Mechanism of dioxin action: receptor – enhancer interactions in intact cells

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ABSTRACT

We have used a ligation-mediated polymerase chain reaction technique to analyze protein – DNA interactions at a dioxin-responsive enhancer upstream of the *CYP1A1* gene in intact mouse hepatoma cells. In its inactive state, the enhancer binds few, if any, proteins within the major DNA groove *in vivo*. Thus, the inactive enhancer is relatively inaccessible to DNA-binding proteins. Exposure of cells to 2,3,7,8-tetrachlorodibenzo-p-dioxin leads to the binding of the liganded Ah receptor at six sites within the major DNA groove of the enhancer. The receptor – enhancer interactions occur rapidly and do not require ongoing transcription, consistent with their role in regulating *CYP1A1* gene expression. The liganded receptor, which is a heteromer composed of at least two basic helix-loop-helix proteins, is probably the only DNA-binding transcription factor necessary to activate the enhancer *in vivo*. The small size and irregular distribution of receptor binding sites suggest that chromatin structure imposes substantial steric constraints upon the function of the receptor – enhancer system in intact cells.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is the prototypical halogenated aromatic hydrocarbon; such compounds are ubiquitous environmental contaminants that may pose a threat to human health (1). TCDD elicits biochemical, immunologic, teratogenic, and neoplastic effects in experimental animals (1–3). The possibility that TCDD produces similar adverse health effects in humans is of particular concern (4,5). The diversity of dioxin's biological effects is thought to reflect its ability to alter gene expression in species- and tissue-specific fashion (2,3,6). The Ah receptor, an intracellular protein that is composed of several components, mediates the response to dioxin (2,3,6). TCDD binding converts the receptor to its functional, DNA-binding form. Complementation analyses using somatic cell hybrids indicate that at least three genes contribute to receptor function (7,8). Biochemical studies, involving protein–DNA and protein–protein crosslinking, imply that the functional (liganded)

form of the receptor is heteromeric (9,10); these observations are consistent with the idea that the functional form of the receptor contains (at least) a ligand-binding component and a DNA-binding component (6). cDNA cloning experiments indicate that the ligand-binding component of the Ah receptor has a basic helix-loop-helix (bHLH) motif analogous to that identified in other transcription factors (11,12). In addition, a protein termed Arnt (for Ah receptor nuclear translocation), which contributes to the DNA-binding activity of the liganded receptor, also contains a bHLH motif (13,14). Presumably, the ligand-binding protein and the Arnt protein dimerize via their helix-loop-helix domains to generate the functional, DNA-binding, heteromeric form of the receptor, as occurs with other bHLH transcription factors (15).

The TCDD-inducible *CYP1A1* gene in mouse hepatoma cells constitutes a useful experimental system for analyzing the mechanism by which the Ah receptor functions. The liganded Ah receptor induces the transcription of the *CYP1A1* gene (16). The DNA upstream of the gene contains a dioxin-responsive transcriptional enhancer, to which the liganded Ah receptor can bind *in vitro* (17–20). Each receptor binding site contains the core recognition sequence

5' T-GCGTG 3'
3' A-CGCAC 5', (21–23). *In vitro*

studies reveal that the liganded receptor binds within the major DNA groove and contacts each of the four guanine residues of the core sequence (23–25). In a general sense, the dioxin-responsive receptor – enhancer system resembles control systems described for other genes, where protein – DNA interactions at *cis*-acting control elements regulate expression (26). Therefore, analyses of the dioxin-responsive system may reveal aspects of enhancer function that apply to other genes, particularly those that are regulated by bHLH transcription factors.

