Annu. Rev. Pharmacol. Toxicol. 1990. 30:251–77

GENETIC AND MOLECULAR ASPECTS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN ACTION

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KEY WORDS: halogenated aromatic hydrocarbons, gene regulation, environmental health, agent orange, carcinogenesis

INTRODUCTION

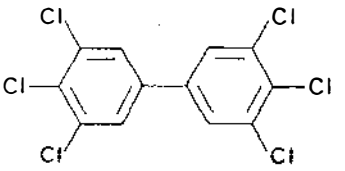
Some halogenated aromatic hydrocarbons[1] (HAHs) such as the polychlorinated biphenyls have been used extensively for commercial purposes. Related compounds, such as the halogenated dibenzo-p-dioxins and dibenzofurans, have no commercial value themselves but are generated during the manufacture or combustion of other HAHs. In general, HAHs are lipophilic and stable; halogenation renders them relatively resistant to enzymatic conversion to water-soluble derivatives. Therefore, HAHs tend to persist in the environment and to accumulate in the food chain, posing a potential risk to human health (1, 2). Toxicologic evaluations reveal that the various HAHs evoke similar responses, although they differ by orders of magnitude in potency; therefore, they are believed to share a common mechanism of action.

[1]Abbreviations used:
HAH, halogenated aromatic hydrocarbon; AHH, aryl hydrocarbon hydroxylase; MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; RFLP, restriction fragment length polymorphism; TCDF, 2,3,7,8-tetrachlorodibenzofuran; SAR; structure-activity relationship; QSAR, quantitative structure-activity relationship.

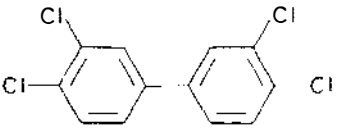
0362-1642/90/0415-0251$02.00

The prototypical HAH is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Figure 1); it has been studied more intensively than other HAHs because it is the most potent. TCDD has gained notoriety as a contaminant in the herbicide Agent Orange and because of its release into the environment during industrial accidents and the improper disposal of chemical wastes. In experimental animals, TCDD elicits a broad spectrum of effects, ranging from changes in various enzyme activities to thymic atrophy to the production of birth defects and cancer. TCDD has generated controversy among the lay public, the scientific community, and regulatory agencies because of its presence as an environmental contaminant, its toxic potency in experimental animals, and its potential as a teratogen and carcinogen for humans. Animal species vary, both in their relative susceptibilities to TCDD and in the pattern of responses that the dioxin elicits (1–5). This variation complicates assessment of the risk that TCDD poses for humans. Furthermore, difficulties in documenting actual exposure to TCDD have limited the ability of epidemiological studies to reveal dioxin-related adverse human health effects. Thus, the risk that dioxin poses to humans remains uncertain. In the future, analyses of the mechanism of TCDD action may reduce this uncertainty by identifying the cellular components that mediate the response to TCDD. Such knowledge could then be used to identify individuals at increased risk from exposure to TCDD and related compounds.

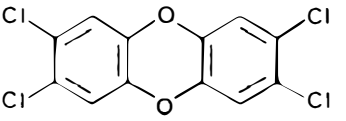
Models for TCDD action must account for the diversity of its biological effects (1–3). Based upon analyses of a response that occurs in many tissues (the induction of aryl hydrocarbon hydroxylase (AHH) activity), we envision



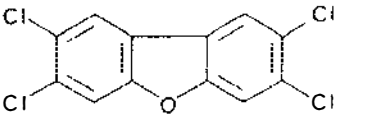
3,3',4,4',5,5' - Hexachlorobiphenyl



3,3',4,4', - Tetrachlorobiphenyl



2,3,7,8 - Tetrachlorodibenzo-p-dioxin



2,3,7,8 - Tetrachlorodibenzofuran

Figure 1 Structures of several halogenated aromatic hydrocarbons.

that TCDD produces many responses by altering the expression of specific genes (3, 6). The mechanism involves the binding of TCDD to an intracellular protein (the Ah receptor), followed by the binding of the liganded receptor to a specific DNA recognition sequence in the vicinity of the target gene (see below). Other (as yet undiscovered) regulatory components function in concert with the dioxin-responsive system to generate the diversity characteristic of TCDD action.

GENETIC ASPECTS OF TCDD ACTION

The Ah *Locus*

The *Ah* locus, discovered as a genetic polymorphism in mice, governs the biological responses to certain *a*romatic *h*ydrocarbons. For example, 3-methylcholanthrene (MC) induces hepatic microsomal AHH activity in the prototypical "responsive" C57BL/6 inbred mouse strain, but not in the prototypical "nonresponsive" DBA/2 inbred strain (7, 8). [AHH activity reflects the oxygenation of the environmental carcinogen benzo(a)pyrene (9), catalyzed by a form of cytochrome P450 that has been designated cytochrome P_1-450, cytochrome P450c and, more recently, cytochrome P450IA1 (10)]. In crossbreeding studies among certain mouse strains, the inducible (responsive) phenotype segregates as a dominant trait, which is under the control of a single autosomal gene (7, 8). The alleles governing the responsive and nonresponsive phenotypes are designated Ah^b and Ah^d, respectively. Other studies, using crosses between responsive and nonresponsive strains, reveal that additional aromatic hydrocarbon-induced toxic responses, such as thymic involution, cleft palate production, and hepatic porphyria, segregate in a pattern identical to that for AHH inducibility (3). Similar findings have been observed in studies of congenic mouse strains that differ only at the *Ah* and closely linked loci (11–13). These findings indicate that the *Ah* locus influences a diverse set of responses to aromatic hydrocarbons. Therefore, the locus is considered to be regulatory and to encode a protein(s) that determines the susceptibility of the organism to such compounds.

