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Mechanism of dioxin action: Ah receptor-mediated increase in promoter accessibility *in vivo*

(2,3,7,8-tetrachlorodibenzo-*p*-dioxin/gene regulation/chromatin/protein–DNA interactions/DNA methylation)

LENA WU AND JAMES P. WHITLOCK, JR.*

Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5332

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ABSTRACT **We have analyzed dioxin-inducible, Ah receptor-dependent changes in protein–DNA interactions at the *CYP1A1* transcriptional promoter in intact mouse hepatoma cells. Our findings indicate that in uninduced cells, the promoter is inaccessible to its cognate binding proteins, which are known to be expressed constitutively. Dioxin induces, in Ah receptor-dependent fashion, an increase in promoter accessibility, which occurs rapidly and does not require ongoing transcription of the *CYP1A1* gene. The change in promoter accessibility is not due to an altered pattern of cytosine methylation at the promoter; it probably reflects a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced change in the chromatin structure. These findings provide new insight into the mechanism of dioxin action and contribute to a better understanding of the regulation of inducible gene transcription in mammalian cells.**

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is the prototype for a class of halogenated aromatic hydrocarbons that are widespread and persistent environmental contaminants. In animals, TCDD elicits various biochemical, immunological, teratogenic, and neoplastic effects, depending upon the species and tissue studied (1, 2). Dioxin poses an unknown risk to human health, with birth defects and cancer being of particular concern (3, 4). An intracellular protein, designated as the Ah receptor, binds TCDD saturably and with high affinity and is assumed to mediate the dioxin's biological effects, because receptor-defective cells respond poorly to TCDD (1, 2). More complete knowledge of the mechanism of dioxin action may increase our understanding of the health hazard it poses and may help in identifying individuals who are particularly susceptible to dioxin's toxic effects.

The induction of aryl hydrocarbon hydroxylase activity is an experimentally useful response for analyzing the mechanism of dioxin action (5). The cytochrome P4501A1 enzyme catalyzes hydroxylase activity, which results in the oxygenation of aromatic substrates such as the environmental carcinogen benzo[*a*]pyrene (6). In mouse hepatoma cells, TCDD induces hydroxylase activity by activating the transcription of the corresponding *CYP1A1* gene. Induction of transcription requires the Ah receptor, because it fails to occur in receptor-defective cells. Induction involves the formation of a TCDD–Ah receptor complex, followed by the binding of the liganded receptor to DNA (3, 4).

Transfection experiments reveal that the DNA upstream of the *CYP1A1* gene contains two types of cis-acting transcriptional control element. The more distal element has the functional properties of a dioxin-responsive, Ah receptor-dependent transcriptional enhancer; it spans about 400 base pairs (bp), is centered about 1000 bp upstream of the *CYP1A1* transcription start site, and contains several binding sites for the liganded Ah receptor (7–10). The more proximal element has the functional properties of a transcriptional promoter; it spans about 150 bp immediately upstream of the transcription start site and contains binding sites for several known transcription factors (11). The promoter contains no binding sites for the liganded Ah receptor. Fig. 1 is a schematic representation of the enhancer and promoter and the protein-binding sites that are relevant to the experiments described below. Expression of the *CYP1A1* gene requires that the enhancer and promoter function jointly, because neither control element can, by itself, activate transcription in the absence of the other (11).

