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THE ARYL HYDROCARBON RECEPTOR COMPLEX

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ABSTRACT

The heteromeric unliganded aryl hydrocarbon receptor complex (AHRC) contains the aryl hydrocarbon receptor monomer (AHR). Binding of polycyclic or halogenated aromatic hydrocarbon (PAH and HAH) ligand causes release of AHR, which then associates with the AHR nuclear translocator protein (ARNT) to generate the heterodimeric "transformed" AHRC. AHR and ARNT belong to a novel subclass of basic helix-loop-helix–containing transcription factors. The transformed AHRC binds xenobiotic responsive elements in responsive genes and turns on their transcription. Certain of these genes encode enzymes involved in the metabolic activation of PAHs to mutagenic derivatives. HAHs are not genotoxic: Their pathogenicity depends on the AHRC but not on their metabolism. Current research includes investigations directed towards delineating the pathways of HAH pathogenesis, ascertaining whether AHR can mediate signal transduction independently of DNA binding, understanding the mechanism of transcriptional activation, and investigating the potential roles of AHR and ARNT in development.

Introduction

The aryl hydrocarbon receptor complex (AHRC)[1] mediates most if not all of the toxicological effects of certain halogenated aromatic hydrocarbons (HAHs)

[1]AHR refers to the ligand-binding subunit (the product of the *AHR* gene), which has also been called the Ah, the dioxin, or the TCDD receptor. AHRC refers to any multimeric protein complex containing AHR.

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that are widely disseminated in the environment. Ligands for the AH receptor also include polycyclic aromatic hydrocarbons (PAHs), which are found in cigarette smoke and smog, and aromatic amines; the receptor is directly involved in carcinogenesis by these compounds. The receptor is therefore of considerable interest to toxicologists. As assayed by ligand binding, the AHR protein apparently evolved about 450 million years ago, early in vertebrate evolution and prior to the divergence of bony and cartilagenous fishes (1).

The principal mechanism whereby PAHs cause cancer and the involvement of the AHRC in this process are only understood in broad terms. PAHs are metabolized by several cytochrome P450s to electrophilic derivatives that can mutate DNA, thereby activating protooncogenes or inactivating tumor suppressor genes. The principal cytochrome P450 involved is CYP1A1 [CYP1B1 may also contribute (2)]. CYP1A1 is induced many-fold by PAHs via the AHRC. Ligands for AHR also induce several phase II enzymes, such as certain forms of UDP-glucuronosyltransferase and glutathione *S*-transferase, which can conjugate the electrophilic derivatives with other small molecules, thereby inactivating them. The sensitivity of a particular tissue to PAH carcin- ogenesis depends to a considerable extent on the balance between activation by the cytochrome P450s and inactivation by the phase II enzymes; the AH receptor is involved in both processes.

In contrast, the mechanism(s) whereby HAHs cause cancer and produce their other pathological effects are obscure. Unlike the PAHs, the prototypic and most potent HAH, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is not genotoxic, but it is a very powerful tumor promoter. The extreme potency of TCDD is partially attributable to its resistance to metabolism and its long half-life in the organism. Although no endogenous ligand for AHR has been identified, one working model for tumor promotion by TCDD envisages that TCDD mimics an endogenous ligand, but because of its persistence, it causes transformation[2] of the AHRC in too sustained a fashion or in an inappropriate place or time. The relevant target(s) for the transformed, nuclear AHRC may be genes or gene products (not yet necessarily identified) that are involved in the regulation of cell growth (3). The many other toxic effects of TCDD and other HAHs include lethality, teratogenesis, fetal toxicity, suppression of the immune system, and modulation of various hormonal effects.

The cloning of cDNAs for the two subunits of the transformed AHRC—the AHR nuclear translocator protein (ARNT) and the ligand-binding AHR protein itself—in 1991 and 1992, respectively, has opened up the field of research on

[2]The term transformation is applied to the ligand-dependent process whereby AHR translocates to the nucleus and acquires high binding affinity for DNA in vivo. The term also applies to in vitro treatment with ligand, leading to the acquisition of high binding affinity of the AHRC for DNA (particularly for the xenobiotic responsive element, discussed below).

the AHRC. Contrary to what many investigators had expected, the subunits of the transformed complex are not members of the steroid-thyroid-retinoic acid receptor superfamily of proteins, and they are totally different in structure. This review includes insights into the AHRC that have emanated from these findings.

