Binding of Transformed Ah Receptor Complex to a Dioxin Responsive Transcriptional Enhancer: Evidence for Two Distinct Heteromeric DNA-Binding Forms†

Hollie I. Swanson,‡,§ Kathryn Tullis,‖ and Michael S. Denison*,‖

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, andDepartment of Environmental Toxicology, University of California, Davis, California 95616-8588

Received June 3, 1993; Revised Manuscript Received August 27, 1993®

ABSTRACT: Guinea pig hepatic Ah receptor (AhR) complex was transformed *in vitro* to its DNA-binding form by incubation with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin). Transformed TCDD–AhR was covalently cross-linked by UV-irradiation to a bromodeoxyuridine-substituted oligonucleotide containing its specific DNA recognition site, the dioxin responsive element (DRE). Denaturing gel electrophoresis and autoradiography identified four TCDD-inducible protein–DNA complexes, with molecular masses of approximately 97, 105, and 115 kDa and a somewhat broader complex at 247 kDa. The 247-kDa complex appears to contain two distinct protein–DNA complexes of approximately 232 and 256 kDa and represents two proteins covalently cross-linked to a single DRE oligonucleotide, while the 97, 105, and 115-kDa complexes represent single protein–DRE cross-links. UV cross-linking to DRE oligonucleotides containing variable numbers of BrdU residues revealed that the 105-kDa protein, identiied as the AhR ligand-binding subunit by photoaffinity labeling with a radioiodinated AhR agonist, cross-links to the DRE core consensus (5′-GCGTG-3′); the 97- and 115-kDa non-ligand-binding proteins differentially cross-link immediately 5′-ward of the core. Overall, our results not only demonstrate that the critical protein–DNA contacts which occur between the AhR complex and the DRE are made primarily by the ligand-binding subunit but also indicate that the AhR complex exists as two distinct heteromeric DNA-binding forms, containing one 105-kDa ligand-binding subunit and either one 115- or one 97-kDa non-ligand-binding subunit.

Exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD,[1] dioxin) and related halogenated aromatic hydrocarbons results in a wide variety of species- and tissue-specific toxic and biological responses (Poland & Knutson, 1982; Safe, 1986). Many of these responses appear to be mediated by a solute intracellular receptor protein, the Ah receptor (AhR), which binds these compounds with high affinity (Poland & Knutson, 1982; Safe, 1986; Whitlock, 1986; Poland et al., 1986). The AhR, like steroid hormone receptors, is a ligand-dependent transcription factor that modulates gene expression through a high-affinity interaction with a specific DNA enhancer element upstream of TCDD-responsive genes (Whitlock, 1986; Whitlock et al., 1990; Carson-Jurica et al., 1990; Beato, 1991).

The induction of cytochrome P4501A1,[2] a microsomal enzyme important in the metabolic detoxification and activation of many environmental chemicals (Poland & Knutson, 1982; Safe, 1986), has been used as a model system to examine the molecular mechanisms by which TCDD and the AhR modulate gene expression. Following binding of TCDD to the AhR, the TCDD–AhR complex undergoes a poorly understood process termed transformation[3] that involves, at least in part, the dissociation of hsp90 (a heat shock protein of 90 kDa) and the subsequent conversion of the TCDD–AhR complex to its DNA-binding form (Perdew, 1988; Denis et al., 1988; Henry et al., 1989). Transformed TCDD–AhR complexes translocate into the nucleus and bind with high affinity to specific DNA sequences (termed dioxin responsive elements, DREs) adjacent to the CYPIA1 gene (Denison & Yao, 1991; Fujisawa-Sehara et al., 1987; Denison et al., 1988a,b). Biochemical and genetic evidence indicates that formation of the TCDD–AhR–DRE complex is a critical step in transcriptional activation of the CYPIA1 gene (Whitlock, 1986, 1990; Legraverend et al., 1982).

We have previously utilized gel retardation analysis to examine the binding of transformed TCDD–AhR complex to the DRE (Denison et al., 1988a,b, 1989; Denison & Yao, 1991; Yao & Denison, 1992). Transformed TCDD–AhR complex recognizes and binds to a core DNA consensus sequence, 5′-GCGTG-3′, specifically and with high affinity (Denison et al., 1989; Denison & Yao, 1991; Shen & Whitlock, 1992; Yao & Denison, 1992). DRE mutagenesis studies have

† This work was supported by the American Cancer Society (Grant CN-4B), the International Life Sciences Institute, the Michigan Agricultural Experiment station, and the California Agriculture Experiment Station.

* Address correspondence to this author at the Department of Environmental Toxicology, Meyer Hall, University of California, Davis, CA 95616. Phone: (916) 752-3879.

‡ Michigan State University.

§ Present address: Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60611.

‖ University of California, Davis.

® Abstract published in *Advance ACS Abstracts,* November 1, 1993.

[1] Abbreviations: AhR, aromatic hydrocarbon receptor; $AIBr_2DD$, 2-azido-3-iodo-7,8-dibromodibenzo-*p*-dioxin; bHLH, basic helix–loop–helix: BrdU, 5-bromo-2′-deoxyuridine 5′-triphosphate; DMSO, dimethyl sulfoxide; DRE, dioxin responsive element; DTT, dithiothreitol; HEDG, 25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

[2] Refer to Nebert et al. (1991) for a complete discussion of cytochrome P-450 enzyme and gene nomenclature.

[3] In this report, we have defined transformation as the process by which the TCDD–AhR complex changes to a form which can bind to DNA with high affinity.

0006-2960/93/0432-12841$04.00/0

indicated the importance of nucleotides both within and adjacent to this core sequence in TCDD–AhR–DRE complex formation (Shen & Whitlock, 1992; Yao & Denison, 1992). UV cross-linking studies (Elferink et al., 1990; Gasiewicz et al., 1991) have demonstrated that transformed rat hepatic TCDD–AhR complex binds to DRE-containing DNA as a heterodimer, consisting of ligand- and non-ligand-binding subunits. Recently, Hankinson and co-workers (Hoffman et al., 1991) have reported the cloning of a gene which encodes a protein [termed the Ah receptor nuclear translocator (arnt)] that appears to be necessary for nuclear localization of the AhR complex. Demonstration of the presence of the arnt protein in the nuclear (transformed) form of the murine AhR complex (Reyes et al., 1991) suggests that the non-ligand-binding component of the transformed AhR complex identified by UV cross-linking (Elferink et al., 1990) is arnt or an arnt-like protein.

The UV cross-linking experiments of Elferink et al. (1990) have suggested that the non-ligand-binding (arnt) protein may be the primary DNA-binding component of the transformed AhR heterodimeric complex; however, direct evidence to support this hypothesis is lacking. Here we have utilized gel retardation analysis, UV cross-linking, and DRE mutagenesis to analyze TCDD–AhR–DRE complex formation in greater detail.

EXPERIMENTAL PROCEDURES

Chemicals. TCDD and TCDBF were obtained from Dr. S. Safe (Texas A&M University); [^{125}I]AIBr$_2$DD (2176 Ci/mmol) was from Dr. G. Perdew (Purdue University); [α-^{32}P]-CT (3000 Ci/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were from New England Nuclear; 5-bromo-2-deoxyuridine 5′-triphosphate (BrdU) was from Pharmacia—LKB Biotechnology Inc. Molecular biological reagents were from Bethesda Research Laboratories and Boehringer Mannheim.