Our previous observations impose constraints upon possible mechanisms by which the receptor–enhancer interaction is coupled to the function of the *CYP1A1* promoter, which is situated hundreds of base pairs downstream. For example, DNase I footprinting of naked promoter DNA *in vitro* reveals that the proteins that bind to the TATA box, the NF1 site, and the guanine-rich region (G box) are present in the uninduced cell and are not increased by TCDD; furthermore, TCDD does not appear to increase the affinity of these promoter-binding proteins for their cognate binding sites on naked DNA (11). These observations argue against the possibilities that TCDD either stimulates the synthesis of new promoter-binding transcription factors or increases the affinity of preexisting transcription factors for promoter DNA. Here, we have analyzed protein–DNA interactions at the *CYP1A1* promoter in the intact cell in order to distinguish between two additional possibilities: (*i*) that proteins bind to the promoter in uninduced cells and, therefore, that transcriptional enhancement involves an event(s) subsequent to protein binding or (*ii*) that proteins fail to bind to the promoter in uninduced cells and, therefore, that enhancement involves increased accessibility of promoter DNA *in vivo*. The findings reported here support the latter alternative and thereby provide new insight into the mechanism by which dioxin activates gene transcription. We envision that the mechanism involves a TCDD-induced change in the chromatin structure of the *CYP1A1* promoter.

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, respectively. All other molecular biological reagents were purchased from Bethesda Research Laboratories, New England Biolabs, and Sigma. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. TCDD was obtained from the National Cancer

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMS, dimethyl sulfate; LMPCR, ligation-mediated polymerase chain reaction.

*To whom reprint requests should be addressed.

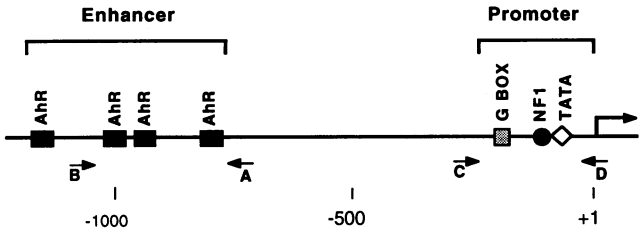

FIG. 1. Transcriptional control elements (enhancer and promoter) upstream of the *CYP1A1* gene and the protein-binding domains associated with each, deduced from *in vitro* studies (6, 8). See text for more details. The boxes designated "AhR" represent recognition motifs for the liganded Ah receptor. The arrows labeled A, B, C, and D indicate the positions of the primer sets used in the ligation-mediated polymerase chain reaction (LMPCR) analyses.

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Cell Culture. Wild-type mouse hepatoma cells (Hepa 1c1c7) and class II variant cells were cultured as described (12). Cells were grown to confluence and induced with 1 nM TCDD for the length of time indicated in the figure legends. Experiments employing actinomycin D were performed as described (13).

Dimethyl Sulfate (DMS) Treatment and DNA Preparation. Approximately 6×10^7 cells were trypsinized and resuspended in 1 ml of serum-free culture medium. Methylation of intact cells was carried out by adding 5 μl of DMS to the cell slurry and incubating for 3 min at room temperature. The reaction was halted 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 described (14). Approximately 200 μg of genomic DNA was digested with *Eco*RI, extracted with phenol/chloroform (1:1), and precipitated. *In vitro* methylation of DNA and piperidine treatment were as described (15).

Analysis of Cytosine Methylation. Cells were treated as described above, except that DMS treatment was omitted. Genomic DNA was prepared as described above. After digestion with *Eco*RI, 50 μg of genomic DNA was subjected to cytosine-specific hydrazine treatment as described (16). Four micrograms of genomic DNA was then analyzed by LMPCR as described below.

LMPCR. For each sample, 4 μg of genomic DNA was analyzed by LMPCR (17), using a nested set of three primers for first strand synthesis (primer 1), PCR (primer 2), and end-labeling (primer 3).

For analyzing the sense strand of the *CYP1A1* enhancer, we used the following primer set (designated "A"): AGTATGGTGGAGGAAAGGGTGGAG (53°C) (primer 1), GAGGAAGGATCCACGCGCCACAGCA (66°C) (primer 2), GAAGGATCCACGCGAGACAGCAGGAGG (69°C) (primer 3).

For analyzing the antisense strand of the enhancer, we used the following primer set (designated "B"): TTGTCGCGCCTTGCAAAGCATAGAT (53°C) (primer 1), AAACCCACCCAACGCCAGGAGAGCT (57°C) (primer 2), CCCAACGCCAGGAGAGCTGGCCCTTTA (66°C) (primer 3).