The discovery that TCDD is much more potent than MC as an inducer of AHH activity led to the search for a target molecule (i.e. a receptor) that mediates the induction response. Using [^{3}H]-TCDD in ligand-binding studies, Poland and his coworkers demonstrated that a responsive mouse strain (C57BL/6) contained an intracellular hepatic protein that binds TCDD saturably and with high affinity and, thus, has the properties of a "TCDD receptor" (14). In contrast, this protein was not detectable in a nonresponsive strain (DBA/2). Thus, the relative inability of aromatic hydrocarbons to induce AHH activity in the DBA/2 strain was associated with a defect in the TCDD

receptor. These observations implied that the receptor is required for the induction of AHH activity by TCDD. Subsequently, Okey and coworkers have shown that the DBA/2 mouse contains a receptor that binds TCDD with about a 10-fold lower affinity than the receptor from the C57BL/6 strain (15). These findings account for the relative inability of the DBA/2 strain to respond to TCDD.

Crossbreeding studies reveal that the segregation patterns for the expression of the high-affinity form of the TCDD receptor and the expression of aromatic hydrocarbon responsiveness are identical (16). These findings imply that the *Ah* locus encodes the TCDD receptor. For this reason, the protein has also been designated the "Ah receptor". Studies using an iodinated photoaffinity ligand (11) to covalently label the Ah receptor protein reveal the existence of two "responsive" alleles for the Ah receptor, designated Ah^{b-1} (typified by the C57BL/6J strain) and Ah^{b-2} (typified by the C3H/HeJ strain). Mating experiments imply that these two alleles are expressed codominantly (17). It is not known whether all nonresponsive mouse strains express the same Ah^d allele, because of the technical difficulties associated with analyzing the low-affinity form of the Ah receptor. Thus, the current evidence indicates the existence of at least three alleles at the *Ah* locus in mice. By analogy, we expect in the future to find multiple alleles at the *Ah* locus in humans.

Recombinant inbred (RI) mouse strains have been used to map the chromosomal location of the *Ah* locus. RI strains are generated by inbreeding the F_2 progeny of two inbred parental strains, a procedure that creates new inbred strains containing unique combinations of genes derived from the parents. Unlinked genes become randomized within a set of RI strains, whereas linked genes tend to remain fixed in the same combinations present in the parental strains. Thus, identity in the strain distribution pattern of two genetic loci within sets of RI strains implies that the loci are linked (18). Using this approach, several groups have mapped the *Ah* locus to the centromeric end of mouse chromosome 12, by virtue of its linkage to restriction fragment length polymorphisms (RFLPs) characteristic of this chromosomal region (17, 19). Similar analyses reveal that the *Ah* locus is also linked to the *Apob* locus, which encodes apolipoprotein B (20). The human homologue of *Apob* is located on human chromosome 2. Because other genes assigned to mouse chromosome 12 also have homologues on human chromosome 2, the locus governing AHH induction that has been assigned to human chromosome 2 (21, 22) is probably the human counterpart of the *Ah* locus. Knowledge of its chromosomal location may facilitate the cloning and analysis of the *Ah* locus. In the future, the characterization of human polymorphisms at the *Ah* locus might contribute to the identification of a human subpopulation(s) that is at increased risk from exposure to TCDD and related compounds.

Other Loci

Genetic analyses of AHH induction in mouse hepatoma cells provide evidence for additional loci that influence the response of the cell to TCDD. Wild-type (Hepa 1c1c7) cells, derived from a responsive (Ah^b) mouse strain, contain a normal Ah receptor; TCDD induces a marked increase in AHH activity in these cells. Hankinson, Whitlock and coworkers have characterized variant cells that exhibit poor AHH-induction responses. One phenotype resembles that of Ah^d mice in that the ligand-binding properties of the Ah receptor are altered, and the cell exhibits a decreased response to TCDD. Presumably, this variant contains a mutation at the *Ah* locus. In a second type of variant, the ligand-binding properties of the Ah receptor appear normal, but the liganded receptor fails to accumulate in the cell nucleus; TCDD fails to induce AHH activity in these cells. Cell-fusion experiments indicate that both phenotypes are recessive and that the variants are in different complementation groups. The latter finding implies that a genetic locus other than *Ah* governs the nuclear accumulation of the liganded receptor (23–29). The protein that this second locus encodes is unknown. It could be a DNA-binding subunit of the receptor or an enzyme that modifies the receptor and thereby causes it to accumulate in the nucleus. This issue constitutes an interesting area for future research. For example, if complementation of the receptor defects can be demonstrated in vitro (i.e. using cell extracts), biochemical approaches can be used to characterize the activity responsible for the nuclear accumulation of the liganded receptor. Alternatively, a genetic approach can be taken, using transfected DNA to complement the defect in the variant cells and the procedures devised by van Gurp & Hankinson to select the desired recombinants (30).

Hankinson and colleagues have characterized a third type of variant that responds poorly to TCDD. The cells exhibit alterations both in the ligand-binding properties of the receptor and in its nuclear accumulation; cell fusions indicate that the variant falls into a third complementation group (31). These findings imply that a third genetic locus influences the function of the Ah receptor and the response of the cell to TCDD. Hankinson has also described a nonresponsive variant with a dominant phenotype, in which the ligand-binding properties of the Ah receptor appear normal. This variant might express a repressor protein that inhibits the function of the *Ah* receptor (32). If so, the variant phenotype reflects the existence of a fourth genetic locus, the product of which can block the receptor-mediated response to TCDD. Taken together, these observations imply that, in mice, multiple genetic loci govern the function of the Ah receptor and, therefore, the response to TCDD. We assume that an analogous situation exists for humans. Thus, we expect that humans will be found to differ on a genetic basis in their responses to TCDD and related compounds.

