S) and [$^{14}$C]catalase (*CAT*) (11.3 S), added as internal sedimentation markers. A, Cytosol incubated at 4° for 2 hr; B, cytosol incubated at 22° for 2 hr; C, cytosol incubated at 4° for 2 hr and then warmed to 22° for an additional 2 hr.

possible to determine the time of ligand-dependent transformation independently of the time for ligand binding (Fig. 8). Cytosols were incubated with 20 nM TCDD for 2 hr at 4°; the time was sufficient to allow maximum ligand binding to the AhR but the low temperature prevented ligand-dependent transformation. After this incubation period, the incubation temperature was increased to 22° and at various times thereafter aliquots were incubated with DNA for a total of 10 min [5 min with poly(dI-dC) followed by 5 min with [$^{32}$P]DRE oligonucleotide] and applied to the polyacrylamide gel. Compared with the untreated (no TCDD) control, small but detectable amounts of TCDD-AhR-DRE complexes were seen at the earliest practical time point (10 min at 22°), and amounts increased with longer times of incubation at 22°. Maximum complex formation appeared to occur after about 60 min at 22° and remained constant for at least 180 min. Parallel samples incubated with [$^3$H]TCDD and assayed for 9 S AhR content

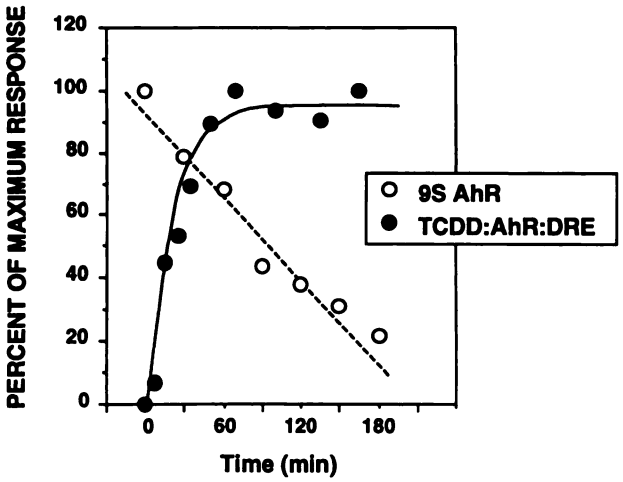


Fig. 8. Time dependence for *in vitro* transformation of human AhR. LS180 cytosol was incubated for 2 hr at 4° with 20 nM TCDD. After 2 hr aliquots were taken, warmed to 22°, and analyzed by gel retardation at various times after warming; the TCDD-AhR-DRE complexes were excised from the dried gel and the amount of radioactivity in each TCDD-AhR-DRE complex was determined by liquid scintillation. Parallel samples incubated with [$^3$H]TCDD in the presence or absence of 100-fold excess TCDF were analyzed by sucrose density gradient to determine the concentration of 9 S AhR.

([$^3$H]TCDD specific binding) showed a steady decrease in 9 S receptor content such that at 60 min about 65% of the 9 S AhR remained, whereas only about 25% was detectable at 150 min.

**Binding of transformed human AhR to DNA- or DRE-Sepharose.** Previous studies (7, 26) demonstrated that transformed rodent AhR can exist as two distinct DNA-binding forms. To determine whether human AhR behaves similarly, we examined the specific binding of transformed human AhR to DNA-Sepharose and DRE-Sepharose. Chromatography of transformed [$^3$H]TCDD-bound AhR complexes from LS180 cell cytosol resulted in three distinct radioactive peaks (Fig. 9). The majority of radioactivity eluted in the flow-through fraction (representing material that did not interact with the column), whereas the remaining radioactivity eluted as two distinct peaks, i.e., peak 1 (at low salt concentration) and peak 2 (at high salt concentration). Further characterization of peak 1 and peak 2 (Fig. 9) indicated that peak 1 represents nonspecific binding and peak 2 represents specific binding to the column, because (i) transformation of AhR by [$^3$H]TCDD in the presence of excess nonradiolabeled competitor abolished the binding of peak 2 but not peak 1 to batch DNA-Sepharose and DRE-Sepharose columns (Fig. 9, A and B) and (ii) peak 1 but not peak 2 was detected in cytosol incubated with [$^3$H]TCDD at 4° (nonpermissive temperature for transformation) (Fig. 9C). In addition, recycling of fractions from peak 2 to sucrose density gradient analysis showed the presence of a 6 S [$^3$H]TCDD-binding AhR complex (Fig. 10), characteristic of transformed AhR (18), whereas no specific binding was detected in fractions recycled from the flow-through fractions or from peak 1. Although quantitatively similar peaks were also obtained when transformed [$^3$H]TCDD-AhR was chromatographed on DNA-Sepharose or on DRE-Sepharose (Fig. 9, A and B), peak 2 eluted at about 420 mM NaCl from DRE-Sepharose, compared with about 320 mM NaCl from DNA-Sepharose. In both instances the radioactivity associated with the nonspecific binding to the column eluted at about 80–90 mM NaCl. These data indicate that the affinity of binding of transformed human AhR was higher to DRE-containing DNA than to bulk nonspecific DNA. Thus, in contrast to the results observed by Henry *et al.* (7), only one form of human AhR ([$^3$H]TCDD specific binding peak) could be bound and subsequently eluted from DNA- or DRE-Sepharose.

