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DNA Sequence Determinants for Binding of Transformed Ah Receptor to a Dioxin-Responsive Enhancer†

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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 100–800-fold; the remaining conserved bases are also important, albeit to a lesser degree (3–5-fold). The 5′-ward thymine, present in the invariant core sequence of all 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.

Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin, TCDD[1]), 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 & Knutson, 1982; Safe, 1986). The mechanism of induction of cytochrome P450IA1[2] 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 & 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 (*a*romatic *h*ydrocarbon) receptor (AhR), which binds TCDD saturably and with high affinity (Poland & 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,[3] 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 & Galeazzi, 1984; Henry et al., 1989; Denison & Yao, 1991). Biochemical and genetic studies (Denison et al., 1988a,b; Whitlock, 1987, 1990) have indicated that transcriptional activation of the cytochrome P450IA1 (CYPIA1)[2] gene is stimulated by the binding of transformed TCDD·AhR complexes to *cis*-acting dioxin-re-

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[1] Abbreviations: AhR, aromatic hydrocarbon receptor; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide. 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.

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

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

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sponsive 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 affinity (Denison et al., 1988a,b; Denison & 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 & 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 & 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/mmol) was from Amersham Corp.

Animals. Male Hartley guinea pigs (200–500 g), 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 & 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 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 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–*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 of 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 was carried out as previously described (Denison & Deal, 1990; Denison & 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-labeled 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 & 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), 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 exper-

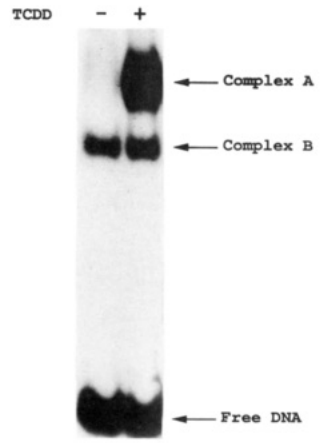

FIGURE 1: Binding of guinea pig hepatic cytosolic proteins to a dioxin-responsive enhancer. Cytosol (16 mg/mL), incubated in the absence (−) or presence (+) of TCDD (20 nM) for 2 h at 20 °C, was mixed with poly(dI·dC) (85 ng) and further incubated for 15 min at 20 °C. ^{32}P-Labeled DRE oligonucleotide (100 000 cpm/0.8–1.0 ng) was added, and the mixture was incubated for an additional 15 min. Protein–DNA complexes were analyzed using the gel retardation assay as described in Materials and Methods. Under these conditions, between 60 and 75% of the total measurable cytosolic AhR is found bound to the DRE. Complex A is the TCDD-inducible complex, and complex B is the constitutive complex.

iments 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 & 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 (Figure 2). Addition of increasing concentrations of the indicated specific and nonspecific competitors effectively decreased formation of the TCDD·AhRCDRE complex (top panel of Figure 2). 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 (bottom panel of Figure 2). 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 & 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 500–1000-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 I). 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 & Yao, 1991) and mouse hepatoma (hepa 1c1c7) cell nuclear extracts (Denison et al., 1988a,b).