MOLECULAR ASPECTS OF TCDD ACTION

The Ah Receptor

BIOCHEMICAL PROPERTIES Many biochemical properties of the Ah receptor remain unknown because it has been difficult to purify. The major methodologic difficulties arise from the apparent tendency of the receptor to aggregate with other proteins during purification and from the use of a reversibly bound ligand to monitor the receptor itself, a situation that eliminates the use of procedures that would denature the protein. The latter problem may be overcome with the use of photoaffinity ligands, which can be used to covalently label the receptor (see below).

The location of the Ah receptor within the intact cell is unknown. In cell homogenates, the unliganded receptor fractionates into the cytosol, and hence is often termed "cytosolic" (14, 33–35). However, redistribution of intracellular components, including the Ah receptor, occurs when the cell is broken open; thus, the current data are amenable to alternative interpretations, including the possibility that the unliganded receptor has a nuclear location in the intact cell (29). Following the binding of TCDD, the liganded Ah receptor is found in the "nuclear" fraction; this finding has been interpreted to mean that the receptor undergoes a "translocation" from cytoplasm to nucleus. Again, because of potential redistribution artifacts, the data are open to several interpretations. The liganded receptor has a higher affinity for DNA than does the unliganded receptor (see below). Therefore, the presence of the liganded receptor in the nuclear fraction of cell homogenates may simply reflect a change in the equilibrium distribution of the receptor produced by ligand binding (36). Potential artifacts associated with tissue homogenization have been discussed with respect to steroid hormone receptors (37). The same issues apply to the Ah receptor.

The ligand-binding properties of the receptor have been studied in some detail (3, 4, 38). In general, a ligand's binding affinity for the Ah receptor correlates with its potency in eliciting a biological response. The most potent ligands are planar (or can assume a planar configuration) and contain halogen atoms in at least three of the four lateral positions (e.g. see TCDD and TCDF in Figure 1). The existence of such structure-activity relationships (SARs) provides pharmacological evidence that the Ah receptor participates in a particular biological response. The SAR approach can be used to study the potential role of the Ah receptor in experimental systems where receptor variants are not available. SAR studies have implicated the Ah receptor in a variety of biochemical, immunologic, morphologic, reproductive, and neoplastic responses to TCDD and other aromatic hydrocarbons (3, 4, 38). These findings lead us to assume that the Ah receptor participates in all of the biological effects that TCDD elicits. The receptor may act either directly (as

exemplified by the TCDD-induced increase in *CYP1A1* gene transcription, described below) or indirectly (i.e. by mediating an early step in a multi-event response). Secondary changes, produced in response to TCDD's direct effects, may complicate the analysis of dioxin action in the intact organism. For example, TCDD-induced alterations in steroid-metabolizing enzyme activities may produce secondary alterations in hormone levels (38–40). The resulting compensatory changes in the levels of trophic hormones may alter the proliferative stimuli reaching the corresponding target organs; this type of mechanism might contribute to TCDD's carcinogenic and anticarcinogenic effects in some hormone-dependent tissues (41). Similarly, TCDD produces a decrease in the number of estrogen receptors in rat liver and uterus by an Ah receptor-dependent mechanism (42). Although its biological significance is unknown, this decrease could be accompanied by compensatory alterations in the levels of other hormones and/or receptors, which subsequently lead to secondary changes not directly mediated by the Ah receptor (43). Likewise, TCDD-induced, Ah receptor-dependent alterations in glucocorticoid receptors (44) or epidemal growth factor receptors (45) might produce secondary effects that do not require the direct participation of the Ah receptor. In addition, TCDD may disrupt calcium homeostasis in certain cells, thereby producing secondary toxic effects (46, 47). Thus, many of TCDD's effects in the intact animal may represent secondary responses that are not directly mediated by the Ah receptor. In addition, the physiological status of the organism may influence its response to TCDD. For example, hormone balance in the mice influences susceptibility to the induction of cleft palate by TCDD, a response that segregates with the *Ah* locus (48, 49). Similarly, ovariectomy decreases the susceptibility of rats to TCDD-induced liver tumors (G. Lucier, personal communication). Furthermore, TCDD produces hepatic tumors in female, but not male, rats (41). Together, these findings imply that estrogens contribute to the carcinogenic action of TCDD in rat liver. These phenomena suggest that, in some instances, TCDD acts in combination with other chemical signals to elicit its biological responses. Thus, some of the diversity in TCDD's effects may result from differences among tissues or species in their endocrine and metabolic status. Such differences complicate the analysis of TCDD action in intact animals but, in turn, provide clues about potentially interesting directions for future research at the cellular level.

TCDD induces tumors in laboratory animals (41, 50). The dioxin is not appreciably genotoxic (51), and probably acts at the stage of tumor promotion, by an Ah receptor-dependent mechanism (52, 53). Given that carcinogenesis is a multi-step process (54), expression of the neoplastic phenotype in response to TCDD probably requires biochemical events subsequent to the Ah receptor-dependent step. Thus, the Ah receptor may be necessary, but not

sufficient, for TCDD to elicit a biological response as complex as cancer. Molecular analyses of such phenomena may require the establishment of less complicated experimental systems to dissect the individual steps that contribute to the overall response. Studies of a simpler response, the induction of AHH activity (described below) have generated potentially useful background information for future experiments, designed to analyze other responses to TCDD.

