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CHARACTERIZATION OF THE INTERACTION OF
TCDD:Ah RECEPTOR COMPLEXES WITH A
DIOXIN-RESPONSIVE TRANSCRIPTIONAL ENHANCER

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Eveline Faith Yao

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Major professor
Dr. Michael S. Denison

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CHARACTERIZATION OF THE INTERACTION OF TCDD:Ah RECEPTOR COMPLEXES WITH A DIOXIN-RESPONSIVE TRANSCRIPTIONAL ENHANCER

By

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ABSTRACT

CHARACTERIZATION OF THE INTERACTION OF
TCDD:Ah RECEPTOR COMPLEXES WITH A
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The mechanism of induction of cytochrome P450IA1 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is mediated by the AhR which binds TCDD saturably and with high affinity. The Ah receptor (AhR) is a ligand-dependent DNA-binding protein which binds to specific *cis*-acting DNA enhancer sequences (dioxin responsive elements (DREs)) adjacent to TCDD-responsive genes. We have examined the DNA binding of transformed guinea pig hepatic cytosolic TCDD:AhR complex utilizing gel retardation and site directed mutagenesis techniques. Using the optimized assay conditions, we have determined that the transformed TCDD:AhR complex binds to the DRE specifically and with high affinity (K_d = 2.5 ± 0.8 nM). In addition, the five murine CYPIA1 upstream DREs were bound by transformed TCDD:AhR complexes with comparable affinity. A common DRE consensus sequence C/GNNNC/GTNGCGTGNC/GA/T was derived from DRE sequence alignment. The DRE consensus contains an invariant core sequence, TNGCGTG, flanked by several variable bases. Utilizing site directed mutagenesis and competitive gel retardation analysis, we have determined that several nucleotides contained within the invariant core sequence (particularly, CGTG) were critical for inducible protein-DNA complex formation, while bases 3′ of the core were important, but to a lesser degree, and those 5′ appeared not to be involved in TCDD:AhR:DRE complex formation. These results suggest that the high affinity DNA-binding of transformed TCDD:AhR complex occurs primarily through an interaction with the invariant core motif of the DRE.

DEDICATION

To my family: Dad, Mom, Yo and Kev, Ro and Kev, and Te
Thanks for all your support and advice.
I love you all.

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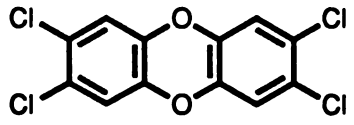
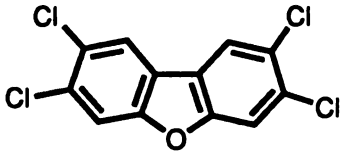
INTRODUCTION

Halogenated Aromatic Hydrocarbons in the Environment

Halogenated aromatic hydrocarbons (HAHs), such as the polychlorinated-dibenzo-*p*-dioxins, biphenyls and dibenzofurans, and related compounds (Figure 1) represent a diverse group of widespread environmental contaminants, many of which are toxic and persistent in the environment. HAH residues have been found in food, water, soil and sediment samples from various regions around the United States and significant concentrations have been identified in a variety of human, wildlife and domestic animal tissues (Firestone 1984; Rappe et al. 1984). Because of their ubiquitous distribution, toxicity, fat solubility, and potential for bioaccumulation and biomagnification, HAHs could have a significant impact on the health and well being of both human and animals (Tanabe et al. 1987).



2,3,7,8-tetrachlorodibenzo-*p*-dioxin



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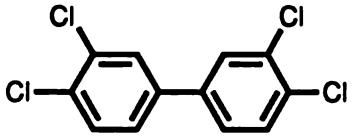

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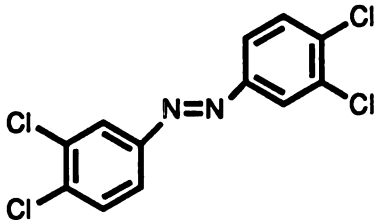

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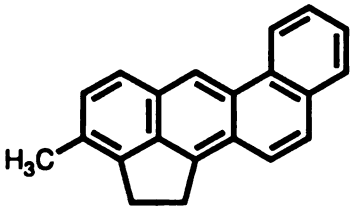

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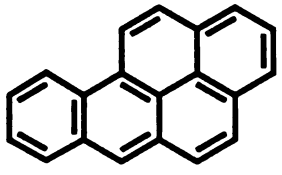

benzo(a)pyrene

Figure 1. Chemical Structure of TCDD and Several Halogenated and Polycyclic Aromatic Hydrocarbons.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent HAH, has gained widespread notoriety in recent years as one of the most toxic environmental contaminants known. TCDD was first identified as an unwanted, toxic byproduct in commercial chlorinated phenol products in the late 1950s (reviewed in Firestone 1984) and was subsequently determined to be the toxic component in the commonly used herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and in Agent Orange, a herbicide mixture (2,4,5-T and 2,4-dichlorophenoxyacetic acid) used as a defoliant during the Vietnam War (Esposito et al. 1980). More recently, TCDD has been found in cigarette smoke, automobile exhaust, commercial incinerator emissions and has been suggested to be produced during most general combustion reactions (reviewed in Firestone 1984).

Exposure to and bioaccumulation of TCDD and related HAHs has been observed to produce a wide variety of species- and tissue-specific toxic and biological effects, including: tumor promotion, lethality, birth defects, hepatotoxicity, immunotoxicity, dermal toxicity and induction of numerous enzymes, such as cytochrome P450IA1 and its associated monooxygenase activity, aryl hydrocarbon hydroxylase (AHH) (Poland and Knutson 1982). P450IA1 enzymatic activity contributes to the metabolic activation and detoxification of polycyclic aromatic hydrocarbons, such as benzo(a)pyrene, many of which are widespread environmental contaminants (Gelboin 1980).

The toxic and biological effects of TCDD and related HAHs vary depending on several factors; such as the dose, route of administration, length of exposure, sex, age, and species of animal. TCDD-induced toxicity is largely species-dependent and with differences in susceptibility varying by as much as 5000-fold, as measured by differences in oral LD_{50} values (the dose which is lethal to 50% of a given population). For example, male guinea pig represents the most sensitive species (with a TCDD LD_{50} value between 0.6 to 2.0 μg/kg body weight) while male hamster is comparitively resistent (with an LD_{50} between 1157 to 5051 μg/kg) (Poland and Knutson 1982; Safe 1986). This large variation cannot be due simply to differences in the rate of *in vivo* metabolism of TCDD, since the whole body half-life of TCDD between guinea pig and hamster differs only by three-fold (Olson et al. 1980). Toxic responses to TCDD of animal species also vary qualitatively. For instance, exposure to

TCDD results in chloracne and dermal lesions in humans, rabbits and hairless mice but not in rats, guinea pigs, hamsters and most strains of mice (Poland and Knutson 1982; Safe 1986). Although many of TCDD's effects are species-specific, the occurrence of thymic involution and/or immunotoxicity and total body weight loss are responses common to all species (Poland and Knutson 1982; Safe 1986). Exposure to TCDD and related HAHs results in a loss of lymphoid tissue; typified by a reduction in normal thymus size by as much as 20% (a condition referred to as thymic involution or thymic atrophy) and is normally accompanied by a significant suppresion of the immune response (Poland and Knutson 1982; Safe 1986). In addition, all species subjected to an acute lethal dose of TCDD experience a latent period of a week or more prior to death. This wasting syndrome is characterized by progressive weight loss (anorexia) in the exposed animal over a period of several days to weeks. The exact mechanism(s) by which TCDD elicits these toxic effects and the target organ(s) involved in lethality are unknown. These variations in species sensitivity and observed toxic responses have complicated the interpretation of TCDD action in animal populations, as well as hindered extrapolation of these data to the human population.

The available information on human sensitivity and responsiveness to TCDD and related compounds suggests that humans are less susceptible to the toxic effect of these compounds than most other animal species. In humans the primary target organ appears to be the skin, where exposure to TCDD results in the appearance of chloracne, a acne-like lesion resulting from enhanced differentiation of skin subaceous glands into keratin producing cells (reviewed in Goldstein and Safe 1980). Although other toxic effects in humans have been reported (McConnell 1984), these "symptoms" do not appear consistently among exposed individuals. A recent epidemiologic study of TCDD-exposed workers (Fingerhut et al. 1991) suggests that TCDD may, in fact, be a human carcinogen, but only at relatively high doses and after a prolonged latency period of about 20 years. Overall, however, the lack of sufficient epidemiologic data to either support or deny the occurrence of adverse TCDD-dependent human health effects (other than transient chloracne) has complicated risk assessment and resulted in a significant amount of controversy in regulatory agencies.

Induction of Cytochrome P450IA1 as a Model HAH-Responsive System

Exposure of laboratory animals to TCDD results in a variety of biological responses (as noted above), including the induction of numerous drug metabolizing enzymes, including: cytochrome P450IA1 (Whitlock 1990), glutathione S-transferase (Rushmore et al. 1990), UDP-glucuronysyl transferase (Poland and Knutson 1982), quinone reductase (Favreau and Pickett 1991) and δ-aminolevulinic acid synthethase (Poland and Knutson 1982). The majority of work to date has focused on the induction of microsomal cytochrome P450IA1 and its associated AHH activity (reviewed in Whitlock 1990). Cytochrome P450IA1 is a member of the P450 superfamily, which currently consists of at least 154 members in 27 different gene families (Nebert et al. 1991). The cytochrome P450s are microsomal membrane-bound hemoproteins which possess wide, and often overlapping, substrate specificitites. In general, the P450s are primarily involved in metabolic detoxification reactions. The P450 monooxygenase system functions to introduce a single oxygen atom (from molecular oxygen) onto a given substrate and represents classical Phase I metabolism of the substrate. This reaction makes the substrate more water-soluble and provides a chemical group which can be easily conjugated by Phase II enzymes (such as glucuronosyl and glutathione transferases which function to transfer a polar moiety onto the substrate). Consequently, P450 metabolites are more readily excreted from the organism. In addition to detoxicification of lipophilic xenobiotics, P450IA1 is also involved in the metabolic activation of these same compounds (such as benzo(a)pyrene) to more toxic/carcinogenic forms. Consequently, modulation of the levels of specific P450 enzymes could have a significant effect on the metabolic capability of a given species and/or tissue.

In the early 1970's, several groups (Nebert et al. 1972; Thomas et al. 1972) observed that administration of polycyclic aromatic hydrocarbons (such as 3-methylcholanthrene; Figure 1) to certain inbred strains of mice (typified by the C57BL/6 strain) induced AHH activity; while other strains (typified by DBA/2 mice) failed to respond. On the basis of these findings, Poland et al. (1974) postulated that AHH induction in mice was mediated by the binding of the inducer to a specific receptor protein and that the strains of mice that were genetically "non-responsive" to AHH induction had a defective receptor

mechanism. Studies using these "responsive" and "non-responsive" inbred strains resulted in the identification of a protein that bound [^{3}H]-TCDD with high affinity and low capacity (Poland et al. 1976). This TCDD "receptor" has also been designated as the aromatic hydrocarbon receptor (AhR). Although the AhR has been identified by its ability to bind [^{3}H]-TCDD, native AhR has not been purified, nor the gene(s) cloned.

Several lines of evidence imply that the AhR is involved in the induction of AHH activity: first, compounds that induce AHH activity compete with [^{3}H]-TCDD for AhR binding, whereas compounds that do not induce, do not compete (Poland et al. 1976; Okey et al. 1979; Denison et al. 1984); second, the binding affinities of a series of halogenated dibenzo-*p*-dioxins for the AhR correlate with their potencies as AHH inducers (Poland et al. 1976); third, TCDD is less (10-20 fold) potent as an inducer of AHH activity in "non-responsive" DBA/2 mice than in "responsive" C57BL/6 mice (Poland and Knutson 1982). Additional evidence has suggested that the AhR is involved in the toxicity of TCDD and related halogenated aromatic hydrocarbons: first, studies of structure-activity relationships reveal a good correlation between the toxic potency of a compound and its affinity for the AhR (Poland et al. 1979; Knutson and Poland 1980; Safe 1986); and second, "non-responsive" mice strains which exhibit decreased AhR binding of TCDD show decreased toxicity compared to the "responsive" mouse strains which exhibit normal binding of TCDD (Poland and Glover 1980). Although no endogenous ligand for the AhR has been identified, it has been proposed that the endogenous ligand may have a role in the regulation of cell differentiation and proliferation (Poland and Knutson 1982); however, this remains to be determined. Interestingly, many of the "toxic" responses to TCDD and related compounds involve alterations in differentiation and/or cell division (Poland and Knutson 1982; Greenlee et al. 1987). Thus, it seems likely that TCDD produces many, if not all, of its biological and toxic effects by differentially altering gene expression in susceptible cells.

The current model for the mechanism of induction of cytochrome P450IA1 by these compounds (Figure 2) is similar to that described for steroid hormone receptors and steroid-responsive genes (Yamamoto 1985). Following ligand (TCDD) binding, the AhR undergoes a poorly defined process of

transformation, during which hsp90 (a heat shock protein of 90 kDa) dissociates from the TCDD:AhR complex (Perdew 1988; Wilhelmsson et al. 1990) and the AhR acquires the ability to bind to DNA with high affinity (Denison and Yao 1991). The accumulation of transformed TCDD:AhR complexes within the nucleus has been shown to correlate with the induction of cytochrome P450IA1 mRNA (Tukey et al. 1982). Nuclear run-off experiments (Whitlock 1987) have confirmed that treatment with TCDD increases the rate of transcription of the CYPIA1 gene, the gene which encodes cytochrome P450IA1. The translation of this message into active cytochrome P450IA1 enzyme can be easily measured by changes in the amount of cytochrome P450IA1-specific AHH activity.

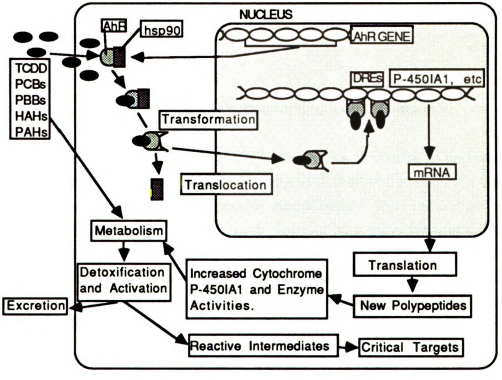

Figure 2. Molecular Mechanism of TCDD:Ah Receptor Action.

Two independent research groups have generated several AhR-defective variant mouse hepatoma (hepa1c1c7) clonal cell lines (Legraverend et al. 1982; Whitlock 1987; Karenlampi et al. 1988) useful for studying the mechanism of TCDD action at the molecular level. At least three distinct classes of variants

have been isolated which exhibit altered responsiveness to TCDD. Class 1 variants contain less (10% of wild type) AhR and exhibit a corresponding decreased response to TCDD. Class 2 variants exhibit normal binding of TCDD to the AhR; however, the strength to which TCDD:AhR binds to the nucleus is greatly decreased (Legraverend et al. 1982; Whitlock 1987). Class 3 variant cells appear to have a defect(s) which affects both ligand and DNA-binding (Karenlampi et al. 1988). Cell fusion studies (Hankinson 1983; Whitlock 1987) of these variants with wild-type cells results in a hybrid with a wild-type phenotype; suggesting the recessive nature of these defects. Fusions between variant cells have also revealed that each of these "defects" were associated with separate genes (or complementation groups) suggesting that the function of the AhR requires at least three distinct gene products. Additionally, the results from studies utilizing these receptor defective variants have implied a requirement for functional AhR in the P450IA1 induction response.