Gel retardation analyses and transfection experiments provide important *in vitro* information about enhancer organization and function. However, such studies require that the enhancer be removed from its native chromosomal context and be analyzed under non-physiological conditions, which might produce misleading results. In particular, chromatin structure makes important contributions to gene expression (27–29); transfection and gel retardation studies do not adequately control for this variable. For example, the *CYP1A1* transcriptional promoter is inaccessible to its cognate binding proteins in intact, uninduced

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cells, even though those proteins are expressed constitutively (30). This reflects the positioning of nucleosomes over the promoter *in vivo* (31). Similarly, chromatin structure may influence protein–DNA interactions at the enhancer *in vivo*. To address this issue, we have used a ligation-mediated polymerase chain reaction technique (32) to analyze protein binding to the dioxin-responsive enhancer in intact cells.

MATERIALS AND METHODS

Materials

DNA Taq polymerase, Sequenase 2.0, and T4 DNA ligase were purchased from Perkin Elmer Cetus, United States Biochemical, and Promega Corporation, respectively. All other molecular biological reagents were purchased from Bethesda Research Laboratories, New England Biolabs and Sigma. [γ - 32 P]ATP (3000 Ci/mmol) was from Amersham Corporation. TCDD was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository.

Cell culture

Wild-type (Hepa 1c1c7) and receptor-defective (BP^c1) mouse hepatoma cells were cultured as monolayers and induced with 1 nM TCDD (in DMSO) as previously described (8). The BP^c1 cells are defective in the Arnt protein (13,14). In these cells, the liganded Ah receptor fails to bind to DNA, and the *CYP1A1* gene is inactive and does not respond to TCDD (7,8).

DMS treatment and DNA preparation

Approximately 6×10^7 cells were trypsinized and resuspended in 1 ml of serum-free culture medium. Methylation was carried out by adding 5 μ l of dimethylsulfate (DMS) to the cell slurry and incubating for 3 min at room temperature. The reaction was placed on ice and stopped by the addition of 10 ml of ice-cold phosphate-buffered saline (PBS), and the cells were pelleted and washed again with PBS. Nuclei were prepared and genomic DNA was isolated as previously described (30). Approximately 200 micrograms of genomic DNA was digested with EcoR1, extracted with phenol:chloroform (1:1), precipitated with ethanol, and treated with piperidine, as previously described (30). Preparation of *in vitro* methylated DNA was as previously described (30).

Ligation-mediated PCR

For each sample, 4 micrograms of genomic DNA was analyzed by LMPCR (32), using a nested set of three primers for first strand synthesis (primer 1), PCR (primer 2) and end-labeling (primer 3). Oligonucleotides were synthesized using an Applied Biosystems synthesizer and were purified by polyacrylamide gel electrophoresis.

For footprinting the sense strand of the *CYP1A1* enhancer, we used the following primer sets (designated 'IX' and 'X'):

IX: primer 1, 5'-GGGTGCAGAGCTATCTAAGTATG-3' (53°C),
primer 2, 5'-TATGGTGGAGGAAAGGGTGGAGGAA-3' (66°C),
primer 3, 5'-GGTGGAGGAAAGGGTGGAGGAAGGATCC-3' (69°C).
X: primer 1, 5'-GTAAGCCTGCTCCATCTCT-3' (47°C),
primer 2, 5'-AGTTGCGTGAGAAGAGCCTGGAGGC-3' (66°C),
primer 3, 5'-TTGCGTGAGAAGAGCCTGGAGGCCCG-3' (69°C).

For footprinting the antisense strand of the enhancer, we used the following primer sets (designated 'II', 'V', and 'VII'):

II: primer 1, 5'-TTGTCGCGCCTTGCAAAGCATAGAT-3' (53°C),
primer 2, 5'-AAACCCACCCAACGCCAGGAGAGCT-3' (57°C),
primer 3, 5'-CCCAACGCCAGGAGAGCTGGCCCTTA-3' (66°C).
V: primer 1 5'-ATCCATCCCCACCTCTAGATGAAG-3' (56°C),
primer 2, 5'-CGAACTTCGCCGATACCCAATTG-3' (63°C),
primer 3, 5'-GGCCGATACCCAATTTGTGGGGCACAG-3' (66°C).
VII: primer 1, 5'-CTTACACTAATCTCACTCTGGGAG (47°C),
primer 2, 5'-GGCCAGAGAGCACCTGCAAAACAGC-3' (66°C),
primer 3, 5'-AGCACCTGCAAAACAGCCAGCTAGCGTG-3' (69°C).

