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Final Project Summary

Molecular Mechanism of Dioxin Action:

Molecular Cloning of the Ah Receptor Using a DNA

Recognition Site Probe

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INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs) represent a large group of widely distributed, persistent and highly toxic environmental contaminants. In experimental animals, exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), the prototypical and most potent HAH, results in a wide variety of species- and tissue-specific toxic and biological effects, including: teratogenesis, immuno- and hepato-toxicity, tumor promotion and induction of numerous enzymes, including microsomal cytochrome P450IA1 (Poland and Knutson, 1982; Safe, 1986).

Numerous biochemical and genetic studies (Poland et al., 1976; Legraverend et al., 1982; Whitlock et al., 1986) have indicated that the induction of cytochrome P450IA1 is mediated by a soluble, intracellular protein, the Ah receptor (AhR), which binds TCDD saturably and with high affinity. Mechanistically, induction of the cytochrome P450IA1 (Fig. 1) involves the binding of TCDD to the AhR protein, "transformation" of the TCDD:AhR complex to its DNA-binding form, and subsequent accumulation of transformed TCDD:AhR complexes within the cell nucleus (Whitlock, 1986). The binding of these transformed TCDD:AhR heterodimeric complexes to specific cis-acting dioxin responsive enhancers (DREs) adjacent to the CYPIA1 gene results in enhanced transcription of the CYPIAI gene (Whitlock, 1986, 1990; Denison et al., 1988a,b). Additionally, recent studies have suggested that many, if not all, of the observed TCDD-inducible responses are AhR-dependent (Safe, 1986; Whitlock, 1986).

Partial purification of a protein(s) (presumably the AhR, or its ligand binding subunit), affinity labeled with $[^{125}I]$-2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin, a high affinity agonist for the AhR, has recently been reported (Bradfield et al., 1988). Since the partially purified pr in(s) was isolated in a denatured condition, further biochemical and fu tional analyusis to confirm the presence of the AhR in this fraction was not possible. To date, the native AhR has not been purified nor the AhR gene(s) cloned.

Recent studies (Denison et al., 1989; Elferink et al., 1990) have indicated that the DRE-binding form of the AhR is a heterodimer, containing only one ligand-binding subunit and possibly a distinct DNA-binding subunit. In fact, a protein factor required for AhR nuclear translocation/DNA binding has been recently cloned (Hoffmann et al., 1991) and may represent the DNA-binding AhR subunit, however, this remains to be determined. The isolation and characterization of AhR genes and their gene products is of considerable interest.

SPECIFIC AIMS

The overall goals of our research are to gain an understanding of the molecular mechanisms by which 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) regulates gene expression. We are utilizing a mouse hepatoma cell system to study the mechanism by which TCDD regulates the expression of the cytochrome P450IA1 gene and the induction of aryl hydrocarbon hydroxylase (AHH) activity. TCDD induces the rate of transcription of the cytochrome P450IA1 gene and the induction is mediated by an intracellular protein (the Ah receptor (AhR)) which binds TCDD with high affinity. After TCDD binding, TCDD:AhR complexes accumulate within the nucleus. Biochemical and genetic evidence no only implies that the TCDD:AhR complex activates gene transcription through an interaction with specific DNA sequences (dioxin-

responsive enhancers (DREs)) upstream of the promoter of TCDD-responsive genes but also suggests that many, if not all, of the toxic and biological effects of TCDD are mediated by the AhR and involve alterations in gene expression. Thus, overall, our system is useful for studying the molecular mechanisms of TCDD action and the mechanism by which the TCDD:AhR complex regulates eukaryotic gene expression.

Here we proposed to:

1. Identify the DRE sequence determinants necessary for formation of the TCDD:AhR:DRE complex binding as well as those required for TCDD-inducible enhancer function.

2. Detect and purify AhR gene clones by screening a λgt11 cDNA expression library with a DRE recognition site probe. Since the AhR has been refractory to purification, utilization of this procedure would enable us to clone the AhR gene directly, without first having to purify the AhR protein.

MATERIALS AND METHODS

The specific materials and methodology used in our studies are described in detail in the supporting manuscript and original proposal.

PROPOSED RESEARCH AND PROGRESS

Our overall research aim is to understand the mechanism by which TCDD and the AhR modulate gene expression. We prepared a series of DNA oligonucleotides which contained single or multiple nucleotide mutations in the recognition sequence and analyzed them, relative to their ability to bind to the TCDD:AhR complex (in gel retardation assays) and exhibit TCDD-inducible enhancer function(in transient expression assays). We will use the optimal oligonucleotide from these studies (the one with the lowest nonspecific DNA binding, yet the highest affinity for the TCDD:AhR complex) as a probe in experiments designed to clone the AhR gene. Concatenated oligonucleotides were will be labeled and used as to probe replicate protein filters of a λgt11 cDNA expression library and any recombinant clone encoding at least the DNA-binding domain of the AhR will be identified. cDNAs from the screening procedure were be isolated and the fusion proteins characterized (i.e., cloning of the AhR gene and production of functional AhR protein will be confirmed). Further details of the experimental design and methodology are included in the body of the original proposal.

The original grant proposal was to be submitted as a three yeart project. As agreed upon with the AFOSR Office (Lt. Col. Cerveny, Program Manager) this proposal was submitted with the understanding that only one year of funding was available. The "seed money" for this project would allow us to adequately screen the DNA oligonucleotides, identify the DRE oligonucleotide with the highest binding affinity, optimize the screening protocol and begin the actual library screening. The techniques to be used for characterization and sequencing of the positive fusion proteins and cDNAs, respectively, were included in the original proposal in the event that we identified positive clones early in the screening procedure.

As proposed, we have completed our mutational analysis of AhR binding to the DRE (see attached manuscript an the following brief description of these results) and have begun the library screening. Although this project has not

yet been submitted to the AFOSR for continued funding, we are in the process of preparation of a continuation proposal.

RESEARCH ACCOMPLISHMENTS

Significant progress has been made in our proposed research and is described in the following subsections.