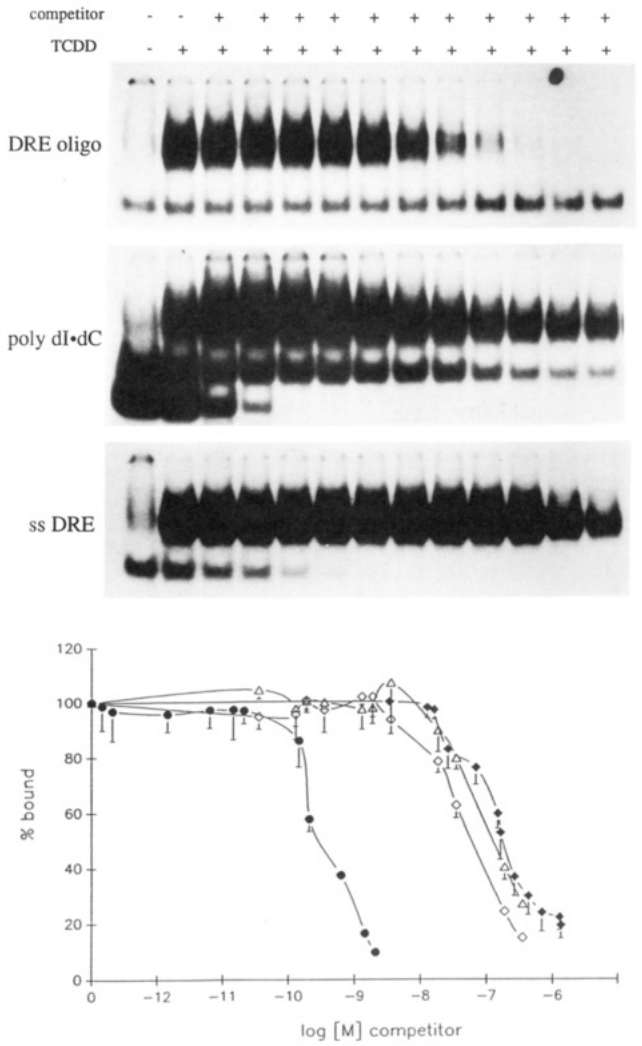

FIGURE 2: Relative affinity of transformed TCDD·AhR complexes for nonspecific and single-stranded DNA. (Top) Cytosol (16 mg/mL) was incubated in the absence (−) or presence (+) of TCDD (20 nM) for 2 h at 20 °C. Increasing concentrations of DRE oligonucleotide, poly(dI·dC), single-stranded DRE oligonucleotide (noncoding strand) was added to the DNA-binding reaction, and the protein–DNA complexes were resolved by gel retardation analysis. (Bottom) Competitive gel retardation analysis binding curves. Increasing concentrations of DRE oligonucleotide (●), poly(dI·dC) (◆), single-stranded DRE (coding strand) (◇), or single-stranded DRE (noncoding strand) oligonucleotide (△) were added to the DNA-binding reaction, and the protein–DNA complexes were resolved by gel retardation analysis. The amount of specific TCDD-inducible protein–DNA complex formed was determined as described in Materials and Methods. The molar amount of poly(dI·dC) was calculated assuming that each 22 bp represents the start of a different nonspecific binding site. The standard error of all values was less than 10%.

Table I: Comparison of the Relative Binding Affinity of Transformed TCDD·AhR Complexes to Specific and Nonspecific DNA

competitor	binding affinity[a] (nM)	competitor	binding affinity[a] (nM)
DRE oligomer[b]	2.5 ± 0.5	ssDRE noncoding strand	2600 ± 700[c]
ssDRE coding strand	1200 ± 110[c]	poly(dI·dC)	3400 ± 1070[c]

[a] Values are expressed as the mean ± SD of the relative binding affinity (K_d) as estimated from three separate experiments. [b] Wild-type DRE oligonucleotide. [c] Indicated value is significantly different from that of the wild-type DRE oligonucleotide ($p < 0.05$).