Annealing temperatures for each primer are given in parenthesis. All samples were denatured for 3 minutes at 95°C immediately prior to the first PCR cycle. Conditions for 15 cycles of PCR amplification using primer 2 were: 1 minute at 94°C for denaturation, 2 minutes at the annealing temperature, and 3 minutes at 76°C for extension. The length of the extension reaction was increased an additional 5 seconds per cycle. The conditions for extension of the end-labeling primer 3 were: 3 minutes at 95°C for denaturation, 2 minutes at the annealing temperature, and 10 minutes at 76°C for extension. The magnesium concentration for the PCR reactions was 2.5 mM for all primer sets. Primer 3 was end-labeled using [γ - 32 P]ATP and T4 polynucleotide kinase (33); 4–10 picomoles of the end-labeled primer were added to each extension reaction.

Following amplification, the DNA fragments were extracted with phenol:chloroform (1:1) and precipitated as previously described (30). DNA fragments were separated on an 8% sequencing gel and visualized by autoradiography. Each experiment was performed at least three times using independently methylated DNA samples, with consistent results.

Fig. 1 shows the positions of the primer sets used in these studies and the observed locations of protein binding sites (see Results).

RESULTS

Contacts between the Ah receptor and DNA in intact cells

Methylation protection studies *in vitro* reveal that the liganded receptor contacts the four G residues within the core sequence 5' T-GCGTG 3' (25). To verify that we can, in fact, detect the 3' A-CGCAC 5' binding of the receptor to DNA *in vivo*, we initially analyzed the DNA domain (designated here as 'D', see Fig. 1) previously studied *in vitro*. Our findings reveal that domain D is not protected

Figure 1. Protein binding sites upstream of the *CYP1A1* gene *in vivo*. Boxes (■) represent binding sites for the liganded Ah receptor. Circle (○) represents a G-rich domain. See text for more details. Arrows and Roman numerals indicate the positions of the primer sets used for LMPCR (see Methods).

Figure 2. Protein–DNA interactions at an Ah receptor recognition sequence *in vivo*. Uninduced (–TCDD) and induced (+TCDD: 1nM, 4 hr) wild-type and receptor-defective mouse hepatoma cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA were analyzed by LMPCR. The lanes designated ‘CON’ contain genomic DNA methylated *in vitro*. The arrowheads indicate the guanine residues that exhibit decreased (▽) or increased (▼) susceptibility to methylation. The orientation of the G ladder is 5' to 3' from top to bottom. The Ah receptor binding sequence, designated in this paper as site ‘D’, is located between nucleotides –985 and –979, upstream of the *CYP1A1* gene.

from methylation in uninduced wild type cells (Fig. 2). Therefore, in the absence of TCDD, we find no evidence *in vivo* for protein binding in the major DNA groove at or near this recognition sequence for the receptor. However, TCDD induces changes in the methylation pattern at each of the four G residues previously implicated in receptor binding. Three of the four G residues become protected, while methylation of the fourth G is enhanced. All of these TCDD-inducible changes are Ah receptor-dependent, because they do not occur in the receptor-defective cells (Fig. 2). Because the altered methylation pattern is (a) TCDD-inducible, (b) receptor-dependent, and (c) occurs at the core recognition sequence, we infer that it reflects the binding of the liganded Ah receptor to its enhancer *in vivo*. The reason for the increased methylation of one G is unknown. Possibly, the receptor–enhancer interaction bends (or otherwise distorts) the DNA *in vivo*, increasing the susceptibility to chemical modification of a G at the boundary of the receptor’s binding site. This idea is consistent with other observations that the binding of the liganded receptor to DNA generates a change in chromatin structure (see Discussion).