I attempt to cover the broad field of research on the AHRC, from the molecular analysis of the different forms of the AHRC to the processes whereby the AHRC mediates toxicity of its ligands in the whole organism. I emphasize the mechanisms involved and accentuate more recent findings. The discovery and earlier work on the AHRC has been discussed in another review (4). Because of limitations in space I sometimes refer to reviews or the most recent relevant papers, rather than citing the appropriate original articles. I apologize to those individuals whose work, because of this, may seem to have been given short shrift. Recent reviews covering various aspects of AHRC research include Refs. 4–11.

Cloning and Structure of ARNT and AHR

Certain mutants of the mouse hepatoma cell line Hepa-1 (mutants in complementation group C) have a functionally defective AHRC and are phenotypically defective in ligand-dependent translocation of the AHR from cytoplasm to nucleus, as assessed by the conventional subcellular fractionation procedure. A portion of a human gene capable of restoring AHRC function to the mutant cells was cloned. The corresponding human cDNA encodes a protein (the AHR nuclear translocator protein, or ARNT) of 87 kDa, which is not capable of binding ligand (12). Evidence was subsequently obtained that the transformed, DNA-binding AHRC is a dimer of ARNT and AHR (although the presence of additional proteins has not been rigorously excluded) (13).

For many years AHR resisted purification, owing, among other reasons, to its low abundance and its lability. A key to the successful purification of the receptor was the development of a photoaffinity ligand that could be used to monitor the receptor under denaturing conditions (14). The amino acid sequence of a short segment of purified mouse AHR (15) was utilized by two research groups to clone the corresponding cDNA (16, 17).

Mouse ARNT (18) and human AHR (19, 20) have also been cloned. ARNT and AHR of each species are approximately 20% identical in amino acid sequence and show a striking resemblance in overall structure (Figure 1). Both proteins contain basic helix-loop-helix (bHLH) motifs near their amino termini. Such motifs are found in other transcription factors that bind specific DNA sequences as homodimers and heterodimers, where they function in both protein dimerization and DNA binding (reviewed in 21). Near their carboxy termini, the proteins contain a segment of homology that is also found in two regulatory proteins of *Drosophila melanogaster,* PER and SIM, and that has

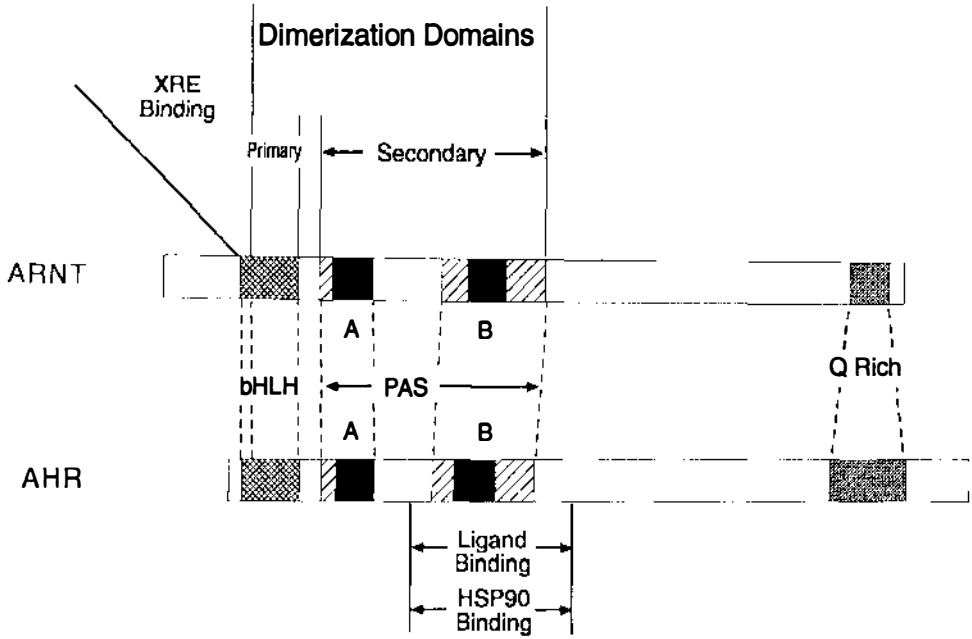

Figure 1 Functional domains of mouse AHR and ARNT. The solid boxes represent the PAS A and PAS B degenerate direct repeats. The hatched areas flanking the repeats include the remainder of the PAS A and PAS B segments.