For analyzing the sense strand of the *CYP1A1* promoter, we used the following primer set (designated "C"): ACTGAAGTGAAGAGTGTTCTCTAGG (50°C) (primer 1), TCTCTAGGACCCTAGGGAGGATCGG (60°C) (primer 2), CCTAGGGAGGATCGGGGAAGCTCCAAG (63°C) (primer 3).

For analyzing the antisense strand of the promoter, we used the following primer set (designated "D"): CCTCAGTGGGATTATGCACTGT (47°C) (primer 1), CTGTCCATGGAGGACCTTGAAAGTG (63°C) (primer 2), CTGTCCATGGAGGACCTTGAAAGTGTGGG (69°C) (primer 3).

Annealing temperatures for each primer are given in parentheses. All samples were denatured for 3 min at 95°C immediately prior to the first PCR cycle. Conditions for 15 cycles of amplification were 1 min at 94°C for denaturation, 2 min at the primer 2 annealing temperature for hybridization, and 3 min at 76°C for extension. The time period of the extension reaction was increased an additional 5 sec for every cycle. The conditions for primer extension of the end-labeled primer 3 were 3 min at 95°C for denaturation, 2 min at the primer 3 annealing temperature for hybridization, and 10 min at 76°C for extension. The final magnesium concentration for PCR reactions was 1.0 mM for primer set A and 2.5 mM for primer sets B, C, and D.

Following amplification and precipitation, DNA fragments were separated on an 8% sequencing gel and visualized by autoradiography. Each of the experiments presented was performed at least three times from independently methylated DNA samples, with consistent results.

RESULTS

To analyze protein–DNA interactions in intact cells, we measured the susceptibility of guanine residues to modification by DMS, using a LMPCR technique (17) to study specific regions of the *CYP1A1* promoter. As a positive control, we first analyzed a receptor–enhancer interaction *in vivo*, because we know from *in vitro* studies that the liganded receptor contacts the four guanine residues of the core recognition motif $\begin{matrix}5'\ \text{T–GCGTG}\ 3' \\ 3'\ \text{A–CGCAC}\ 5'\end{matrix}$ (18, 19). Our findings reveal that, in wild-type cells, TCDD induces a change in the susceptibility of these same four guanine residues to methylation by DMS (Fig. 2). The change is dependent upon the Ah receptor, because it does not occur in receptor-defective cells (Fig. 2). These results indicate the formation of a TCDD-inducible, receptor-dependent protein–DNA interac-

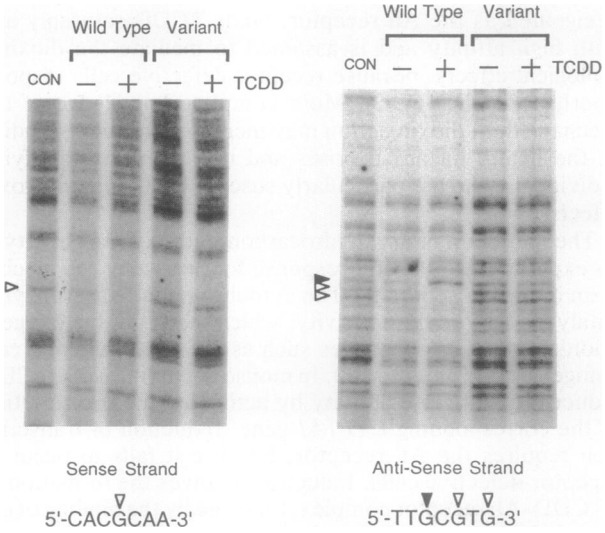

FIG. 2. Protein–DNA interactions *in vivo*, at a binding site for the liganded Ah receptor. Uninduced (− TCDD) and induced (+ TCDD: 1 nM, 4 hr) wild-type and receptor-defective variant mouse hepatoma cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA was analyzed by LMPCR. The lanes designated "CON" contain genomic DNA methylated *in vitro*. The Ah receptor-binding site is located at nucleotides −985 to −979. The arrowheads indicate the guanine residues that exhibit decreased (▽) or increased (▼) susceptibility to methylation. The orientation of the G ladder is 5′ → 3′ from top to bottom.