To determine whether the receptor binds at other enhancer sites *in vivo*, we analyzed the protein–DNA interactions along approximately 700 base pairs of DNA. Our findings reveal five additional domains (designated here as ‘A’, ‘B’, ‘C’, ‘E’, and ‘F’, see Fig. 1) that exhibit patterns of methylation similar to that of domain D. Each domain contains a core recognition sequence for the liganded Ah receptor. On the strand containing three G residues, two Gs are protected and one G is

Figure 3. Protein–DNA interactions at Ah receptor binding sites upstream of the *CYP1A1* gene. Uninduced (–TCDD) and induced (+TCDD: 1nM, 4 hr) wild-type and receptor-defective mouse hepatoma cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA were analyzed by LMPCR. The lanes designated ‘CON’ contain genomic DNA methylated *in vitro*. The arrowheads indicate the guanine residues that exhibit decreased (▽) or increased (▼) susceptibility to methylation. The orientation of the G ladder is 5' to 3' from top to bottom. **Panel A:** Receptor binding site located between nucleotides –494 and –485 designated in this paper as site ‘A’; **panel B:** receptor binding site located between nucleotides –875 and –864, designated in this paper as site ‘B’; **panel C:** receptor binding site located between nucleotides –898 and –889, designated in this paper as site ‘C’; **panel D:** receptor binding site located between nucleotides –1062 and –1053, designated in this paper as site ‘E’; **panel E:** receptor binding site located between nucleotides –1218 and –1209, designated in this paper as site ‘F’.

Figure 4. Protein–DNA interactions at base pairs adjacent to the core recognition sequence for the Ah receptor. Uninduced (–TCDD) and induced (+TCDD: 1nM, 4 hr) wild-type and receptor-defective mouse hepatoma cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA were analyzed by LMPCR. The lanes designated ‘CON’ contain genomic DNA methylated *in vitro*. The arrowheads (▽) indicate the guanine residues that exhibit decreased susceptibility to methylation. The orientation of the G ladder is 5' to 3' from top to bottom. **Panel A:** analysis of receptor binding site ‘F’, located between nucleotides –1218 and –1209; **panel B:** analysis of receptor binding site ‘E’, located between nucleotides –1062 and –1053.

hypermethylated; the pattern is TCDD-inducible and Ah receptor-dependent (Fig. 3). On the strand containing one G residue, that residue is protected in TCDD-inducible, receptor-dependent fashion at domains A, C, E, and F (data not shown for domains A and C; see Fig. 4 for domains E and F); we have not analyzed the single-G strand at domain B. Thus, we observe a consistent pattern of TCDD-inducible, receptor-dependent methylation at each of six recognition sites for the liganded Ah receptor. These findings indicate that the liganded receptor can bind to all of these sites *in vivo*. The binding of the receptor at domain B was unexpected, because we had not previously observed an analogous protein–DNA interaction *in vitro* (21).

At domains B and C the protection from methylation is noticeably weaker than at the other four receptor-binding domains (Fig. 3, panels B and C). Presumably, the diminished protection reflects a weakened interaction between the liganded receptor and DNA. Domain B contains a 5' G-GCGTG 3' sequence, instead of the usual 5' T-GCGTG 3' core sequence. We have observed

previously that mutating the T/A base pair decreases the affinity of the liganded receptor for its recognition sequence *in vitro* (34). Therefore, our findings suggest that the T/A base pair also contributes to the strength of the receptor–enhancer interaction *in vivo*. Domain C contains the usual core recognition sequence; nevertheless, it exhibits diminished protection from DMS. This finding implies that base pairs outside of the core sequence affect the strength of the receptor–enhancer interaction *in vivo*. Additional observations at two other receptor binding sites support this concept. At domains E and F, we observe TCDD-inducible,

Figure 5. Contacts between the liganded Ah receptor and DNA in intact cells. Summary of methylation protection results at six receptor binding sites *in vivo*. The open G's represent guanines protected from methylation. The bold-face, underlined G's represent guanines exhibiting increased susceptibility to methylation. See text for more details.

receptor-dependent protection of G residues immediately adjacent to the core sequence (Fig. 4). Thus, these guanines contact the liganded receptor in the intact cell, indicating that the receptor binding site extends beyond the core sequence. These findings were not expected, because previous methylation protection studies *in vitro* had suggested that the receptor contacts only the four guanine residues of the core sequence (23–25). Furthermore, other studies of receptor–DNA interactions *in vivo* also failed to reveal these G contacts outside the core sequence, because the enhancer was not analyzed completely in earlier experiments (35). Our observations also serve to define the size of the receptor binding site *in vivo*; based on methylation protection, it spans 6 base pairs of DNA (see Fig. 5).