Analyses of quantitative structure-activity relationships (QSAR) provide information about the possible structure of the ligand-binding site on the Ah receptor, as well as the forces that contribute to the ligand-receptor interaction. These findings imply that the ligand-binding site is hydrophobic, and that the hydrophobicity of a potential ligand is a major determinant of its binding affinity. In addition, steric and other factors influence the ligand-receptor interaction (4). QSAR analyses also reveal that the ligand properties optimal for receptor binding differ from those that are optimal for the induction of AHH activity. This observation suggests that ligand-binding, per se, is not sufficient to convert the receptor to a functional form and that an additional step(s) is necessary (4). The nature of this hypothetical step (possibly a conformational change) and its relationship to the phenomenon of receptor "transformation" (see below) remain to be determined. Knowledge of the structure-binding relationships for the Ah receptor may help in predicting the relative potencies of other aromatic hydrocarbons, in terms of "TCDD equivalents." Likewise, structure-binding information may facilitate the design of potential antagonists for the receptor. In principle, antagonists might be useful in (*a*) determining whether a particular response is receptor-mediated, (*b*) analyzing Ah receptor function (55, 56) and (*c*) blocking a receptor-dependent toxic response (57, 58). These could well be interesting areas for future research.

Treatment of rodent liver cytosol with agents that modify sulfhydryl groups alters the TCDD-receptor interaction, suggesting that the conformation of the receptor is important for ligand binding (59, 60). Treatment of cytosol with alkaline phosphatase has no detectable effect on the binding of TCDD to the Ah receptor; under the same conditions, the binding of dexamethasone to the glucocorticoid receptor is substantially diminished (61). These findings point to an interesting difference between the Ah and glucocorticoid receptors and suggest that the Ah receptor's phosphorylation status does not directly affect its ligand-binding properties. On the other hand, depletion of cellular ATP does inhibit the formation of liganded receptor, implying that the ligand-binding event is energy-dependent (62). The availability of purified Ah receptor in the future should facilitate the follow-up of these observations.

According to Scatchard analyses of [^{3}H]-TCDD binding data, the apparent equilibrium dissociation constants for the hepatic Ah receptor are in the

0.1–1.0 nM range in various experimental animals (63) and in humans (64). The human Ah receptor also can exhibit about a 10-fold lower binding affinity for TCDD (65, 66), associated with about a 10-fold decrease in the sensitivity of human cells to TCDD, as measured by AHH induction (66). Thus, at least one human Ah receptor phenotype resembles that of the relatively unresponsive DBA/2 mouse strain. Therefore, some humans may be relatively insensitive to the potentially toxic effects of TCDD and related compounds. Using an [^{125}I]-labeled dioxin of high specific activity, Poland and coworkers estimate that, at infinite dilution, the equilibrium dissociation constant for the C57BL/6 mouse Ah receptor is actually in the 1–10 pM range (67, 68). This observation implies that a TCDD concentration of less than 1 part per trillion (ppt) could produce a biological effect. TCDD is resistant to metabolic breakdown (69) and accumulates to the ppt level (i.e. pg/g) in humans. However, tissue concentrations of TCDD in the ppt range do not consistently produce adverse health effects (70–73). This probably means that the dioxin partitions into lipophilic compartments of the cell (e.g. membranes), where it is not available to interact with the Ah receptor. Therefore, TCDD needs to accumulate to some higher threshold concentration to elicit a biological response.

Several investigators have compared the ligand-binding and hydrodynamic properties of the Ah receptor from various species and tissues, to find differences that might explain the diversity of TCDD's biological effects. The differences that exist appear relatively minor, and do not easily account for the variation in TCDD's effects (63). Most tissues contain the Ah receptor, as measured either directly (by ligand-binding) or indirectly (by AHH induction, a receptor-dependent response). The number of receptors per cell varies but, in general, does not account for most tissue- or species-specific differences in TCDD responsiveness; however, this issue has not been studied extensively.

The sedimentation coefficients and estimated molecular weights of the hepatic Ah receptor from various rodents vary to some extent (63). The hydrodynamic properties of the human receptor are similar to those of rodents (64–66). Variation in the molecular mass of the receptor is also observed when denatured, photoaffinity-labeled proteins are analyzed on polyacrylamide gels (74). In general, the data indicate that the gross structural properties of the Ah receptor vary somewhat among species. It is too early to say whether this structural heterogeneity is associated with substantial differences in receptor function. The hydrodynamic behavior of the Ah receptor resembles that of the glucocorticoid receptor, suggesting that both could belong to the same receptor family (75).

The binding of TCDD to the Ah receptor is associated with the accumulation of the liganded receptor in the nucleus in vivo and with the acquisition of DNA-binding ability in vitro (76–80). These observations imply that ligand

binding converts the receptor to a DNA-binding protein, a process that has been termed "transformation." Experiments involving partial proteolysis of the liganded receptor imply that its DNA-binding and ligand-binding domains are distinct (73, 74). The transformation of rodent liver receptor in vitro involves several steps (81, 82), one of which may be the dissociation from the unoccupied receptor of another macromolecule, possibly the 90-kDa heat-shock protein (83, 84). This step appears to be accompanied by a conformational change, because it is associated with alterations in receptor thermostability, sensitivity to sulfhydryl reagents, overall surface charge, and hydrodynamic properties (14, 59, 60, 78, 82, 85). These alterations increase the affinity of the liganded receptor for DNA in vitro. A subsequent step, possibly involving an association with another protein(s), increases its affinity for DNA still more (82, 86). Although the properties of the liganded receptor that is transformed in vitro resemble those of the liganded receptor that is extracted from the cell nucleus, it is not yet clear that the in vitro process faithfully mimics that which occurs in vivo. However, the in vitro studies provide clues about the receptor's interactions with other macromolecules, thereby suggesting areas for future research.

Purification of the Ah receptor has been hampered by the difficulties inherent in using a reversibly bound, hydrophobic ligand to monitor a protein that is present in low abundance within the cell. To overcome this problem, Poland and coworkers synthesized [^{125}I]-2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin, a ligand useful as a photoaffinity reagent to covalently label the protein (11). More recently, Safe and colleagues have used [^{3}H]-TCDD itself as a photoaffinity ligand (87). The photoaffinity approach permits the use of denaturing conditions and may lead to substantial advances in receptor purification (88).