been termed the PAS domain. The PAS domain contains two copies of an approximately 50–amino acid degenerate direct repeat, referred to as the PAS A and PAS B repeats (22) (SIM but not PER, also possesses a bHLH motif). Although the molecular mechanisms of action of PER and SIM remain largely obscure, Huang et al have shown that the PAS domains of PER and SIM mediate heterodimerization between these two proteins and that the PAS domain of PER also mediates homodimerization (23). AHR, ARNT, and SIM all contain glutamine-rich regions near their carboxy termini. The AHR-ARNT complex therefore represents a new class of transcription factor that differs from other bHLH-containing transcription factors in a number of important ways: (*a*) AHR is unique among bHLH proteins in that its activity is dependent on the binding of a ligand; (*b*) the specific DNA-binding sequence for the AHR-ARNT heterodimer [the xenobiotic-responsive element (XRE)] differs from the E-box sequence, which is the core sequence recognized by nearly all other bHLH proteins; and (*c*) besides SIM, no other known bHLH protein contains a PAS domain.

No other genes closely related to ARNT appear to exist in the human (12). Equivalent information concerning AHR is not available. *ARNT* maps to a

conserved linkage group located on human chromosome 1q21 and mouse chromosome 3, about 40 cM from the centromere (24). *AHR* is located on human chromosome 7 within bands p21 and p15 (25) and in the centromeric region of mouse chromosome 12 (25–27).

Genetics of the AHRC

Certain inbred mouse strains are noninducible for CYP1A1-dependent aryl hydrocarbon hydroxylase (AHH) activity by PAHs in liver. In crosses between one such "nonresponsive" strain, DBA-2, and a prototypic responsive strain, C57BL-6, inducibility was shown to segregate as a dominant or codominant trait. Whereas the DBA-2 mouse is nonresponsive to PAHs, it is inducible by TCDD (28). However, approximately tenfold higher concentrations of TCDD are required to achieve induction in DBA-2 mice equivalent to that in C57BL-6 mice. This is explained by the observation that the receptor in DBA-2 mice has an apparent affinity (i.e. K_d) for [^{3}H]TCDD that is about tenfold lower than that in C57BL-6 mice (29). The lack of induction by PAHs in the livers of nonresponsive mice is probably due to the rapid metabolism and elimination of PAHs (by constitutive forms of cytochrome P450), such that a sufficiently high concentration of these compounds for binding to the low-affinity receptor is not achieved. Recently, the Ah^d allele from DBA-2 mice has been sequenced. Compared with the Ah^{b-1} allele in C57BL-6 mice (which encodes an 89-kDa protein of 805 amino acids), the Ah^d allele encodes a protein with an additional 43 amino acids at the carboxy terminus and also differs from the Ah^{b-1} allele at four other amino acid positions within the protein (30). One (conservative) difference occurs in the PAS B repeat, within the ligand-binding domain (see below). However the amino acid difference(s) responsible for reduced ligand-binding affinity of the Ah^d allele has not been determined. By using crosses between different inbred mouse strains, Robinson et al obtained evidence for a second locus affecting AHH inducibility and for a dominant gene conferring noninducibility (31). However, these observations have not been followed up in the literature, and their molecular bases are unknown.

A genetic polymorphism has been identified in the coding region of the *CYP1A1* gene in humans that appears to be associated with differences in susceptibility to cigarette-induced lung cancer among the Japanese (32) but not necessarily among Northern Europeans (33). AHH activity is inducible by PAHs and TCDD in monocytes, alveolar macrophages, and mitogen-stimulated lymphocytes. A number of studies suggest that either maximally induced AHH activity or the degree of AHH induction in these cells differs between individuals, that the differences observed are partly genetic in origin, and that high activity or inducibility may be a risk factor for cigarette-induced lung cancer (reviewed in 6 and 34). However, the relatively low induction ratios and the relatively poor reproducibility of the AHH-activity measurements

obtained with these cells have limited their usefulness, and the gene(s) responsible for the variation in inducibility have not been identified (35, 36).