Figure 5 summarizes the G contacts made by the liganded Ah receptor in intact cells, as determined by methylation protection. Our observation that the receptor contacts DNA base pairs beyond its core recognition sequence *in vivo* is consistent with the results of transfection experiments, which indicate that such base pairs contribute to enhancer function (36). The possible implications of the relatively small size of the receptor binding sites, as well as their irregular distribution, are discussed below.

Characteristics of the receptor–enhancer interaction in intact cells

We performed additional experiments in order to relate the observed receptor–enhancer interactions to the activation of *CYP1A1* gene transcription by TCDD. For example, at domain D, time-course studies reveal that the receptor–enhancer interaction is fully established within 30 minutes of exposure to TCDD; furthermore, the interaction persists for at least 16 hours (Fig. 6). We made similar observations at each of the other five receptor binding sites (data not shown). Thus, the time course for receptor–enhancer binding *in vivo* is rapid and mirrors that for the induction of *CYP1A1* transcription, which is also maximal within 30 minutes of exposure to TCDD and persists for at least 16 hours (16). This temporal relationship tends to implicate the receptor–enhancer interaction in the activation of gene

Figure 6. Time course of the Ah receptor–enhancer interaction *in vivo*. Wild-type mouse hepatoma cells were exposed to TCDD (1nM) for the indicated times. Cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA were analyzed by LMPCR. The lanes designated 'CON' contain genomic DNA methylated *in vitro*. The orientation of the G ladder is 5' to 3' from top to bottom. The arrowheads indicate the guanine residues that exhibit decreased (▽) or increased (▼) susceptibility to methylation. The receptor binding site analyzed (site 'D') is located between nucleotides –985 and –979.

transcription. The persistence of the interaction suggests that the binding of the receptor to DNA also contributes to the maintenance of the induced state. These findings, together with other observations (30, 31), lead us to envision that the receptor–DNA interactions generate and maintain an alteration in chromatin structure that is necessary for the activation of *CYP1A1* transcription (see Discussion).

In a second type of experiment, we used actinomycin D to further clarify the relationship between receptor–enhancer binding and *CYP1A1* transcription in intact cells. At a concentration that inhibits transcription of the *CYP1A1* gene by >95% (37), actinomycin D fails to block the receptor–enhancer interaction (Fig. 7). We observed similar results at each of the other five receptor binding sites (data not shown). Therefore, the receptor–enhancer interaction *in vivo* does not require ongoing transcription and does not occur secondary to the induction of *CYP1A1* gene expression. In fact, our observations support the contrary hypothesis, namely, that the receptor–enhancer interaction is a necessary component of the induction mechanism and precedes the activation of *CYP1A1* transcription by TCDD.

Other protein–DNA interactions at the dioxin-responsive enhancer in intact cells

Our analyses reveal one additional TCDD-inducible, receptor-dependent protein–DNA interaction at the dioxin-responsive enhancer *in vivo*. The interaction occurs at a G-rich domain located between nucleotides –1075 and –1066 (Fig. 8). The DNA sequence of this domain resembles that of a G-rich region previously implicated in the function of the *CYP1A1* promoter (38). Therefore, it is possible that the protein–DNA interaction

Figure 7. Effect of actinomycin D on the Ah receptor–enhancer interaction *in vivo*. Wild-type mouse hepatoma cells were exposed to actinomycin D (2μg/ml, 2.5 hr) and/or TCDD (1nM, 2 hr), as indicated. Cells were exposed to actinomycin D 30 min prior to exposure to TCDD. Cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA were analyzed by LMPCR. The lanes designated 'CON' contain genomic DNA methylated *in vitro*. The orientation of the G ladder is 5' to 3' from top to bottom. The arrowheads indicated the guanine residues that exhibit decreased (▽) or increased (▼) susceptibility to methylation. The receptor binding site analyzed (site 'D') is located between nucleotides –985 and –979.