tion at the Ah receptor's recognition motif within the intact cell. The altered methylation pattern is localized to the receptor's DNA recognition motif and does not represent a general change in the susceptibility of enhancer DNA to modification by DMS. We observe an identical pattern of TCDD-inducible, receptor-dependent changes at other receptor recognition motifs within the dioxin-responsive enhancer (data not shown). These findings imply that, in the intact cell, the liganded receptor recognizes the nucleotide sequence deduced from *in vitro* studies. These control experiments indicate that we can, in fact, measure dioxin-inducible, Ah receptor-dependent alterations in protein–DNA interactions at specific genomic sites within the intact cell.

We next used this experimental approach to analyze protein–DNA interactions at the *CYP1A1* promoter. (Note that we cannot study interactions at the TATA box, because it is not susceptible to modification by DMS). Fig. 3 reveals no protection of guanine residues at either the NF1 site or the G box in uninduced wild-type cells; thus, in the absence of TCDD, transcription factors fail to bind at these sites *in vivo*, even though the proteins are present in uninduced cells. In contrast, TCDD induces the protection of guanine residues at the NF1 site and at the G box *in vivo*; transfection and mutagenesis experiments previously have implicated both sites in promoter function (11). These observations indicate that exposure of the cell to dioxin enables preexisting proteins to bind at specific sites on the *CYP1A1* promoter.

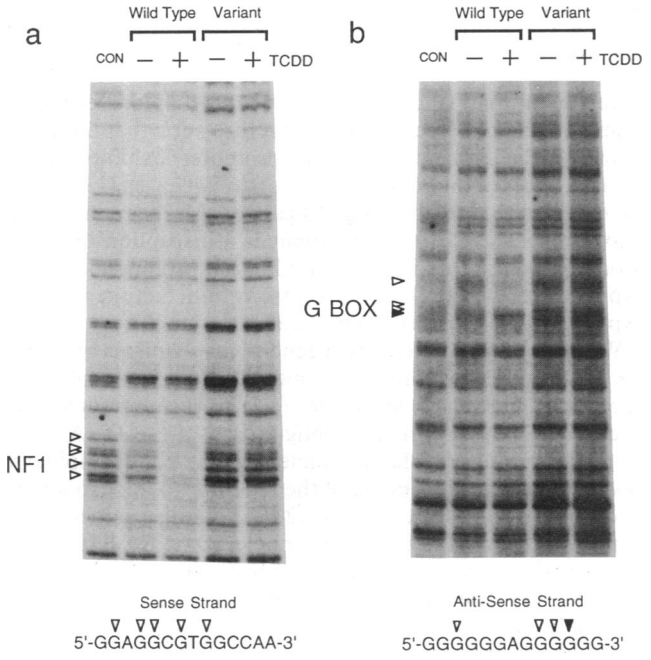

FIG. 3. Protein–DNA interactions at the *CYP1A1* transcriptional promoter *in vivo*. Uninduced (− TCDD) and induced (+ TCDD: 1 nM, 4 hr) wild-type and receptor-defective variant mouse hepatoma cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA was analyzed by LMPCR. The lanes designed "CON" contain genomic DNA methylated *in vitro*. The orientation of the G ladder is 5′ → 3′ from top to bottom. (*a*) Analysis of the NF1-binding site, on the "sense" DNA strand, which contains the indicated nucleotide sequence. The NF1-binding site is located at nucleotides −58 to −42. The arrowheads indicate the guanine residues that exhibit decreased (▽) susceptibility to methylation. (*b*) Analysis of the G box, on the "antisense" DNA strand, which contains the indicated nucleotide sequence. The G box is located at nucleotides −128 to −115. The arrowheads indicate the guanine residues that exhibit decreased (▽) or increased (▼) susceptibility to methylation.