The LD_{50} for TCDD differs by greater than 300-fold between two particular strains of rat. Resistance to the high levels of TCDD appears to be conferred by dominant alleles at two or three loci. The difference in susceptibility is not ascribable to differences in AHR levels, AHRC-dependent transcriptional activation, TCDD metabolism, or TCDD disposition between the two strains (37, 38). Therefore it appears likely that the genetic loci responsible for resistance to lethality encode proteins involved in the pathway of TCDD lethality downstream from the steps involving the AHRC.

CYP1A1-dependent AHH activity is highly inducible in the cultured mouse hepatoma cell line Hepa-1, and these cells are very sensitive to the toxicity of the PAH benzo(a)pyrene (BP). I isolated rare BP-resistant (BP^r) clones of Hepa-1 after a single exposure to the carcinogen; they were defective in induction of CYP1A1 (39). Evidence was obtained that the clones were mutational in origin. Analysis of somatic cell hybrids formed between individual mutants and the wild-type Hepa-1 demonstrated that a few of the mutants are dominant, while the majority are recessive. Somatic cell fusion analysis allowed the latter to be assigned to four complementation groups, probably representing four different genes. The characteristics of the different mutant classes are presented in Table 1 (reviewed in 40).

Complementation group A corresponds to the *CYP1A1* gene. Some mutants defective in this gene, and thereby lacking AHH activity, express high levels of mRNA for CYP1A1 even when they are grown without TCDD (41). These mutants also constitutively express high levels of other proteins subject to AHRC regulation (42, 43). One interpretation of these results is that CYP1A1 activity converts a hypothetical prorepressor protein to a molecule that represses CYP1A1 transcription, thereby autoregulating its own synthesis and the synthesis of other proteins subject to AHRC regulation (reviewed in 6). An alternative and simpler explanation is that the CYP1A1-deficient mutants accumulate an agonist for AHR that is also a substrate for CYP1A1. The agonist could be an intermediary metabolite of the cells (i.e. an endogenous ligand) or a component of the medium (41).

In mutants of group B the level of ligand binding to AHR and the amounts of AHR protein and AHR mRNA are much reduced, although the mutants are not defective in translocation of liganded AHR to the nucleus (44; LK Durrin, J Zhang, MR Probst & O Hankinson, unpublished data). Whether these mutants are mutated in the *AHR* gene is unknown. Group C mutants are deficient in the ligand-dependent translocation of AHR to the nucleus, as assessed by conventional subcellular fractionation procedures, and in binding of the AHRC to DNA. However, AHR probably does translocate to the nucleus in these mutants but is apparently bound within the nucleus with reduced avidity (45;

Table 1 Properties of the AHH-deficient mutants of Hepa-1[a]

Type of strain	AHH-specific activity in TCDD-treated cells	CYP1A1 mRNA levels in Uninduced cells	CYP1A1 mRNA levels in TCDD-treated cells	Levels of [^{3}H]TCDD binding after treatment of cells *in vivo*, and subcellular fractionation: Cytosol	Levels of [^{3}H]TCDD binding after treatment of cells *in vivo*, and subcellular fractionation: Nucleus	Basis
Hepa-1	100	2	100	100	100	
A$^-$[b]	0	50	50	100	100	*CYP1A1* gene
B$^-$ (Class I)	2	<2	2	2	2	Reduced levels of AHR
C$^-$ (Class II)	0	<2	<2	120	0	Deficient in ARNT function
D$^-$	3	ND[c]	3	20	3	AHRC cannot be transformed
Dominant	■	<2	2	100	100	Repressor

[a] All values are given as a percentage of those in Hepa-1 treated with TCDD.
[b] Data shown for the constitutive A$^-$ mutant c1.
[c] ND, not determined.