Specific Aim I   Identify the DRE sequence determinants necessary for formation of the TCDD:AhR:DRE complex binding as well as those required for TCDD-inducible enhancer function.

We have completed the DRE mutational analysis and have submitted a manuscript involving this work to the journal "Biochemistry (see attached copy). A brief synopsis of the results are included here (please see the manuscript itself for more details). The optimal DRE sequence is currently being utilized for screening of λgt11 cDNA libraries (see specific aim II). In addition, the transcriptional enhancer activity of each mutant DRE is currently being evaluated in transient transfection assays (described in the original proposal). Alignment of known rat and mouse DREs (Figure 4 of manuscript) has allowed us to derive a putative DRE consensus sequence (G/CNNNG/CTNGCGTGNG/CT/ANNNG/C) from which we carried out the DRE mutagenesis experiments designed to examine nucleotide-specificity of TCDD:AhR:DRE complex formation.

Effect of Single Nucleotide Substitutions on Inducible Complex Formation (see attached manuscript for figures and experimental details)

To determine the importance of each conserved DRE nucleotide in TCDD:AhR:DRE complex formation, we prepared a series of single nucleotide-substituted DRE oligonucleotides based on the sequence of mouse DRE3 (Table 1). The binding of transformed TCDD:AhR:DRE complex to each oligonucleotide was determined both by direct labeling of the oligomers and subsequent gel retardation analysis as well as by the use of competitive gel retardation analysis. The relative binding affinity (Kd) of transformed AhR for the wild type and mutant DRE oligonucleotides are presented in Table 1. The estimated Kd values for each mutant oligonucleotide were consistent with the results of the direct binding experiments (see figure 5 of manuscript), in that those mutations which caused the greatest decrease in binding affinity exhibited little or no inducible complex formation. Mutation of the four "core" nucleotides (CGTG of the "core") decreased the relative AhR DNA binding affinity by 200- to 2,000-fold. A significant decrease in binding affinity was also observed with substitution of only two of the five identified variable consensus bases (positions 15 and 19). The results of our single nucleotide substitution experiments demonstrate that the majority of the nucleotides contained within the core consensus appear to be involved (are important in TCDD:AhR:DRE complex formation), while those bases 5'-ward of the "core" were 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. The results of these experiments also indicate that formation of the TCDD-inducible protein-DNA complex appears to be dependent upon the relative affinity of the transformed TCDD:AhR complex for each DRE.

Effect of Multiple Nucleotide Substitutions on Inducible Complex Formation

The results of the single nucleotide substitution experiments indicated that changes in the variably conserved bases had either a moderate effect (5- to 10-fold) or no significant effect on inducible complex formation, compared to substitution of certain "core" nucleotides. To examine the role of these bases in complex formation in greater detail, we also prepared and tested several mutant DRE oligonucleotides which contained multiple base substitutions. Substitution of any or all of the conserved 5' nucleotides at positions 1, 5 and 6 had no significant effect on inducible complex formation, supporting 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 also in agreement with the results of the single base substitution experiments.

Conclusionsof the Results from Specific Aim I

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,b; Denison and Yao, 1991). Sequence alignment of the mouse CYPIA1 upstream DREs has revealed a consensus sequence 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 now identified those nucleotides important for TCDD:AhR:DRE complex formation and have derived a putative DNA-binding consensus sequence of GCGTGNNA/TNNNC/G. The four "core" nucleotides, CGTG, are important for inducible complex formation while the remaining conserved bases are also important, albeit to a significantly lesser degree. The results of our binding experiments are consistent with methylation interference studies (Shen and Whitlock, 1989; Saatcioglu et al., 1990), in that methylation of these nucleotides blocked TCDD-inducible complex formation. Although our results indicate that the primary interaction of transformed TCDD:AhR complex with the DRE occurs specifically with the CGTG sequence of the "core" motif, we have previously observed that nucleotides outside of the "core" motif are also required for DRE enhancer function (Denison et al., 1988b). We are currently examining the effect of these mutations on transcriptional enhancer activity and 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 and enhancer function is currently unknown, but the identification of additional DRE sequences may increase understanding of their importance/function.

In the attached manuscript we have also described the significance of DRE DNA structure in TCDD:AhR DNA binding, interaction of the AhR with other endogenous CYPIA1 5'-flanking DREs and other aspects regarding AhR DNA binding

Specific Aim II Detect and purify AhR gene clones by screening a λgt11 cDNA expression library with a DRE recognition site probe.

The majority of our work during this period was focused on DRE mutational analysis and consequently we have only begun to optimize the λgt11 DRE-binding screening assay. A double-stranded DRE consensus oligonucleotide will be used

to screen expression libraries for the AhR gene(s). A recombinant clone encoding the particular DRE sequence-specific DNA-binding protein (or at least its DNA-binding domain) should be identified by probing replicate protein filters of a λgt11 expression library with labeled DRE oligonucleotide. This technique has been used successfully for cloning of a number of different sequence-specific DNA-binding proteins (Singh et al., 1988, 1989; Vinson et al., 1988). Although the experimental protocols in this section describe cloning of the AhR gene, we are aware that other DRE-binding proteins may also be cloned. The optimal double stranded DRE oligonucleotide (5'-CGGAGTTGCGTGATAAGAG-3') has been phosphorylated and ligated to produce a concatenated DRE oligomer of 150bp (consisting of 6 DRE oligonucleotides ligated end to end). This concatenated DRE will be utilized for the screening assay. In addition, to confirm that the identified positive plaques actually represent AhR gene product(s) we will utilize mutant DRE oligonucleotide #5 (Table 1) which binds AhR with a 2,000-fold lower affinity. A positive plaque will bind radiolabeled concatenated DRE but not radiolabeled mutant DRE concatamer. In this way, any positive clones can be characterized relatively rapidly as to whether they represent AhR clones or not.