One possible mechanism that could account for these observations is that TCDD induces a change in DNA methylation, thereby facilitating the binding of proteins to the promoter. It is known, for example, that NF1 fails to bind DNA that is methylated at cytosine (14). Therefore, we analyzed TCDD's effect on cytosine methylation within the *CYP1A1* promoter. Because 5-methylcytosine is resistant to chemical cleavage, such residues appear as gaps in the cytosine ladder. Our findings (Fig. 4) reveal that TCDD does not induce changes in cytosine methylation at either the NF1 site or the G box *in vivo*. At both protein-binding sites, all cytosines are unmethylated in uninduced and induced wild-type cells. As a positive control, we note that, in uninduced and induced receptor-defective variant cells, a CpG dinucleotide is methylated at cytosine, indicating that we could, in fact, detect 5-methylcytosine in the wild-type promoter, if it were present. We do not know if the observed cytosine methylation in the variant cells contributes to the variant phenotype.

TCDD does not induce changes in cytosine methylation that could account for increased protein binding at the *CYP1A1* promoter. Therefore, the results of the DMS protection studies (Fig. 3) imply that TCDD increases the accessibility of promoter DNA, which facilitates the binding of transcription factors. The increase in accessibility is Ah receptor-dependent, because it fails to occur in receptor-defective cells (Fig. 3). However, the promoter itself contains no binding sites for the Ah receptor; thus, we envision that the increase in accessibility is generated from a distance, by an action of the receptor at the enhancer, as discussed in more detail below.

Experiments employing actinomycin D [at a concentration that inhibits *CYP1A1* transcription by >95% (13)] reveal that the TCDD-induced increase in promoter accessibility does not require ongoing transcription (Fig. 5). Thus, the altered

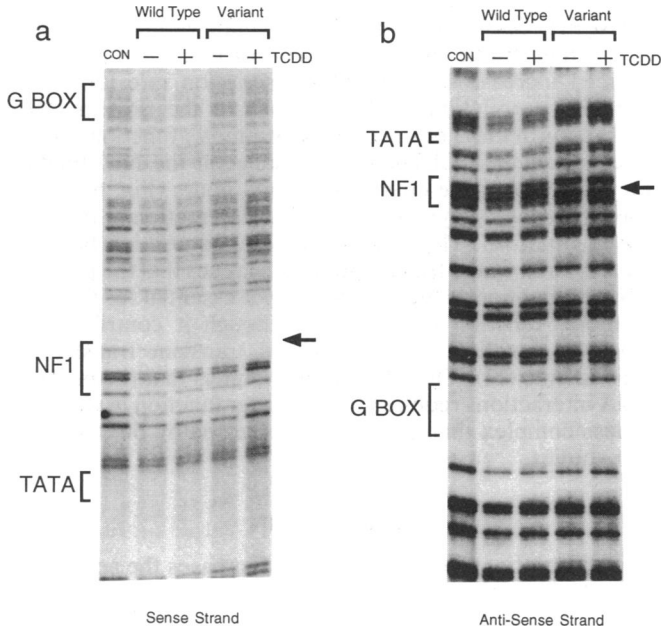

FIG. 4. Analysis of cytosine methylation at the *CYP1A1* transcriptional promoter *in vivo*. Uninduced (− TCDD) and induced (+ TCDD: 1 nM, 4 hr) wild-type and receptor-defective variant mouse hepatoma cells were harvested and genomic DNA was isolated. Four micrograms of genomic DNA was analyzed by LMPCR. The lanes designated "CON" contain genomic DNA from untreated cells. The brackets indicate the location of transcriptional control elements at the promoter. The arrow indicates the position of a 5-methylcytosine residue. The orientation of the C ladder is 5′ → 3′ from top to bottom.