Animals and Preparation of Cytosol. Male Hartley guinea pigs (250–300 g), obtained from the Michigan Department of Public Health (Lansing, MI), and male Sprague-Dawley rats (200 g), obtained from Charles River Breeding Laboratories (Wilmington, DE), were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water. Hepatic cytosol was prepared in HEDG buffer [25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol] as previously described (Denison et al., 1986) and was stored at −80 °C until use. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Synthetic Oligonucleotides and Preparation of BrdU-Substituted DNA. A complementary pair of synthetic oligonucleotides containing the sequences 5′-GATCTGG-CTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGT-TGCGTGAGAAGAGCCA-3′ [corresponding to the AhR-binding site of DRE3 (Denison et al., 1988a) and designated here as the wild-type DRE oligonucleotide] were synthesized, purified, annealed, and radiolabeled with [γ-^{32}P]ATP as previously described (Denison et al., 1988b). Annealing generates a double-stranded DNA fragment with a *Bam*HI cohesive end at its 3′ terminus and a *Bgl*II cohesive end at its 5′ terminus, relative to its normal orientation with respect to the CYPIA1 gene promoter (Jones et al., 1986). For cross-linking studies, the synthetic oligonucleotide 5′-TCGGCTCT-TCTCACGCAACTCCGTGCTC-3′ (also containing the AhR-binding site of DRE3) was annealed to the 8 bp complementary primer, 5′-GAGCACGG-3′. The annealed template was end-filled with the Klenow fragment of DNA polymerase in the presence of 50 μM each of dGTP, dATP, and BrdU and 10 μM [^{32}P]dCTP as described previously (Sambrook et al., 1989) and was designated as the BrdU-substituted wild-type DRE oligonucleotide. Nonradioactive DNA was synthesized by replacing the [^{32}P]dCTP with 50 μM dCTP in the fill-in reaction. Mutant DRE oligonucleotide templates and primers for cross-linking studies were synthesized, annealed, filled-in, and radiolabeled as described above. The resulting wild-type and mutant BrdU-substituted DRE oligonucleotides used in our studies are shown in Figure 1.

Gel Retardation Assay. Gel retardation analysis was performed as previously described (Helferich & Denison, 1991). Briefly, guinea pig hepatic cytosol (16 mg of protein/mL) was incubated with either DMSO (20 μL/mL) or TCDD (20 nM) for 2 h at 20 °C. Cytosol (80 μg of protein) was mixed with 225 ng of poly(dI·dC) and added to an incubation mixture containing 25 mM Hepes, 1 mM EDTA, 1 mM DTT, 10% glycerol (v/v), and 80 mM KCl (final concentration). Following incubation for 15 min at 20 °C, the desired ^{32}P-labeled DNA oligonucleotide (100 000 cpm, ~1 ng) was added to the mixture, and the sample was incubated for an additional 15 min. Samples were loaded onto a 4% nondenaturing polyacrylamide gel, the products separated by electrophoresis, and the gels dried and analyzed following autoradiography.

UV Cross-Linking. The gel retardation assay mixture (containing BrdU-substituted ^{32}P-labeled DNA) was UV-irradiated on ice (four 15-W lamps, >205 nm, UV Products) at a distance of 4–5 cm from the source. Irradiation times are indicated in the figure legends. Samples were then mixed with SDS-loading buffer, heated to 100 °C for 4 min, and then subjected to electrophoresis on 5–10% SDS–polyacrylamide gradient gels (Laemmli, 1970). Protein–DNA complexes were resolved by autoradiography of the dried gel.

Photoaffinity Labeling of AhR. Guinea pig cytosol (5 mg of protein/mL) was incubated with 0.94 nM [^{125}I]AIBr$_2$DD in the absence or presence of a 200-fold molar excess of TCDBF for 30 min at room temperature. After incubation, the samples were cooled on ice for 5 min, mixed with charcoal/dextran (1.0%/0.1% final concentration), and further incubated for 15 min. Following centrifugation at 3000*g* for 10 min, the supernatant was UV-irradiated [two 15-W UV lamps, >302 nm (UV Products)] for 4 min at a distance of 8 cm from the source. The proteins were precipitated overnight at −20 °C with acetone (90% final concentration); the pellets was collected by centrifugation, resuspended in SDS sample buffer, and analyzed by SDS–PAGE. [^{125}I]AIBr$_2$DD–protein complexes were detected by autoradiography of the dried gels.

RESULTS

UV Cross-Linking of Transformed TCDD–AhR Complex to the DRE. UV irradiation of protein–DNA complexes results in low-efficiency cross-linking of proteins to thymine residues with which the proteins are in close contact (Markowitz, 1972). The efficiency of UV-induced cross-linking of nucleic acids to DNA-bound proteins can be significantly increased by the incorporation of BrdU residues into DNA fragments in place of thymine (Ogata & Gilbert, 1977; Hillel & Wu, 1978). Incorporation of BrdU residues into the primed wild-type DRE template produces a double-stranded oligonucleotide which contains three BrdU residues adjacent to and within the DRE AhR-binding core consensus, GCGTG (Figure 1). Gel retardation analysis using ^{32}P-labeled BrdU-substituted wild-type DRE oligonucleotide resulted in the formation of a TCDD-inducible protein–DNA complex

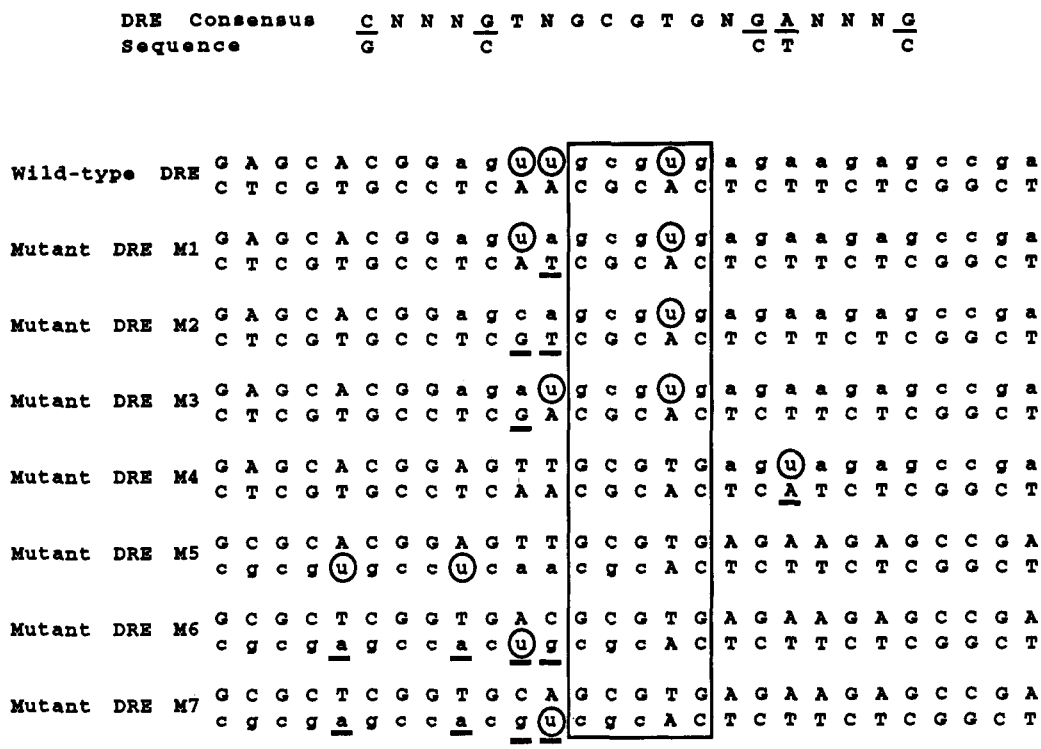

FIGURE 1: Wild-type and mutant DRE oligonucleotides. The conserved and variable nucleotides contained within the murine DRE DNA consensus sequence are shown. The DNA sequence used in these studies corresponds to that we have previously defined as DRE3 (Denison et al., 1988b). BrdU-substituted wild-type and mutant DRE oligonucleotides used in these studies are as indicated. The nonsubstituted wild-type DRE oligomer was identical to the BrdU-substituted wild-type oligomer except that thymine was incorporated instead of BrdU. The double-stranded oligonucleotides were prepared as described under Experimental Procedures. The synthesized, annealed primed DNA is indicated by capital letters, the filled-in nucleotides are shown lower case letters, the BrdU residues are circled, the specific mutations are underlined, and the DRE "core" consensus sequenced is boxed.

comparable to that produced using ^{32}P-labeled unsubstituted wild-type DRE oligomer (Figure 2A). These data indicate that substitution of thymine residues with BrdU does not adversely affect TCDD–AhR–DRE complex formation.