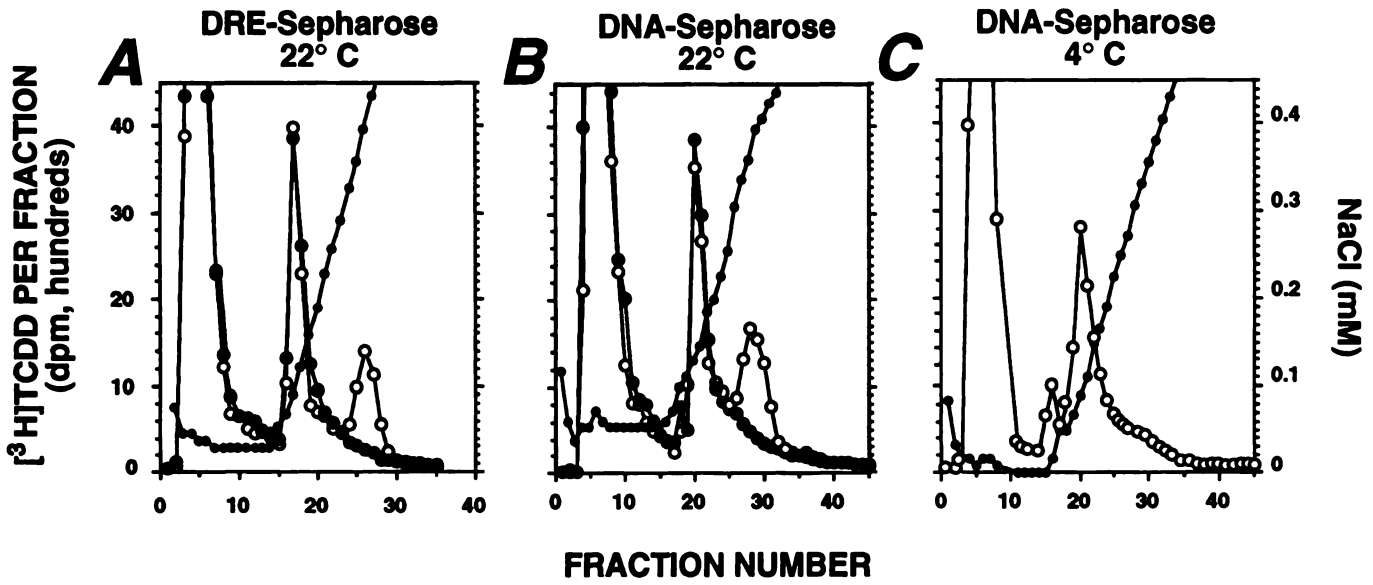


**Fig. 9.** DNA-Sepharose and DRE-Sepharose chromatography of transformed cytosolic AhR. LS180 cytosol (≈10 mg/ml cytosolic protein, 2 ml total) was incubated with [$^3$H]TCDD in the absence or presence of excess nonradiolabeled competitor for 2 hr at 22° and then applied directly to a DNA-Sepharose column. The column was washed with 2 column volumes of HEGD containing 50 mM NaCl, followed by 50 ml of a NaCl gradient (50 mM to 1000 mM in HEGD). Fractions were collected, and the amounts of radioactivity and conductivity were determined. A, Cytosol was incubated at 22° for 2 hr with 20 nM [$^3$H]TCDD (○) or [$^3$H]TCDD plus 100-fold excess TCDF (●) and applied to a DRE-Sepharose column. B, Cytosol was incubated at 22° for 2 hr with 20 nM [$^3$H]TCDD (○) or [$^3$H]TCDD plus 100-fold excess TCDF (●) and applied to a calf thymus DNA-Sepharose column. C, Cytosol was incubated at 4° for 2 hr with 20 nM [$^3$H]TCDD (○) and applied to a calf thymus DNA-Sepharose column.