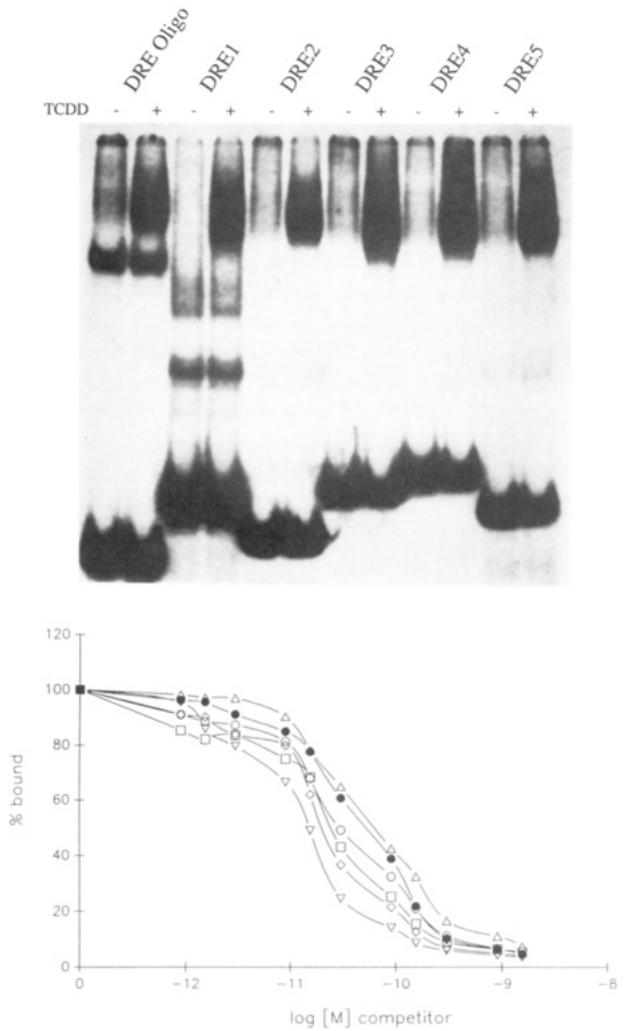

FIGURE 3: Binding of transformed TCDD·AhR complexes to endogenous mouse CYPIA1 DREs. (Top) The five mouse DREs were isolated by restriction enzyme digestion and radiolabeled with ^{32}P. Cytosol (16 mg/mL), treated in the absence (−) or in presence (+) of TCDD (20 nM), was incubated with the indicated radiolabeled DRE, and protein–DNA complexes were resolved by gel retardation analysis. (Bottom) Increasing concentrations of unlabeled DRE oligomer (●), DRE1 fragment (○), DRE2 fragment (△), DRE3 fragment (◇), DRE4 fragment (□), or DRE5 fragment (▽). 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.

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 (Figure 3, top panel). Comparative gel retardation analysis with these DNA fragments indicated a relatively similar degree of competitive binding with each DRE (Figure 3B). Comparison of the estimated relative binding affinity of all five mouse CYPIA1 upstream DREs (Table II) revealed that DNA fragments containing DREs 1, 3, 4, or 5 were significantly more effective (1.5–3.8-fold) as competitors than the DRE oligonucleotide itself. These small, but significant,

Table II: Comparison of Binding Affinities of Transformed TCDD·AhR Complexes to Mouse CYPIA1 DREs

DNA fragments	binding affinity[a] (nM)	DNA fragments	binding affinity[a] (nM)
DRE3 oligo[b]	2.5 ± 0.5[c]	DRE4 fragment	1.5 ± 0.03[c]
DRE1 fragment	1.7 ± 0.5[c]	DRE5 fragment	0.7 ± 0.1[c]
DRE2 fragment	3.5 ± 0.5	DRE5 oligo	3.3 ± 1.0
DRE3 fragment	1.1 ± 0.01[c]		

[a] Values are expressed as the mean ± SD of the relative binding affinity (K_d) as estimated from three separate experiments. [b] Wild-type DRE3 oligonucleotide. [c] Indicated value is significantly different from that of the wild-type DRE oligonucleotide ($p < 0.05$).

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.