FUTURE STUDIES Better understanding of the molecular mechanism of dioxin action requires purification of the Ah receptor and analysis of its structure and function. Two relatively recent developments may facilitate receptor purification. First, the photoaffinity approach should generate homogeneous preparations of (denatured) receptor protein. It remains to be seen whether the receptor is amenable to renaturation and whether the yields are adequate for biochemical characterization of the protein. At a minimum, this approach should allow determination of the (partial) amino acid sequence of the receptor. The sequence information should lead to the design of synthetic peptides, against which antireceptor antibodies can be raised, and synthetic oligonucleotides, which can be used to screen DNA libraries for receptor cDNA(s) and/or gene(s).

DNA-recognition site affinity chromatography (89, 90) constitutes a second novel approach to receptor purification. The liganded Ah receptor is a

DNA-binding protein, which recognizes a specific double-stranded nucleotide sequence (see below). A DNA-recognition sequence can be used to purify the cognate DNA-binding protein. In principle, the technique appears applicable to the liganded Ah receptor, and has the advantage that the protein need not be subject to denaturing conditions and that the potential yields are good. Together, the photoaffinity and DNA-affinity approaches complement each other and appear likely to overcome the impasse to date in receptor purification.

Antireceptor antibodies will facilitate the analysis of receptor structure and function. For example, immunohistochemical techniques can be used to determine the location of the unliganded receptor within the intact cell. Such information will impose constraints on the nature of the transformation process. Antibodies will also be useful in analyzing the possible enzymatic modification of the receptor, its possible heterogeneity among tissues, its rates of synthesis and degradation, and possible changes in receptor concentration in response to TCDD and other chemical or hormonal signals. Antireceptor antibodies can also be used to identify cDNA clones that express the receptor protein, a prelude to the process of generating mutants for analyzing receptor function.

An alternative strategy for analyzing the receptor is to identify and express the corresponding cDNA, bypassing the protein purification process. Again, two approaches are possible. The first involves the use of cDNA to complement the defect in receptor-defective cells, employing the selection techniques developed by Hankinson and colleagues (30) and/or the fluorescence-activated cell sorter (27) to identify TCDD-responsive recombinants. Although this approach appears feasible, in practice the complexity of the selection methods, combined with the spontaneous reversion of the receptor-defective cells, renders it technically difficult.

A second potential way to identify receptor cDNA uses the receptor's DNA-recognition sequence to screen an expression library. This screening approach has been used previously to identify cDNAs for other regulatory DNA-binding proteins and, in principle, appears applicable to the Ah receptor (91, 92). However, this technique too will require adaptation to identify a cDNA encoding a ligand-dependent DNA-binding protein and is unlikely to be successful if the receptor requires a post-translational modification or an interaction with another protein(s) for its DNA-binding activity. Nevertheless, the technique merits consideration in view of its relative simplicity and the overall importance of receptor analysis.

Expression of receptor cDNA will permit the mutational analysis of receptor function. We presume that the receptor contains at least three functional domains: first, a ligand-binding domain interacts with TCDD and converts the protein to a DNA-binding species; second, a DNA-binding domain recognizes

a particular nucleotide sequence and positions the liganded receptor at specific regulatory sites along the genome; third, a protein-binding domain(s) interacts with other transcription factors and induces the expression of the target gene. Functional analyses of altered receptor proteins, expressed from mutated receptor cDNAs, will allow us to test these hypotheses in the future.

The Ah receptor is similar in certain respects to receptors for steroid hormones. For example, both receptor types have similar hydrodynamic properties; both are intracellular, ligand-dependent DNA-binding proteins; both activate gene transcription by an enhancer-dependent mechanism (see below). These similarities suggest that the Ah receptor might be a member of the steroid/thyroid/retinoic acid family of receptors (74, 93–95). On the other hand, there are differences between them. For example, complementation analyses imply that several genes contribute to Ah receptor function, whereas analogous glucocorticoid receptor variants fall into a single complementation group (93). The DNA recognition motifs for several steroid receptors are palindromic, and the corresponding proteins bind as dimers (95); in contrast, the recognition motif for the Ah receptor is not symmetric, and the liganded protein appears to bind to DNA as a monomer (see below). Although the biological significance of these differences is not yet clear, it would be premature to assume that the Ah and steroid receptors are members of the same family. Several classes of transcription factors exist as families of proteins (96–99). Thus, if the Ah receptor is not a member of the steroid/thyroid/retinoic acid receptor family, it may represent the prototype for a new class of regulatory proteins. Differences among members of this hypothetical family might account, in part, for the variety of responses evoked by TCDD and other aromatic hydrocarbons. Purification and characterization of the receptor will allow us to address these issues in the future.

Why we have an Ah receptor is unknown. The receptor evolved long before HAHs were introduced into the environment (100, 101); therefore, TCDD presumably mimics a naturally occurring ligand(s) for the receptor. TCDD's teratogenic and neoplastic effects indicate that it can disrupt fundamental mechanisms of cell proliferation and differentiation, perhaps by inappropriately mimicking an endogenous chemical signal. However, receptor-defective cells and animals grow and reproduce without apparent difficulty; therefore, an endogenous ligand for the Ah receptor, if it exists, may not govern a vital function. In addition, variation among species in the molecular mass of the Ah receptor suggests the absence of strong evolutionary pressure to conserve receptor structure (75). This finding argues against the existence of an endogenous ligand. Plants contain substances that are high-affinity ligands for the receptor (102–104). Thus, the receptor may have evolved as part of a mechanism that enables the cell to metabolize certain aromatic compounds present in the diet.