As mentioned above, we have only recently begun the library screening. We have obtained a λgt11 cDNA clone for the DNA binding protein C/EBP from Dr. S. McKnight (Baltimore, MD) as well as a DNA fragment which contains its DNA recognition site. We are in the process of utilizing this clone as a positive control for optimization of the DNA screening assay system and expect no problem in setting up the system. As discussed above we will be submitting a grant to the AFOSR for continuation of this work.

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Table 1. DRE substitution mutant oligonucleotides used in direct binding and competitive binding experiments.

DRE Nucleotide Position

Mutant Oligo	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Binding Affinity[a]
(base)	C	G	G	A	G	T	T	G	C	G	T	G	A	G	A	A	G	A	G	
WT[b]	.				.				.						.			.		2.5
1	A				.				.						.			.		3.0
2	.				T				.						.			.		3.2
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 (Kd) estimated from at least three separate experiments (in nM).

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

c. Indicated value is significantly different from the wild-type DRE oligonucleotide ($p < 0.05$).

DNA SEQUENCE DETERMINANTS

FOR BINDING OF TRANSFORMED Ah RECEPTOR

TO A DIOXIN RESPONSIVE ENHANCER[†]

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Footnotes

1. The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of the Air Force Office of Scientific Research or the U.S. Government.

2. Abbreviations used:

AhR, aromatic hydrocarbon receptor.

BSA, bovine serum albumin.

DMSO, dimethylsulfoxide.

DRE, dioxin responsive element.

DTT, dithiothreitol.

HEDG, 25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT. 10% (v/v) glycerol.

TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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

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

ABSTRACT

We have utilized gel retardation analysis and DNA mutagenesis to examine the specific interaction of transformed guinea pig hepatic cytosolic TCDD:AhR complex with a dioxin responsive element (DRE). Sequence alignment of the mouse CYPIA1 upstream DREs has identified a common invariant "core" consensus sequence of TNGCGTG flanked by several variable nucleotides. Competitive gel retardation analysis using a series of DRE oligonucleotides containing single or multiple base substitutions has allowed identification of those nucleotides important for TCDD:AhR:DRE complex formation. A putative TCDD:AhR DNA-binding consensus sequence of GCGTGNNA/TNNNC/G has been derived. The four core nucleotides, CGTG, appear to be critical for TCDD-inducible protein-DNA complex formation since their substitution decreased AhR binding affinity by 200- to 2000-fold; the remaining conserved bases are also important, albeit to a lesser degree (3- to 5-fold). The 5'-ward thymine, present in the invariant core sequence of all of the DREs identified to date, appears not to be involved in DNA binding of the AhR. The results obtained here indicate that although the primary interaction of the TCDD:AhR complex with the DRE occurs with the conserved "core" sequence, nucleotides flanking the core also contribute to the specificity of DRE binding.

INTRODUCTION

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin, TCDD[2]), 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[3] 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, 1987, 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[4], during which hsp90 (a 90 kDa 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, 1987, 1990) have indicated that transcriptional activation of the cytochrome P450IA1 (CYPIA12) 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 *in vivo* or *in vitro*, can bind to a DRE oligonucleotide specifically and with high aff.·'ty (Denison et al., 1988a, 1988b; Denison and Yao, 1991). Four functional DRE sequences have 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 variably conserved 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 (Neuhold and Nebert, 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) and [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 (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'-GATCCGGAGTTGCGTGAGAAGAGCCA-3' (corresponding to the 20 bp AhR binding site of DRE3 (Denison et al., 1988a; 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 $[\gamma-^{32}P]ATP$ as previously described (Denison et al., 1988b).

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 ‐ .rt site of transcription (Gonzalez et al., 1985)). DRE1 is an *Eco*RI-*Hp*ŀ fragment isolated from the plasmid pGEMLS5.28 and spans the region from -933 to -869; DRE2 is an *Eco*RI-*Ppu*MI fragment isolated from the plasmid pGEMLS5.30, and spans the region from -1076 to -1048; DRE3 is an *Eco*RI-*Ppu*MI 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 plasmid 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 and Deal, 1990; 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 in the absence of 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 (Fig. 1), one of which (complex A) was TCDD-inducible (observed only in the

presence 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. Moreover, 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* and bind to the DRE, 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 (A and B) are comparable to those previously observed using rat hepatic cytosol (Denison and Yao, 1991) and 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

(Fig. 2). Addition of increasing concentrations of the indicated specific and nonspecific competitors effectively decreased formation of the TCDD:AhR:DRE complex (Fig. 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 (Fig. 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 saturation binding experiments, analogous to that we have described previously (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} values 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. 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 (Denison et al., 1989) resulted in comparable levels of inducible complex formation (Fig. 3A). Competitive gel retardation analysis with these DNA fragments indicated a relatively similar degree of competitive binding with each DRE (Fig. 3B). Comparison of the estimated relative binding affinity of all five mouse CYPIA1 upstream DREs (Table 1) 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 1). These results 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 1). 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. 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 is in support of the recent work of Fisher et al. (1990) which demonstrated that the transcriptional enhancer activity of DREs 1-4 were also similar (DRE5 was not tested).

Effect of Single Nucleotide Substitutions on Inducible Complex Formation

Sequence alignment of these 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 Fig. 4. The derived DRE consensus sequence (G/CNNNC/GTNGCGTGNG/CA/TNNNC/G) contains an invariant "core" sequence (underlined) which is 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 2). To test the ability and extent to which the TCDD:AhR complex recognizes and binds to these transversion mutant DREs, double-stranded 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 (Fig. 5). No TCDD-inducible complex was formed when certain of the "core" consensus bases were substituted (specifically the bases CGTG at positions 9, 10, 11 and 12 (Table 2)). Substitutions of several of the variably conserved flanking 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 (Fig. 5).