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 II). 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 II). Additionally, the binding affinity of the DRE2-containing DNA fragment (30 bp) was not significantly different from the DRE oligomer, while that of the large 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 are 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 Figure 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 on the basis of the sequence of mouse DRE3 (Table III). To test the ability and extent to which the TCDD·AhR complex recognizes and binds to these mutant DREs, double-stranded wild-type and mutant DRE oligonucleotides were radiolabeled with ^{32}P and the ability of transformed TCDD·AhR complex

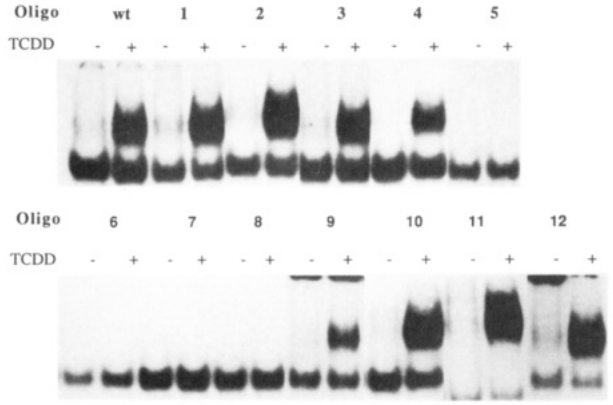

FIGURE 5: Effect of single nucleotide substitution on formation of the TCDD-inducible protein–DNA complex. Cytosol (16 mg/mL), incubated in the absence (−) or presence (+) of TCDD (20 nM) for 2 h at 20 °C, was mixed with the indicated ^{32}P-labeled wild-type (WT) or mutant DRE oligonucleotide, and protein–DNA complexes were resolved by gel retardation analysis as described in Materials and Methods. Only the protein–DNA complexes are shown (the results using mutant oligomer 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 III).

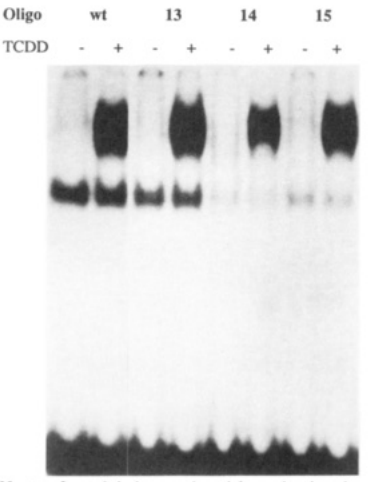

FIGURE 6: Effect of multiple nucleotide substitutions on formation of the TCDD-inducible protein–DNA complex. Cytosol (16 mg/mL), incubated in the absence (−) or presence (+) of TCDD (20 nM), was mixed with ^{32}P-labeled wild-type (WT) or multiply-substituted DRE oligonucleotide, and protein–DNA complexes were resolved by gel retardation analysis. The specific substitutions in each mutant DRE oligomer are indicated in Table III).

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
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/G	T	N	G	C	G	T	G	N	N	A/T	N	N	N	N
DRE OLIGO BINDING CONSENSUS		N	N	N	N	N	N	N	G	C	G	T	G	N	N	A/T	N	N	N	C/G

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 DRE-binding consensus generated from our studies is indicated for comparison. Nucleotides in boldface indicate those bases which deviate from the DRE consensus sequence, and asterisks indicate those which differ between the two derived sequences.

to bind DNA directly was analyzed by gel retardation (Figure 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 III)]. 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 (Figure 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 III). The estimated K_d values for each mutant oligonucleotide were consistent with the results of the direct binding experiments (Figure 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 100–800-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 (Figure 5) and binding affinity (Table III), this increase was not statistically significant. 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". On the basis of our mutagenesis experiments, we have deduced an optimal TCDD·AhR DNA-binding consensus sequence of GCGTGNNA/TNNNC/G (Figure 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 (5–10-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, is presented in Table III. 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 supports 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

Table III: 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	**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 (K_d) 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 that of the wild-type DRE oligonucleotide ($p < 0.05$).

