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2,3,7,8-Tetrachlorodibenzo-P-Dioxin Receptors Regulate Transcription of the Cytochrome P_1-450 Gene

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The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dioxin, produces a diverse set of biological responses which, in some cases, reflects the altered expression of specific genes. An intracellular receptor protein binds TCDD saturably and with high affinity and mediates several of TCDD's biological effects. In mouse hepatoma cells, TCDD induces aryl hydrocarbon hydroxylase activity by activating the transcription of the cytochrome P_1-450 gene. Studies of receptor-defective variant cells indicate that the activation of cytochrome P_1-450 gene transcription requires functional TCDD receptors. Analysis of the DNA that flanks the 5'-end of the mouse cytochrome P_1-450 gene reveals at least three control regions: a promoter, an inhibitory element, and a dioxin-responsive element (DRE). Therefore, expression of the cytochrome P_1-450 gene represents a balance between negative and positive control. The DRE contains two discrete, non-overlapping DNA domains that respond to TCDD. Each TCDD-responsive domain acts independently of the other, each requires TCDD receptors for function, and each has the properties of a transcriptional enhancer. For example, the function of the DREs is relatively independent of both their location and their orientation with respect to the promoter. Together, the DREs and the TCDD-receptor complex constitute a dioxin-responsive enhancer system. Exposure of cells to TCDD results in the protection of a specific DNA domain from exonuclease digestion. This protection requires TCDD receptors. The protected domain maps to a DRE. This observation implies that the TCDD-receptor complex interacts with the DRE to activate the transcription of the cytochrome P_1-450 gene.

Abbreviations used: TCDD, dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin; CAT, chloramphenicol acetyltransferase; DRE, dioxin-responsive element; HAV, high-activity variant; bp, base pairs; AHH, aryl hydrocarbon hydroxylase; HAH, halogenated aromatic hydrocarbon; 3 MC, 3-methylcholanthrene; Exo III, exonuclease III.

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During the past three decades, halogenated aromatic hydrocarbons (HAHs) (eg, polychlorinated/polybrominated biphenyls, dibenzofurans, dibenzo-p-dioxins) have generated intense public and scientific concern because of their widespread occurrence as environmental contaminants, their resistance to degradation, and their biological potency. The prototypical HAH, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin; Fig. 1), produces a diverse set of responses in experimental animals, including a wasting syndrome, immunological alterations, teratogenic effects, tumor promotion, epithelial hyperplasia/metaplasia, and the induction of several drug-metabolizing enzymes [1,2]. In humans, TCDD produces chloracne and subtle alterations in cellular immunity [3–5]; its action as a teratogen or carcinogen for humans is somewhat controversial [6–8]. The ability of TCDD to produce a broad spectrum of effects suggested that the compound might act by altering the expression of particular genes in a species-specific and/or tissue-specific fashion [2,9].

CYTOCHROMES P-450

In many cell types, TCDD induces the activity of aryl hydrocarbon hydroxylase (AHH), which is primarily catalyzed by an isozyme of cytochrome P-450 that is designated cytochrome P-450c in the rat and cytochrome P_1-450 in the mouse [10]. The cytochromes P-450 are enzymes that catalyze the oxygenation of many endogenous and exogenous lipophilic substrates and are involved in a variety of metabolic activities [11,12]. For example, the AHH system is responsible for the metabolic activation and detoxification of polycyclic aromatic hydrocarbons, such as the environmental carcinogen benzo(a)pyrene [13]. TCDD is the most potent known inducer of AHH activity, and many investigators have utilized AHH induction as a model response by which to study the mechanism of TCDD action. In addition, studies of TCDD congeners reveal that their potencies as AHH inducers correlate with their toxic potencies. Therefore, an understanding of AHH induction might also provide insights into the mechanisms of TCDD toxicity [2].