To quantitatively examine the effect of each DRE mutant, we performed competitive gel retardation analysis. Competitive displacement curves were generated for each mutant DRE oligomer (data not shown), and the relative affinity of each oligomer for the transformed TCDD:AhR complex was calculated from the IC_{50} values of each competitive displacement curve (Table 2). The estimated Kd values for each mutant oligonucleotide were consistent with the results of the direct binding experiments (Fig. 5), in that those mutations which caused the greatest decrease in binding affinity exhibited little or no inducible complex formation. Mutation of the same four "core" nucleotides indicated above (CGTG of the "core") decreased the relative DNA binding affinity by 100- to 800-fold. A significant decrease in binding affinity was also observed with substitution of only two of the five identified variable consensus bases (positions 15 and 19). Although substitution at position 5 appeared to result in a slight increase in complex formation (Fig. 5) and binding affinity (Table 2), this increase was not statistically significantly. The results of our single nucleotide substitution 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, while those bases 5'-ward of the "core" were 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 (Fig. 7). The results of these experiments also indicate that formation of the TCDD-

inducible protein-DNA complex appears to be dependent upon the relative DNA binding affinity of the transformed TCDD:AhR complex.

Effect of Multiple Nucleotide Substitutions on Inducible Complex Formation

The results of the single nucleotide substitution experiments above indicated that changes in the variably conserved bases had either a moderate effect (5- to 10-fold) or no significant effect on inducible complex formation, compared to substitution of certain "core" nucleotides. To examine the role of these bases in complex formation in greater detail, we also prepared and tested several mutant DRE oligonucleotides which contained multiple base substitutions. Gel retardation analysis to determine the ability of each multiply-substituted DRE to directly bind to transformed TCDD:AhR complex is shown in Figure 6 and an estimation of the relative binding affinities of these mutant DREs, derived from competitive displacement curves, are presented in Table 2. Substitution of any or all of the conserved 5' nucleotides at positions 1, 5 and 6 had no significant effect on inducible complex formation and support the apparent lack of involvement of these three conserved nucleotides in TCDD:AhR:DRE complex formation as described above. 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 also in agreement with the results of the single base 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 (Fig. 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 now identified those nucleotides important for TCDD:AhR:DRE complex formation and have derived a putative DNA-binding consensus sequence of GCGTGNNA/TNNNC/G. The four "core" nucleotides, CGTG, are important for inducible complex formation while the remaining conserved bases are also important, albeit to a significantly lesser degree. The results of our binding experiments are consistent with methylation interference studies (Shen and Whitlock, 1989; Saatcioglu et al., 1990), in that methylation of these nucleotides blocked TCDD-inducible complex formation. Although our results indicate that the primary interaction of transformed TCDD:AhR complex with the DRE occurs specifically with the CGTG sequence of the "core" motif, we have previously observed that nucleotides outside of the "core" motif are also required for DRE enhancer function (Denison et al., 1988b). We are currently examining the effect of these mutations on transcriptional enhancer activity and 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 and enhancer function is currently unknown, but the identification of additional DRE sequences may increase understanding of their importance/function.

One unexpected finding of our study was that substitution of the thymine at position 6 had no apparent effect on DNA binding. This specific nucleotide is not only present in the invariant core sequence described above for mouse DREs but it is present in all of the functional DREs identified to date. We envision that this base plays a role in the transcriptional enhancer activity of the DRE but it is not involved in high affinity DNA binding. Other investigators have reported variant DNA binding sites which can bind a transcription factor 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 would suggest that specific nucleotides within the recognition site of a DNA-binding factor could be critical for transcriptional activation but not DNA binding.

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 rat 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 from 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 this activity.

Recent studies have indicated that the DNA-binding form of the AhR is a heterodimer, containing only one ligand-binding subunit per complex (Denison et al., 1989; Elferink et al., 1990; Gasiewicz et al., 1991). The UV-crosslinking experiments of Elferink et al (1990) have indicated that the non-ligand subunit of the transformed AhR complex appears to be the primary DNA-binding component. These data, combined with our results, which demonstrate that the most significant protein-DNA interaction between the AhR and the DRE occurs with the core motif, suggests that the DNA-binding of transformed TCDD:AhR complex occurs primarily through a specific, high affinity interaction between the non-liganded subunit and the "core" motif.

How the AhR specifically interacts with the DRE and whether both subunits of the AhR contribute to high affinity DNA binding is currently a matter of speculation. What is apparent, however, is the critical requirement of several of the invariant "core" nucleotides. It is possible that for high affinity DNA binding to occur several distinct interactions between the AhR and the DRE core motif must occur and that substitution of any one of these bases disrupts this interaction. Alternatively, 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). Although DNA sequences containing 8 bp segments of alternating purine-pyrimidines have previously been shown to form Z-DNA structures upon negative supercoiling, whether the five murine CYPIA1 DREs, which contain between 5 and 9 bp of alternating purine and pyrimidines (Table 1), can form these structures or contain some small, yet significant, structural configuration remains to be determined. The DRE core mutations reported here represent transversion substitutions (purine ⇔ pyrimidine) which would disrupt this alternating pattern. Interestingly, a single transition substitution within the core motif (GCGTG to GTGTG) resulted in a modest decrease in relative affinity (38-fold) compared to the 2,000-fold with the transversion substitution (Table 3)). The effect of other 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 nucleotides may decrease DRE flexibility and thus reduce

formation of the additional contacts. Although it is difficult to determine whether one or more of the mechanisms is involved in the high affinity binding of liganded AhR to the DRE, site-directed mutagenesis and DNA-binding analysis provides an avenue to examine this interaction. Final confirmation of the exact mechanism, however, will require the use of purified AhR preparations.

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

Competitor	Binding Affinity (nM)[a]
DRE oligomer[b]	2.5
ssDRE coding strand	1200[c]
ssDRE non-coding strand	2600[c]
poly dI·dC	3400[c]

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

b. Wild-type DRE oligonucleotide.

c. Indicated value is significantly different form the
   wild-type DRE oligonucleotide (P < 0.05).