Identification of Dioxin Responsive Transcriptional Enhancers

Whitlock and coworkers isolated DNA from the 5'-flanking region of the mouse cytochrome P450IA1 (CYPIA1) gene and inserted it directly upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene (Jones et al. 1985). Transfection of the recombinant plasmid into wild-type and receptor-defective variant hepa1c1c7 cells revealed that CAT expression was TCDD-inducible in wild-type cells, while absent or diminished in the two classes of receptor defective cells (Jones et al. 1985; Whitlock 1987). Thus, the DNA insert contained a domain(s) with properties expected of a TCDD-responsive DNA element. The results of extensive deletion analysis (Jones et al. 1985, 1986a, 1986b; Denison et al. 1988) revealed that the 5'-flanking region of the CYPIA1 gene (Figure 3) contains a relatively strong promoter located near the transcriptional start site, an inhibitory element (whose presence blocks constitutive promoter function) and at least four spatially distinct and functionally independent TCDD-responsive elements (or dioxin-repsonsive elements (DREs)). Transient transfection studies have revealed that these DREs can function independently of each other, can confer TCDD-responsiveness upon a heterologous gene and its promoter, require

functional AhR for activity and exhibit the properties of a transcriptional enhancer (Jones et al. 1986a, 1986b; Denison et al. 1988b).These results also suggested that these DREs contained the binding site(s) for the transformed TCDD:AhR complex.

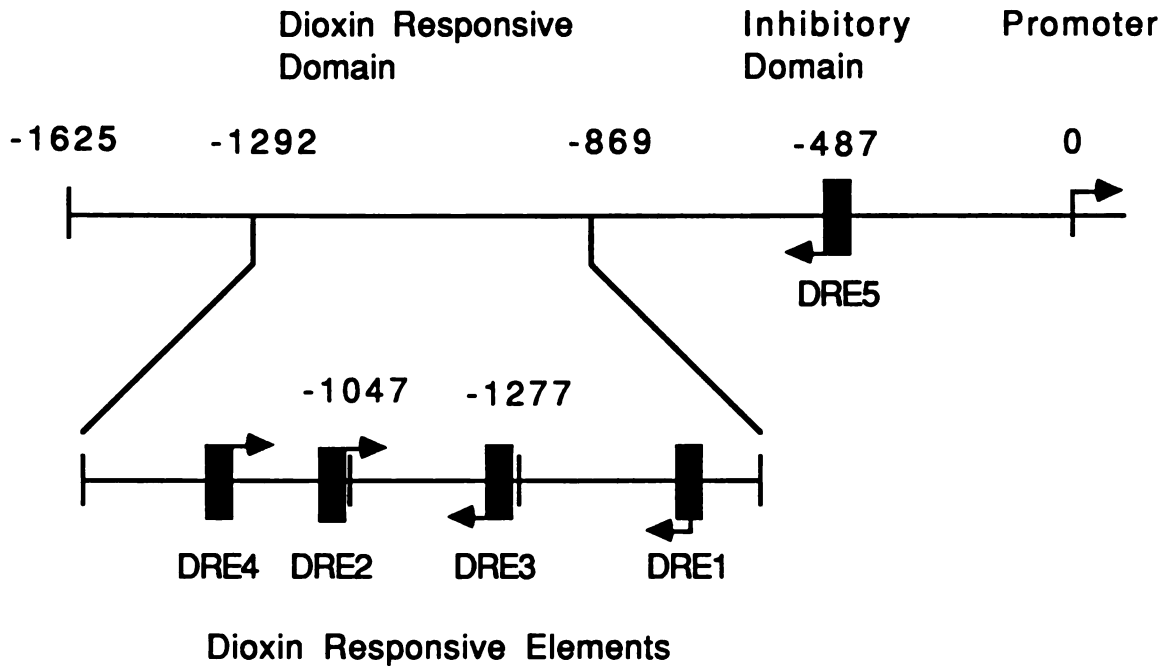

Figure 3. 5'-flanking Region of the Mouse CYPIA1 Gene.
(the arrows indicate the direction of the consensus sequence GCGTG and whether the sequence is on the upper or lower strand)

Utilizing a sensitive gel retardation assay (Garner and Revzin 1981), Denison et al. (1988b) have shown that nuclear extracts from TCDD-treated wild-type hepa 1c1c7 cells contained a protein(s) that bound to a [^{32}P]-DRE oligonucleotide in a TCDD-inducible, AhR-dependent and DNA sequence specific manner. The presence of the AhR in the TCDD-inducible complex was recently demonstrated in experiments utilizing radiolabeled TCDD (Fujisawa-Sehara et al. 1988) or a TCDD agonist (Denison et al. 1989). Subsequent studies have resulted in the identification of at least 5 DREs contained within the 5'-flanking region of the murine CYPIA1 gene which

can specifically interact with transformed TCDD:AhR complexes (Denison et al. 1989).

Alignment of the five murine CYPIA1 upstream DRE sequences has revealed a common consensus sequence of:

```
5'-  G N N N C T N G C G T G N G A N N N G  -3'
     C       G                 C T       C
```

A review of the literature has not revealed any known DNA-binding protein which contains the DRE consensus sequence as all or part of its DNA recognition site. The consensus sequence for the DRE is characterized by an invariant core sequence of 5'- TNGCGTG -3' with several variably conserved bases flanking this core. Some of these flanking sequences appear to be required for DRE function since their deletion significantly decreases AhR binding and transcriptional enhancer activity (Denison et al. 1989). DRE consensus sequences have also been identified in the 5'-flanking region of the TCDD-inducible human (Nebert and Jones 1989) and rat (Fujisawa-Sehara et al. 1987) CYPIA1 gene and the rat glutathione S-transferase (Rushmore et al. 1990) and quinone reductase (Favreau and Pickett 1991) genes and they appear to be functionally similar to those previously characterized (Whitlock 1990).

AhR Transformation and DNA-Binding

After ligand binding the TCDD:AhR complex undergoes transformation and binds with high affinity to DNA. DNA-binding of the AhR *in vitro* occurs strictly in a ligand-dependent manner, unlike that observed with steroid hormone receptors (Pratt 1990). "Transformation" is defined as the conversion of the non-DNA binding form of the TCDD:AhR complex to a form which interacts specifically with DNA. Transformation of the AhR appears to require subunit dissociation, with at least the loss of the hsp90 subunit, and a change in molecular weight from 250-300 kDa to 150-200 kDa (Perdew 1988; Prokipcak and Okey 1988; Henry et al. 1989). Since the dissociated ligand binding subunit of the AhR ranges in size between 95-120 kDa in size (Poland and Glover 1987), transformation must represent the

association of at least one additional protein to the ligand binding subunit. Whether this additional protein(s) is present in the cytosolic form of the AhR or whether it binds to the liganded AhR subunit after dissociation remains to be determined.

DNA-binding of transformed TCDD:AhR has been examined in protein-DNA cross-linking experiments utilizing a 5-bromo-2'-deoxyuridine (BrdU) substituted DRE-containing oligonucleotide. Separation of UV-cross-linked TCDD-inducible protein-DNA complexes by SDS-PAGE revealed the presence of three protein-DNA complexes of approximately 100, 110 and 220 kDa (Elferink et al. 1990; Gasiewicz et al. 1991). Since it was previously known that the transformed nuclear TCDD:AhR complex had an apparent molecular weight of between 150-200 kDa (Prokipcak and Okey 1988; Henry et al. 1989), these results were interpreted to mean that the 220 kDa complex represented the cross-linking of the two smaller proteins to a single DRE; while the smaller complexes represented the cross-linking of either monomer to the DRE. Time course analysis (Elferink et al. 1990) indicated that the cross-linking of the two smaller proteins to DNA occurred more rapidly than that of the 220 kDa complex, with the 110 kDa complex forming prior to that of the 100 kDa complex. These results suggested that the primary (or initial) DNA-binding occurred between the 110 kDa subunit and the DRE. Photoaffinity and UV-cross-linking experiments subsequently revealed that only the 100 kDa complex (subunit) bound ligand supporting a heterodimeric nature for the transformed TCDD:AhR complex. More recently, Gasiewicz *et al.* (1991) demonstrated that the 100 kDa ligand-binding subunit alone would not bind to the DRE whereas the heterodimer would, suggesting that the multimeric form of the AhR must contain an additional component(s) that confers the ability of the AhR to bind DNA.

The identity of the presumed "DNA-binding" subunit of the AhR has not yet been resolved, however, a recent report by Hankinson and coworkers (Hoffman et al. 1991) has described cloning of a gene for an AhR-associated protein, which may be this subunit. This "Ah Receptor Nuclear Translocation" (*arnt*) gene product appears to be necessary for nuclear localization and DNA binding of the AhR. Expression of this gene in Class 2 variants mouse hepatoma cells, which contain AhR defective in nuclear

translocation and DNA binding, restored AhR function. The predicted size of the Arnt protein is 87 kD and analysis of its deduced amino acid sequence has revealed the presence of a segment with similarity to the basic helix-turn-helix motif present in many DNA-binding proteins (Brennan and Matthews 1989). Whether the *arnt* gene product is the "DNA-binding" subunit of the transformed AhR remains to be determined.

Comparison Between the Ah and Steroid Hormone Receptors

The mechanism of induction of cytochrome P450IA1 by TCDD is similar to induction of specific responsive genes by steroid hormone receptors (Yamamoto 1985) and suggests that it is a member of the steroid and thyroid hormone receptor superfamily (Evans 1988). However, comparison of the biochemical properties of the AhR and its cognate DNA recognition sequence with that of the steroid hormone receptors has revealed several striking differences (Denison 1991, in press (Table 1)). The most significant differences are discussed below (see Denison 1991 for specific references). Although the cytosolic form of these receptors groups are similar in size and shape, transformed (nuclear) AhR is significantly larger than that of the steroid family and appears to be a heterodimer. Additionally, while the molecular weight of the ligand binding subunit of the AhR varies significantly among species (Poland and Glover 1987), the molecular weight of steroid hormone receptors are relatively conserved. Steroid hormone receptors appear to bind to DNA as homodimers (Kumar and Chambon 1988; Wrange et al. 1989), consistent with the observed dyad (or partial dyad) symmetry of hormone receptor DNA recognition sequences (Beato et al. 1989). In contrast, the DRE recognition sequence has no obvious dyad or palindromic symmetry (Denison et al. 1988a) and the AhR complex appears to be a heterodimer composed of distinct ligand- and DNA-subunits (Elferink et al. 1990). Although, all of the steroid hormone receptor genes isolated to date encode all of the functional determinants for hormone binding, nuclear translocation/localization, DNA binding, transcriptional enhancement and transcriptional repression in a single gene or complementation group (Pfahl and Bourgeois 1980; Evans 1988), the function of the AhR appears to involve at least three genetically distinct complementation groups (Legraverend et al.

1982; Karenlampi et al. 1988; Whitlock 1990). One of these affects the ability of the protein to bind TCDD, another, the ability of the TCDD:AhR to bind DNA, and, the third affects both AhR ligand binding and DNA binding. Taken together, these and other observations (Table 1) suggest that the Ah receptor is similar to, yet distinctly different, from members of the steroid/thyroid hormone receptor superfamily and that it may represent an unique class of ligand-dependent transcriptional activators.

Table 1. Comparison of AhR and Hormone Receptor Systems.[1]

Characteristic	AhR/DRE	SHR/HRE
Receptor		
1. Sedimentation Coefficient		
Low Salt (0.1M)	8-10S	8-10S
High Salt (0.4M)	4-6S	3.5-4.5S
2. Molecular Weight		
A. Cytosolic:		
Low Salt (0.1M)	250-300K	250-310K
High Salt (0.4M)	100-130K	90-100K
B. Nuclear:	~176K	100-130K
C. Ligand Binding Subunit (SDS PAGE)	95-124K	~100K
3. hsp90 Bound to Receptor	Yes	Yes
4. p59 Bound to Receptor	No	Yes
5. Molybdate Stabilization	+/-	+
6. Reactive SH Groups	Yes	Yes
7. Complimentation Groups	3+	1
8. Phosphorylation Required for Ligand Binding	No	Yes/No
9. DNA Binding Inhibited by Metal Chelators	No	Yes
10. DNA Binding Form	heteromer	homodimer
DNA Recognition Site		
1. Sequence Motif	single site	dyad symmetry
2. Numbers of Receptors Bound per recognition motif	one	two

[1] for references to specific differences, see text and Denison (1991)

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CHAPTER 1

CHARACTERIZATION AND OPTIMIZATION OF TRANSFORMED GUINEA PIG TCDD:AhR COMPLEXES

CHAPTER 1

INTRODUCTION

Exposure to halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) results in a variety of species- and tissue-specific toxic and biological effects (Poland and Knutson 1982; Safe 1986). Many, if not all of these effects, are mediated by the Ah receptor (AhR), a soluble, intracellular protein which binds TCDD, and related compounds, saturably and with high affinity (Poland et al. 1976). The most studied of these responses to TCDD has been the induction of cytochrome P450IA1 (reviewed in Whitlock 1990). The enzymatic activity of cytochrome P450IA1 is involved in the metabolic detoxification and activation of numerous polycyclic aromatic hydrocarbons (Gelboin 1971). The mechanism of induction of cytochrome P450IA1 is in many ways similar to that described for steroid hormone receptors and steroid responsive genes (Yamamoto 1985; Pratt 1987; 1990). TCDD enters the cell, presumably via passive diffusion, where it is then bound by the AhR. After ligand binding, the TCDD:AhR complex undergoes a poorly defined process known as transformation, during which hsp90 (a heat shock protein of 90 kDa) dissociates from the TCDD:AhR complex and the AhR is converted to a form which binds DNA with high affinity (Perdew and Poland 1988; Wilhelmsson et al. 1990; Denison and Yao 1991). The transformed complexes then translocate into the nucleus and bind to *cis* -acting dioxin-responsive enhancers (DREs) upstream of the cytochrome P450IA1 (CYPIA1) gene. The binding of transformed TCDD:AhR complexes to these DREs stimulates transcription of the CYPIA1 gene ultimately increasing the amount of measurable cytochrome P450IAI enzymatic activity (Whitlock 1990).

In vivo studies of AhR ligand binding and transformation to the DNA binding form have been studied using nuclear extract from mouse hepatoma cells (Whitlock 1987; Denison et al. 1988a). Utilizing a sensitive gel retardation assay, we have recently reported transformation of rat hepatic AhR *in vitro* and have characterized its binding to DNA (Denison and Yao 1991). In the studies presented here, we have used liver cytosol from a variety

of species in an effort to optimize TCDD-inducible complex formation to enhance the analysis of the effect of DRE mutagenesis (Chapter 2). Of the species surveyed, guinea pig hepatic cytosol produced the greatest amount of TCDD-inducible signal. Moreover, we have characterized and optimized the conditions for *in vitro* transformation and DNA binding of guinea pig hepatic cytosolic AhR as a prelude to the studies in Chapter 2.