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,b; Denison & 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 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 DRE mutagenesis binding experiments, using in vitro transformed AhR, are consistent with methylation interference studies using in vivo transformed AhR (Shen & Whitlock, 1989; Saatcioglu et al., 1990), in that methylation of these nucleotides blocked TCDD-inducible complex formation. This similarity in binding, combined with the apparent lack of difference in the apparent size and nucleotide-specific DRE binding of in vitro or in vivo transformed AhR (Denison, unpublished observations), suggests that AhR transformation in vitro appears to closely mimic that which occurs in vivo (although minor differences between them cannot be eliminated). Although the primary interaction of transformed TCDD·AhR complex with the DRE appears to occur 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 "nonconsensus" 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. Not only is this specific nucleotide 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 & Evans, 1988; Sakai et al., 1988; Kim & 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 & Jones, 1989) CYPIA1 gene and rat glutathione *S*-transferase (Rushmore et al., 1990) and quinone reductase (Favreau & 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-cross-linking experiments of Elferink et al. (1990) have indicated that the nonligand subunit of the transformed AhR complex appears to be the primary DNA-binding component. Preliminary experiments have revealed that the DNA-binding form of guinea pig AhR, like that of the rat, is a heterodimer (Swanson and Denison, unpublished observations). Thus, overall, 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, might suggest that the DNA binding of transformed TCDD·AhR complex occurs primarily through a specific, high-affinity interaction between the nonliganded 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 are currently matters 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 & 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 II), 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 $\rightleftarrows$ pyrimidine) 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 & 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 provide 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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Functional Reconstitution of the Nicotinic Acetylcholine Receptor by CHAPS Dialysis Depends on the Concentrations of Salt, Lipid, and Protein†

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ABSTRACT: The detergent CHAPS was found to be the preferable surfactant for the efficient purification and reconstitution of the *Torpedo californica* nicotinic acetylcholine receptor (AChR). The main result is that the incorporation of the AChR proteins into lipid vesicles by CHAPS dialysis was strongly dependent on the salt and protein concentrations. As monitored by sucrose gradients, by electron microscopy, and by agonist-induced lithium ion flux, the best reconstitution yields were obtained in 0.5 M NaCl at a protein concentration of 0.5 g/L and in 0.84 M NaCl at 0.15 g/L protein. Electron micrographs of receptor molecules, which were incorporated into vesicles, showed single, nonaggregated dimer (M_r = 580000) and monomer (M_r = 290000) species. CHAPS dialysis at NaCl concentrations <0.5 M largely reduced the receptor incorporation concomitant with *protein aggregation*. Electron micrographs of these preparations revealed large protein sheets or ribbons not incorporated into vesicles. The analysis of static and dynamic light scattering demonstrated that the *detergent-solubilized* AChR molecules aggregate at low lipid contents (≤500 phospholipids/AChR dimer), *independent* of the salt concentration. AChR proteins eluted from an affinity column with a solution containing 8 mM CHAPS (but no added lipid) still contained 130 ± 34 tightly bound phospholipids per dimer. The aggregates (about 10 dimers on the average) could be dissociated by readdition of lipid and, interestingly, also by increasing the CHAPS concentration up to 15 mM. This value is much higher than the CMC of CHAPS = 4.0 ± 0.4 mM, which was determined by surface tension measurements. The data clearly suggest protein–*micelle* interactions in addition to the association of *monomeric* detergents with proteins. Furthermore, the concentration of the (free) monomeric CHAPS at the vesicle–micelle transformation in 0.5 M NaCl ($[D_w]^c$ = 3.65 mM) was higher than in 50 mM NaCl ($[D_w]^c$ = 2.8 mM). However, it is suggested that the main effect of high salt concentrations durig the reconstitution process is an increase of the fusion (rate) of the ternary protein/lipid/CHAPS complexes with mixed micelles or with vesicular structures, similar to the salt-dependent fusion of vesicles.

A necessary and essential step in the detailed characterization of intrinsic membrane proteins is the detergent solubilization and the reconstitution of the protein species in artificial lipid membranes. Impressive examples are channel proteins where the channel characteristics of defined protein species may be studied in planar bilayers or in lipid vesicles of defined composition.

It has been recognized that it is the actual solubilization and reconstitution process which determine the protein content and the channel function in the membrane. The choice of detergent for the solubilization of the membrane proteins and the type of lipid to be used for vesicle formation are crucial and often

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