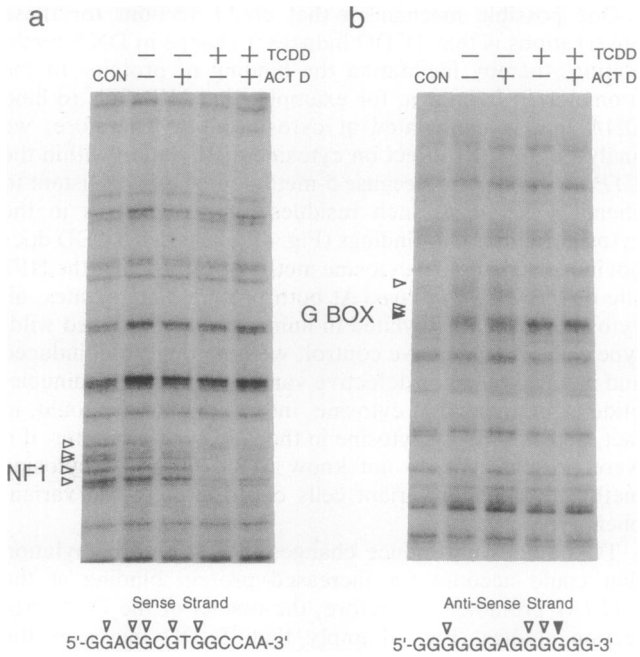

FIG. 5. Effect of actinomycin D on protein–DNA interactions at the *CYP1A1* transcriptional promoter *in vivo*. Wild-type mouse hepatoma cells were exposed to solvent, actinomycin D (ACT D) (2 μg, 2.5 hr), and/or TCDD (1 nM, 2 hr), as indicated. Cells were harvested and treated with DMS (0.5%, 3 min) and genomic DNA was isolated. Four micrograms of genomic DNA was analyzed by LMPCR. The lanes designed "CON" contain genomic DNA methylated *in vitro*. The orientation of the G ladder is 5′ → 3′ from top to bottom. (*a*) Analysis of the NF1-binding site, as in Fig. 3*a*. (*b*) Analysis of the G box, as in Fig. 3*b*.

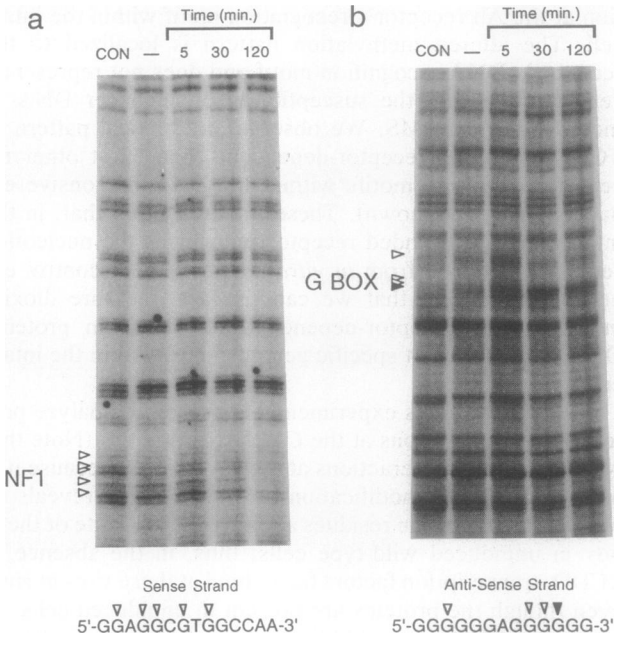

FIG. 6. Time course of TCDD-induced protein–DNA interactions at the *CYP1A1* transcriptional promoter *in vivo*. Wild-type mouse hepatoma cells were exposed to TCDD (1 nM) for the indicated times. Cells were harvested and treated with DMS (0.5%, 3 min), and genomic DNA was isolated. Four micrograms of genomic DNA was analyzed by LMPCR. The lanes designated "CON" contain genomic DNA methylated *in vitro*. The orientation of the G ladder is 5′ → 3′ from top to bottom. (*a*) Analysis of the NF1-binding site, as in Fig. 3*a*. (*b*) Analysis of the G box, as in Fig. 3*b*.

protein–DNA interactions at the promoter do not result from a preceding increase in *CYP1A1* gene expression.