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

DNA fragments	Binding Affinity (nM)[a]
DRE3-oligo[b]	2.5[c]
DRE1 fragment	1.7[d]
DRE2-fragment	3.5
DRE3-fragment	1.1[d]
DRE4-fragment	1.5[d]
DRE5-fragment	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. Indicated value is significantly different from
   the wild-type DRE oligonucleotide (p < 0.05).

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

DRE Nucleotide Position

Mutant Oligo	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Binding Affinity[a]
	C	G	G	A	G	T	T	G	C	G	T	G	A	G	A	A	G	A	G	
WT[b]	.					.						.						.		2.5
1	A					.						.						.		3.0
2	.				T	.						.						.		3.2
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 (Kd) estimated from at least three separate experiments (in nM).

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

c. Indicated value is significantly different from the wild-type DRE oligonucleotide ($p < 0.05$).

Figure Legends

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 2 h at 20°C, was mixed with poly dI•dC (85 ng) and further 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. Protein-DNA complexes were analyzed using the gel retardation assay as described in Materials and Methods. Complex A is the TCDD-inducible complex and complex B is the constitutive complex.

Figure 2. Relative affinity of transformed TCDD:AhR complexes for nonspecific and single-stranded DNA. **A.** Cytosol (16 mg/ml) was incubated in the absence (-) or presence (+) of TCDD (20 nM) for 2 h at 20°C. Increasing concentrations of DRE oligonucleotide, poly dI•dC, single-stranded DRE oligonucleotide (non-coding strand) 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. Only the protein-DNA complexes are shown. The molar amount of poly dI•dC was calculated assuming that each 22 bp represent the start of a different nonspecific binding site. The concentrations of the specific competitors are as indicated in Fig. 2B. **B.** Typical competitive gel retardation experiments used in the generation of competitive binding curves. In addition to the above competitors, single-stranded DRE oligomer (coding strand) was also included. The standard error of all values was less than 10%.

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.** Increasing concentrations of the indicated 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.

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

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 2 h at 20°C, was mixed with the indicated [^{32}P]-labeled wild-type (WT) or mutant DRE oligonucleotide and protein-DNA complexes resolved by gel retardation analysis as described in Materials and Methods. Only the protein-DNA complexes are shown (the results using mutant oliogmer 10 were taken from a different experiment and thus the constitutive binding signal which migrated further is not shown). The specific nucleotide substitution in each mutant DRE oligomer is indicated in Table 2.

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 oligonucleotide and protein-DNA complexes resolved by gel retardation analysis. The specific substitutions in each mutant DRE oligomer are indicated in Table 3.