MATERIALS AND METHODS

Materials

[^{3}H]TCDD (37 Ci/mmole), unlabeled TCDD and TCDBF were obtained from Dr. S. Safe (Texas A&M University). These compounds are extremely toxic substances and were handled with special precautions, including the use of disposable benchtop paper, gloves, plasticware and glassware.

Animals

Male Sprague-Dawley rats (250-500g), Golden Syrian hamsters (125g) and C57Bl/6N and C3HeN mice (20g) were obtained from Charles River Breeding Laboratories (Wilmington,DE); male Hartley guinea pigs (250-300g) were from the Michigan State Department of Health (Lansing, MI); and male New Zealand White rabbits (2 Kg) were from Baileys (Alto, MI). Ovine and bovine hepatic tissue samples were obtained from the Michigan State University Meat Science Laboratory (East Lansing, MI); rainbow trout (200g) from Dr. J. Giesy (Dept. Natural Resources, Michigan State University) and white leghorn chicken (100g) from Michigan State University Laboratory Animal Care Services (East Lansing, MI). Laboratory animals were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water.

Cell Culture

The human intestinal cell line, LS180, was obtained from the American Type Tissue Culture Collection (Rockville, MD) and maintained as described by the supplier.

Preparation of Cytosol

Cytosol was prepared in HEDG buffer (25 mM Hepes, pH 7.5, 1mM EDTA, 1mM DTT and 10% (v/v) glycerol) from the indicated tissue as described by Denison et al. (1986) and from LS180 cells as described by Prokipcak and Okey (1988). Sample aliquots were stored at -80°C until use. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

AhR Ligand-Binding Assay

Specific binding of [^{3}H]TCDD to cytosol was measured using the hydroxylapatite adsorption assay as described previously (Gasiewicz and Neal 1982; Denison et al. 1986).

Oligonucleotides and Gel Retardation Analysis

A complementary pair of synthetic oligonucleotides containing the sequence 5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCGTG-AGAAGAGCCA-3' (corresponding to the wild type AhR binding site of DRE3 (Denison et al. 1988a) and designated as the DRE oligonucleotide) were synthesized, purified, annealed and radiolabeled with [γ-^{32}P]-ATP as 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 promoter).

Gel Retardation assay

Cytosol (16 mg protein/ml for tissue and 2-4 mg protein/ml for cell extracts) was incubated with DMSO (20 µl/ml) or 20 nM TCDD, in DMSO, for 2 h at 20°C. An aliquot (5 µl, 80 µg protein) was then mixed with poly dI•dC (85 ng for guinea pig; 225 ng for all others) and incubated for 15 min at 20°C. [^{32}P]-labeled DRE oligonucleotide (100,000 cpm; 0.1-0.5 ng) was added and the mixture incubated for an additional 15 min at 20°C. The DNA-binding reaction was loaded onto a 4% non-denaturing polyacrylamide gel and the protein-DNA complexes resolved as previously described (Denison and Yao 1991). To determine the amount of protein-DNA complex formed, the specific radiolabeled band was excised from the dried gel and radioactivity quantitated by liquid scintillation. The amount of [^{32}P]-DRE specifically bound in the TCDD- inducible complex was estimated by measuring the

amount of radioactivity in the inducible protein-DNA complex, isolated from a TCDD-treated sample lane, and subtracting the amount of radioactivity present in the same position in a DMSO-treated sample lane. The difference in radioactivity between these samples represents the TCDD-inducible specific binding of [^{32}P]DRE and was expressed as the amount of TCDD:AhR:DRE complex formed.

RESULTS

Formation of the TCDD:AhR:DRE complex

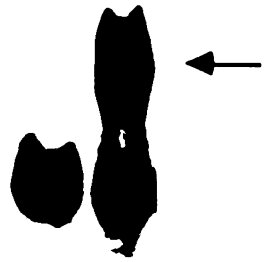
Preliminary studies were performed with AhR obtained from hepatic cytosol of rats. Liver was used in these experiments because it contains the greatest concentration of AhR (Gasiewicz and Rucci 1984). Incubation of the rat liver hepatic cytosol, transformed *in vitro*, with [^{32}P]-labeled DRE oligomer resulted in the formation of two protein-DNA complexes (Figure 1), one of which was TCDD-inducible. We have previously characterized the DNA binding of rat hepatic AhR in detail (Denison and Yao 1991).

Species Variation in Ah Receptor Transformation and DNA Binding

To assess the ability of TCDD:AhR complexes from different species to bind to the DRE oligonucleotide, we carried out gel retardation analysis using hepatic cytosol from various species. Incubation of TCDD-treated cytosol from rat, rabbit, guinea pig, hamster, mouse, sheep, cow, and chicken liver, as well as cytosol from the human cell line LS180, with the [^{32}P]-labeled DRE oligonucleotide results in the formation of two protein-DNA complexes (Figure 2). No TCDD-inducible complex was observed using hepatic cytosol from rainbow trout (Figure 2) or blue gill sunfish, chinook salmon or yellow perch (data not shown). Comparison of the relative amount of TCDD-inducible protein-DNA complex formed to the concentration of AhR ([^{3}H]-TCDD specific binding) present in the same cytosolic preparation, as determined by hydroxylapatite-binding, is presented in Table 1. The apparent lack of TCDD-inducible protein-DNA complex in rainbow trout presumably is due to the low level of AhR in the liver and/or the inability of fish AhR to

Figure 1. Binding of rat hepatic cytosolic AhR to a dioxin responsive enhancer. Cytosol (16 mg protein/ml) was incubated in the absence (-) or presence (+) of TCDD (20 nM) for 2h at 20°C. An aliquot (5 µl, 80 µg protein) was mixed with poly dI•dC (225 ng), incubated for 15 min at 20°C followed by the addition of [^{32}P]-labeled DRE oligonucleotide (100,000 cpm/0.1-0.5 ng) and the mixture incubated for an additional 15 min. Specific protein-DNA complex formation was determined by gel retardation analysis as described in Materials and Methods. The arrow indicates the position of the TCDD-inducible protein-DNA complex.

TCDD - +



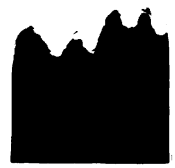


Figure 2. TCDD-inducible protein-DNA complex formation using TCDD-treated cytosol from various species. Cytosol (16 mg/ml or 2-4 mg/ml for cell extract) prepared from liver of the indicated species or from human LS180 cells was incubated in the absence (-) or presence (+) of TCDD and protein-DNA complexes resolved by gel retardation analysis as described in Materials and Methods.

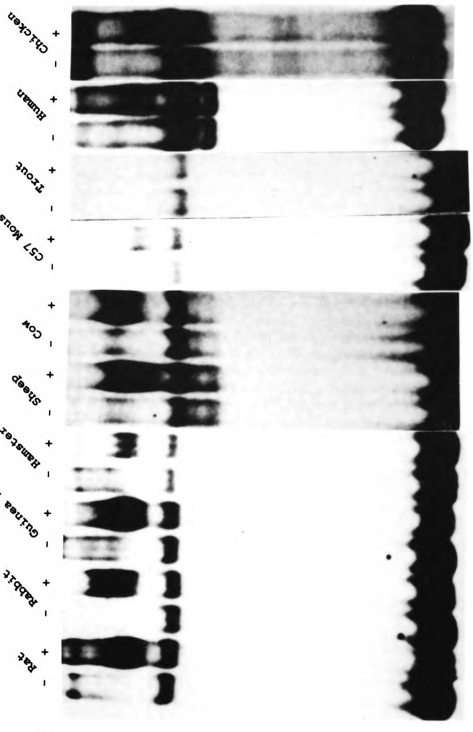

TCDD
-
Rat
+
-
Rabbit
+
-
Guinea Pig
+
-
Hamster
+
-
Sheep
+
-
Cow
+
-
C57 Mouse
+
-
Trout
+
-
Human
+
-
Chicken
+

Table 1. Specific Binding of [^{3}H]TCDD to Hepatic Cytosol from Various Species.

Species	[^{3}H]TCDD Specific Binding (fmoles/mg protein[a])	Relative DRE Binding[b]
Sprague Dawley Rat	51.0 ± 2.0	+++
New Zealand White Rabbit	75.1 ± 3.5	+++
Hartley Guinea Pig	43.2 ± 4.5	++++
Hamster	50.4 ± 5.4	++
Sheep	42.1 ± 10.0	+++
Cow	35.9 ± 4.4	+++
C57 Mouse	41.2 ± 1.7	+
Rainbow Trout	5.1 ± 2.6	-
Human LS180 Cells	260.0 ± 3.0	+++
Chicken	41.3 ± 2.3	+

a. Values are expressed as the mean ± SD of duplicates of at least four determinations.

b. Relative DRE binding is expressed as the relative amount of TCDD-inducible protein-DNA complex formed in gel retardation analysis (see Fig. 2). The symbols represent the relative amount of TCDD-inducible complex formed, ranging from no inducible complex (-) to relatively high amounts of inducible complex (+++).

bind to the DRE. The small amount of inducible protein-DNA complex formed using C57 mouse hepatic cytosol, which contains AhR levels comparable to most other species (Table 1), is most likely due to the relatively slow transformation/dissociation of mouse AhR to a DNA binding form when compared to other species (Denison et al. 1986; Denison and Vella 1990). These results, in combination with the observation that the migration of the TCDD-inducible protein-DNA complex in the gel retardation assay is the same regardless of whether the TCDD:AhR complexes are transformed *in vitro* or *in vivo* (Denison et al. 1988a; Denison and Yao 1991), imply that AhR transformation in vitro appears to faithfully mimic that which occurs *in vivo*. Based on the above results, we selected guinea pig hepatic cytosol as the source of Ah receptor for further studies as it yielded the greatest amount of TCDD-inducible protein-DNA complex formation.

Our results indicate that cytosolic AhR, from most of the species we studied, can be transformed *in vitro* to a form(s) that binds to the DRE oligonucleotide and suggests that the DNA binding domain of the AhR is highly conserved among species. Additionally, all of the "factors" necessary for these events to occur must be present in the cytosolic fraction. Species variation in the relative migration of the TCDD-inducible protein-DNA complex were also readily apparent (Figure 2) and may be due to previously reported species differences in the molecular weight of the AhR (Poland and Glover 1987) and/or the presence of additional proteins in the inducible protein-DNA complex (Denison et al 1989; Elferink et al. 1990; Gasiewicz et al. 1991). These results demonstrate that some species differences in the ability of hepatic AhR to transform to its DNA binding form and/or bind to the DRE do exist. In general, however, our results imply that transformation and DNA binding of hepatic AhR appear to be similar among species, and they support the ligand-dependent nature of AhR transformation and DNA binding.

Characterization and Optimization of Guinea Pig TCDD:AhR:DRE Complex Formation

We have examined several properties of the interaction between the transformed guinea pig TCDD:AhR complex and the DRE oligonucleotide.

Incubation of TCDD-treated guinea pig cytosol with [^{32}P]-labeled DRE oligonucleotide resulted in the formation of two complexes (Figure 3), a TCDD-inducible protein-DNA complex (complex A); and a constitutive protein-DNA complex (complex B). We have previously reported similar complexes using rat hepatic cytosol (Denison and Yao 1991). Studies utilizing a variety of competitor DNAs were employed to define the binding specificity of the transformed hepatic TCDD:AhR complex for the DRE. Formation of [^{32}P]-labeled complexes A and B were inhibited by excess DRE oligomer (10-fold excess) but not by excess nonspecific DNA (10-fold excess) which lacks a DRE consensus sequence (Figure 3). Competitive displacement of [^{32}P]-DRE from complex B by excess unlabeled single stranded DRE oligonucleotide but not by double-stranded DRE-containing DNA (isolated as a plasmid fragment) indicates that complex B may represent a single-stranded DNA binding protein(s) which binds to the small amount of single-stranded oligonucleotide remaining after reannealing. What role, if any, this protein(s) may play in TCDD:AhR:DRE complex formation is unknown. Thus, these studies reveal that the transformed TCDD:AhR preferentially binds, in a sequence specific manner, to double-stranded DRE-containing DNA.

Initial optimization experiments using guinea hepatic cytosol were performed as previously detailed for rat cytosol (Denison and Yao 1991). Cytosol was incubated at 20°C for 2 hours with TCDD and optimal conditions for TCDD-inducible complex formation were determined by varying single parameters. Under these conditions TCDD:AhR:DRE complex formation increased with respect to protein concentration (Figure 4). Binding was optimal at 5 nM TCDD (Figure 5), 80 mM KCl (Figure 6) and 85 ng poly dI•dC (Figure 7). Addition of $MgCl_2$, known to stimulate the DNA-binding of some proteins (Chodosh et al. 1986), resulted in inhibition of TCDD:AhR:DRE complex formation (Figure 8). This inhibition appeared to be due to an adverse effect of $MgCl_2$ on DNA-binding, rather that transformation or ligand (TCDD) binding, since $MgCl_2$ was added to the incubation mixture immediately prior to DNA binding. Incubation of cytosol with TCDD at any temperature above 20°C resulted in a significant decrease in inducible complex formation than that observed at 20°C (Figure 9), presumably due to greater instability of unoccupied AhR at elevated temperature (Kester and

Figure 3. Binding specificity of proteins to the DRE. [^{32}P]-labeled DRE oligonucleotide was mixed with untreated (-) or treated (+) cytosol and the protein-DNA complexes resolved by gel retardation analysis. After ligand incubation, an aliqout containing transformed TCDD:AhR complexes was mixed with poly dI•dC, followed by the addition of the indicated excess of unlabeled competitor DNA fragments prior to the addition of the [^{32}P]-labeled DRE oligonucleotide. DNA competitors were as follows: 10x DRE is unlabeled double-stranded 26 bp DRE oligonucleotide; non-specific is double-stranded 28 bp oligonucleotide which lacks a DRE consensus sequence; plasmid DRE is a double stranded restriction fragment containing DRE2 (a 30 bp *Eco*RI-*Ppum*I fragment isolated from pGEM5.30 (Denison et al, 1989) and ssDRE is the 26 bp single-stranded DRE oligonucleotide (coding strand).