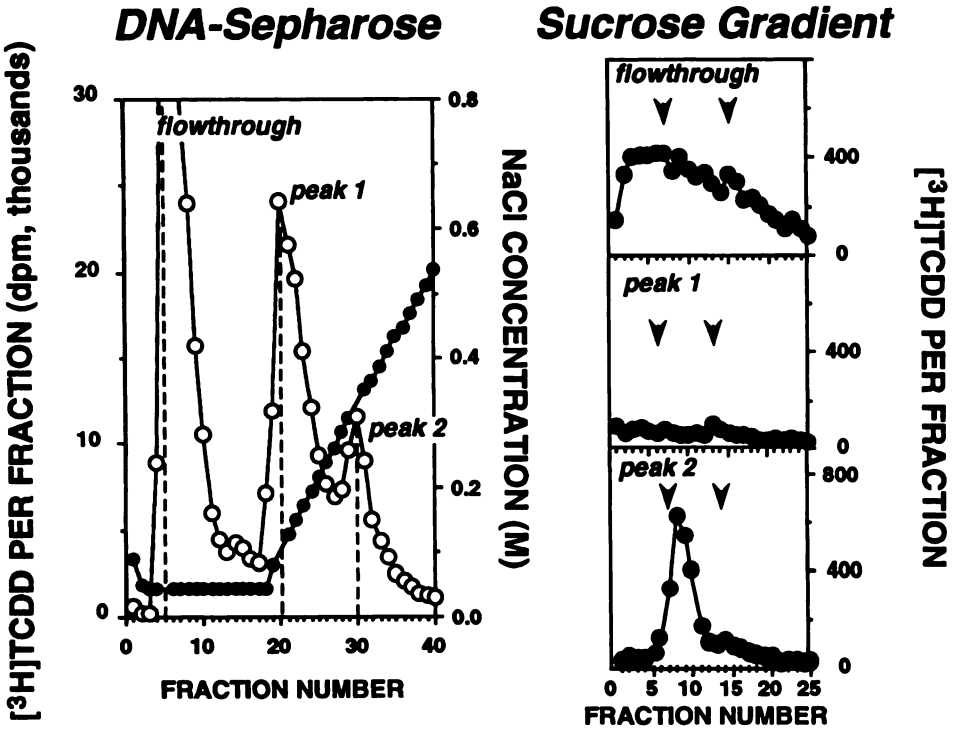


**Fig. 10.** Sequential analysis of *in vitro* transformed AhR by DNA-Sepharose chromatography followed by sucrose gradient sedimentation. *In vitro* transformed AhR was applied to DNA-Sepharose columns and then eluted as described in Materials and Methods. *Dashed lines* in the DNA-Sepharose profile indicate the elution position of fractions that were removed and transferred onto sucrose gradients. Sucrose gradients for flow-through fractions were prepared in HEGD only, sucrose gradients for peak 1 fractions were prepared in 0.1 M NaCl, and sucrose gradients for peak 2 fractions were prepared in 0.32 M NaCl. *Arrowheads*, positions of [$^{14}$C]formaldehyde-labeled BSA (4.4 S, about fraction 7) and catalase (11.3 S, about fraction 14), included in each gradient as internal sedimentation markers.

## Discussion

Many biochemical and toxicological actions of TCDD are mediated by the AhR, a soluble, intracellular, multimeric protein complex that binds to DNA and stimulates gene expression in a ligand-dependent manner (6–8, 27–29). We previously examined the physiochemical properties of the AhR from human LS180 cells (18) and now extend those observations to examine and characterize transformation and specificity of DNA binding of human AhR.

Using gel retardation analysis, we have shown that human LS180 cytosolic AhR can transform *in vitro* to a form that specifically binds to a DRE-containing oligonucleotide, in a ligand-dependent manner. The presence of the AhR in the TCDD-inducible protein-DNA complex was demonstrated by the co-migration of a [$^3$H]TCDD-specific binding component in the same position in the gel. The specificity of DRE binding of human AhR was demonstrated using DRE-containing and non-DRE-containing competitor DNA fragments, as well as a

mutant DRE oligonucleotide. More exhaustive DRE mutational analysis revealed no significant difference in nucleotide-specific DRE binding of human AhR, compared with that determined for other animal species (25). These results confirm that the inducible protein-DNA complex truly represents specific binding of transformed human TCDD-AhR complex to the DRE.