The Dioxin-responsive Enhancer

Screening studies for TCDD's potential effects on microsomal enzyme activities indicate that the dioxin is a potent inducer of AHH activity (105, 106). We now know that the cytochrome P450IA1 enzyme catalyzes AHH activity (10); the corresponding gene is designated *CYP1A1* (107). AHH activity is present and inducible in many tissues, including cells in culture; furthermore, it is measured using a simple, sensitive fluorescence technique. Thus, from a technical standpoint, the induction of AHH activity is a convenient TCDD-dependent response to study. In addition, recent progress in the analysis of cytochrome P450 genes has been substantial (108). These factors have facilitated the application of molecular genetic techniques to analyzing the mechanism by which TCDD induces AHH activity. For this discussion, we assume that the basic regulatory components identified for AHH induction also govern other responses to TCDD. Thus, we envision that the Ah receptor and the dioxin-responsive enhancer (see below) contribute to the regulation of numerous TCDD-responsive genes; however, this hypothesis remains to be tested.

TCDD induces AHH activity by increasing the rate of transcription of the *CYP1A1* gene (109, 110). The transcriptional response occurs within minutes of exposure to TCDD and does not require ongoing protein synthesis; therefore, it represents a direct effect of the dioxin (110, 111). Studies of Ah receptor-defective mouse hepatoma cells reveal that the activation of *CYP1A1* transcription requires the binding of TCDD to the receptor, followed by the accumulation of the liganded receptor in the cell nucleus (22, 110, 112).

The observations that (*a*) the response to TCDD is transcriptional, (*b*) the response requires the nuclear accumulation of liganded receptor, and (*c*) the liganded receptor is a DNA-binding protein led to the search for a DNA "switch" that mediates the induction response. The regulation of genes (such as *CYP1A1*) that are transcribed by RNA polymerase II involves the binding of specific proteins to DNA control elements in the vicinity of the target gene (113–115). The resulting protein-DNA interactions either activate or repress transcription, depending upon the system. Some DNA control elements (known as "promoters") are located relatively close to the target gene (i.e. within 100–200 base pairs) and ensure that transcription is initiated at the correct site. The proteins that bind to promoters are often constitutively expressed and are present in many different cell types. In contrast, other DNA elements (known as "enhancers") can be located large distances from the target gene (i.e. hundreds to thousands of base pairs), function relatively independently of their 5'–3' orientation with respect to the gene, and act to increase the rate of transcript initiation from the promoter. The proteins that bind to enhancers are often cell-specific, may be developmentally regulated

and, in some cases (such as the Ah receptor), are activated by a specific chemical signal.

By analogy with other inducible systems, a TCDD-responsive DNA control element, if it existed, would likely be found upstream of the *CYP1A1* gene. To examine this possibility, several groups constructed hybrid genes, in which potential TCDD-responsive DNA domains were ligated to a heterologous "reporter" gene, whose product was convenient to assay. Transfection of the hybrid gene into cells that contained the Ah receptor, followed by measurement of the reporter gene product, determined whether the gene could respond to TCDD. Transfections into receptor-defective cells revealed whether the TCDD-inducible response was also dependent upon the Ah receptor. This gene-transfer approach indicated the existence of a DNA domain, upstream of the *CYP1A1* gene, that responds to TCDD in Ah receptor-dependent fashion (116–118). The TCDD-responsive domain has the properties of an enhancer, in that it functions at a distance from and independent of its orientation to the target gene, can utilize a heterologous promoter, and activates the rate of gene transcription (119–123). Analogous studies reveal that the dioxin-responsive region is composed of multiple subdomains, each of which functions independently in TCDD-inducible, Ah receptor-dependent fashion and contributes to overall enhancer activity (121, 124, 125).

The dioxin-responsive enhancer contains four binding sites for the liganded Ah receptor (see below), as well as potential binding sites for other transcription factors. Thus, like other enhancers, the dioxin-responsive system has a modular organization, suggesting that it may have evolved as a combination of smaller subunits (113–115). The organization of the enhancer associated with the *CYP1A1* gene is similar in rodents and humans, implying that the arrangement of protein-binding sites along the DNA has some advantage for the cell (126, 127). For example, the existence within the enhancer of multiple receptor binding sites may increase the probability that exposure to aromatic hydrocarbons will activate *CYP1A1* gene expression. This would seem advantageous, because the cytochrome P450IA1 enzyme catalyzes the initial step in a pathway that primarily results in detoxification (9). For example, prior exposure to TCDD can protect experimental animals against the tumorigenic effects of certain polycyclic aromatic hydrocarbons, which are metabolized by the cytochrome P450IA1 enzyme (128, 129). Thus, exposure to TCDD can have a protective effect under certain circumstances. It will be interesting to determine whether other dioxin-responsive enhancers are organized like the *CYP1A1* enhancer and, if not, whether differences in enhancer organization are associated with differences in the responsiveness of the target gene to TCDD. In addition, the binding of other proteins to the enhancer could either augment or diminish the response generated by the

binding of the liganded receptor to the DNA. Thus, differences in enhancer organization among various dioxin-responsive genes could contribute to the diversity of TCDD's biological effects. Therefore, analyses of additional dioxin-responsive genes may be a productive area for future research.