UV irradiation, SDS–PAGE, and autoradiography of incubation mixtures containing control or TCDD-treated cytosol and [^{32}P]BrdU-substituted wild-type DRE oligonucleotide revealed the presence of four TCDD-inducible, UV-cross-linked protein–DNA complexes (Figure 2B). Although several other protein–DNA complexes were also observed, they were not TCDD-inducible and thus represented proteins which bind to DNA nonspecifically or with low affinity. A small amount of each of the proteins labeled in the presence of TCDD was also observed in the absence of added TCDD (Figure 2B). This "background" signal could be predicted, since gel retardation analysis of control cytosol (Figure 2A) also revealed a small amount of a protein–DNA complex which migrated to the same position as the TCDD-inducible complex. These complexes may represent AhR complex transformed after occupancy by an endogenous ligand and/or a small fraction of AhR complex transformed in the absence of ligand. The increased number of protein–DNA complexes observed by UV cross-linking compared to gel retardation analysis likely results from covalent cross-linking of low-affinity protein–DNA complexes that occur in solution but that are not stable enough to be resolved by gel retardation analysis. The DNA specificity of inducible protein–DNA binding was revealed in competition experiments which demonstrated that the TCDD-inducible protein complexes can be effectively eliminated by an excess of unlabeled wild-type DRE oligonucleotide (Figure 2C) but not by an excess of nonspecific DNA oligonucleotide (data not shown).

Comparison of the relative mobilities of the TCDD-inducible protein–DNA complexes to those of known protein standards has allowed calculation of the apparent molecular masses of the inducible complexes [97 ± 2, 105 ± 2, and 115 ± 3 kDa and a somewhat broader complex at about 247 ± 12 kDa (n = 5)]. Treatment of the protein–DNA complexes with micrococcal nucleus did not significantly alter the migration of any of the detected protein–DNA complexes, although the DNA oligonucleotide was progressively digested (data not shown). Previous studies have similarly observed that when the DNA used in protein–DNA-binding studies is of relatively small size, little or no alteration in the relative mobility (apparent molecular mass) of the protein–DNA complex was detected, even upon nuclease digestion (Elferink et al., 1990; Ogata & Gilbert, 1977; Hillel & Wu, 1978).

UV irradiation times and gel analysis conditions which have been optimized for resolution of the three smaller complexes result in formation of the somewhat broader complex at 247 kDa as described above. Occasionally, when samples are separated further on the denaturing gel and/or irradiation times are increased, the larger 247-kDa complex can be resolved into two distinct complexes of approximately 232 ± 3 and 256 ± 4 kDa (Figure 3). Since both of these complexes formed in relatively similar amounts with increasing time of irradiation, we conclude that these complexes do not form from each other. Thus, the broader 247-kDa complex resolved in the standard cross-linking is accounted for by the fact that it is actually composed of two complexes. In the remaining experiments, we utilized standard analysis conditions which favor formation of the smaller inducible complexes.

Previous studies with rat hepatic cytosol reported the formation of three TCDD-inducible UV-cross-linked protein–DRE complexes (Elferink et al., 1990; Gasiewicz et al., 1991). In order to determine whether the number of TCDD-inducible protein–DNA complexes observed in our cross-linking studies is unique to guinea pig hepatic cytosol, we also cross-linked

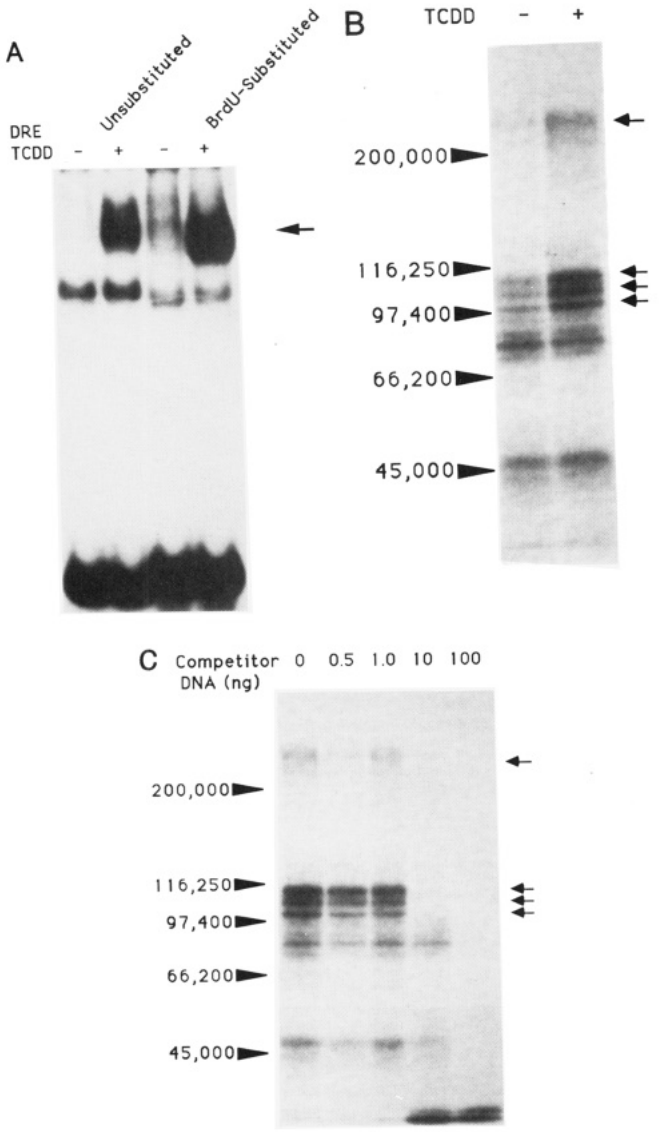

FIGURE 2: TCDD-inducible, UV cross-linking of the transformed TCDD–AhR complex to DNA. Guinea pig hepatic cytosol (16 mg of protein/mL) was incubated in the absence (–TCDD) or presence (+TCDD) of 20 nM TCDD for 2 h at 20 °C. Aliquots were then incubated with ^{32}P-labeled unsubstituted or BrdU-substituted (100 000 cpm, 0.5–1.0 ng) wild-type DRE oligonucleotides as described under Experimental Procedures. Duplicate samples were analyzed directly by gel retardation (A) or UV-irradiated for 30 min and subsequently analyzed by SDS–PAGE (B) and protein–DNA complexes resolved by autoradiography. The arrows indicate the positions of the TCDD-inducible protein–DNA complexes. (A) Gel retardation analysis of DMSO-treated (–TCDD) and TCDD-treated (+TCDD) guinea pig cytosol incubated with wild-type ^{32}P-labeled DRE oligonucleotide or ^{32}P-labeled BrdU-substituted wild-type oligonucleotide. (B) TCDD-inducible, UV cross-linking of proteins to BrdU-substituted wild-type oligonucleotide. The positions of protein standards are indicated. (C) Specificity of DNA binding of proteins inducibly cross-linking to BrdU-substituted wild-type oligonucleotide. TCDD-treated cytosol was incubated in the absence or presence of increasing amounts of wild-type DRE oligonucleotide.