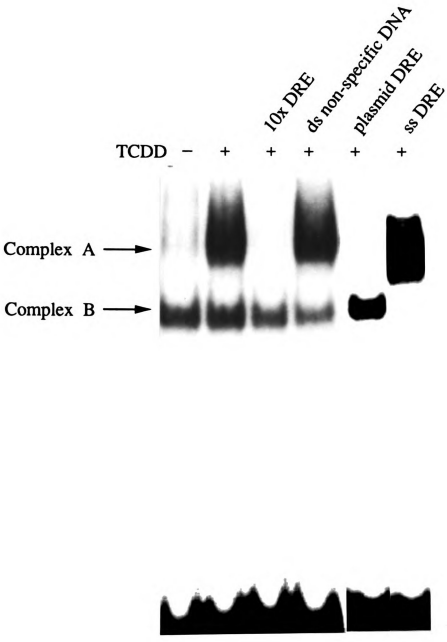
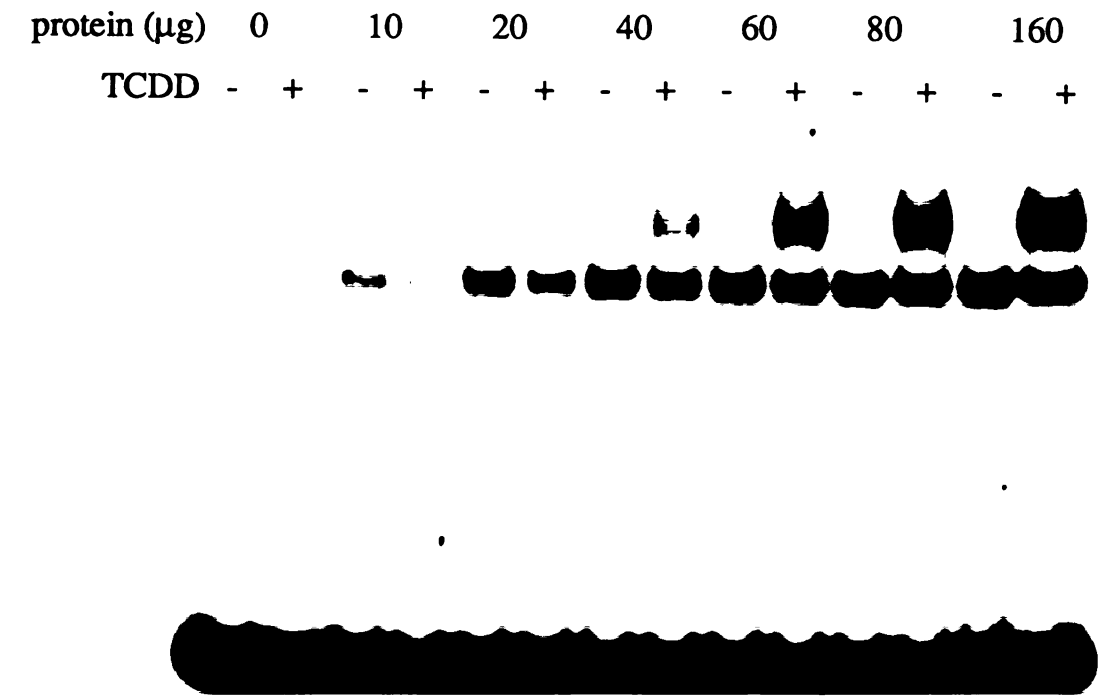

10x DRE
ds non-specific DNA
plasmid DRE
ss DRE
TCDD – + + + + +
Complex A
Complex B

Figure 4. Dependence of TCDD-inducible protein-DNA complex formation on protein concentration. Cytosol (concentrations as indicated) was treated with (+) or without (-) TCDD (20 nM) for 2h at 20°C. An aliquot (5 µl) was analyzed for the formation of protein-DNA complexes by gel retardation as described in Materials and Methods. The amount of [^{32}P]-DRE specifically bound in the TCDD- inducible complex was estimated by measuring the amount of radioactivity in the inducible protein-DNA complex, isolated from a TCDD-treated sample lane, and subtracting the amount of radioactivity present in the same position in a DMSO-treated sample lane. The difference in radioactivity between these samples represents the TCDD-inducible specific binding of [^{32}P]-DRE.


protein (μg) 0 10 20 40 60 80 160
TCDD - + - + - + - + - + - + - +

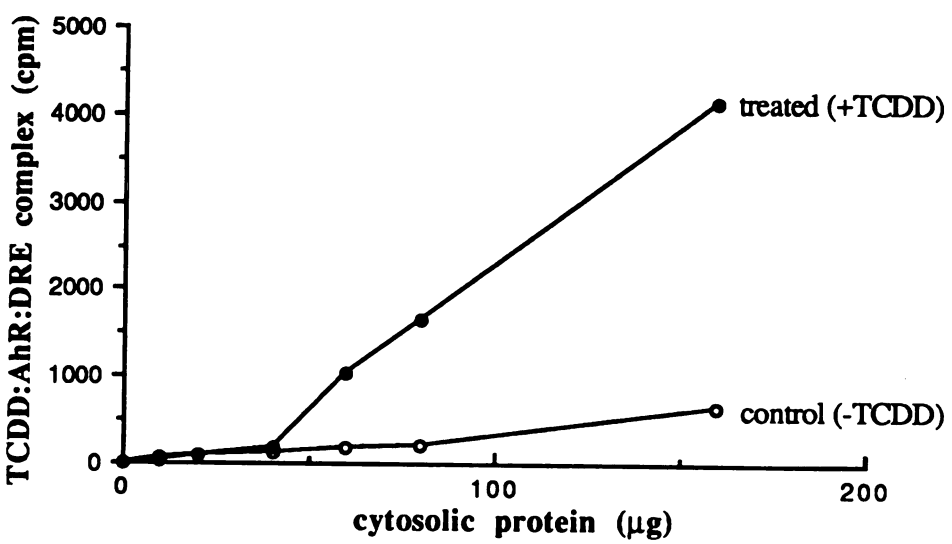
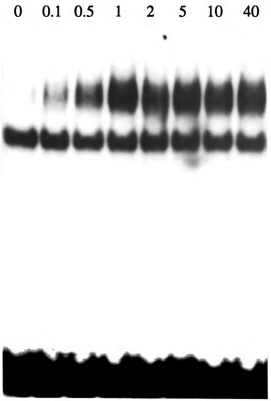

TCDD:AhR:DRE complex (cpm)
5000
4000
3000
2000
1000
0
0
100
200
cytosolic protein (μg)
treated (+TCDD)
control (-TCDD)

Figure 5. Dose-dependence of TCDD-inducible protein-DNA complex formation. Cytosol (16 mg/ml) was incubated with the indicated concentration of TCDD for 2h at 20°C and an aliquot added to the standard binding reaction and analyzed by gel retardation. The amount of [^{32}P]-DRE present in the TCDD-inducible complex was estimated as described in the legend to Figure 4.


TCDD (nM) 0 0.1 0.5 1 2 5 10 40

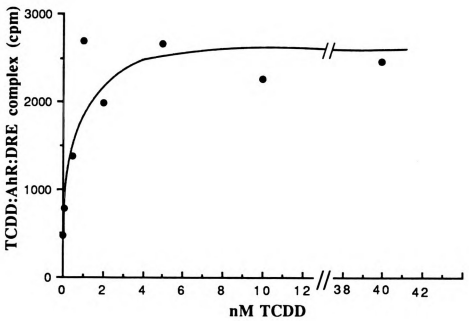

TCDD:AhR:DRE complex (cpm)
3000
2000
1000
0
0 2 4 6 8 10 12 38 40 42
nM TCDD

Figure 6. Effect of KCl on TCDD-inducible protein-DNA complex formation. TCDD-treated cytosol was incubated in the binding reaction with the indicated concentrations of KCl for 15 min just prior to addition of [^{32}P]-labeled DRE oligonucleotide and then analyzed by gel retardation as described in Materials and Methods. The amount of specific TCDD-inducible complex formed was determined as described in the legend to Figure 4.

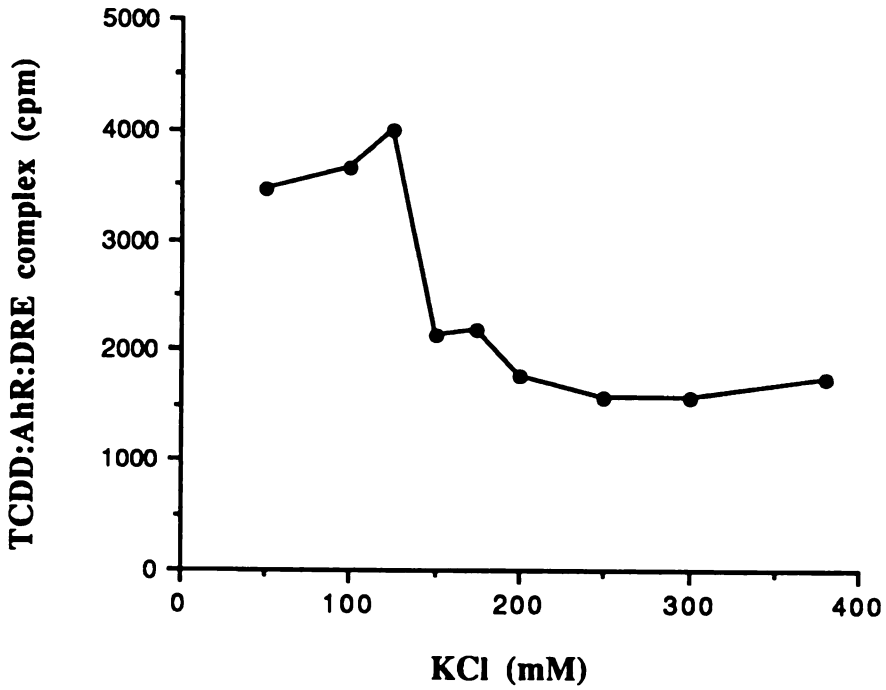
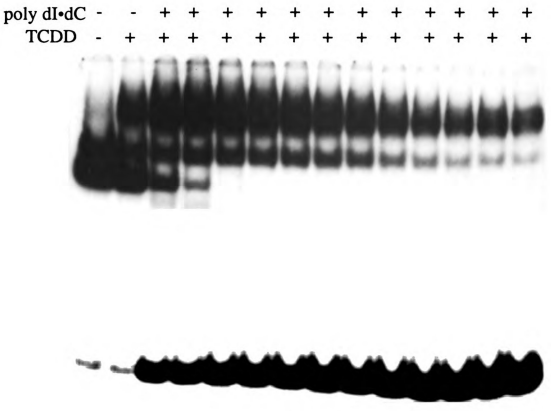

TCDD:AhR:DRE complex (cpm)
5000
4000
3000
2000
1000
0
0
100
200
300
400
KCl (mM)

Figure 7. Effect of poly dI•dC on TCDD-inducible protein-DNA complex formation. TCDD-treated cytosol was incubated with increasing amounts of poly dI•dC for 15 min.prior to addition of [^{32}P]-labeled DRE oligonucleotide and the protein-DNA complexes resolved by gel retardation as decribed in Materials and Methods. The amount of specific TCDD-inducible complex formed was determined as described in the legend toFigure 4.


poly dI•dC - - + + + + + + + + + + +
TCDD - + + + + + + + + + + + +

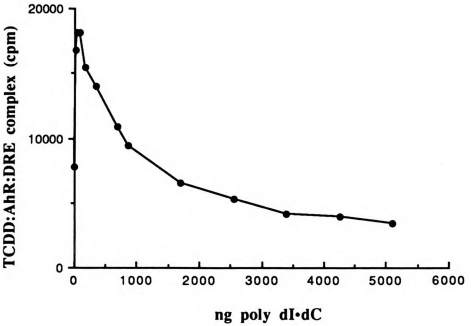

TCDD:AhR:DRE complex (cpm)
20000
10000
0
0
1000
2000
3000
4000
5000
6000
ng poly dI•dC

Figure 8. Effect of $MgCl_2$ on TCDD-inducible protein-DNA complex formation. TCDD-treated cytosol was incubated with the indicated concentrations of $MgCl_2$ in the binding reaction for 15 min prior to addition of [^{32}P]-labeled DRE oligonucleotide and then analyzed by gel retardation as described in Materials and Methods. The amount of specific TCDD-inducible complex formed was determined as described in the legend to Figure 4.

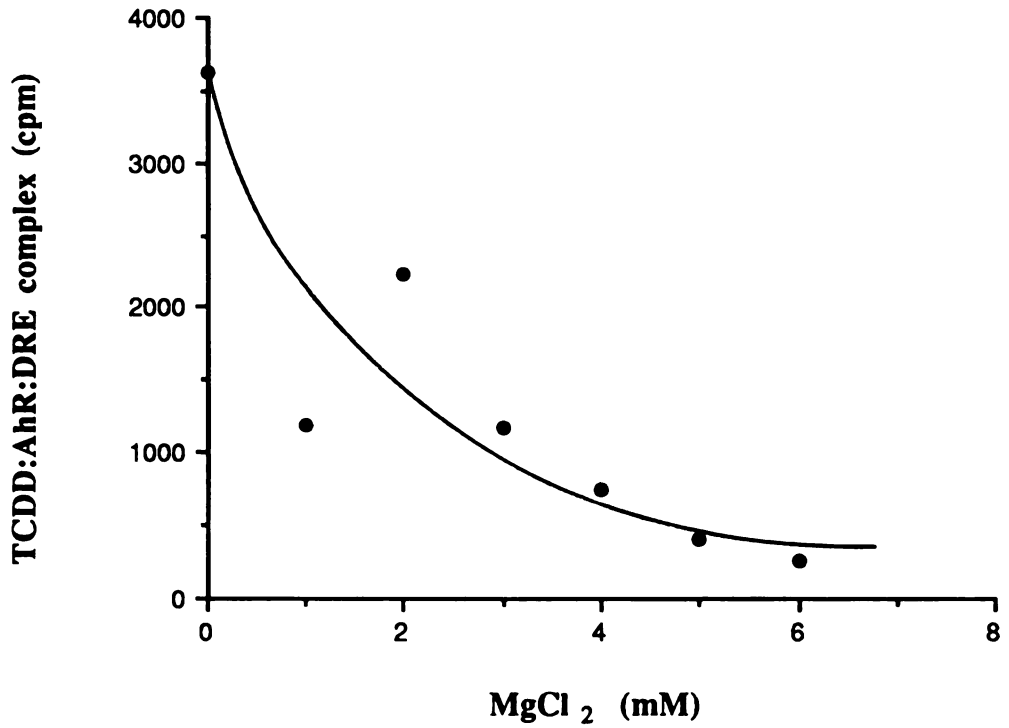
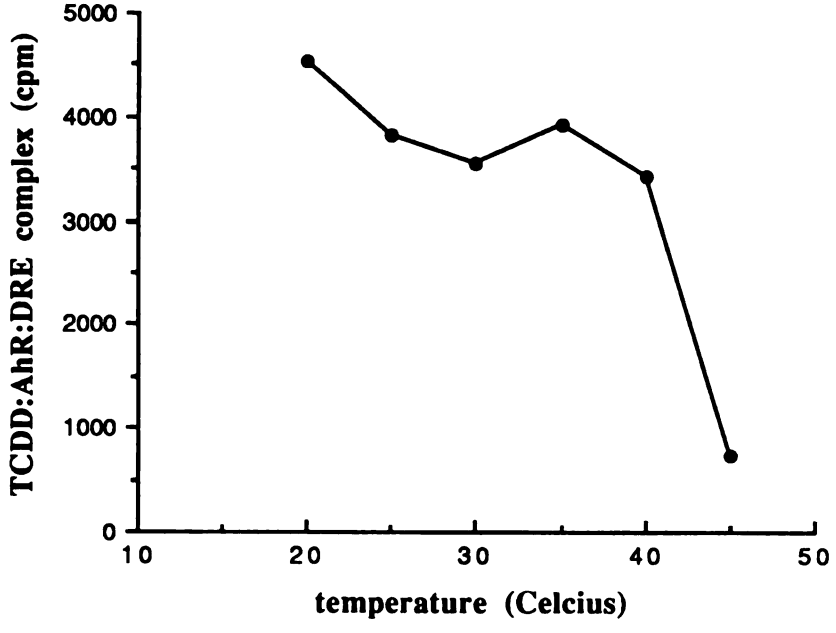

TCDD:AhR:DRE complex (cpm)
4000
3000
2000
1000
0
0
2
4
6
8
MgCl 2 (mM)

Figure 9. Effect of temperature on TCDD-inducible protein-DNA complex formation. TCDD-treated cytosol (16 mg/ml) was incubated at various temperatures and protein-DNA complexes analyzed by gel retardation. The amount of specific TCDD-inducible complex formed was determined as described in the legend to Figure 4.