DIOXIN RECEPTORS

TCDD is about 10,000 times as potent as other compounds (eg, 3-methylcholanthrene [3MC], β-naphthoflavone) that induce AHH activity [14]. Studies of inbred mice revealed differences in their responsiveness to 3MC and TCDD, as measured by AHH induction [15]. In crosses between "responsive" strains (eg, C57BL/6) and "nonresponsive" strains (eg, DBA/2), the responsive phenotype segregated as an

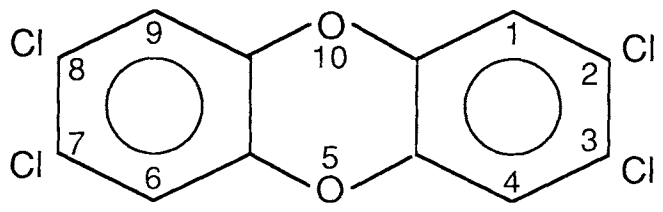

Fig. 1. 2,3,7,8-Tetrachlorodibenzo-p-dioxin.

autosomal dominant trait [16,17]. The locus responsible for this phenotype was designated *Ah* (for *a*romatic *h*ydrocarbon responsiveness) [18] and was postulated to encode a specific induction "receptor" for TCDD [15]. Subsequently, Poland and his co-workers identified in hepatic cytosol from C57BL/6 mice a protein that had properties appropriate for a TCDD receptor [19]. This macromolecule exhibited (1) reversible, high-affinity, and saturable binding of ^{3}H-TCDD; (2) binding affinities for other halogenated dibenzo-p-dioxins that correspond to their potencies as inducers of AHH activity in vivo; and (3) the ability to bind 3MC and other polycyclic aromatic hydroacarbons that induce AHH activity. Subsequent studies have demonstrated that the hydrodynamic properties of TCDD receptors and their apparent mechanism of signal transduction are similar to those of steroid receptors [20,21]. For example, both TCDD and steroid receptors (1) are acidic proteins with salt-dissociated monomeric molecular weights around 90,000–120,000 [see 20,21 for a more thorough discussion of hydrodynamic properties]; (2) bind DNA-cellulose and heparin-Sepharose [21]; (3) have distinct ligand-binding and DNA-binding domains as demonstrated by limited proteolysis [21,22]; (4) exist in the absence of ligand; as soluble intracellular proteins with a low affinity for the cell nucleus [23,24]; (5) undergo ligand-induced transformation to a state with high affinity for the nucleus [23,25–27]; and (6) act at the level of transcription to induce the expression of a target gene [28–30].

TCDD receptors differ from steroid receptors in their ligand-binding domain, because steroids do not bind TCDD receptors, nor does TCDD bind to steroid receptors [31,31]. Also, genetic analyses of TCDD receptor function using cell fusion reveal that receptor-defective variant cells compose at least two complementation groups [33–35]. Analogous studies of glucocorticoid receptor variants reveal that they fall into a single complementation group [36]. Thus, despite their biochemical similarities, it is unclear at present whether TCDD receptors belong to the family of steroid receptors that is related to the *erb A* gene [37].

DIOXIN-RESPONSIVE GENOMIC ELEMENTS

TCDD induces AHH activity in mouse hepatoma cells by increasing the rate of transcription of the cytochrome P_1-450 gene [29]. This response requires both the formation of TCDD-receptor complexes and an interaction between the inducer-receptor complex and the cell nucleus, because transcriptional activation does not occur in receptor-defective variant cells [29]. Furthermore, the TCDD-receptor complex is a DNA-binding protein [22]. By analogy with the mechanism of action of steroid-receptor complexes [30], it appeared that the TCDD-receptor complex might activate gene expression by interacting with a genomic regulatory element located upstream of the transcriptional promoter for the cytochrome P_1-450 gene. To test this idea, several groups have isolated putative control regions from cloned cytochrome P-450 DNA and linked these sequences to a heterologous reporter gene (usually the chloramphenicol acetyltransferase [CAT] gene). The function of these hybrid genes was tested by transfection.

Using this approach, Jones et al [38] identified a dioxin-responsive element (DRE) that is located more than 1,500 bp upstream of the cytochrome P_1-450 promoter in mouse hepatoma cells. Deletion analysis of these upstream sequences established that at least two DREs are present within a 480-bp segment of DNA [39]. The results of transfection experiments using both wild-type and receptor-defective

variant cells indicated that the induction of CAT activity (1) had the correct inducer specificity, (2) had the expected sensitivity to TCDD, and (3) required functional TCDD-receptor complexes.

Gonzalez and Nebert [40] reported observations for the cytochrome P_1-450 gene in C57BL/6 mouse liver that are qualitatively similar to those found for the mouse hepatoma cytochrome P_1-450 gene. DREs also have been identified upstream of the rat and human genes that correspond to the mouse cytochrome P_1-450 gene [41,42]. In the rat, two DREs were identified further upstream from the region examined in the mouse cytochrome P_1-450 gene. Whether additional DREs exist at equivalent positions upstream of the mouse gene has yet to be established.