The affinity of a given compound for the AhR is a major determinant of the potency of that compound as an inducer of *CYP1A1* (27, 30). Recently, Cuthill *et al.* (31) postulated that the difference in biological potency between halogenated (TCDD) and nonhalogenated [MC and benzo(*a*)pyrene] compounds observed *in vivo* was due in part to the differential ability of these compounds to transform AhR. They demonstrated, in the rodent cell line Hepa-1, that the relative ability of TCDD and MC to induce DRE-specific DNA-binding *in vitro* was directly proportional to their AhR binding affinity (determined *in vitro*) and was correlated with their potencies *in vivo* (with TCDD being several thousand times more potent than MC). In contrast, in our experiments the relative biological potency of TCDD and MC as inducers of *CYP1A1* in human cells is not well correlated with their affinity for the AhR or with their ability to induce AhR transformation. There was no significant difference between the AhR binding affinity of TCDD and MC in human LS180 cell cytosol *in vitro* ($K_d$ = 5.2 nM and 5.3 nM, respectively) (18), nor was there any dose-dependent difference in TCDD-inducible protein-DNA complex formation with each compound (Fig. 3). However, TCDD is a significantly more potent inducer of *CYP1A1*-dependent aryl hydrocarbon hydroxylase activity (32, 33). Thus, at least in human cells, the difference in biological potency between TCDD and MC is not simply due to a difference in their apparent affinity for the AhR or to any apparent difference in their ability to induce AhR transformation. The difference between TCDD and MC may be related more to differences in their cellular distribution and/or rate of metabolism.

A significant amount of information is available regarding the binding of ligand to the AhR; however, little is known about the events that occur during the transformation of AhR to its DNA-binding form. It is clear that AhR transformation is ligand dependent and involves at least the loss of an hsp90 molecule(s) from the liganded cytosolic AhR complex. Analogous studies with steroid hormones and their receptors would suggest that the loss of heat shock protein hsp90 is induced by a ligand-dependent conformational change in the receptor protein(s). Transformation of human AhR is accompanied by a decrease in sedimentation and apparent molecular weight from 8–10 S and ~285,000 to 6 S and ~176,000 (18), respectively. Because the molecular weight of the ligand-binding subunit of the human AhR is about 110,000 (18, 19, 34), the transformed AhR must represent a complex of at least two proteins. Several recent studies have, in fact, demonstrated that transformed rat AhR exists as a heterodimer containing both ligand-binding and non-ligand-binding subunits (29, 35). More recently, Hoffman *et al.* (36) isolated a human cDNA clone that encodes a non-TCDD-binding 80-kDa protein, the AhR nuclear translocation factor, which appears to be a component of the nuclear AhR complex (37). Thus, the transformed human AhR appears to be a heterodimer, consisting of at least one ligand-binding subunit and one AhR nuclear translocation factor subunit. Whether other proteins or factors are present in the DNA-binding form of the AhR remains to be determined.

Transformed human TCDD-AhR complexes bound to DNA-Sepharose or DRE-Sepharose columns can be eluted as a single specific binding peak (peak 2) by high salt (0.3–0.4 M NaCl). Analysis of peak 2 on sucrose density gradients showed a single 6 S binding species, characteristic of transformed heteromeric human AhR (18). In addition, the lack of specific [$^3$H]TCDD binding to Sepharose alone (data not shown) confirmed that transformed TCDD-AhR complex bound specifically to the DNA rather than to the column matrix itself. In contrast to our results, Henry *et al.* (7) reported that two distinct rodent cytosolic [$^3$H]TCDD-specific binding peaks can be salt-eluted from calf thymus DNA-Sepharose columns. Their subsequent analysis revealed that one of these peaks represented ligand-occupied AhR monomer, which bound to DNA with relatively low affinity (eluted from the column with 0.19 M NaCl), and the second represented a ligand-occupied multimeric AhR form, which bound to DNA-Sepharose with relatively higher affinity (eluted with 0.28 M NaCl). More recently, they demonstrated that of these two forms only the "high affinity" multimeric form could specifically bind to a DRE-containing oligonucleotide (29). Although the high affinity multimeric AhR form identified by Gasiewicz and co-workers (7) appears to be comparable to our single specific binding peak (peak 2), in no instance have we observed a lower affinity human AhR form eluting from DNA-Sepharose or DRE-Sepharose. It is possible, however, that a small amount of a lower affinity form exists but is masked by the large nonspecific binding peak (peak 1) observed with human cytosol.