Receptor-enhancer Interactions

The conditional (i.e. Ah receptor-dependent) nature of enhancer function, together with the observation that the liganded receptor is a DNA-binding protein, suggested that the liganded receptor binds to the enhancer during the activation of *CYP1A1* gene transcription by TCDD. Two kinds of evidence support this idea. First, in intact nuclei, a region of the enhancer becomes resistant to exonuclease digestion in TCDD-inducible, Ah receptor-dependent fashion. The effect is rapid, and does not require new protein synthesis. These findings imply that a protein(s) interacts with the dioxin-responsive enhancer in vivo during the activation of the *CYP1A1* gene by TCDD (130). Second, gel retardation analyses, using enhancer DNA and extracts from uninduced or TCDD-induced cells, reveal the existence of a TCDD-inducible, Ah receptor-dependent in vitro protein-DNA interaction, with characteristics expected for the binding of the liganded Ah receptor to DNA (125, 131–133). Furthermore, the use of a high-affinity, iodinated ligand for the Ah receptor indicates that the liganded receptor participates in the formation of a nucleoprotein complex with enhancer DNA (134). These findings imply that the liganded Ah receptor binds to enhancer DNA in vitro. Each individual dioxin-responsive subdomain of the enhancer participates in a TCDD-inducible protein-DNA interaction and contains a copy of the sequence

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5' T-GCGTG 3'
3' A-CGCAC 5',
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which represents the "core" DNA recognition motif for the liganded Ah receptor (134, 135). The recognition motif lacks obvious symmetry, in contrast to the DNA-binding sites for several steroid receptors (95, 136). Gel retardation studies using both [^{32}P]-labeled DNA and [^{125}I-dioxin]-labeled protein imply that the receptor binds as a monomer to its recognition motif (135). This finding differs from those for steroid receptors, which appear to bind as dimers to their cognate DNA recognition sequences (95, 136). In addition, the liganded Ah receptor preferentially binds to double-stranded DNA (135). This observation puts constraints on the possible DNA structure that the liganded receptor is likely to recognize in vivo. In addition, it illustrates a possible difference between the liganded Ah receptor and the liganded estrogen receptor, which appears to preferentially bind single-stranded DNA (136). Methylation protection and methylation interference

analyses imply that the liganded receptor binds within the major groove of the DNA helix and contacts the four guanine residues of the core recognition motif (133, 138). In this respect, the liganded Ah receptor resembles other regulatory proteins in that it binds within the major DNA groove (96, 97, 139). The configuration of the receptor's DNA-binding domain is unknown. Preliminary experiments, involving the use of chelating agents to analyze the role of divalent cations in the receptor-enhancer interaction, have yielded contradictory results (J. Fagan, personal communication; M. Denison, personal communication). Thus, it is not yet clear whether the liganded Ah receptor uses a "zinc finger" or some other motif in its interaction with DNA (140, 141). More detailed analyses of the receptor-DNA interaction and the configuration of the resulting protein-DNA complex await purification of the Ah receptor.

Methylation of DNA can influence gene expression; in particular, cytosine methylation at CpG dinucleotides is associated in several systems with the tissue-specific inhibition of gene transcription. The effect of methylation may reflect either the stabilization of chromatin in an "inactive" configuration or inhibition of the binding of a transcription factor to its cognate DNA recognition site (142, 143). Methylation of the core recognition motif for the liganded Ah receptor at its two CpG dinucleotides diminishes the receptor-enhancer interaction, as measured by gel retardation. This is accompanied by a corresponding decrease in enhancer function (138). These preliminary observations reveal a potential role for DNA methylation in generating tissue-specific differences in the biological responses to TCDD. Furthermore, studies using 5-azacytidine in rat hepatoma cells suggest that DNA methylation might also influence expression of the Ah receptor gene (142). Therefore, more detailed analyses of DNA methylation and TCDD action may be an interesting area for future research.

Gel retardation analyses reveal that proteins other than the Ah receptor interact in vitro with the dioxin-responsive enhancer in TCDD-independent fashion (125, 133–135). These findings suggest that other proteins may contribute to enhancer function in vivo. These other proteins may modulate the Ah receptor-enhancer interaction, thereby contributing to tissue-specific differences in responsiveness to TCDD. This too may be an interesting area for future research.

The mechanism by which the receptor-enhancer interaction activates the transcription of the *CYP1A1* gene is unknown. Models for enhancer function propose that enhancer-binding proteins increase the formation of initiation complexes at the transcriptional promoter by (*a*) facilitating additional protein-protein interactions, with the "looping-out" of intervening DNA and/or (*b*) altering chromatin structure, such that the transcriptional promoter is more accessible to its cognate protein factors (113–115, 145–147). The binding of

the liganded Ah receptor to its recognition motif bends the DNA in vitro (unpublished observations). Thus, the receptor-enhancer interaction could distort the configuration of the DNA in vivo. In addition, in intact nuclei, the chromatin upstream of the *CYP1A1* gene undergoes a TCDD-inducible, Ah receptor-dependent alteration that increases the accessibility of the DNA to nucleases (148). These findings imply that the receptor-enhancer interaction produces a change in chromatin structure that is propagated to the promoter. This increased accessibility of promoter DNA likely enhances the formation of initiation complexes and, hence, transcription of the *CYP1A1* gene (Figure 2).

For this discussion, we assume that the receptor-enhancer system regulates the response of numerous genes to TCDD, a reasonable assumption given that the receptor-enhancer system can function in a chromosomal context distinct from that of the *CYP1A1* gene (122). Heterogeneity among various tissues in the properties of the Ah receptor, combined with heterogeneity among various target genes in the organization of the dioxin-responsive enhancer, could possibly account for the variety of responses that TCDD elicits. However, we envision that the receptor-enhancer system functions in concert with other regulatory systems and that the particular combination of controls determines the pattern of expression of a given target gene (93). For example, the dioxin-responsive system of the *CYP1A1* gene may function in concert with other control elements (133, 149, 150). In addition, TCDD's receptor-dependent action as a skin tumor-promoter in mice involves an interaction between the *Ah* and *hr* loci; mice that are susceptible to the tumor-promoting effect are homozygous for a recessive mutation at the *hr* locus (52). This situation is reminiscent of that for tumor-suppressor genes (151) and suggests that the product of the *hr* locus prevents the expression of TCDD's tumor-promoting action. Thus, the *hr* locus (152) merits additional study, because it may encode a protein(s) that controls susceptibility to TCDD-induced neoplasia. Likewise, TCDD's immunologic and teratogenic effects are restricted to