TCDD:AhR:DRE complex (cpm)
5000
4000
3000
2000
1000
0
10
20
30
40
50
temperature (Celcius)

Gasiewicz 1987). TCDD-inducible complex formation was also maximal by 2 hours (Figure 10). Interestingly, some AhR transformation occurred extremely rapidly following the addition of TCDD (Figure 10). Comparison of these results to the time course (rate) of specific ligand binding of [^{3}H]-TCDD (Bunce et al. 1988) suggests that transformation occurs rapidly following ligand binding.

To determine the equilibrium dissociation constant (K_d) of the TCDD:AhR complex binding to the DRE, we generated a saturation binding curve utilizing a constant amount of TCDD-treated cytosol and increasing amounts of specific [^{32}P]-labeled DRE (Figure 11A). After correction for the presence of single-stranded DRE in each incubation, the kinetics of binding were determined using a Woolf plot of the saturation data (Figure 11B) , in which the slope of the line is equal to $1/B_{max}$ and the *y* intercept equal to K_d/B_{max} (Cressie and Keightley 1981). The estimated K_d from five separate experiments of this type (using two different cytosolic preparations) was determined to be $2.5 \pm 0.8 \times 10^{-9}$ M (Table 2). The linearity of both Woolf (Figure 11B) and Scatchard (data not shown) plots were suggestive of a single DRE-binding species. These results do not, however, rule out the possibility of multiple DRE-binding species with similar affinities.

DISCUSSION

We have previously utilized gel retardation analysis to demonstrate the interaction of nuclear TCDD:AhR complexes, isolated from TCDD-treated mouse hepatoma cells, with a dioxin responsive DNA element (Whitlock 1987; Denison et al. 1988a). Here, we have extended our observations to show that cytosolic TCDD:AhR complexes from a variety of species can be transformed *in vitro* to a form(s) which specifically binds to a DRE oligonucleotide with high affinity. The ability of cytosolic AhR to bind ligand, undergo transformation, and bind to the DRE, implies that all constituents necessary for these processes must be available in the cytosol. In addition, no significant increase in DRE binding was observed following the addition of nuclear extract to these cytosolic preparations, implying that no

additional nuclear proteins were necessary for formation of the inducible complex (data not shown).

Saturation binding gel retardation analysis of guinea pig hepatic cytosolic AhR was suggestive of a single DRE-binding site with an apparent K_d of 2.5 ± 0.8 nM. Competitive binding experiments indicated that transformed TCDD:AhR complexes bound to the DRE with a 1300-fold greater DNA binding affinity than the non-specific DNA competitor, poly dI•dC. A K_d in the nanomolar range is consistent with values obtained for the binding of steroid receptors to their cognate DNA recognition sequences. A K_d of 2.5 nM for DRE binding of transformed crude cytosolic TCDD:AhR complexes is comparable to the values estimated for binding of partially purified estrogen and glucocorticoid receptor to their specific DNA recognition sites (0.5 nM (Peale et al. 1989) and 1.4 nM (Wrange et al. 1989), respectively). It is currently not known exactly how the AhR interacts with the DRE site; however, recent evidence suggests that the DNA-binding form of the AhR is at least a heterodimer (Elferink et al 1990; Gasiewicz et al. 1991) with only one ligand-binding subunit. Whether both AhR subunits are required for high affinity DNA binding of transformed AhR is not known. The lack of palindromic DNA recognition site in the DRE (Denison et al. 1988a) combined with the fact that the DNA-binding form of the AhR appears to be at least a heterodimer suggests several different scenarios for this interaction. It is possible that both AhR subunits directly contact the DRE or that only one subunit binds specifically to DNA and the other is bound to this subunit by protein-protein interactions. The experiments desribed in Chapter 2 examine TCDD:AhR:DRE complex formation using site directed DRE mutagenesis and will discuss these DNA-binding models in greater detail.

Figure 10. Time dependence on TCDD-inducible protein-DNA complex formation. Cytosol (16 mg/ml) was incubated in the absence or presence of TCDD (20 nM) for the indicated time, mixed in the standard binding reaction prior to the addition of the [^{32}P]-labeled DRE oligonucleotide and was analyzed for the formation of inducible complex by gel retardation analysis. TCDD-inducible protein-DNA complex formation was quantitiated as described in the legend to Figure 4.

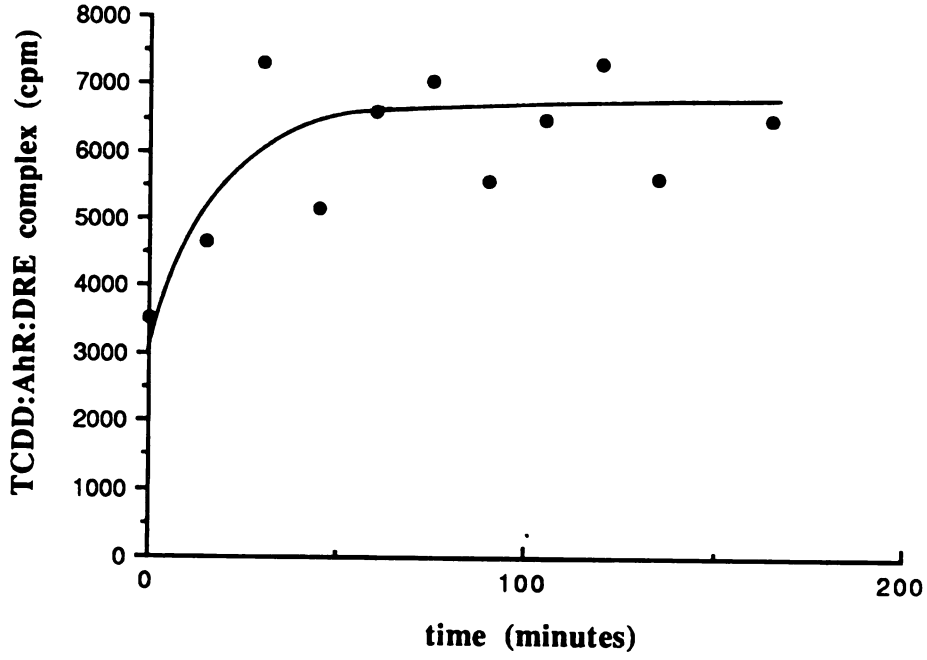
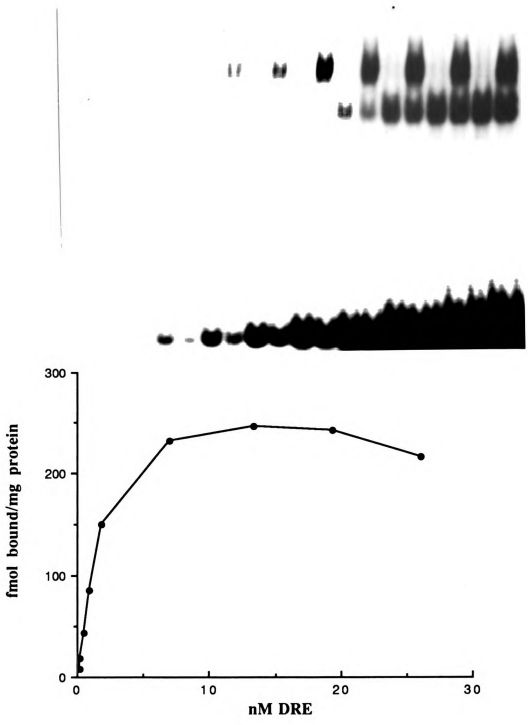

TCDD:AhR:DRE complex (cpm)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
100
200
time (minutes)

Figure 11. Equilibrium binding of TCDD:AhR complexes to the DRE. **A.** Increasing amounts of [^{32}P]-labeled DRE oligonucleotide were added to the standard binding reaction and incubated for 15 min. at 20°C and subsequently analyzed by gel retardation. The amount of DRE specifically bound in the TCDD-inducible complex was determined as described in the legend to Figure 4. **B.** The data were analyzed on a Woolf plot, in which the slope of the line is equal to $1/B_{max}$ and the *y*-intercept equal to K_d/B_{max} (Cressie and Keightley 1981). Combined results from six separate experiments indicated that the K_d for binding of the TCDD:AhR complex to the DRE was 2.5 ± 0.8 nM and the B_{max} was 255 ± 39 fmol/mg.

A.



B.

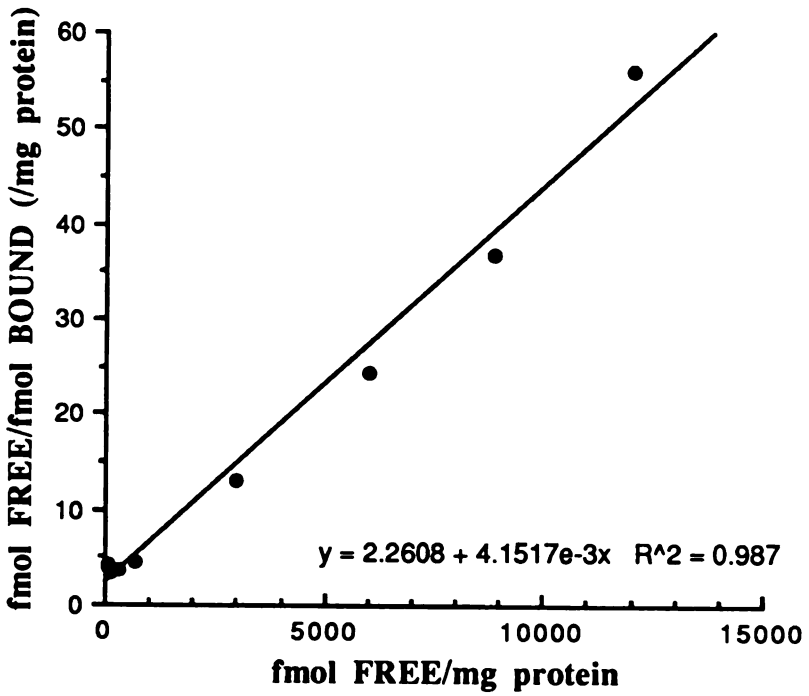

fmol FREE/fmol BOUND (/mg protein)
60
50
40
30
20
10
0
y = 2.2608 + 4.1517e-3x R^2 = 0.987
0
5000
10000
15000
fmol FREE/mg protein

Table 2. Saturation binding analysis of transformed guinea pig TCDD:AhR complex to the DRE oligonucleotide.

Experiment #	Bmax (fmol/mg)	Kd (nM)
1	206.7	1.8
2	312.4	3.6
3	242.7	1.8
4	266.0	2.6
5	249.2	2.9
mean ± SD	255.4 ± 38.5	2.5 ± 0.8

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CHAPTER 2

DNA SEQUENCE DETERMINANTS FOR BINDING OF TRANSFORMED Ah RECEPTOR TO A DIOXIN RESPONSIVE ENHANCER

CHAPTER 2

INTRODUCTION

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD), the most potent member of a large group of halogenated aromatic hydrocarbons (HAHs), results in numerous species- and tissue-specific toxic and biological effects, including tumor promotion, immunotoxicity, hepatotoxicity, teratogenesis, and enzyme induction (Poland and Knutson 1982; Safe 1986). The mechanism of induction of cytochrome P450IA1[1] and its associated monooxygenase activity, the most widely studied response to TCDD, is in many ways similar to that described for steroid hormone receptors and steroid-responsive genes (Poland and Knutson 1982; Yamamoto 1985; Safe 1986; Whitlock 1990). Induction by TCDD and other related HAHs is mediated by a soluble intracellular protein, the Ah (aromatic hydrocarbon) receptor (AhR), which binds TCDD saturably and with high affinity (Poland and Knutson 1982; Poland et al. 1986; Safe 1986; Whitlock 1990). Following ligand (TCDD) binding, the AhR, like steroid hormone receptors, undergoes a poorly defined process of transformation[2], during which hsp90 (a 90 kD heat shock protein (Denis et al. 1988; Perdew 1988) dissociates from the TCDD:AhR complex and the AhR acquires the ability to bind to DNA with high affinity (Whitlock and Galeazzi 1984; Henry et al. 1989; Denison and Yao 1991). Biochemical and genetic studies (Denison et al. 1988a; 1988b; Whitlock 1990) have indicated that transcriptional activation of the cytochrome P450IA1 (CYPIA1) gene is stimulated by the binding of transformed TCDD:AhR complexes to *cis*-acting dioxin-responsive enhancers (DREs) located upstream of the gene.

Previously, we have shown that transformed TCDD:AhR complexes, formed

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

[2] We have defined transformation as the process by which the ligand:AhR complex changes to a form which binds to DNA with high affintiy.

in vivo or *in vitro,* can bind to a DRE oligonucleotide specifically and with high affinity (Denison et al. 1988a; 1988b; Denison and Yao 1991). Four functional DRE sequences have currently been identified in the 5' flanking region of the mouse CYPIA1 gene (Fisher et al. 1990) and their alignment has revealed the presence of an invariant core sequence,TNGCGTG, flanked by several variable nucleotides (Denison et al. 1988a; 1989). The results of methylation interference studies (Shen and Whitlock 1989; Saatcioglu et al. 1990) have demonstrated that several of these "core" nucleotides are critical for TCDD:AhR:DRE complex formation. Additionally, although several studies (Nebert and Jones 1989; Saatcioglu et al. 1990; Cuthill et al. 1991) have examined the effect of DRE mutagenesis on AhR DNA binding, the role of specific nucleotides within the DRE consensus could not be established since these studies utilized DRE oligonucleotides which contained multiple substitutions. Here we have utilized gel retardation analysis and DRE mutagenesis in order to examine the DNA binding of transformed AhR in greater detail and to identify those nucleotides important in TCDD:AhR:DRE complex formation.

MATERIALS AND METHODS

Materials

Molecular Biological reagents were from New England Biolabs and Bethesda Research Laboratories. TCDD was obtained from Dr. S. Safe (College Station, TX). [γ-^{32}P]-ATP (>6000 Ci/mmole) was from Amersham Corp.

Animals

Male Hartley guinea pigs (200-500g), obtained from the Michigan Department of Health (Lansing, MI), were exposed to 12 h of light and 12 h of dark daily and were allowed free access to food and water.