Figure 8. Other protein–DNA interactions at the dioxin-responsive enhancer *in vivo*. Uninduced (–TCDD) and induced (+TCDD: 1nM, 4 hr) wild-type and receptor-defective mouse hepatoma cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA were analyzed by LMPCR. The lanes designated 'CON' contain genomic DNA methylated *in vitro*. The orientation of the G ladder is 5' to 3' from top to bottom. The arrowheads (▽) indicate the guanine residues that exhibit decreased susceptibility to methylation. The G-rich sequence is located between nucleotides –1075 and –1066.

at the G-rich region contributes to enhancer function. The TCDD-inducible, receptor-dependent characteristics of the interaction are consistent with this idea.

DISCUSSION

We have used a ligation-mediated PCR technique to analyze protein–DNA interactions at approximately 700 base pairs of DNA in the vicinity of a dioxin-responsive enhancer upstream of the *CYP1A1* gene. Our findings reveal that the inactive enhancer binds very few proteins within the major DNA groove *in vivo*. This observation has several implications. 1) The paucity of protein–DNA interactions suggests that the inactive enhancer is relatively inaccessible to DNA-binding proteins. For example, mouse hepatoma cells constitutively express a protein(s) that binds to naked, G-rich DNA *in vitro* (39). However, such binding does not occur at the *CYP1A1* promoter *in vivo*, unless the gene has been activated by TCDD. Thus, the inactive promoter is inaccessible in intact cells (30). Similarly, we observe no protein–DNA interaction at the G-rich domain of the inactive enhancer, implying that it is also inaccessible. The resistance of the inactive enhancer to restriction endonuclease digestion is another indicator of its inaccessibility (37). The nucleosomal configuration of the inactive enhancer may account for its inaccessibility *in vivo* (31). 2) The absence of protein binding at the inactive enhancer puts constraints on the possible mechanisms of TCDD action. For example, our findings argue against the possibility that the liganded Ah receptor acts by displacing a specific repressor protein, which might maintain the enhancer in an inactive configuration. If such a repressor exists, it must not contact G residues within the major DNA groove. 3) Gel retardation analyses reveal that uninduced cells contain proteins that bind to naked enhancer DNA *in vitro*; the structure and possible function of these proteins are unknown (21, 23, 39, 40). Our analyses reveal no evidence for the corresponding protein–DNA interactions at the inactive enhancer *in vivo*. This inconsistency raises doubts about the biological relevance of the interactions that are observed only *in vitro*.

Enhancer activation is associated with the appearance of several protein–DNA interactions *in vivo*. Watson and Hankinson previously observed TCDD-inducible, receptor-dependent protein–DNA interactions at regions corresponding to domains D and E and the G-rich domain, and they commented upon the modular organization of the enhancer in intact cells (35). The observations reported here agree with those of Watson and Hankinson and extend their findings in several ways. We find, for example, that the liganded Ah receptor binds at six DNA domains *in vivo*, including one that was not identified using *in vitro* measurements. In addition, we find that the receptor makes contact with guanine residues immediately adjacent to its core recognition sequence and that the binding site *in vivo* spans six base pairs, as measured by methylation protection. Furthermore, we show that the TCDD-induced receptor–enhancer interactions occur rapidly and independently of RNA synthesis, characteristics that are consistent with the receptor's role in the control of *CYP1A1* transcription. Another potential regulatory protein–DNA interaction occurs at a G-rich domain. However, by analogy with the situation at the *CYP1A1* promoter (30), protein binding at the G-rich domain probably reflects its increased accessibility induced by the receptor–enhancer interaction. Therefore, the liganded Ah receptor appears to be

an example of a bHLH transcription factor that can activate transcription without a requirement for other enhancer-binding proteins.