- Diffusion into cell
- Formation of liganded Ah receptor (LR)
- Transformation of LR
- Binding of LR to DNA recognition motif
- Altered DNA configuration
- Altered chromatin structure
- Enhanced accessibility of promoter DNA
- Increased binding of transcription factors
- Enhanced transcription of target gene
- Biological response
- Compensatory response(s)

Figure 2 Working model for TCDD action.

certain cell types (153, 154). This tissue-specificity implies the existence of additional controls, either positive or negative, that modulate the response of the receptor-enhancer system to TCDD. Further characterization of regulatory systems that can inhibit or augment the TCDD-responsive system could identify additional factors that influence the susceptibility of the cell to TCDD's biological effects. In addition, because of TCDD's ability to elicit neoplastic and teratogenic effects, analyses of combinatorial gene regulation could reveal novel aspects of cell proliferation and differentiation.

FUTURE DIRECTIONS

Lack of information about the structure and function of the Ah receptor constitutes a major obstacle to understanding the mechanism of TCDD action. As outlined above, photoaffinity labeling and DNA-recognition site affinity chromatography offer new approaches to receptor purification, and we anticipate substantial progress in this area in the next few years.

The TCDD-responsive system uses specific protein-DNA interactions to regulate *CYP1A1* gene expression; in this respect, it resembles other transcriptional control systems (113–115, 145, 146). Thus, more detailed analyses of the receptor-enhancer interaction are likely to generate information relevant to the general issue of transcriptional regulation.

The potential teratogenic and neoplastic effects of environmental chemicals are of particular public health concern; the study of dioxin action could provide insight into the mechanisms by which such effects occur. A major challenge will be to establish experimental systems in which such complex phenomena are amenable to molecular analyses. Success in such endeavors could lead to a better understanding of such fundamental phenomena as cell proliferation and differentiation and intercellular communication.

Analysis of the *CYP1A1* system provides clues about the possible mechanisms by which TCDD may activate the expression of other genes. Studies using two-dimensional electrophoresis suggest that numerous genes can respond to TCDD and other aromatic hydrocarbons (155). The study of other TCDD-inducible genes such as quinone reductase (156–158), glutathione S-transferase (159), and aldehyde dehydrogenase (160) should reveal additional aspects of dioxin action, e.g. whether differences in enhancer organization affect the responsiveness of the target gene to TCDD. In addition, analyses of genes that respond to TCDD only in a particular tissue may reveal new aspects of tissue-specific gene expression and the mechanism by which the TCDD-responsive receptor-enhancer system functions in combination with other control components. Such information should be of relatively broad interest.

In some cases, TCDD might inhibit gene expression. For example, expo-

sure of male rats to aromatic hydrocarbons decreases the hepatic levels of mRNA encoding a constitutive form of cytochrome P450 (161). Presumably, this reflects the inhibition of transcription of the corresponding gene. The mechanism governing this response is unknown. Perhaps the liganded Ah receptor can also function as a repressor of transcription. This possibility appears worthy of additional study. TCDD may also act at the post-transcriptional level in primary hepatocyte cultures (162, 163); such an effect may also occur in vivo (162, 164). Perhaps TCDD induces the production of a protein that protects certain mRNAs from degradation. Conversely, the dioxin might inhibit the expression of a particular nuclease. In either case, analysis of the mechanism should reveal interesting aspects of TCDD action.

Analyses of receptor-enhancer interactions in vitro provide important clues about the mechanism of TCDD action. A challenge for future research will be to establish a TCDD-responsive in vitro transcription system with which to analyze the functional importance of these protein-DNA interactions. It will be equally important to analyze such protein-DNA interactions in vivo, and to examine the contributions that chromosomal proteins and chromatin structure make to the TCDD-responsiveness of a particular gene (165). Again, the results of such studies would be of relatively broad interest.

From a public health standpoint, TCDD is interesting because it is prevalent, potent, and persistent. Its prevalence implies that many humans are exposed to TCDD; its potency implies that low concentrations of TCDD may produce biological effects; and its persistence implies that TCDD accumulates and that our body burdens increase with age. These characteristics suggest that further contamination of the environment with TCDD should be avoided. On the other hand, epidemiological studies have not yet determined that exposure to TCDD is associated with adverse health effects, with the exception of chloracne (166–172). Still, certain human subpopulations may be at increased risk on a genetic basis (173). However, the problem is complicated because other environmental factors may also influence the response to dioxin. For example, TCDD's action as a tumor promoter presumably requires prior exposure of the cell to an initiator. In addition, exposure to other chemicals can augment or antagonize the biological response to TCDD (174–176). Thus, assessing the risk that TCDD poses to human health is a formidable task, given our daily exposure to complex mixtures of chemicals. Epidemiological studies may be unable to detect subpopulations at increased risk, if they exist. Analyses of dioxin action at the molecular level will, in our view, reveal the factor(s) that governs the sensitivity of a given tissue or organism to the biological effects of TCDD. Ultimately, this knowledge may help in assessing the health risk that TCDD poses to humans. Equally important, the molecular and genetic analyses of dioxin action appear likely to reveal important mechanisms governing mammalian gene expression.

ACKNOWLEDGMENTS

I thank numerous colleagues for communicating interesting observations prior to their publication. I am grateful to Drs. M. S. Denison and T. A. Gasiewicz, who made thoughtful comments on earlier versions of this review. I thank Shirley Kruk for typing the manuscript. The research in my laboratory was supported by the National Institutes of Health and the American Cancer Society.

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