Preparation of Cytosol

Guinea pig hepatic cytosol was prepared in ice cold HEDG (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 stored at -80°C until use. Protein concentrations

were measured by the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

Synthetic oligonucleotides

A complementary pair of synthetic DNA fragments containing the sequence 5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and 5'-GATCCGGAGTTGCG-TGAGAAGAGCCA-3' (corresponding to the 20 bp AhR binding site of DRE3 (Denison et al. 1986; Denison and Yao 1991) and designated here as the "DRE oligonucleotide") or complementary pairs of DRE oligonucleotides containing single or multiple nucleotide substitutions (see text for details) were synthesized using an Applied Biosystems DNA synthesizer, purified by polyacrylamide gel electrophoresis or HPLC techniques, annealed and radiolabeled with [γ-^{32}P]ATP (Denison et al. 1988b). Annealing of the oligonucleotides 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 the normal orientation of the DRE with respect to its promoter.

Isolation of DNA fragments

The following DNA fragments were isolated from the indicated plasmids by restriction digestion using standard procedures (the numerical values indicate their normal position in the 5' flanking region of the mouse CYPIA1 gene, relative to the start site of transcription (Gonzalez et al. 1985). DRE1 is an *Eco*RI-*Hph*I fragment isolated from the plasmid pGEMLS5.28, and spans the region from -933 to -869; DRE2 is an *Eco*RI-*Ppum*I fragment isolated from the plasmid pGEMLS5.30, and spans the region from -1076 to -1048; DRE3 is an *Eco*RI-*Ppum*I fragment isolated from the plasmid pGEMLS3.2, and spans the region from -997 to -977; DRE4 is an *Eco*RI-*Stu*I fragment isolated from the plasmid pGEMLS3.19, and spans the region from -1227 to -1146; and DRE5 is a *Mnl*I-*Pvu*II fragment isolated from the plamid pMcat5.D8S, and spans the region from -509 to -448.

Gel Retardation analysis

Cytosol (16 mg protein/ml) was incubated with DMSO (20 µl/ml) or 20 nM TCDD, in DMSO, for 2 h at 20°C and gel retardation analysis carried out as previously described (Denison et al. 1988b; Denison and Yao 1991) using [^{32}P]-labeled DRE or mutant DRE oligonucleotides. To determine the relative

binding affinity of transformed TCDD:AhR complexes for various DRE-containing fragments and mutant DRE oligomers, we carried out competitive gel retardation analysis. In these experiments, increasing concentrations of competitor DNA were added to the incubation mixture, prior to [^{32}P]-DRE oligonucleotide addition, and after separation by electrophoresis, the specific radiolabeled band was excised from the dried gel and quantitated by liquid scintillation. The amount of [^{32}P]-DRE specifically bound in the TCDD-inducible complex was estimated by measuring the amount of radioactivity in the inducible protein-DNA complex, isolated from a TCDD-treated sample lane, and subtracting the amount of radioactivity present in the same position in a non-TCDD-treated sample lane. The difference in radioactivity between these samples represents the TCDD-inducible specific binding of [^{32}P]-DRE and was expressed as the amount of TCDD:AhR:DRE complex formed. Competitive displacement curves were generated by plotting the log of the molar concentration of added competitor versus the percent of [^{32}P]-oligomer specifically bound in the TCDD-inducible complex, with 100% bound representing the amount with no competitor DNA. Comparison of the IC_{50} value of the DRE oligonucleotide (competitor concentration which reduces inducible complex formation by 50%) to that obtained with a specific competitor DNA allowed estimation of the relative binding affinity of the specific competitor.

Analysis of data

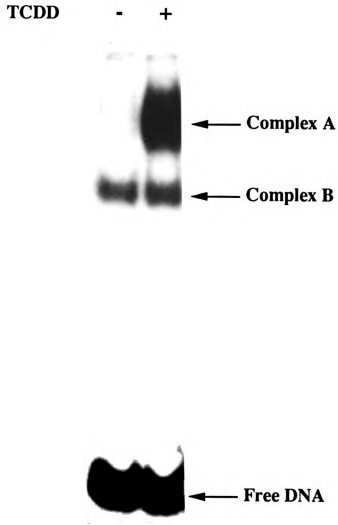
Data were analyzed by a one-way analysis of variance (ANOVA) when data were homogeneous; homogeneity was assessed using the *F max* test (Steel and Torrie 1980). When data were not homogeneous, a log transformation was performed. Individual means were compared using the least significant difference test and the results are expressed as means ± SE. In all cases, $p < 0.05$ was set as the criterion for statistical significance.

RESULTS

Formation of the TCDD:AhR:DRE Complex

Incubation of guinea pig hepatic cytosol with [^{32}P]-labeled DRE oligonucleotide resulted in the formation of two protein-DNA complexes

Figure 1. Binding of guinea pig hepatic cytosolic proteins to a dioxin responsive enhancer. Cytosol (16 mg/ml), incubated in the absence (-) or presence (+) of TCDD (20 nM) for 2h at 20°C, was mixed with poly dI•dC (85 ng) and further incubated for 15 min at 20°C. The [^{32}P]-labeled DRE oligonucleotide (100,000 cpm; 0.1-0.5 ng) was added and the mixture incubated for an additional 15 min. Protein-DNA complexes were analyzed using the gel retardation assay as described in Materials and Methods. Complex A is a TCDD-inducible complex and complex B is a constitutive (observed in the absence and presence of TCDD).


TCDD
-
+
Complex A
Complex B
Free DNA

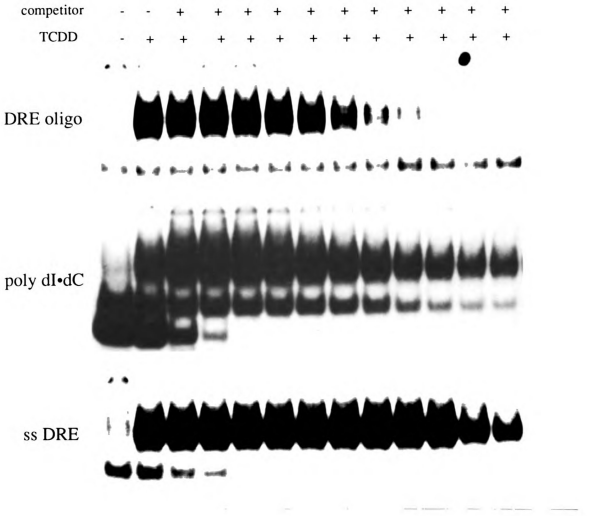
(Figure 1), one of which (complex A) was TCDD-inducible (observed only in thepresence of TCDD) and the other (complex B) was constitutive (observed in the absence and presence of TCDD). Previous studies have indicated that the TCDD-inducible protein-DNA complex represents the binding of transformed TCDD:AhR to the DRE (Denison et al. 1988a; 1989). In some experiments, a small amount of complex A was observed in control cytosol and may represent some nonspecific protein-DNA complex, transformed AhR occupied by an endogenous ligand and/or a small fraction of AhR transformed in the absence of ligand. We have recently observed that some lots of DMSO will induce formation of a protein-DNA complex which migrates similarly to that of complex A, in a DMSO dose-dependent manner (data not shown). Thus, whether this protein-DNA complex is due to the presence of a contaminant(s) in the DMSO which can bind to the AhR and induce transformation and DNA binding or whether it actually represents a different protein-DRE complex is unknown. These experiments demonstrate, however, that cytosolic guinea pig hepatic TCDD:AhR complexes can transform *in vitro*, implying that all constituents necessary for AhR transformation and binding must be present in the cytosol preparation.

Specificity of TCDD:AhR Complex in Binding to DNA

The DNA-binding specificities of both complexes are comparable to that previously observed in rat hepatic cytosol (Denison and Yao 1991). Formation of both complexes was inhibited by excess DRE oligomer but not by excess nonspecific DNA which lacks a DRE consensus sequence (data not shown). Relative binding of transformed AhR to specific versus nonspecific DNA was assessed utilizing competitive gel retardation analysis (Figure 2). Addition of increasing concentrations of the indicated specific and nonspecific competitors effectively decreased formation of the TCDD:AhR:DRE complex (Figure 2A). Quantitation of the amount of specific TCDD:AhR:DRE complex formed in the presence of increasing concentrations of the indicated competitor DNA was determined and competitive displacement curves were generated (Figure 2B). Comparison of the IC_{50} value of a given DNA competitor to that determined using the DRE oligonucleotide provides a measure of its relative potency as a competitor and allows calculation of its relative binding affinity compared to that for the DRE oligonucleotide. In

Figure 2. Relative affinity of transformed TCDD:AhR complexes for nonspecific and single-stranded DNA. **A.** Typical competitive gel retardation experiments used in the generation of competitive binding curves. The concentrations of specific competitors are as indicated as plotted on 2B. **B.** Cytosol (16 mg/ml) was incubated in the absence (-) or presence (+) of TCDD (20 nM) for 2h at 20°C. Increasing concentrations of DRE oligonucleotide (●), poly dI•dC (◆), single-stranded DRE oligonucleotide (non-coding strand (Δ) was added to the DNA-binding reaction and the amount of specific TCDD-inducible protein-DNA complex formed determined as described in Materials and Methods. The molar amount of poly dI•dC was calculated assuming that each 22 base pairs is the start of a different nonspecific binding site. Table 1 indicates the relative affinity of the TCDD:AhR complex for each DNA competitor, as estimated from the IC_{50} values.

A.



B.

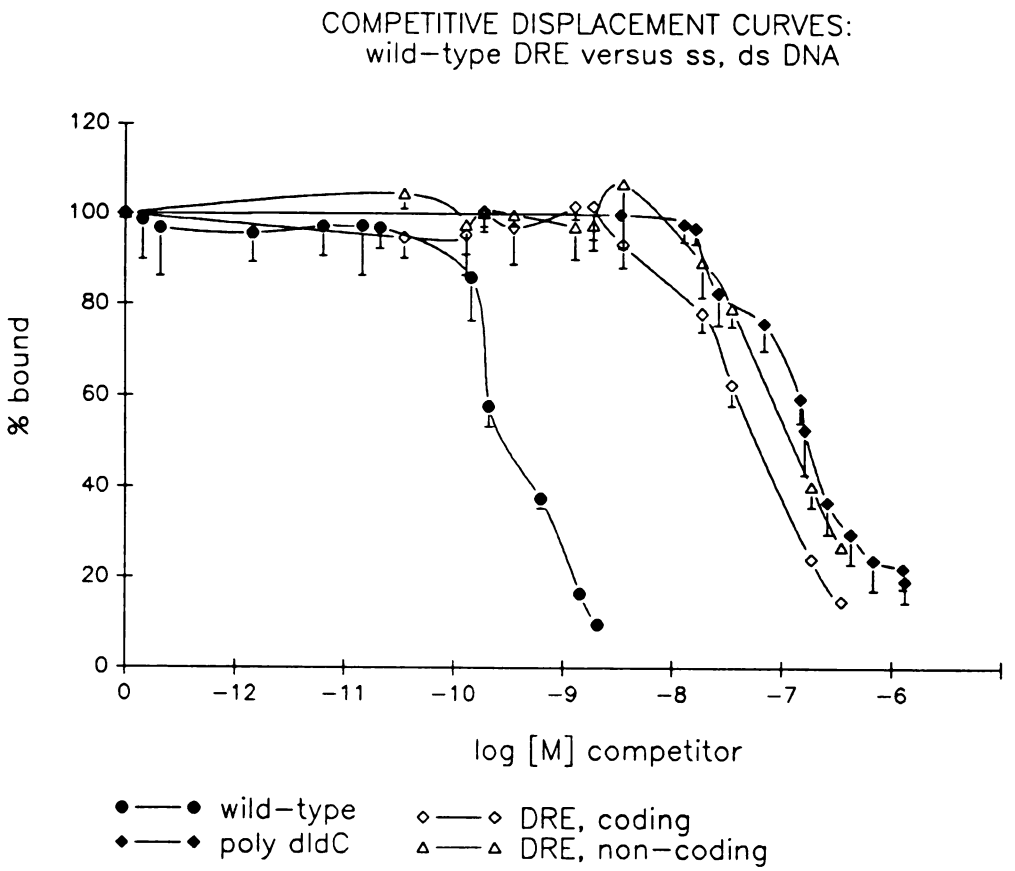

COMPETITIVE DISPLACEMENT CURVES:
wild-type DRE versus ss, ds DNA
% bound
120
100
80
60
40
20
0
0
-12
-11
-10
-9
-8
-7
-6
log [M] competitor
wild-type
poly dIdC
DRE, coding
DRE, non-coding

Table 1. Comparison of binding affinities of transformed TCDD:AhR complexes to specific and nonspecific DNA.

Competitor	Relative Binding Affinity (nM)	Fold Difference
DRE oligomer	2.5	1
ssDRE coding strand	1200	457x lower
ssDRE non-coding strand	2600	1037x lower
poly dI•dC	3400	1337x lower

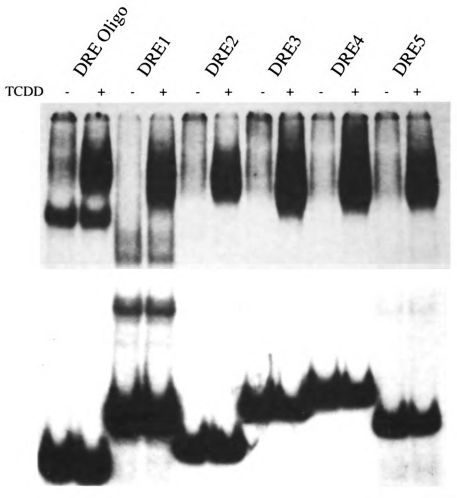
saturation binding experiments, analogous to that described by Denison and Yao (1991), we have determined that the affinity of DRE-binding of transformed guinea pig hepatic cytosolic TCDD:AhR complexes is 2.5 ± 0.8 nM (Bank, Yao and Denison, manuscript in preparation). Comparison of the relative IC_{50}'s has revealed that transformed TCDD:AhR complexes bind to double-stranded DRE oligonucleotide with a 500- to 1000-fold greater affinity than that of single-stranded DRE DNA oligomers; nonspecific DNA (poly (dI•dC)) displayed approximately a 1300-fold lower affinity for the TCDD:AhR complex, relative to the double stranded DRE oligonucleotide (Table 1). These results demonstrate that the DNA binding of transformed guinea pig TCDD:AhR complexes is specific and of high affinity, consistent with our previous studies using rat hepatic cytosol (Denison and Yao 1991) and mouse hepatoma (hepa 1c1c7) cell nuclear extracts (Denison et al. 1988a; 1988b).