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 (mDRE1-4), rat (rXRE1-2), human (hXRE1) CYPIA1, rat glutathione S-transferase Ya (YaDRE) and quinone reductase (QRDRE) genes. The RE 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 indicate those which differ between the two derived sequences.
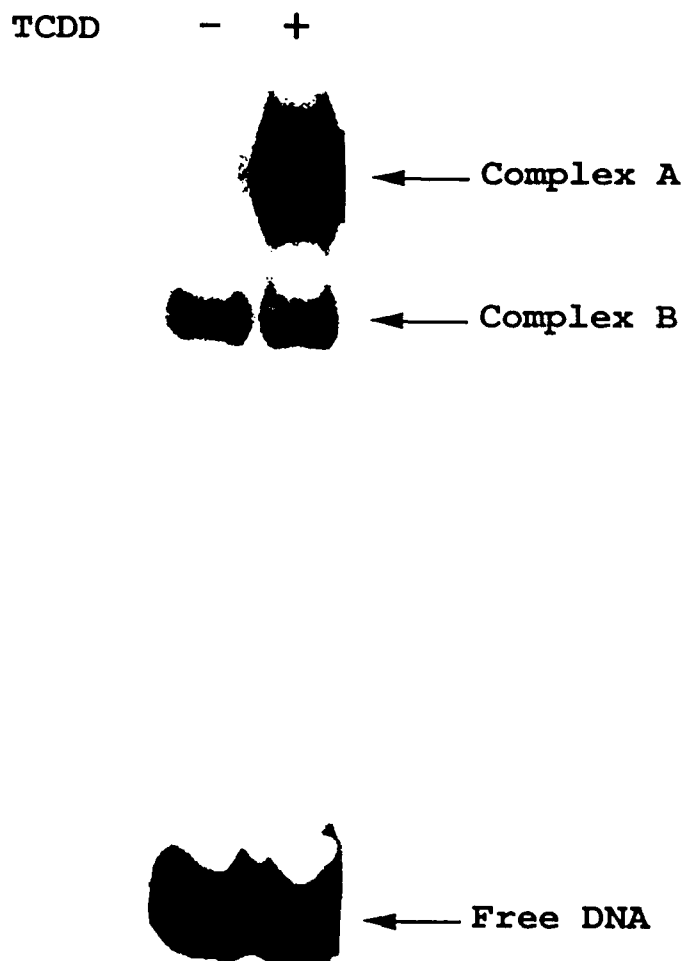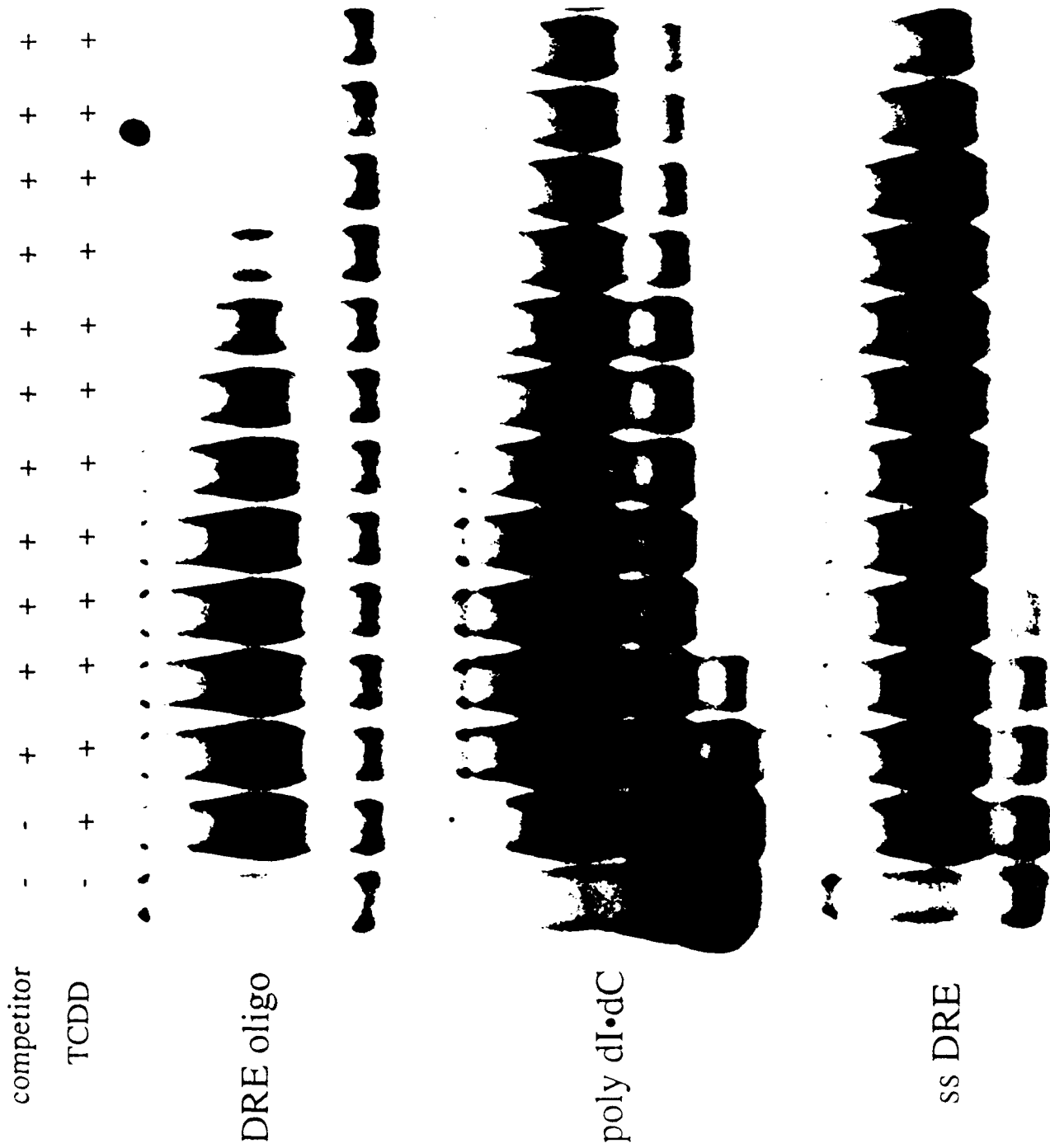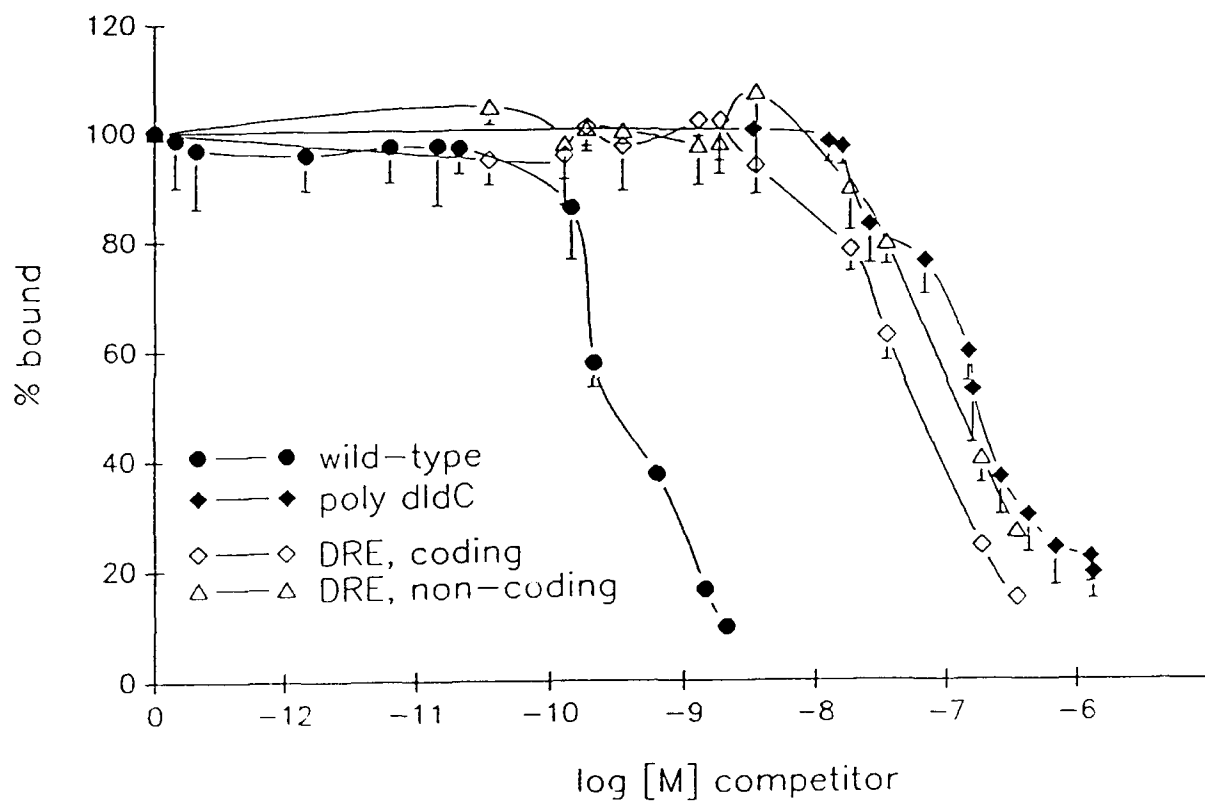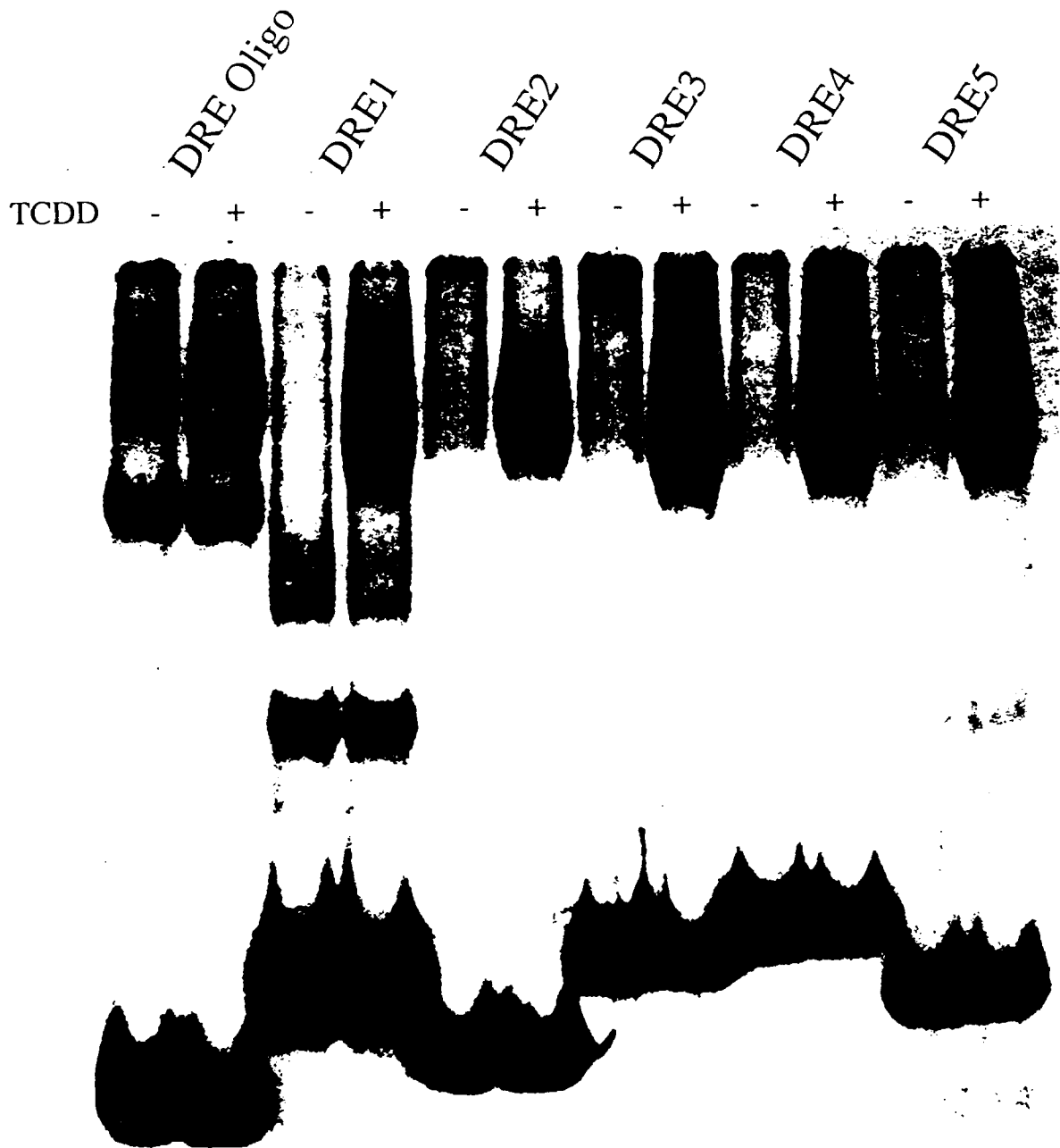
FIGURE 1