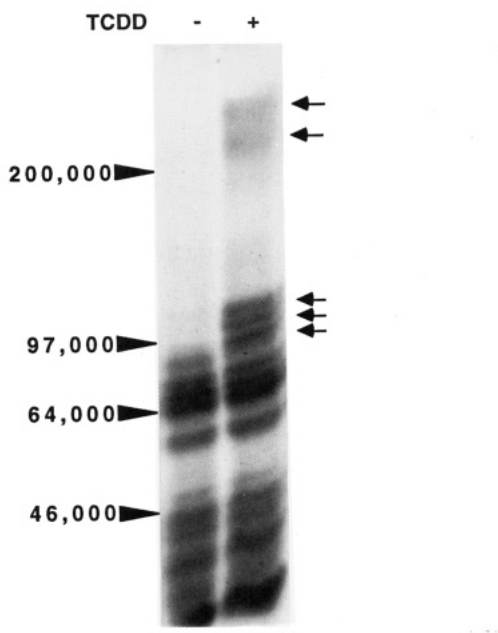

FIGURE 3: Identification of two distinct high molecular mass TCDD-inducible, UV-cross-linked protein–DNA complexes. Guinea pig hepatic cytosol (16 mg of protein/mL) was incubated with DMSO (–TCDD) or 20 nM TCDD (+TCDD) for 2 h at 20 °C. Aliquots were then incubated with ^{32}P-labeled BrdU-substituted wild-type DRE oligonucleotide, irradiated for the indicated time, and analyzed by SDS–PAGE and autoradiography as described under Experimental Procedures. The arrows indicate the positions of the TCDD-inducible protein–DNA complexes. The apparent molecular masses of the two higher complexes was calculated to be approximately 232 ± 3 and 256 ± 4 kDa (n = 3).

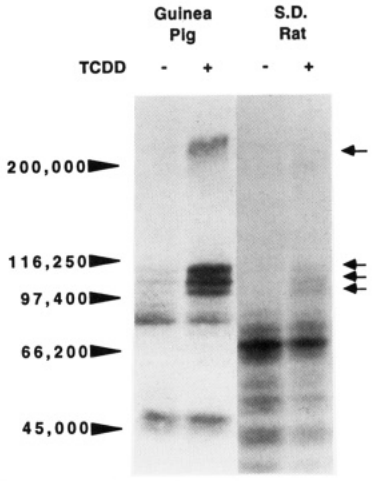

FIGURE 4: TCDD-inducible, UV cross-linking of the transformed guinea pig and rat hepatic cytosolic TCDD–AhR complex to DNA. Hepatic cytosol (16 mg of protein/mL) from the indicated species was incubated in the absence (–TCDD) or presence (+TCDD) of 20 nM TCDD for 2 h at 20 °C. Aliquots were then incubated with ^{32}P-labeled unsubstituted or BrdU-substituted (100 000 cpm, 0.5–1.0 ng) wild-type DRE oligonucleotides as described under Experimental Procedures. The arrows indicate the positions of the inducible protein–DNA complexes.

TCDD-treated Sprague-Dawley rat hepatic cytosol to the BrdU-substituted DRE oligonucleotide. These experiments revealed formation of four TCDD-inducible, UV-cross-linked protein–DNA complexes using rat cytosol (Figure 4), similar to that observed with guinea pig cytosol. Comparable results were also obtained using C3H/HeN mouse hepatic cytosol (data not shown). In these experiments, the low amount of TCDD-inducible protein–DNA complex formation observed using UV-irradiated rat cytosol, compared to guinea pig cytosol, was due to the low amount of inducible TCDD–AhR–DRE complex formed using this rat cytosolic sample, as determined by gel retardation analysis (data not shown). Overall, these results confirm that the TCDD-inducible protein–DNA complexes observed here are not unique to guinea pig hepatic cytosol.

Identification of the Ligand-Binding (AhR) Subunit by UV Cross-Linking with [^{125}I]AIBr$_2$DD. Previous studies have suggested that the DNA-binding form of the AhR is a heterodimer, containing one ligand-binding and one non-

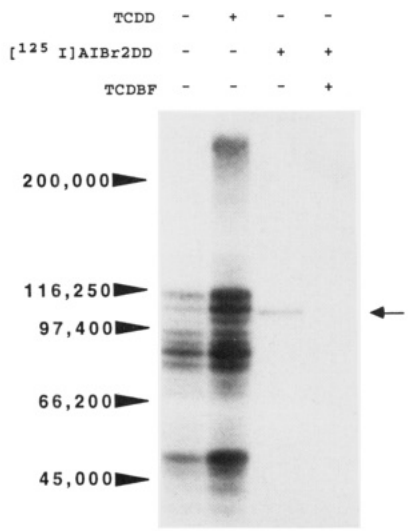

FIGURE 5: Identification of the ligand-binding subunit of the AhR complex. Guinea pig hepatic cytosol (16 mg of protein/mL) was incubated with DMSO (–TCDD) or 20 nM TCDD (+TCDD) for 2 h at 20 °C. Aliquots were then incubated with ^{32}P-labeled BrdU-substituted wild-type DRE oligonucleotide, irradiated for 30 min, and analyzed by SDS–PAGE and autoradiography as described under Experimental Procedures. Cytosol (5 mg of protein/mL) was incubated with 0.94 nM [^{125}I]AIBr$_2$DD in the absence (–TCDBF) or presence (+TCDBF) of a 200-fold molar excess of TCDBF for 30 min at room temperature, charcoal-treated, and irradiated for 4 min, and the proteins were precipitated and analyzed by SDS–PAGE and autoradiography as described under Experimental Procedures. The [^{125}I]AIBr$_2$DD–AhR complex (arrow) migrates with an apparent molecular mass of 104.4 2 0.8 kDa (n = 5).

ligand-binding (arnt) subunit (Elferink et al., 1990; Gasiewicz et al., 1991). To identify which of our three smaller protein–DNA complexes (97, 105, and 115 kDa) contains the ligand-binding subunit of the AhR complex, we utilized a photoaffinity radioiodinated TCDD agonist, [^{125}I]AIBr$_2$DD (Poland & Glover, 1987). UV irradiation of [^{125}I]AIBr$_2$DD-occupied AhR complex covalently cross-links the [^{125}I]AIBr$_2$DD to the ligand-binding subunit, resulting in the formation of a single ^{125}I-labeled protein (Figure 5) with an apparent molecular mass of 104.4 ± 0.83 kDa [comparable to the 103-kDa size previously reported for the guinea pig AhR ligand-binding subunit (Poland & Glover, 1987)]. Competitive inhibition of the formation of the ^{125}I-labeled complex by TCDBF, another high-affinity AhR ligand, identified this complex as the ligand-binding subunit of the AhR complex (Figure 5, lane 4). Although the calculated molecular mass of the ^{125}I-labeled AhR complex was not significantly different from that of the 105-kDa TCDD-inducible protein–[^{32}P]DNA complex, a slight difference in the relative mobility of each complex is apparent. We attribute this minor shift in the relative migration of the ^{125}I-cross-linked AhR complex, compared to that of the protein–[^{32}P]DNA cross-linked complex, to the presence of covalently bound DRE oligonucleotide within the latter cross-linked complex and not the former. Although we have been unable to obtain satisfactory cross-linking of the [^{125}I]AIBr$_2$DD–AhR complex to the BrdU-substituted [^{32}P]DRE oligonucleotide in the same experimental conditions, our results support the identity of the 105-kDa protein–DNA complex as the AhR ligand-binding subunit covalently bound to the DRE oligonucleotide.