Binding of Transformed TCDD:AhR Complexes to Mouse CYPIA1 upstream DREs

We have previously identified five discrete DREs present in the upstream region of the mouse CYPIA1 gene which specifically interact with nuclear TCDD:AhR complexes from mouse hepatoma cells, in a ligand-dependent manner (Denison et al. 1989). Although the results of this study were suggestive of subtle differences in the affinity with which transformed TCDD:AhR complex could bind to each of these DREs, further analysis was not performed. Gel retardation analysis of the binding of DNA fragments containing [^{32}P]-labeled DREs 1 to 5 (Figure 3A) resulted in comparable levels of inducible complex formation. Competitive gel retardation analysis with these DNA fragments revealed a relatively similar degree of TCDD-inducible protein-DNA complex formation (Figure 3B). Comparison of the estimated relative binding affinity of all five mouse CYPIA1 upstream DREs (Table 2) revealed that DNA fragments containing DREs 1, 3, 4, or 5 were significantly more effective (1.5- to 3.8-fold) as competitors than the DRE oligonucleotide itself. These small, but significant, differences may be due to variations in the size of the competitor DNA fragment rather than to real differences in DNA-binding affinity. This is supported by the results of additional competitive binding experiments using the DRE5-containing DNA fragment above (165 bp) and a DRE5-containing oligonucleotide (26 bp) (Table 2). These results

Figure 3. Binding of transformed TCDD:AhR complexes to endogenous mouse CYPIA1 DREs. **A.** The five mouse DREs were isolated by restriction enzyme digestion, and radiolabeled with [^{32}P]. Cytosol (16 mg/ml), treated in the absence (-) or presence (+) of TCDD (20 nM), was incubated with the indicated radiolabeled DRE and protein-DNA complexes resolved by gel retardation analysis. **B.** The indicated concentrations of competitor DNA was added to the standard incubation and the amount of specific TCDD-inducible protein-DNA complex formed was quantitated as described in Materials and Methods and plotted versus molar concentration of DRE competitor.

A.



B.

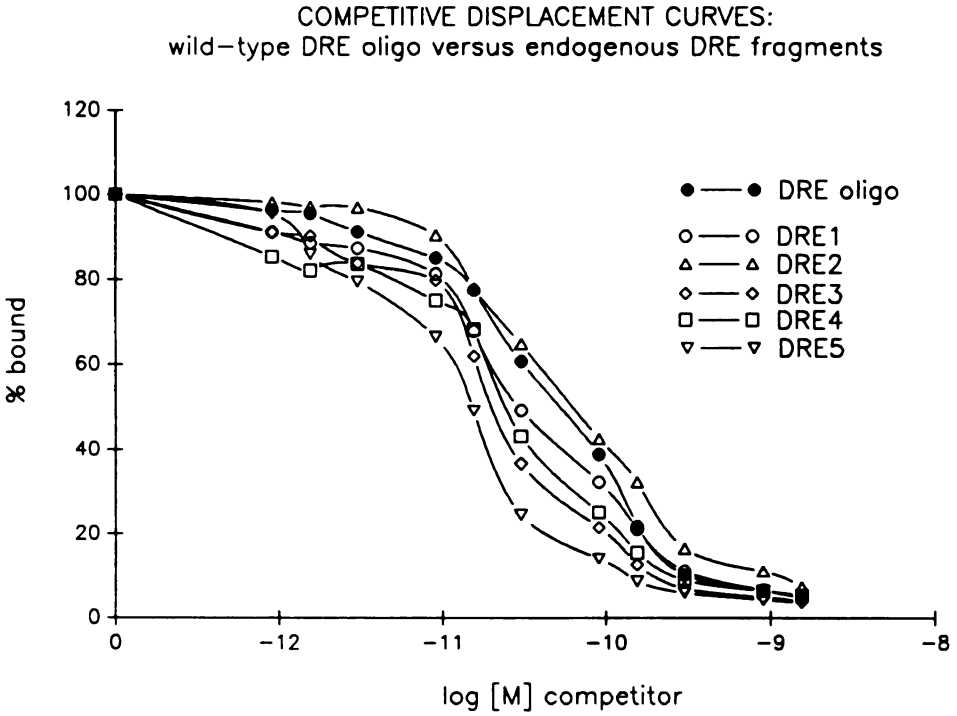

COMPETITIVE DISPLACEMENT CURVES:
wild-type DRE oligo versus endogenous DRE fragments
% bound
120
100
80
60
40
20
0
0
-12
-11
-10
-9
-8
log [M] competitor
DRE oligo
DRE1
DRE2
DRE3
DRE4
DRE5

Table 2. Comparison of Binding Affinities of Transformed TCDD:AhR Complexes to Mouse CYPIA1 DREs.

DNA fragments	Relative Kd (nM)[a]
DRE3-oligo[b]	2.5[c]
DRE1	1.7[d]
DRE2	3.5
DRE3	1.1[d]
DRE4	1.5[d]
DRE5	0.7[d]
DRE5-oligo	3.3

[a]Values are expressed as the mean relative binding affinity (Kd) as estimated from three separate experiments.

[b]Wild-type DRE3 oligonucleotide.

[c]Data from Chapter 1.

[d]Indicates values that are significantly different from the wild-type DRE oligonucleotide ($p < 0.05$), based on calculations as described in Materials and Methods.

indicated that while the DRE5-containing DNA fragment was 3.8-fold better as a competitor than the DRE oligonucleotide, the relative binding affinity of the DRE5-containing oligonucleotide was not significantly different from that of the DRE oligonucleotide (Table 2). Additionally, the binding affinity of the DRE2-containing DNA fragment (30 bp) was not significantly different from the DRE oligomer, while that of the larger DNA fragments containing DRE1, DRE3, DRE4 and DRE5 were significantly better; consistent with their increased size. The recent work of Fisher et al. (1990) has demonstrated that the transcriptional enhancer activity of DREs 1-4 are very similar (DRE5 was not tested). Thus, the results presented here demonstrate that the endogenous DREs flanking the murine CYP1A1 gene can each be recognized and bound by TCDD:AhR complexes with a similar affinity and suggest that each DRE may contribute equally to the TCDD responsiveness of the CYPIA1 gene.

Effect of Single Nucleotide Substitutions on Inducible Protein-DNA Complex Formation

Sequence alignment of the DREs contained within the upstream region of the rat and mouse CYPIA1 gene which have been observed to bind transformed TCDD:AhR complex (by gel retardation analysis) are presented in Figure 4. The derived DRE consensus sequence (G/CNNNC/GTNGCGTGNG/CA/T-NNNC/G) contains an invariant "core" sequence (underlined) flanked on either side by several variable nucleotides. To determine the importance of each of these conserved nucleotides in TCDD:AhR:DRE complex formation, we prepared a series of single nucleotide-substituted DRE oligonucleotides based on the sequence of mouse DRE3 (Table 3). To test the ability and extent to which the TCDD:AhR complexes recognize and bind to these mutant double stranded DREs, wild type and mutant DRE oligonucleotides were radiolabeled with [^{32}P] and the ability of transformed TCDD:AhR complex to bind DNA directly analyzed by gel retardation analysis (Figure 5). No TCDD-inducible complex was observed when several of the "core" consensus bases were substituted (specifically the nucleotides CGTG,positions 9, 10, 11 and 12 (Table 3)). Substitutions of several of the variably conserved nucleotides (positions 8 and 15) resulted in a modest decrease in complex formation while others (positions 1, 5 ,6 and 19) had no apparent effect on complex formation

Figure 4. Nucleotide sequence alignment of DREs identified in the mouse (Denison et al. 1988) and rat CYPIA1 (Fujisawa-Sehara et al. 1987) genes. The DRE consensus sequence shown below was derived from the alignment of these DREs (shown above).

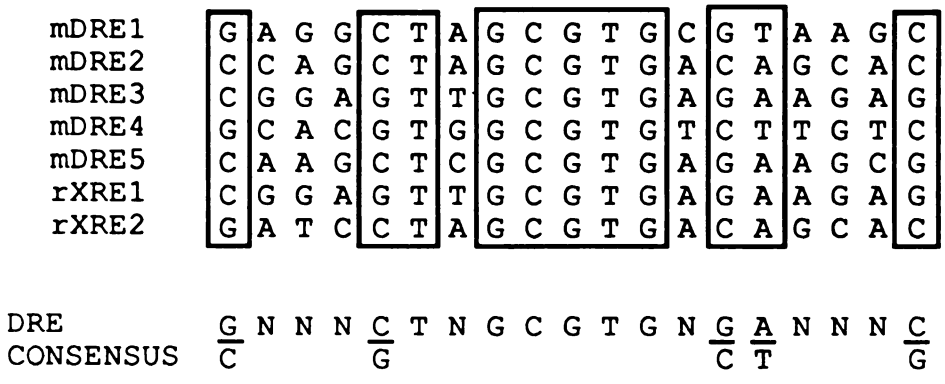

mDRE1 G A G G C T A G C G T G C G T A A G C
mDRE2 C C A G C T A G C G T G A C A G C A C
mDRE3 C G G A G T T G C G T G A G A A G A G
mDRE4 G C A C G T G G C G T G T C T T G T C
mDRE5 C A A G C T C G C G T G A G A A G C G
rXRE1 C G G A G T T G C G T G A G A A G A G
rXRE2 G A T C C T A G C G T G A C A G C A C
DRE CONSENSUS G/C N N N C/G T N G C G T G N G/C A/T N N N C/G

Table 3. DRE substitution mutant oligonucleotides used in direct binding and competitive binding experiments.

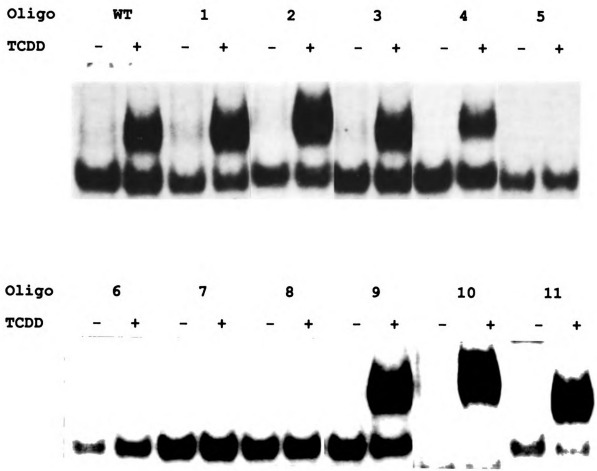
	DRE Nucleotide Position																		
Mutant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Binding
Oligo	C	G	G	A	G	T	T	G	C	G	T	G	A	G	A	A	G	A	G	Affinity[a]
WT[b]	.					.						.							.	2.5
1	T					.						.							.	2.0
2	.				A	.						.							.	0.3
3	.					G						.							.	3.0
4	.					.		T				.							.	11[c]
5	.					.			A			.							.	2000[c]
6	.					.				T		.							.	1600[c]
7	.					.					G	.							.	460[c]
8	.					.						T							.	240[c]
9	.					.						.		T					.	1.9
10	.					.						.			C				.	5.8[c]
11	.					.						.							T	7.4[c]
12	T				A	G						.							.	3.2
13	.					.						.		T	C				.	14[c]
14	.					.						.		T					T	7.4[c]

[a] Values are expressed as the mean relative binding affinity (K_d) estimated from at least three separate experiments (in nM).

[b] Wild type (WT) DRE oligonucleotide containing no nucleotide substitution.

[c] Values are significantly different from the wild-type DRE oligonucleotide ($p < 0.05$), based on calculations as described in Materials and Methods.

Figure 5. Effect of single nucleotide substitution on formation of the TCDD-inducible protein-DNA complex. Cytosol (16 mg/ml), incubated in the absence (-) or presence (+) of TCDD (20 nM) for 2h at 20°C, was mixed with the indicated [^{32}P]-labeled wild-type (WT) and mutant oligonucleotides and protein-DNA complexes resolved by gel retardation analysis as described in Materials and Methods. The specific nucleotide substitution in each mutant DRE oligomer is indicated in Table 2. Figure 5 represents a composite audoradiogram from several different experiments resulting in variations in the position of the inducible and constitutive bands.


Oligo WT 1 2 3 4 5
TCDD - + - + - + - + - + - +
Oligo 6 7 8 9 10 11
TCDD - + - + - + - + - + - +

(Figure 5).To quantitatively examine the effect of each DRE mutant, we performed competitive gel retardation analysis as described above. Competitive displacement curves were generated for each mutant DRE oligomer, as described above (data not shown), and relative affinity for transformed TCDD:AhR complex calculated from the determined IC_{50} values from each competitive displacement curve (Table 3). The estimated K_d values were consistent with the results from direct binding experiments (Figure 5) in that those mutations which had the greatest decrease in binding affinity exhibited little or no inducible complex formation. Mutation of the same four "core" nucleotides described above (CGTG of the "core") decreased the relative binding affinity by 240- to 2000-fold. The results in these experiments are also consistent with methylation interference studies (Shen and Whitlock 1989; Saatcioglu et al. 1990), in that these nucleotides blocked formation of the TCDD:AhR:DRE complex when methylated. Interestingly, substitutions of the remaining two invariant "core" nucleotides had either no effect (mutation at position 6) or a moderate effect (a 10-fold decrease with mutation at position 8) on DRE-binding affinity (Table 3). Similarly, methylation at position 8 had only a moderate effect on complex formation (Shen and Whitlock 1989). A significant decrease in binding affinity was observed with substitution of only two of the five identified variable consensus bases (positions 15 and 19). Substitution at position 5 appeared to result in a slight increase in complex formation (Figure 5) and binding affinity (Table 3), although not significantly. The results of our binding experiments demonstrate that the majority of the nucleotides contained within the core consensus appear to be involved or are important in TCDD:AhR:DRE complex formation. Of the variable nucleotides, those 5'-ward of the "core" are involved to a lesser degree than those 3'-ward of the conserved "core". Based on our mutagenesis experiments, we have deduced an optimal TCDD:AhR DNA-binding consensus sequence of GCGTGNNA/TNNNC/G (Figure 7). Thus, the results of these experiments indicate that formation of the TCDD-inducible protein-DNA complex, as resolved by gel retardation analysis, appears to be dependent upon the relative affinity to which the transformed TCDD:AhR complex binds to the DRE.

Effect of Multiple Substitutions on TCDD:AhR:DRE Complex Formation

The results of the single nucleotide substitution experiments above indicated that changes in the variably conserved bases had a moderate (5- to 10-fold) or no significant effect on inducible complex formation. To examine the role of these bases in complex formation in greater detail, we prepared and tested several DRE oligonucleotides containing multiple nucleotide substitutions. Gel retardation analysis to determine the ability of each multiply substituted DRE to bind to transformed TCDD:AhR is presented in Figure 6 and an estimation of the relative binding affinities of these mutant DREs, derived from competitive displacement curves, are presented in Table 3. These results are similar to those above which indicate that the substitution of any or all of the conserved 5′ nucleotides at positions 1, 5 and 6 had no significant effect on inducible complex formation. These data support the apparent lack of involvement of these three conserved nucleotides in TCDD:AhR:DRE complex formation. In contrast, multiple substitution of the bases 3′ of the core consensus sequence (positions 14, 15 and 19) resulted in a significant decrease in complex formation and DNA binding affinity and are in agreement with the results of the single substitution experiments.