Other regulatory elements, in addition to the DREs, are present in the DNA that flanks the 5′-end of the cytochrome P_1-450 gene in mouse hepatoma cells. A promoter element, situated between 25 and 30 bp upstream of the transcription start site, confers constitutive expression when linked to the CAT gene in the absence of other upstream sequences [38]. Inclusion of an additional 600–1,000 bp of DNA inhibits CAT expression, suggesting the presence of an element that may bind a trans-acting repressor molecule [38,40]. Inclusion of additional upstream DNA containing the DREs confers TCDD-responsiveness upon the CAT gene [38]. Thus, expression of the cytochrome P_1-450 gene reflects the combination of a constitutive component, an inhibitory component, and a stimulatory component (Fig. 2).

A DIOXIN-RESPONSIVE ENHANCER SYSTEM

Enhancers are DNA sequences that bind specific proteins, thereby activating transcription from cognate promoters [43]. Enhancer elements increase transcriptional efficiency relatively independently of their orientation and distance with respect to the promoter. Also, enhancers function when linked to either their natural promoter or with a heterologous promoter [44]. The ability of the cytochrome P_1-450 DRE to activate transcription from a distance suggested that it might function as a transcriptional enhancer. Jones et al [45] inserted the cytochrome P_1-450 DRE into a CAT expression vector, which was designed to evaluate the enhancer properties of the insert. Analysis of the recombinants by transfection revealed that the DREs stimulate CAT expression from a heterologous promoter and require functional TCDD-receptor complexes. The DRE functions in *cis* at several positions relative to the promoter and

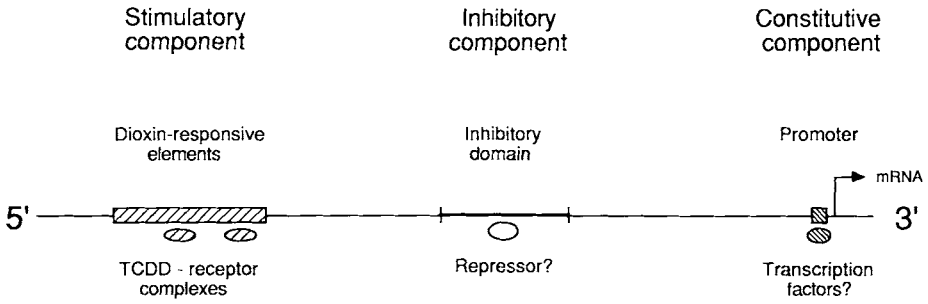

Fig. 2. Genomic control elements located upsteam of the cytochrome P_1-450 gene. See text for discussion.

in either orientation. Thus, the DRE, together with the TCDD-receptor complex, constitutes a dioxin-responsive enhancer system.

Neuhold et al [46] prepared stable Hepa-1 cell transfectants, using fragments of the C57BL/6 mouse cytochrome P_1-450 regulatory region linked to the CAT gene. They identified at least two DREs (in the same regions previously shown to be DREs by Jones et al [38,39]), but they concluded that only one of these DRE's functions as an enhancer element. Sogawa et al [41] demonstrated that the 3′ DRE near the rat cytochrome P-450c gene is also a transcriptional enhancer. They have not yet examined the upstream DREs for enhancer properties.

The mechanism(s) by which enhancers augment transcription from a distance is unknown [see 47]. Two plausible models envision that the binding of the *trans*-acting factor (ie, the TCDD-receptor complex) to the *cis*-acting element (ie, the DRE) produces (1) a change in chromatin structure that is propagated to the promoter and converts the nucleoprotein to a "transcriptionally active" form, or (2) "looping" of the DNA that brings the receptor binding site close to the promoter and results in the activation of gene expression. Further studies will be necessary to distinguish between these possibilities.