UV Cross-Linking of the AhR Complex to Oligonucleotides Containing BrdU Residues within the Conserved DRE Core Sequence. We synthesized various mutant DRE oligonucleotides such that the positions of the BrdU residue(s) within the DRE core sequence were varied (Figure 1). These BrdU-substituted DRE mutants allow identification of the nucleotide residue(s) in close contact with the various subunits of the ArH complex. To identify the nucleotide(s) within the DRE core consensus sequence which cross-link(s) to each of the three AhR complex protein subunits, we used BrdU-substituted wild-type and mutant (M1–M3) oligonucleotides (Figure 1). Analysis of the binding of the transformed TCDD–AhR complex to these BrdU-substituted ^{32}P-labeled DRE oligonucleotides revealed formation of a TCDD-inducible protein–DNA complex with the mutant oligomers M1 and M3 (which contain single-nucleotide substitutions) similar to that of the wild-type DRE oligomer (Figure 6A). TCDD-inducible protein–DNA complex formation with mutant M2 oligomer (which contained two nucleotide substitutions and only one BrdU residue) was significantly reduced, when compared to the wild-type oligonucleotide (presumably due to reduced affinity for the transformed AhR complex). These results indicate that although mutation of one nucleotide (in M1 and M3) had no significant effect, substitution of both of these

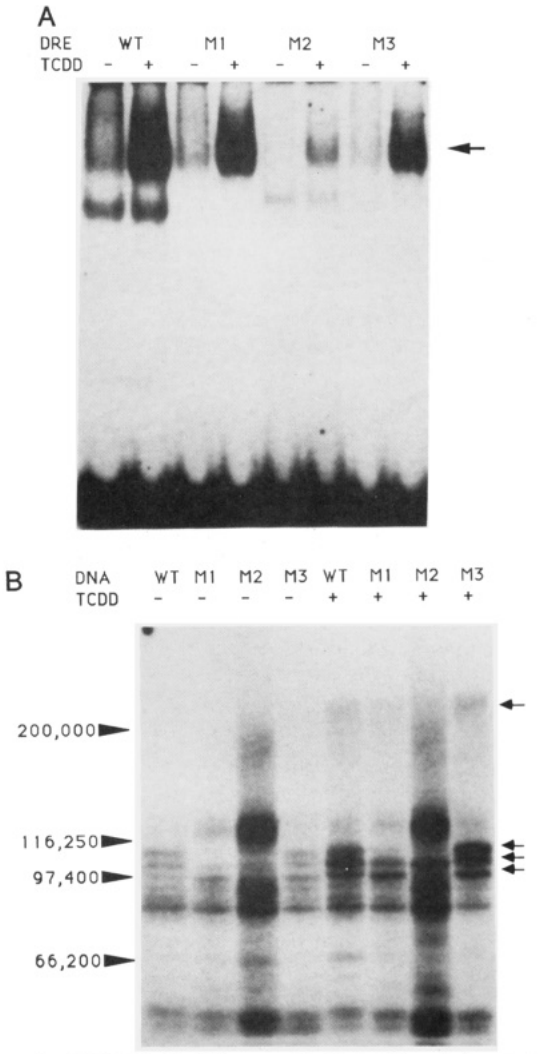

FIGURE 6: TCDD-inducible cross-linking of proteins to DRE oligonucleotides containing variable numbers of BrdU residues substituted within the DRE "core" region. Guinea pig hepatic cytosol (16 mg of protein/mL) was incubated in the absence (–TCDD) or presence (+TCDD) of 20 nM TCDD for 2 h at 20 °C. Aliquots were then incubated with ^{32}P-labeled BrdU-substituted wild-type or mutant (mutants 1–3, see Figure 1) DRE oligonucleotide. Protein–DNA complexes were then directly resolved by gel retardation (A), or samples were irradiated for 30 min and cross-linked protein–DNA complexes resolved by SDS–PAGE (B) as described under Experimental Procedures. The arrows indicate the positions of the inducible protein–DNA complexes.

nucleotides (in M2) adversely affected DNA binding of the transformed TCDD–AhR complex.

UV irradiation, SDS–PAGE, and autoradiography of these incubations revealed the specific BrdU residues to which the various protein subunits of the transformed TCDD–AhR complex can be cross-linked. Four TCDD-inducible protein–DNA complexes (97, 105, 115, and approximately 247 kDa) were observed with both wild-type and mutant M3 oligonucleotides in a TCDD-dependent manner (Figure 6B). Given that the M3 mutant oligomer contains only two BrdU residues which can form at most two cross-links (in contrast to the wild type which contains three BrdUs and can form three cross-links), the similarity in size of the larger (247 kDa) protein–DNA complex with each oligonucleotide suggests that this (these) complex(es) represent(s) a single DNA oligomer cross-linked to two proteins rather than three.

In contrast to the above results, only the 97-, 105-, and 247-kDa protein–DNA complexes were inducibly formed using DRE mutant M1, and only the 105-kDa protein–DNA complex was inducibly formed using DRE mutant M2 (Figure 6B). The results with the M1 mutant oligomer demonstrate that the site of covalent attachment of the 115-kDa subunit occurs only with the first BrdU residue (indicated in boldface type) directly 5′-ward of the five base pair DRE core, **BB**GCGBG, while that of the 105-kDa (ligand-binding) subunit occurs with the BrdU residue (indicated in boldface type) contained within the five base pair DRE core, GCG**B**G. Although gel retardation analysis indicated a significant decrease in AhR complex binding to the M2 oligomer (Figure 6A), TCDD-inducible protein–DNA cross-linking was observed (Figure 6B). Additionally, given that the 97-kDa protein complex was formed using DRE mutants M1 and m3 (but not M2), this implies that this protein can cross-link to either of the two BrdU residues (indicated in boldface) directly upstream of the five base pair DRE core, **BB**GCGBG. These results demonstrate an overlapping DNA-binding/contact specificity of the 97- and 115-kDa AhR complex subunits with the DRE oligonucleotide.

UV Cross-Linking of the AhR Complex to Oligonucleotides Containing BrdU Residues Outside of the Conserved DRE Core Sequence. To examine the sites of contact/cross-linking of the three AhR complex protein subunits outside of the DRE core sequence, we used BrdU-substituted wild-type and mutant (M4–M7) DRE oligonucleotides (Figure 1). Analysis of binding of the transformed TCDD–AhR complex to these BrdU-substituted ^{32}P-labeled DRE oligonucleotides revealed formation of a TCDD-inducible protein–DNA complex with all DRE oligomers (Figure 7A). UV cross-linking analysis, however, revealed little or no TCDD-inducible protein–DNA complex formation with the M4–M7 mutant oligomers (Figure 7B), compared to that obtained with the wild-type substituted DRE oligonucleotide (Figure 6B). These results indicate that transformed TCDD–AhR complexes do not cross-link to BrdU residues at these positions to any significant degree. Although the 97- and 115-kDa proteins bind to BrdU residues directly upstream of the core sequence on the coding strand (Figure 6B), they fail to cross-link to BrdU residues at these same positions (M6 and M7 oligomers) on the noncoding strand (Figure 7B), demonstrating tht the two non-ligand-binding AhR proteins preferentially contact these base pairs only on one strand of the DRE oligomer. The lack of cross-linking of the 105-kDa protein to the BrdU located immediately 3′-ward of the conserved GCGTG sequence on the coding strand (M4 oligomer) indicates that the AhR subunit does not come in close enough contact to this residue to allow cross-linking.