DISCUSSION

We have previously used gel retardation analysis to demonstrate the specific interaction of the TCDD:AhR complex, transformed *in vivo* or *in vitro*, with the DRE (Denison et al. 1988a; 1988b; Denison and Yao 1991). Sequence alignment of the mouse CYPIA1 upstream DREs has revealed a consensus sequence (Figure 5) which contains an invariant 6 bp core sequence, TNGCGTG, and several variable nucleotides flanking this core that we have previously shown to be important for TCDD:AhR:DRE complex formation (Denison et al. 1988a). Using a series of DRE oligonucleotides containing single or multiple base substitutions, we have identified those nucleotides important for TCDD:AhR:DRE complex formation and derived a putative DNA-binding consensus sequence of GCGTGNNA/TNNNC/G (Figure 7). Four core nucleotides CGTG appear to be critical for complex formation while the remaining conserved bases are important, albeit to a lesser degree.

Figure 6. Effect of multiple nucleotide substitutions on formation of the TCDD-inducible protein-DNA complex. Cytosol (16 mg/ml), incubated in the absence (-) or presence (+) of TCDD (20 nM), was mixed with [^{32}P]-labeled wild-type (WT) or multiply substituted DRE oligonucleotides and protein-DNA complexes resolved by gel retardation analysis as described in Materials and Methods. The specific substitutions in each mutant DRE oligomer are indicated in Table 2.

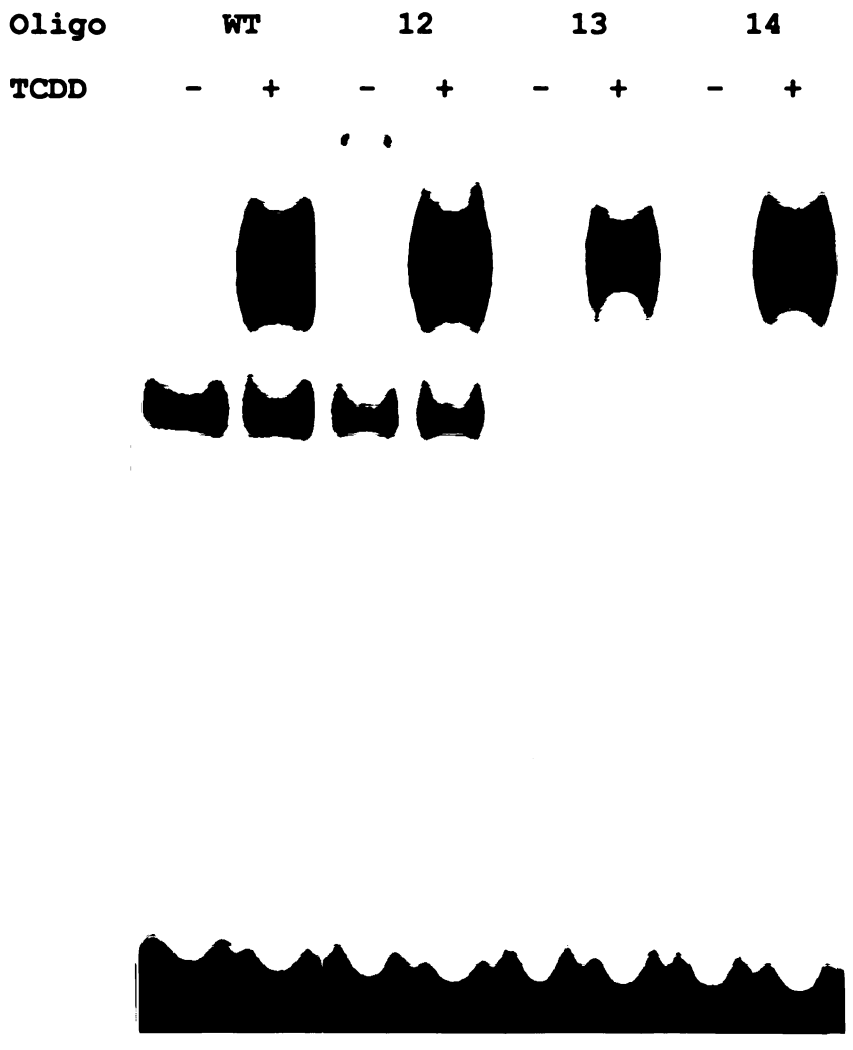
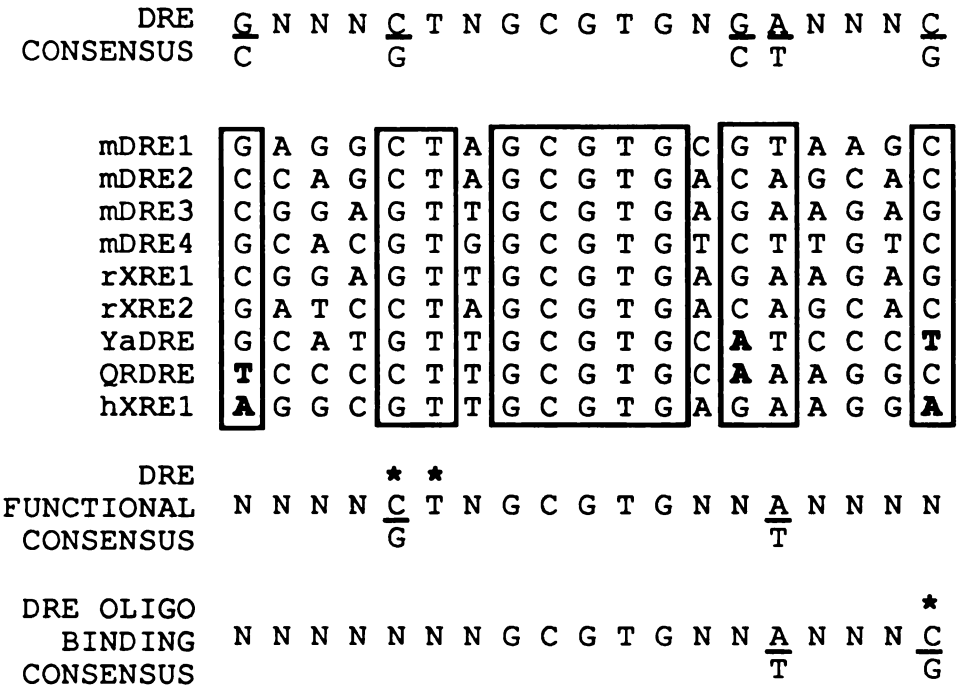

Oligo
WT
12
13
14
TCDD
- + - + - + - +

Figure 7. Alignment of the currently identified functional DRE sequences. Generation of a putative DRE functional consensus sequence from the alignment of functional DREs identified in the flanking regions of the mouse CYPIA1 (mDRE1-4), rat CYPIA1 (rXRE1-2), human CYPIA1 (hXRE1), glutathione S-transferase Ya (YaDRE) and quinone reductase (QRDRE) genes. The DRE binding consensus generated from our studies is indicated for comparison. Nucleotides in bold face indicate those bases which deviate from the DRE consensus sequence and asterisks, those which differ between the two derived sequences.


DRE
CONSENSUS
G N N N C T N G C G T G N G A N N N C
C G C T A
mDRE1 G A G G C T A G C G T G C G T A A G C
mDRE2 C C A G C T A G C G T G A C A G C A C
mDRE3 C G G A G T T G C G T G A G A A G A G
mDRE4 G C A C G T G G C G T G T C T T G T C
rXRE1 C G G A G T T G C G T G A G A A G A G
rXRE2 G A T C C T A G C G T G A C A G C A C
YaDRE G C A T G T T G C G T G C A T C C C T
QRDRE T C C C C T T G C G T G C A A A G G C
hXRE1 A G G C G T T G C G T G A G A A G G A
DRE
FUNCTIONAL
CONSENSUS
N N N N C T N G C G T G N N A N N N N
G T
DRE OLIGO
BINDING
CONSENSUS
N N N N N N N G C G T G N N A N N N C
T G

Although the thymine at position 6 of the DRE is present in the invariant core sequence described above and present in all of the functional DRE identified to date, its substitution had no apparent effect on DNA binding. We envision that this base may play a role in the transcriptional enhancer activity of the DRE but is not critical for high affinity binding. In fact, several investigators have reported variant DNA binding sites which can bind transcription factors with affinity similar to that of the wild-type sequence but which do not activate transcription (Hollenberg and Evans 1988; Sakai et al. 1988; Kim and Guarente 1989). These studies suggest that specific interactions between the protein and DNA results in a conformational change in the protein that activated the complex and substitution of a critical "functional nucleotide(s)" prevents or reduces this change.

Although other investigators have reported comparable effects of multiple DRE mutations on complex formation (Saatcioglu et al. 1990; Neuhold et al. 1989), the studies reported here utilized DRE oligonucleotides containing single base substitutions which allowed us to identify specific nucleotides involved in DNA binding. The results of our analysis have extended previous studies and demonstrate that the primary interaction of transformed TCDD:AhR complex with the DRE occurs specifically at the CGTG sequence of the "core" motif. We are currently examining the effect of these mutations on transcriptional enhancer activity. We expect that decreased AhR DNA binding will coincide with decreased enhancer activity as has been observed with other transcriptional factors (Glass et al. 1988; Schule et al. 1990). The contribution, if any, of other "non-consensus" nucleotides to the high affinity Ah receptor-DNA interaction is currently unknown, but the identification of additional DRE sequences may increase understanding of their importance/function.

Functional DREs which confer TCDD-responsiveness upon an adjacent promoter and gene have been identified in the upstream region of the mouse (Fisher et al. 1990), rat (Fujisawa-Sehara et al. 1987), and human (Nebert and Jones 1989) CYPIA1 gene and glutathione S-transferase (Rushmore et al. 1990) and quinone reductase (Favreau and Pickett 1991) genes. Alignment of these DREs and a putative functional consensus sequence derived form this alignment is presented in Figure 7. Comparison of this consensus with the

binding consensus derived in our studies reveals one nucleotide (position 19) which appears to be important in DNA binding but is not conserved in the functional DREs. In contrast, our mutagenesis results have also identified two nucleotides (positions 5 and 6) which do not appear to be important for DNA binding but are highly conserved among the functional DREs. It is likely that these bases play a role in DRE transcriptional enhancer function and that their interaction with the AhR (or another protein in the TCDD:AhR:DRE complex) may be important for transcriptional enhancer activity (as described above).

It has been proposed that the AhR may be a member of the steroid/thyroid hormone receptor superfamily because of the similarities between their overall mechanism of action and physiological properties (Evans 1988). However, evidence is accumulating which strongly indicates that the AhR is distinctly different from steroid receptors and that it may belong to a separate class of ligand dependent transcription factors (Denison et al. 1989; Elferink et al. 1990; Denison 1991). Steroid hormone receptors, appear to bind to their cognate palindromic DNA recognition sites as homodimers with each monomer binding to one half of the dyad recognition site (Kumar and Chambon 1988; Beato et al. 1989). Unlike steroid receptors, however, the DNA binding form of the AhR appears to be a heterodimer, containing only one ligand-binding subunit per complex (Denison et al. 1989; Elferink et al. 1990) which binds to the non-palindromic DRE specifically and with high affinity (Denison and Yao 1991) Additionally, our results demonstrate that the most significant protein-DNA interaction between the AhR and the DRE occurs within the core motif. Recent UV-cross-linking experiments of Elferink et al. (1991) have indicated that the non-ligand subunit of the transformed AhR complex appeared to be the primary DNA-binding component. Taken together, these results suggest that the DNA-binding of transformed TCDD:AhR complex occurs through the specific high affinity interaction between the non-liganded subunit and the "core" motif. However, more than just the core DRE motif is required for high affinity binding, since we have previously observed that DNA fragments containing only the core DRE motif bind transformed AhR with significantly lower affinity (Denison et al. 1988a).

The identity of the presumed "DNA-binding" AhR subunit has not yet been resolved, however, a recent report by Hankinson and coworkers (Hoffman et al. 1991) has described cloning of a gene for an AhR-associated protein which may represent this subunit. This "Ah receptor nuclear translocation" (*arnt*) gene product appears to be important in both AhR nuclear translocation and DNA binding, in that it complements variant AhRs defective in these functions (Legraverend et al. 1982; Whitlock 1987). Analysis of the deduced amino acid sequence of the *arnt* gene product has also revealed a segment of the protein which exhibits similarity to the basic helix-loop-helix motif present in many DNA-binding proteins (Brennan and Matthews 1989; Murre et al. 1989; Weintraub et al. 1991). Whether the *arnt* protein represents the "DNA-binding" subunit of the transformed TCDD:AhR complex, however, remains to be determined.

How the AhR interacts with the DRE and whether both subunits of the AhR contribute to the high affinity DNA binding is currently a matter of speculation. However, the results of our mutagenesis experiments, combined with UV-cross-linking results of Elferink and Whitlock (1991) allow us to suggest possible models for this interaction. One hypothesis is that for high affinity DNA binding to occur four distinct interactions must occur between the AhR and the DRE core motif and substitution of any one of these bases disrupts this interaction. A somewhat analogous situation has been reported in studies examining the effect of DNA mutagenesis on the enhancer activity of the sequence TATAAAG (Wobbe and Struhl 1990), which binds the transcription factor TFIID. In that study, T to C and A to G transversions (comparable to those made in our studies) eliminated the transcriptional enhancer activity of this element. These results suggests that the activity of this element reflects its ability to interact with a single TATA-binding factor (TFIID) and the decreased enhancer activity was due to decreased DNA binding affinity. By analogy, the DNA binding of AhR occurs primarily, at least, via an interaction of the DRE "core" with a single AhR subunit and transversion substitutions of the core eliminate DNA-binding.

In addition to the potential electrostatic interactions between the AhR and the DRE, it is possible that the AhR recognizes some structural feature contained within the core motif and that mutagenesis of the core disrupts this structure

and decreases AhR binding affinity. Examination of the core motif of DRE3 reveals six alternating purine and pyrimidine bases, a characteristic found in sequences which can potentially form Z-DNA (Nordheim and Rich 1983). DNA sequences containing 8 bp segments of alternating purine-pyrimidines have been shown to form Z-DNA structures upon negative supercoiling. Although it is unknown whether the DREs, which contain between 5 and 9 bp of alternating purine and pyrimidines (Table 1), can form these structures or whether this alternating pattern produces some small, yet significant, structural configuration. The mutations reported here represent purine to pyrimidine transversions which would disrupt this alternating pattern. The effect of transition substitutions within the core motif on TCDD:AhR:DRE complex formation is currently being examined. Changes in the flexibility/bendability of the DRE before and after AhR binding may also be involved in high affinity inducible complex formation. A recent study has demonstrated that binding of liganded AhR to the DRE resulted in bending of the DNA at (or near) the site of protein-DNA interaction (Elferink and Whitlock 1990). If DRE bending is required for the formation of additional protein-DNA contacts which are necessary for stabilization of the high affinity TCDD:AhR:DRE complex, then substitution of one of the core bases might decrease DRE flexibility and thus prevent formation of the additional contacts. Although it is difficult to determine whether one or more of these mechanisms is involved in the high affinity binding of liganded AhR to the DRE, these models can be tested using site-directed mutagenesis and cross-linking techniques. Final confirmation of the specific mechanism(s) involved in TCDD:AhR:DRE complex formation, however, will require the use of purified receptor preparations.

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