The six receptor binding sites are arranged in an irregular pattern (Fig. 1). The absence of regular spacing between the sites suggests that enhancer activation does not require protein–protein interactions between adjacent, DNA-bound receptors. Instead, the multiplicity of sites may reflect the constraints imposed by chromatin structure on receptor–DNA interactions in the intact cell. For example, studies using micrococcal nuclease imply that the inactive enhancer is organized into nucleosomes, which occupy random positions along the DNA (31). Therefore, enhancer activation requires that the liganded Ah receptor bind to nucleosomes. As the DNA helix wraps around the histone core of the nucleosome, the major groove is periodically accessible and inaccessible. Therefore, increasing the number of receptor binding sites at irregular intervals increases the probability that at least one site will be accessible even when the DNA is nucleosomal. In addition, the liganded receptor contacts a relatively short (6 base pair) DNA segment, increasing the probability that the entire binding site will be accessible in the nucleosome. Thus, the multiplicity, irregular distribution, and small size of Ah receptor binding sites may have evolved as a mechanism for overcoming the steric constraint imposed by the nucleosomal organization of the inactive enhancer *in vivo*.

We envision that the binding of one liganded Ah receptor to the enhancer is sufficient to start the transcriptional activation process, by causing a local change in chromatin structure that increases the accessibility of the neighboring DNA. As a result, additional liganded receptors bind to their previously inaccessible sites, producing a more global alteration in chromatin structure, which leads to loss of nucleosomes at the *CYP1A1* promoter and enhanced transcription (30, 31). The mechanism by which the receptor–enhancer interaction alters chromatin structure and DNA accessibility is unknown. One mechanism could involve a receptor-induced alteration in DNA configuration. For example, the receptor–enhancer interaction bends the DNA *in vitro* (41). The TCDD-induced hypermethylation of a G residue at one boundary of each receptor binding site suggests that the receptor–enhancer interaction may also distort the DNA in the intact cell. Such changes in DNA configuration may lead to the local disruption of nucleosomal structure and increased DNA accessibility. A second mechanism could involve a receptor-induced alteration in the histones. For example, the liganded receptor may contain (or recruit) an activity (e.g., histone acetylase) that weakens histone–DNA interactions, thereby increasing the accessibility of the DNA. From a mechanistic standpoint, an alteration in chromatin structure can account for the ability of the receptor–enhancer system to activate the *CYP1A1* promoter from a distance, irrespective of enhancer orientation. Our findings suggest the possibility that other bHLH transcription factors might also bend DNA *in vitro* and alter chromatin structure *in vivo*.

The bHLH class of proteins, to which the components of the Ah receptor belong, are diverse in their effects; for example, they contribute to the regulation of immunoglobulin genes and genes involved in myogenesis and neurogenesis (15). Dimerization of bHLH proteins in different combinations provides a mechanism for generating regulatory diversity. For example, different dimers may exhibit different stabilities, may have different binding affinities for DNA, or may recognize

different DNA sequences. In addition, some HLH proteins, which lack a basic, DNA-binding domain, may act as dominant negative regulators of transcription, contributing further to regulatory diversity (15). It is not yet known if the bHLH components of the Ah receptor are present in multiple forms, which might dimerize in different combinations. Hankinson and co-workers have presented evidence for a dominant negative regulator in the TCDD-responsive system (42); however, it is unknown whether the protein(s) responsible for the dominant negative phenotype is a member of the HLH class. We do know that TCDD typically elicits a broad spectrum of tissue-specific biological effects (1–3). We speculate that some of this biological diversity might reflect differential gene regulation by different combinations of bHLH proteins that comprise and/or interact with the Ah receptor. Future analyses of the TCDD-responsive *CYP1A1* gene may provide clues about the mechanisms by which bHLH transcription factors regulate gene expression in other systems.

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