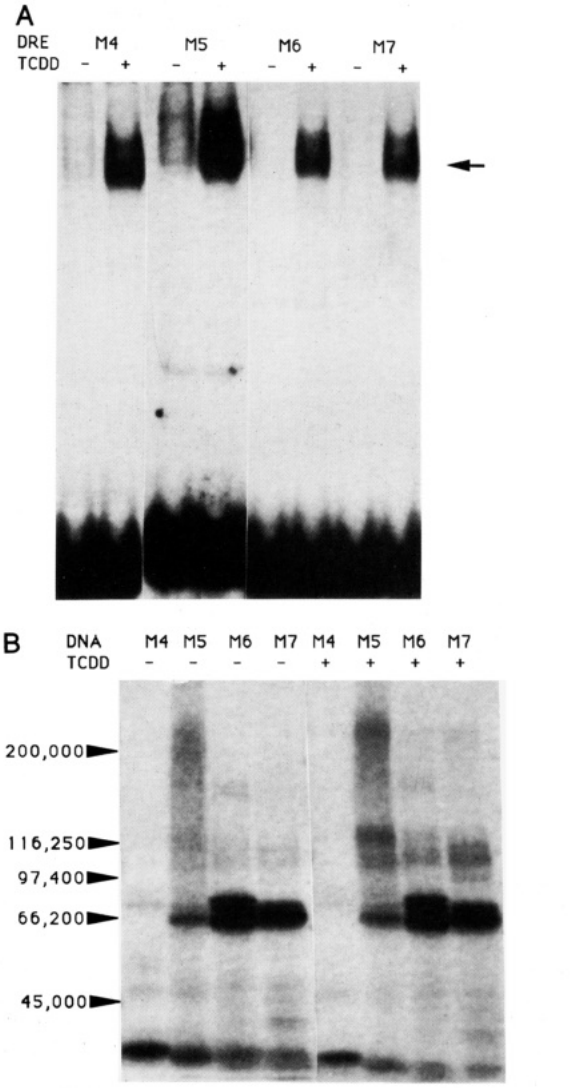

FIGURE 7: TCDD-inducible cross-linking of proteins to DRE oligonucleotides containing variable numbers of BrdU residues substituted outside of the DRE "core" sequence. Guinea pig hepatic cytosol (16 mg of protein/mL) was incubated either in the absence (–TCDD) or in the presence (+TCDD) of 20 nM TCDD for 2 h at 20 °C. Aliquots were then incubated with ^{32}P-labeled BrdU-substituted wild-type or mutant (mutants 4–7, see Figure 1) DRE oligonucleotide. Protein–DNA complexes were resolved by gel retardation (A), or samples were irradiated for 30 min and cross-linked protein–DNA complexes was resolved by SDS–PAGE (B) as described under Experimental Procedures. The arrows indicate the positions of the inducible protein–DNA complexes.

Additionally, the lack of inducible cross-linking to BrdU residues further upstream of the core (on the noncoding strand) indicates the lack of involvement of these bases. Thus, the primary DNA contact sites for the protein subunits of the transformed TCDD–AhR complex(es) appear to be associated with nucleotide contained within the TNGCGTG sequence.

DISCUSSION

We have previously shown that guinea pig hepatic cytosolic TCDD–AhR complexes can transform *in vitro* to a form(s) that bind(s) to DRE-containing DNA in a nucleotide-specific and high-affinity (K_d ~2.5 nM) manner (Yao & Denison, 1992). DNA mutagenesis experiments (Shen & Whitlock, 1992; Yao & Denison, 1992) have revealed that transformed TCDD–AhR complex primarily interacts with the core motif (indicated in boldface) of the DRE consensus sequence (G/CNNNG/CTN**GCGTG**NG/CT/ANNNG/C) derived from

DRE sequence alignment (Denison et al., 1989; Yao & Denison, 1992). In this report, we have utilized a series of BrdU-substituted DRE oligonucleotides and UV cross-linking techniques to determine the relative positioning of individual transformed TCDD–AhR complex subunits on the DRE. Our results reveal the presence of five distinct TCDD-inducible protein–DNA complexes [97, 105, 115, and approximately 247 kDa (the latter complex being composed of 232- and 256-kDa complexes)] and demonstrate that the transformed guinea pig TCDD–AhR complex is composed of multiple protein subunits.

The experiments described here utilize *in vitro* transformed TCDD–AhR complexes rather than those transformed *in vivo*. Previous studies have demonstrated that transformed cytosolic and nuclear TCDD–AhR complexes not only are similar in size (Denison et al., 1991) but also exhibit the same nucleotide-specific DNA-binding specificity (Shen & Whitlock, 1992; Yao & Denison, 1992), indicating that transformation of the AhR complex *in vitro* appears to faithfully mimic that which occurs *in vivo*. Additionally, there are currently no published reports which indicate that the cytosolic TCDD–AhR complex (transformed *in vitro*) and the nuclear TCDD–AhR complex (transformed *in vivo*) produce the same inducible, UV-cross-linked protein–DNA complexes. However, preliminary experiments with hepalclc7 cells (data not shown) indicate that the inducible cross-linking pattern is the same. Thus, our results should be comparable to those obtained utilizing TCDD–AhR complexes transformed *in vivo*.

Recently, other investigators have demonstrated, using UV cross-linking, that the rat cytosolic TCDD–AhR complex, transformed *in vitro*, binds to DNA as a heterodimer, consisting of one ligand- and one non-ligand-binding subunit (Elferink et al., 1990; Gasiewicz et al., 1991). In contrast, our results support the existence of one ligand-binding subunit (105 kDa) and at least two distinct non-ligand-binding subunits (97 and 115 kDa) contained within transformed AhR complexes. The only obvious difference between our experimental procedures and those previously described is in the final denaturing gel conditions (linear gradient gels in our studies compared to single polyacrylamide gel concentration in the others). Perhaps the greater separating power of linear gradient denaturing gels allows resolution of the additional protein–DNA complex seen in our studies. Although it is possible that our additional inducible protein–DNA complex resulted from proteolysis of a larger protein–DNA complex, several observations (data not shown) argue against his: (A) incubation of guinea pig cytosol at 22 °C for various times prior to or after TCDD treatment did not alter the number or relative ratio of TCDD-inducible cross-linked protein–DNA complexes; (B) consistently similar ratios of each cross-linked protein–DNA complex are observed using different cytosolic preparations; (C) four TCDD-inducible UV-cross-linked protein–DNA complexes were observed using hepatic cytosol from species other than guinea pig.

Our cross-linking results might at first be interpreted to suggest that the transformed TCDD–AhR complex consists of four or five protein subunits; however, the largest inducible protein–DNA complex(es) observed was (were) only about 247 kDa. Since the larger 247-kDa protein–DNA complex was observed to form more slowly than the smaller complexes (data not shown), we conclude that formation of the larger complex probably involves more than one cross-linking event, with the smaller protein–DNA complexes representing single proteins cross-linked to the DRE. While it is possible that the 247-kDa protein–DNA complex represents cross-linking of all three smaller proteins to the same DNA oligonucleotide, several points argue against this possibility: (1) the size of the largest complex is only about 247 kDa rather than >300 kDa (which would be expected if all three of the smaller proteins were cross-linked to the DRE); (2) if the 247-kDa protein–DNA complex represented a bound trimer, then we would expect to see inducible cross-linked complexes corresponding to DNA covalently bound to two and to three proteins, and these additional complexes were not observed; and (3) the larger 247-kDa complex was observed using a mutant DRE oligonucleotide which contained only two BrdU residues and thus could only form two covalent cross-links. Thus, our results imply that DNA-binding form of the AhR exists as a protein dimer rather than a trimer, and the presence of three distinct smaller inducible protein–DNA complexes suggests the existence of multiple heteromeric DNA-binding forms of the AhR complex.