FIGURE 2A

FIGURE 2B

FIGURE 3A

TCDD labels across lanes: DRE Oligo (− +), DRE1 (− +), DRE2 (− +), DRE3 (− +), DRE4 (− +), DRE5 (− +)

COMPETITIVE DISPLACEMENT CURVES:
wild—type DRE oligo versus endogenous DRE fragments

FIGURE 4

```
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        G N N N C T N G C G T G N G A N N N C
CONSENSUS  C         G                 C T       G
```
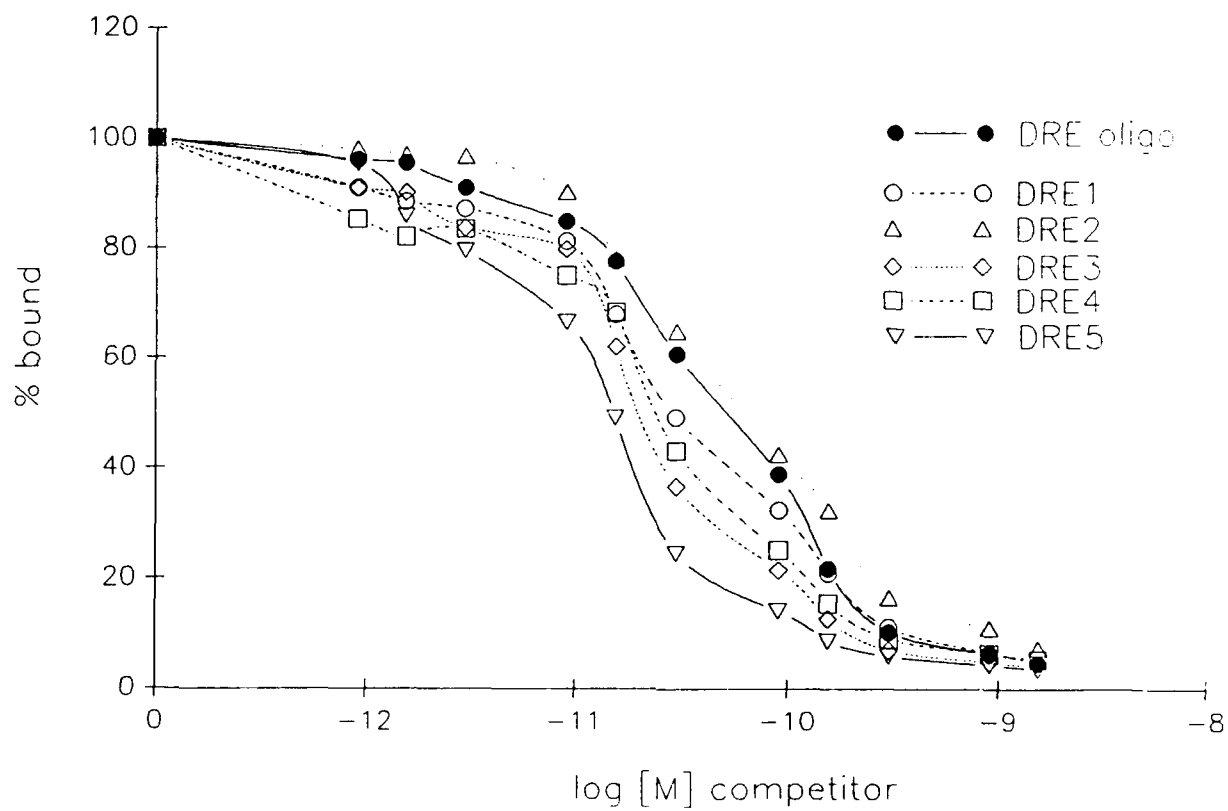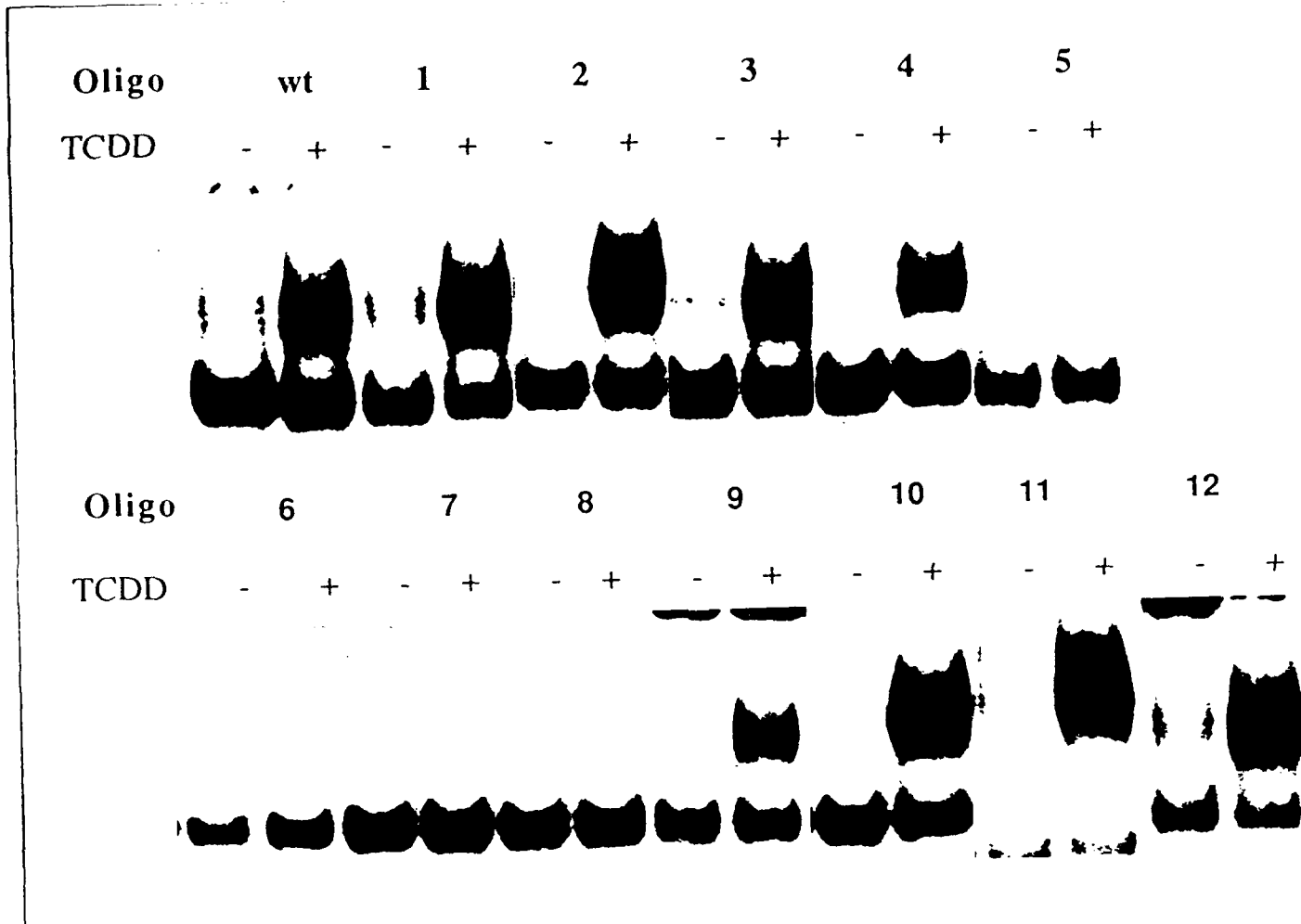
FIGURE 5

FIGURE   6

FIGURE 7

```
DRE        G N N N C T N G C G T G N G A N N N C
CONSENSUS  C         G                 C T       G

          ┌─┐     ┌───┐ ┌─────────┐ ┌───┐       ┌─┐
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  N N N N C T N G C G T G N N A N N N N
CONSENSUS           G                   T

DRE OLIGO                                        *
BINDING     N N N N N N N G C G T G N N A N N N C
CONSENSUS                              T         G
```