Time-course studies reveal that the TCDD-induced increase in accessibility of the G box occurs within 30 min (Fig. 6*B*), a time frame that is the same as that for the TCDD-induced increase in *CYP1A1* transcription rate (20). This temporal correlation tends to implicate the protein–DNA interaction at the G box in *CYP1A1* promoter function *in vivo*. In contrast, the TCDD-induced change at the NF1-binding site occurs over the course of several hours (Fig. 6*A*). The reason for the delayed protein–DNA interaction at the NF1 site is unknown. The lack of temporal correlation between protein binding and transcription rate argues that the protein–DNA interaction at the NF1 site is not essential for *CYP1A1* promoter function *in vivo*, even though it contributes to promoter function as measured in transient transfection experiments (11). This observation implies that the protein–DNA interactions required for promoter function *in vivo* may be less complex than previously suggested by our transfection studies.

DISCUSSION

Our observations imply that TCDD increases the accessibility of the *CYP1A1* promoter, thereby allowing proteins to bind and to activate transcription. The receptor-dependent mechanism by which this change occurs remains to be determined in detail. Our analyses of cytosine methylation imply that changes in DNA modification do not contribute to increased promoter accessibility. Therefore, we envision that the mechanism involves an altered chromatin structure. For example, we have observed that, in uninduced cells, the *CYP1A1* promoter assumes a nucleosomal configuration, which could account for its inaccessibility to transcription factors *in vivo* (unpublished observations). Furthermore, we have shown that TCDD induces, in Ah receptor-dependent fashion, a change in chromatin structure such that the DNA spanning the enhancer and the promoter exhibits an increased accessibility to restriction endonucleases (13). Thus, we envision that the binding of liganded Ah receptors to the dioxin-responsive enhancer produces a disruption in nucleosome structure that is propagated to the promoter, thereby exposing promoter DNA to transcription factors that are expressed constitutively by the cell.

We can envision at least two general mechanisms by which the liganded Ah receptor could induce a more "open" (i.e., accessible) chromatin structure. (*i*) The receptor–enhancer interaction may distort the configuration of the DNA, such that it can no longer fold into nucleosomes. For example, we have shown that the binding of the receptor to its recognition motif bends the DNA *in vitro* (21). A similar effect could occur in chromatin, disrupting nucleosome structure and relieving the repressive effect of the histones on transcription (22, 23). (*ii*) The liganded Ah receptor might contain an enzymatic activity (e.g., a histone acetylase) that could produce a local weakening of histone–DNA interactions, thereby increasing the accessibility of the DNA to other proteins. These and other possibilities remain to be tested. However, our findings imply that the study of dioxin-inducible changes in chromatin structure may be a productive area for future research.

Induction of gene transcription is associated with increased promoter accessibility in some systems but not in others. For genes that are transcribed even in uninduced cells [e.g., tyrosine aminotransferase (14, 24, 25), heat-shock (25), metallothionein (15)], the corresponding promoters bind proteins constitutively; therefore, increased accessibility appears not to contribute substantially to the induction mechanisms in such systems. Instead, the induction of transcription may involve an "activation" event(s), which involves the binding of additional proteins to the chromatin template (14, 15,

24–27). In contrast, increased promoter accessibility may represent a primary mechanism for regulating inducible genes that are not transcribed in the uninduced cell. For example, the steroid-responsive mouse mammary tumor virus gene and the very-low-density apolipoprotein II genes, like the dioxin-responsive *CYP1A1* gene, are silent in the uninduced state, and the corresponding promoters do not bind proteins (28, 29). In such systems, increased promoter accessibility appears to play an important role in the induction mechanism.

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