The DRE mutagenesis and cross-linking experiments not only indicate that the 105-kDa protein subunit is the only protein which inducibly cross-links to the DRE core sequence (GCGTG) but also support its identity as the AhR ligand-binding subunit. In addition, these experiments reveal the presence of two non-ligand-binding proteins (97 and 115 kDa) which bind to (are in close contact with) DNA immediately 5′-ward, but not within, the DRE core (GCGTG). Previous DRE mutagenesis studies (Shen & Whitlock, 1992; Yao & Denison, 1992) have indicated that single-nucleotide substitutions within the core sequence (GCGTG), but not immediately upstream, eliminate TCDD-inducible protein–DNA complex formation. Additionally, methylation interference experiments (Shen & Whitlock, 1989; Saatcioglu et al., 1990) have demonstrated that methylation of the DRE core sequence (on both strands) interferes with TCDD–AhR–DRE complex formation while methylation outside of the core has little effect. These results, combined with the observation that mutation of the specific nucleotides to which the 97- and 115-kDa proteins contact had no significant effect on the DNA-binding affinity of the transformed TCDD–AhR complex (Shen & Whitlock, 1992; Yao & Denison, 1992), suggest that it is the 105-kDa ligand-binding subunit, rather than the 97- or 115-kDa subunits, that makes the critical DNA contacts necessary for high-affinity AhR–DRE complex formation. Additionally, although the AhR may be distinct from members of the steroid hormone receptor superfamily [due to the lack of a zinc finger DNA-binding motif within the AhR cDNA (Burbach et al., 1992; Ema et al., 1992)], it is similar to this class of ligand-dependent transactivators in that both the ligand- and DNA-binding activities of the AhR appear to be contained within the same protein.

Our results are consistent with the presence of two distinct DNA-binding forms of the guinea pig AhR complex, with each containing one 105-kDa ligand-binding subunit and either one 115-kDa or one 97-kDa non-ligand-binding subunit. This interpretation would also account for the larger 232- and 256-kDa inducibly cross-linked complexes. The discrepancy between the apparent molecular masses of the larger complexes and the sum of the apparent molecular masses of our proposed heterodimers is likely due to the fact that the larger complexes do not migrate at their actual molecular mass due to their more complex cross-linking (i.e., two proteins cross-linked to the DNA compared to one for the smaller complexes). Consistent with our interpretation of the UV cross-linking studies are the recent gel retardation analysis results of Gasiewicz et al. (1991) which reported the identification of two distinct TCDD-inducible, protein–DNA complexes using

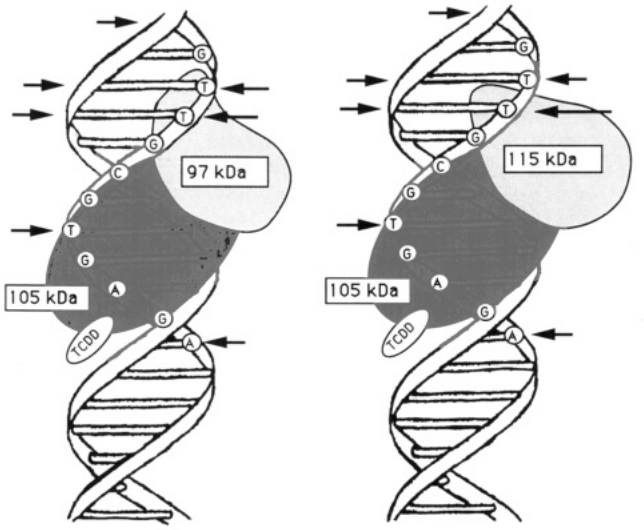

FIGURE 8: Proposed model for the specific interaction of the two transformed heterodimeric TCDD–AhR complexes with DRE-containing DNA. Nucleotides contacted on the coding strand are as shown. The arrows indicate the positions of the BrdU residues in the wild-type and mutant DRE oligonucleotides examined (Figure 1).

TCDD-treated guinea pig hepatic cytosol. The exact subunit composition of these two DNA-binding forms remains to be determined.

The BrdU cross-linking results obtained here are in apparent contrast to the photoaffinity and chemical cross-linking studies of Perdew (1992), which demonstrated that the transformed mouse hepatoma (hepa1c1c7) cell AhR complex exists as a heterodimer composed of one 97-kDa ligand-binding subunit and one 85-kDa non-ligand-binding subunit (presumably arnt). Interestingly, preliminary UV cross-linking experiments utilizing TCDD-treated C57BL/6 mouse hepatic cytosol and a BrdU-containing DRE oligonucleotide resulted in the formation of only two smaller inducible protein–DNA complexes, unlike that obtained using guinea pig hepatic cytosol (data not shown). Given that the mouse hepatoma cells used in the chemical cross-linking studies were originally derived from C57BL/6 mouse liver (Bernhard et al., 1973), it is likely that the discrepancy between our results and those of Perdew (1992) results from species differences in the proteins associated with the transformed TCDD–AhR complex(es).

A hypothetical model for the interaction of the two guinea pig heterodimeric DNA-binding forms of the AhR complex, on the basis of our *in vitro* cross-linking and BrdU distribution binding studies, is presented in Figure 8. Clearly, *in vivo*, the high-affinity DNA binding of transformed AhR complex(es) as well AhR-dependent transcriptional activation will involve the interaction of the AhR and/or its subunits with additional nuclear proteins. In fact, nuclear chromatin-binding factors which specifically interact with the AhR complex have been recently reported (Dunn et al., 1993). Although the role of these specific binding factors in AhR-dependent action remains to be elucidated, nuclear accessory proteins which can modulate the functional activity of a variety of transcription factors, including members of the steroid and thyroid hormone receptor superfamily (Beebe et al., 1991; Yu et al., 1991; Adler et al., 1992; Pearce & Yamamoto, 1993), have been isolated. Thus, it is likely that proteins in addition to those identified here will be involved in the modulation of AhR action in the nucleus.

The identity of the two non-ligand-binding subunits identified here is currently unknown. Recently, Hankison and co-workers (Hoffman et al., 1991; Reyes et al., 1992) have reported cloning of a gene for a human protein (arnt) that appears to be a non-ligand-binding subunit contained within the nuclear (transformed) heteromeric AhR complex. Examination of the DNA sequences of arnt (Hoffmann et al., 1991) and the ligand-binding subunit of the AhR complex (Burbach et al., 1992; Ema et al., 1992) has revealed a putative basic/helix–loop–helix (bHLH) motif contained within each protein. Although bHLH motifs have been shown to be essential for protein–protein and protein–DNA interactions, a functional role for the putative bHLH motifs contained within AhR and arnt in these activities remains to be determined. However, since bHLH motifs have been implicated in both DNA binding and dimerization (John & McKnight, 1989; Murre et al., 1992), one can envision the existence of multiple arnt or arnt-like proteins (which contain compatible bHLH motifs). In fact, the detection of alternative spliced products of arnt mRNA (Hoffman et al., 1991), combined with studies demonstrating functional heterodimer formation by heterologous bHLH-containing proteins (Murre et al., 1992), would support this hypothesis. Whether the 97- and 115-kDa non-ligand-binding proteins identified here are analogous or identical to arnt or each other remains to be determined; however, given that these two proteins are significantly larger than that reported for the human arnt protein [87 kDa (Hoffman et al., 1991)] and that DNA binding by arnt has not been demonstrated, it is likely that these proteins are not arnt. Although Reyes et al. (1992) have demonstrated that arnt is contained within the transformed murine nuclear AhR complex, it is possible that arnt may be bound to the AhR complex via protein–protein interactions and does not directly contact the DNA itself. If this is the case then an inducible cross-linked protein–DNA complex corresponding to the arnt–DNA complex would not be detected by our UV cross-linking technique. The role of arnt and how it specifically interacts with the AhR and/or DNA remain to be elucidated.

Since the 97- and 115-kDa non-ligand-binding subunits appear to contact only a small portion of the DNA, we suggest that these non-ligand-binding subunits have little intrinsic DNA-binding activity themselves. This conclusion is supported by DRE mutagenesis experiments (Shen & Whitlock, 1992; Yao & Denison, 1992) which indicate that mutation of the specific nucleotides in which these proteins contact has little effect on both formation of the TCDD–AhR–DRE complex and transcriptional enhancer activity. We envision that the 97- and 115-kDa non-ligand-binding proteins act as accessory or "chaperone-like" proteins for the ligand-binding subunit, in that they function to maintain the liganded AhR in a conformational necessary for nuclear accumulation and/or high-affinity DNA binding. These accessory proteins may facilitate a conformational change in the ligand-binding subunit, resulting in exposure of its DNA-binding site and/or a nuclear localization signal sequence (in one or more of these proteins). In either instance, we would expect the 97- and 115-kDa proteins to exhibit similar functions.