see below). Although the C⁻ mutants are rescued by *ARNT* cDNA, it has not been ascertained whether the lesions in these mutants are actually located in the *ARNT* gene. The D⁻ mutant possesses reduced levels of AHRC-dependent ligand binding, and the receptor also appears to be defective in nuclear translocation. The AHRC in this mutant cannot be transformed either in vivo or in vitro to the XRE-binding state (46; J Zhang & O Hankinson, unpublished data). The primary genetic defect in the D⁻ mutant has not been identified. One dominant mutant, c31, has been studied in detail. This mutant possesses normal levels of AHR, which can translocate to the nucleus after binding ligand. The transformed AHRC in the mutant possesses full XRE-binding activity in vitro. However, the c31 strain expresses a repressor protein that somehow prevents binding of the transformed AHRC to the XRE in vivo. The repressor is probably not a mutated form of ARNT or AHR (47). A possible explanation for c31 is that it arose from an inappropriate activation of a gene (silenced in the liver-derived Hepa-1 cells) that normally regulated tissue and/or developmental expression of AHRC activity.

Whitlock and coworkers also selected BP-resistant derivatives of the Hepa-1 cell line and identified two complementation groups among their clones (48). The class I mutants they found (49) and the B⁻ mutants are in the same complementation group (46). Although no complementation tests have been carried out between the C⁻ mutants and Whitlock's class II mutants, their nearly identical properties indicate that they are mutated in the same gene.

Ligand Binding

Ligands for AHR are hydrophobic aromatic compounds that are planar or can become coplanar. High-affinity ligands include HAHs (dibenzo-*p*-dioxins, dibenzofurans, and biphenyls), PAHs, aromatic amines (50), rutaecarpine alkaloids (51), and indolocarbazoles and related compounds (52, 53). None of the characterized ligands are normal intermediary metabolites, and therefore no endogenous ligand for the receptor has been identified. Kafafi and coworkers have identified certain electronic and thermodynamic characteristics that appear to be required of high-activity HAH ligands (54). HAH ligands can be accommodated within a rectangular binding site of approximately 3×10 Å (3). However, in order to accommodate other high-affinity ligands, including the PAHs, the site must be increased to 7×14 Å (55). It has been postulated that two different binding sites (of the above dimensions) exist on AHR (56). However, if there are two sites, they are likely to overlap, since the above HAH and PAH ligands compete with each other for binding (57).

The (TCDD) ligand-binding domain of AHR has been shown to be within a 192–amino acid region encompassing the PAS B repeat (58, 59). Sulfhydryl groups are required for binding ligand (60). The equilibrium dissociation constants (K_d) of the receptor for the higher-affinity HAHs and PAHs were

initially estimated to be in the nanomolar (10^{-9} M) range. However, more recent determinations indicate that the K_d for TCDD and the related 2-iodo-7,8-dibromodibenzo-*p*-dioxin is in the picomolar (10^{-12} M) range (61, 62; see discussion in 4). Presumably, the K_d of the receptor for PAHs is also correspondingly lower than originally estimated. The EC_{50} (effective concentration giving a fifty percent response) of high-affinity PAHs, such as 3-methylcholanthrene and benzo(a)pyrene, for induction of cytochrome CYP1A1 enzymatic activity is 1,000 to 10,000 greater than the EC_{50} for TCDD. This is attributable in large part to the rapid metabolism of PAHs but not TCDD by the induced CYP1A1 (63, 64).

Structure of the Unliganded AHRC and Transformation by Ligand

After conventional subcellular fractionation, unoccupied AHR is found in the cytosol of Hepa-1 cells (Figure 2). Analysis of fixed cells by immunofluorescence microscopy using antibodies to AHR also indicates a cytosolic location of this moiety (45). The unoccupied AHR is part of a multimeric protein complex of about 280 kDa (65, 66), which contains, besides AHR, two molecules of about 90 kDa and possibly another protein of about 46 kDa (67). The 90-kDa heat shock protein (HSP90) corresponds to at least one of the