Temperature-dependent AhR transformation and nuclear translocation have been observed in cells in culture from a variety of species and tissues. In these studies [$^3$H]TCDD-AhR complexes failed to accumulate within the nucleus of cells maintained at 4°, whereas those at 20° showed significant nuclear AhR accumulation (5). Decreased AhR transformation/nuclear translocation (DNA binding) at low temperatures may result from inhibition of a critical (temperature-dependent) step in the transformation process, such as a ligand-induced protein conformational change, a phosphorylation or dephosphorylation event, the association or dissociation of AhR and other factors, and/or some other as yet undefined event.

In contrast to observations with rodent cytosol (Ref. 7 and Table 1), transformation of human AhR *in vitro* requires not only ligand but also elevated temperature, similar to that observed in cells in culture. Although incubation of LS180 cell cytosol at low temperature (4°) prevents any TCDD-AhR-DRE complex formation, it does not adversely affect binding of ligand to AhR, nor is there any apparent decrease in AhR sedimentation, indicative of AhR dissociation or transformation. Taken together, these results suggest that AhR ligand binding and transformation are independent events (i.e., ligand binding is an essential first step but it is not sufficient for transformation). Furthermore, because transformation of human AhR is exceptionally temperature dependent, our results suggest that *in vitro* transformation of human AhR, unlike rodent AhR, could be a useful model for examining the temperature-dependent mechanism of AhR transformation to its DNA-binding form in greater detail.

We previously observed that human AhR is extremely labile and is rapidly inactivated at elevated temperature (15). Al-

though increasing the temperature to 22° allowed detection of transformed TCDD-AhR complexes by gel retardation analysis, a significant amount of AhR complex was apparently inactivated (as determined by [$^3$H]TCDD specific binding). In addition, a further increase in temperature (to 37°) resulted in the total loss of the TCDD-inducible protein-DNA complex (data not shown), indicating that the transformed human AhR was less stable at elevated temperature. These results suggest that occupied human AhR, like unoccupied AhR, is extremely labile at elevated temperature, and this may help explain the low level of human AhR reported by other investigators (38).

The inactivation of human AhR and formation of the TCDD-AhR-DRE complex at 22° may, at first, seem contradictory, in that an increase in protein-DNA complex formation occurs concomitantly with AhR inactivation. However, comparison of the time course of TCDD-AhR-DRE complex formation with the amount of [$^3$H]TCDD specific binding in the 9 S region (Fig. 8) shows that complex formation was maximal by 1 hr, whereas 9 S AhR progressively decreased throughout the 3-hr experimental time period. Maximal DNA binding by human AhR occurred by the time that 9 S AhR decreased by only 30%. These results, combined with the data in Table 2, suggest that very few human TCDD-AhR complexes can transform and/or bind to the DRE, although those that do so transform and bind rapidly and are apparently stable at 22° for at least 3 hr. The low level of human TCDD-AhR-DRE complex formation may result from the extreme lability of human AhRs and/or the presence or absence of additional "factors" that may modulate AhR transformation and DNA binding. Recent studies with steroid hormone receptors revealed the presence of nuclear factors necessary for high affinity DNA binding of transformed ligand-receptor complexes (39). It is unknown whether similar factors exist that modulate AhR functionality. Additionally, it could be argued that the majority of human AhR does indeed transform but specifically binds to a DNA sequence distinct from the DRE. Given our DNA- and DRE-Sepharose chromatography results, which indicate no significant difference in the amount of AhR ([$^3$H]TCDD specific binding) eluting from DRE-Sepharose versus DNA-Sepharose (which should bind all transformed AhRs irrespective of their DNA sequence preference), we think this possibility unlikely. The *in vitro* results presented here imply that very little of the total measurable human cytosolic AhR can transform and/or bind to DNA. Interestingly, recent studies examining cytosolic and nuclear AhR concentrations in LS180 cells incubated in the absence or presence of [$^3$H]TCDD revealed that <10% of total cytosolic AhR present in these cells is found associated with the nucleus after TCDD exposure under apparently optimal conditions.[3] Thus, the *in vitro* results obtained here are in reasonably good agreement with those obtained in intact cells. Whether this, in addition to the reduced ligand binding affinity of human AhR, plays a role in the reduced TCDD responsiveness of humans remains to be determined.

The absolute temperature dependence of transformation of human AhR, coupled with the utility of gel retardation analysis to measure binding of transformed AhR to its DNA recognition site, provides us with a tractable system for isolation and characterization of factors necessary for transformation and DRE binding of human AhR.

[3] P. A. Harper and J. V. Giannone, unpublished observations.

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