It is currently unknown what physiological role these two DRE-binding forms of the AhR complex may play in transcriptional activation. We are currently attempting to isolate sufficient quantities of each complex in order both to characterize the protein components within each and to assess the ability of each to activate transcription, *in vitro*. It is possible that the different heterodimeric transformed TCDD–AhR complexes exhibit some differences in functionality, since they exhibit differences in their interaction with DNA.

Modulation of expression of the various subunits of the AhR complexes could not only could affect the relative concentration of each transformed heteromeric AhR complex but also could account for some of the apparent species- and tissue-specific differences in TCDD-responsiveness.

ACKNOWLEDGMENT

We thank Dr. S. Safe for unlabeled TCDD and TCDBF and Dr. G. Perdew for ^{125}I-labeled AIBr$_2$DD. We also thank Drs. David DeWitt and William Smith for critical evaluation of the manuscript.

REFERENCES

Adler, A. J., Danielson, M., & Robins, D. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11660–11663.
Beato, M. (1991) *Faseb J. 5*, 2044–2051.
Beebe, J. S., Darling, D. S., & Chin, W. W. (1991) *Mol. Endocrinol. 5*, 85–93.
Bernhard, H. P., Darlington, G. J., & Ruddle, F. H. (1973) *Dev. Biol. 35*, 83–86.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Burbach, K. M., Poland, A., & Bradfield, C. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8185–8189.
Carson-Jurica, M. A., Schrader, W. T., & O'Malley, B. (1990) *Endocr. Rev. 11*, 201–220.
Denis, M., Cuthill, S., Wikstrom, A.-C., Poellinger, L., & Gustafsson, J.-A., (1988) *Biochem. Biophys. Res. Commun. 155*, 801–807.
Denison, M. S., & Yao, E. F. (1991) *Arch. Biochem. Biophys. 284*, 158–166.
Denison, M. S., Vella, L. M., & Okey, A. B. (1986) *J. Biol. Chem. 261*, 3987–3995.
Denison, M. S., Fisher, J. M., & Whitlock, J. P., Jr. (1988a) *J. Biol. Chem. 263*, 17221–17224.
Denison, M. S., Fisher, J. M., & Whitlock, J. P., Jr. (1988b) *Proc. natl. Acad. Sci. U.S.A. 85*, 2528–2532.
Denison, M. S., Fisher, J. M., & Whitlock, J. P., Jr. (1989) *J. Biol. Chem. 264*, 16478–16482.
Denison, M. S., Phelps, C. L., DeHoog, J., Kim, H. J., Bank, P. A., Harper, P. A., & Yao, E. F. (1991) in *Biological Basis of Risk Assessment of Dioxins and Related Compounds*, Banbury Report No. 35, pp 337–350, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Dunn, R. T., Ruh, T. H., & Ruh, M. F. (1993) *Biochem. Pharmacol. 45*, 1121–1128.
Elferink, C. J., Gasiewicz, T. A., & Whitlock, J. P., Jr. (1990) *J. Biol. Chem. 265*, 20708–20712.
Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsuchita, N., Gotoh, O., Funae, Y., & Fujii-Kuriyama, Y. (1992) *Biochem. Biophys. Res. Commun. 184*, 246–253.
Fujisawa-Sehara, A., Sogowa, K., Yamane, M., & Fujii-Kuriyama, Y. (1987) *Nucleic Acids Res. 15*, 4179–4191.
Gasiewicz, T. A., Elferink, C. J., & Henry, E. H. (1991) *Biochemistry 30*, 2909–2916.
Helferich, W. G., & Denison, M. S. (1991) *Mol. Pharmacol. 40*, 674–678.
Henry, E. C., Rucci, G., & Gasiewicz, T. A. (1989) *Biochemistry 28*, 6430–6440.
Hillel, Z., & Wu, C.-W. (1978) *Biochemistry 17*, 2954–2961.
Hoffman, E. C., Reyes, H., Fong-Fong, C., Sander, F., Conley, L. H., Brooks, B. A., & Hankinson, O. (1991) *Science 252*, 954–958.
John, P. F., & McKnight, S. L. (1989) *Annu. Rev. Biochem. 58*, 799–839.
Jones, P. B. C., Durrin, L. K., Galeazzi, D. R., & Whitlock, J. P., Jr. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2802–2806.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Legraverend, C., Hannah, R. T., Eisen, H. J., Owens, I. S., Nebert, D. W., & Hankinson, O. (1982) *J. Biol. Chem. 257*, 6402–6407.
Markowitz, A. (1972) *Biochim. Biophys. Acta 281*, 522–534.
Murre, C., McCaw, P. S., Vaessin, H., Caury, M., Jan, L. Y., Jan, Y. N., Cabrea, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., Weintraub, H., & Baltimore, D. (1992) *Cell 58*, 537–544.
Nebert, D. W., Nelson, D. R., Coon, M. J., Eastbrook, R. W., Feyereisen, R., Fuji-Kuriyama, Y., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Loper, J. C., Sato, R., Waterman, M. R., & Waxman, D. J. (1991) *DNA Cell Biol. 10*, 1–14.
Ogata, R., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4973–4976.
Pearce, D., & Yamamoto, K. R. (1993) *Science 259*, 1161–1165.
Perdew, G. H. (1988) *J. Biol. Chem. 263*, 13802–13805.
Perdew, G. H. (1992) *Biochem. Biophys. Res. Commun. 182*, 55–62.
Poland, A., & Knutson, J. C. (1982) *Annu. Rev. Pharmacol. Toxicol. 22*, 517–554.
Poland, A., & Glover, E. (1987) *Biochem. Biophys. Res. Commun. 146*, 1439–1449.
Poland, A., Glover, E., Ebetino, F. H., & Kende, A. S. (1986) *J. Biol. Chem. 261*, 6352–6365.
Reyes, H., Reisz-Porszasz, S., & Hankinson, O. (1992) *Science 256*, 1193–1195.
Saatcioglu, F., Perry, D. J., Pasco, D. S., & Fagan, J. B. (1990) *J. Biol. Chem. 265*, 9251–9258.
Safe, S. H. (1986) *Annu. Rev. Pharmacol. Toxicol. 26*, 371–399.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., pp 11.40–11.44, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shen, E. S., & Whitlock, J. P., Jr. (1989) *J. Biol. Chem. 264*, 17754–17758.
Shen, E. S., & Whitlock, J. P., Jr. (1992) *J. Biol. Chem. 267*, 6815–1819.
Vinson, C. R., Sigler, P. B., & McKnight, S. L. (1989) *Science 246*, 911–916.
Whitlock, J. P. (1986) *Annu. Rev. Pharmacol. Toxicol. 26*, 333–369.
Whitlock, J. P., Jr. (1990) *Annu. Rev. Pharmacol. Toxicol. 30*, 251–277.
Whitlock, J. P., Jr., Denison, M. S., Fisher, J. M., & Shen, E. S. (1990) *Mol. Biol. Med. 6*, 169–178.
Yao, E. F., & Denison, M. S. (1992) *Biochemistry 31*, 5060–5067.
Yu, V. C., Delsert, C., Anderson, B., Holloway, J. M., Devreny, O. V., Naar, A. M., Kim, S. Y., Boutin, J., Glass, C. K., & Rosenfeld, M. G. (1991) *Cell 67*, 1251–1266.