SELECTIVE RECEPTOR: DNA INTERACTIONS

The binding of TCDD to its receptor increases the affinity of the receptor for the cell nucleus and DNA [22,25]. The activation of cytochrome P_1-450 gene expression requires both the DRE and functional TCDD-receptor complexes [38,39]. These findings suggest the specific dioxin action on cytochrome P_1-450 gene transcription might reflect the binding of the TCDD-receptor complex to specific DNA sequences near the TCDD-responsive gene. To test this hypothesis, Durrin and Whitlock [48] utilized an in situ exonuclease III (Exo III) protection technique [49,50] to measure TCDD-induced changes in the accessibility of the DRE to nuclease digestion. In wild-type mouse hepatoma cells, an Exo III-resistant site is present approximately 1600 bp upstream of the transcription start site, in a region that functions as a dioxin-responsive enhancer. In wild-type cells, resistance to Exo III digestion requires exposure to TCDD, develops rapidly (within 1 hr) in response to TCDD, and does not occur in a receptor-defective variant cell line in which neclear association of the TCDD-receptor complex does not occur. In high activity variant cells (HAV), which transcribe the cytochrome P_1-450 gene constitutively [51], resistance to Exo III occurs only after exposure to TCDD, indicating that the resistance is not simply a consequence of gene transcription. These results imply that the TCDD-receptor complex interacts with the dioxin-responsive enhancer to activate the transcription of the cytochrome P_1-450 gene.

The precise DNA sequence that is recognized by the TCDD-receptor complex is not known; however, the Exo III-resistant site identified by Durrin and Whitlock [48] is located close to a specific consensus decanucleotide sequence [41] that is present in multiple copies in the DNA upstream of the rat and mouse cytochrome P-450 genes. Sogawa et al [41] proposed that the TCDD-receptor complex recognizes this consensus sequence. However, some copies of the consensus sequence are located in DNA regions that do not exhibit responsiveness to the inducer 3MC. Therefore, an altered chromatin structure may also be required for recognition of the DRE by the TCDD-receptor complex.

EFFECTS OF RECEPTOR BINDING ON CHROMATIN STRUCTURE

The conformation of chromatin associated with the cytochrome P-450 genes has not been examined thoroughly. Durrin and Whitlock [48] observed that the cytochrome P_1-450 gene in TCDD-induced (but not uninduced) mouse hepatoma cell nuclei is more accessible to restriction enzyme digestion. This may reflect an altered chromatin structure that could be important for the binding of regulatory molecules. A 3MC-induced nuclease hypersensitive site is presented approximately 160 bp upstream of the rat cytochrome P-450c gene transcription start site [52], however, in those studies regions further upstream (ie, the DREs) were not examined for hypersensitivity to nuclease digestion.

CONCLUSION

Although we have learned some aspects of the mechanism by which the TCDD-receptor complex regulates cytochrome P-450 gene expression, many areas of research remain to be explored. The regulatory DNA sequence(s) that mediates TCDD-induced cytochrome P-450 gene transcription is not known. Gene transfer experiments using hybrid genes that contain mutated DREs will help to define this sequence more precisely. In vitro mutagenesis technique will also be useful in defining the number of DREs within a regulatory region.

In vitro footprinting experiments will complement the in situ studies of Durrin and Whitlock [48] and may reveal that the TCDD-receptor complex binds to a specific DNA sequence directly. These types of experiments have been hampered by the lack of purified TCDD receptors. However, a crude nuclear extract might be useful for such studies under the appropriate conditions.

The inhibitory and promoter regions are also important in the overall regulation of cytochrome P-450 gene transcription. The use of in vitro mutagenesis technique, as well as in situ and in vitro footprinting methods, to study these control regions should help to generate a mechanism for gene regulation that incorporates stimulatory, inhibitory, and constitutive components. While DNA sequence is undoubtedly important in regulating gene expression, eukaryotic DNA is organized into a nucleoprotein complex. Studies of the chromatin structure of TCDD-induced and -uninduced cytochrome P-450 genes should help to clarify the contribution that nucleoprotein structure makes toward the control of gene expression.

Much information about the regulation of TCDD-responsive genes will be obtained after the receptor is purified and the receptor cDNA is cloned. Purification of the receptor has been hindered by its low abundance in target tissues. The ability to covalently label the receptor with an affinity reagent [53] will allow the use of denaturing conditions during the isolation of the TCDD-binding protein and should result in a greater degree of purification than previously possible. Antibodies prepared against the purified receptor can then be used to study structural and functional domains of the receptor.

Purification of the cDNA that encodes the receptor protein may also be feasible. Theoretically, it should be possible to construct wild-type cDNA libraries in an expression vector, transfect these libraries into receptor-defective variant cell lines, and select recombinants in which the receptor defect has been complemented. Mutagenesis and transfection experiment with the cloned TCDD receptor cDNA will

provide a great deal of information about the structural and functional domains of the TCDD receptor and should provide further insights into the mechanism by which the receptor regulates gene transcription.

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