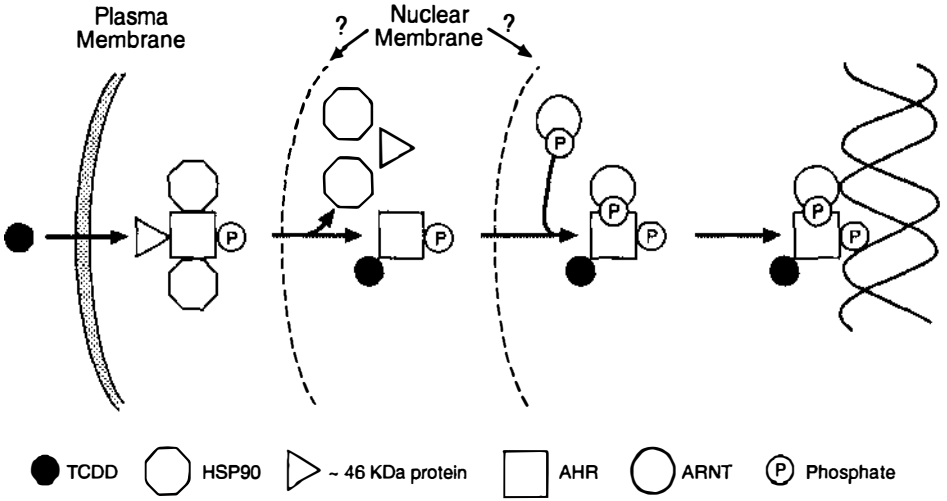

Figure 2 Mechanism of transformation of the AHRC. The unoccupied AHRC is shown containing two molecules of HSP90, although it has not been established whether the complex contains one or two molecules of this protein. An ~46-kDa protein may also be a component of the unliganded AHRC (67), as indicated. The lack of resolution as to where dissociation of the ~280-kDa complex takes place is indicated by the two alternative positions for the nuclear membrane in the figure.

90-kDa proteins (68, 69). Both forms of HSP90 (which are products of different genes) can apparently complex with AHR (70). In vitro mutagenesis of AHR has shown that the binding site for HSP90 appears to be contained within a segment of 132 amino acids overlapping the ligand-binding site (59). Whether or not HSP90 is absolutely required for binding of ligand to AHR is unresolved (71, 72). Release of HSP90 decreases the rate of dissociation of ligand from AHR (61, 73). ARNT is not a component of the unoccupied receptor complex (74, 75). The half-life of the unliganded AHR protein in Hepa-1 cells is about eight hours (76).

Ligand binding triggers the translocation of AHR from cytoplasm to nucleus and dissociation of the 280-kDa AHRC (not necessarily in that order, as discussed below) (45, 77, 78). Nuclear translocation in vivo is apparently an energy-dependent processes (77). Cuthill et al (79) and Harper et al (80) obtained conflicting results regarding the energy dependence of ligand-induced transformation of the AHRC in vitro. The form of the transformed AHRC that can be extracted from nuclei contains both AHR and ARNT and is probably a dimer (13). Both helices of the bHLH region of ARNT are absolutely required for dimerization with AHR. Deletion of either the A or B segments of the PAS region of ARNT slightly affects dimerization with AHR, while deletion of the complete PAS region severely affects dimerization. Thus ARNT (and presumably AHR) possesses multiple domains for maximal heterodimerization (18). Free AHR can be detected in cells after ligand treatment and appears to be an intermediate in the transformation process (81). It has been postulated that ARNT is required for ligand-induced dissociation of the 280-kDa complex (82). However, this is at odds with the observation that the free AHR monomer appears to be an intermediate in the dissociation process and with evidence that TCDD can trigger dissociation of HSP90 from AHR in ARNT-deficient mutants of Hepa-1 cells (59).

After conventional subcellular fractionation of untreated Hepa-1 cells, ARNT was found both in the cytosol and the nucleus (13, 45). However, when fixed, whole Hepa-1 cells were subjected to immunofluorescent staining with antibodies to ARNT, nearly all ARNT molecules were located in the nucleus both before and after TCDD treatment. In contrast, immunocytochemical analysis of whole mouse embryo mounts demonstrated ARNT in both cytosols and nuclei (BD Abbot & MR Probst, personal communication). The cellular location of ARNT has therefore not been resolved. Surprisingly, as assessed by immunostaining, ARNT-deficient mutants of Hepa-1 cells are proficient in ligand-dependent nuclear translocation of AHR. Despite its name, ARNT does not appear to be directly involved in translocating AHR into the nucleus (45).

AHR is too large to diffuse through nuclear pores. Free AHR may be actively transported, unidirectionally, across the nuclear membrane or transported bidirectionally across the membrane but trapped in the nucleus after dimerizing

with ARNT and binding DNA. Some investigators have detected the 280-kDa, HSP90-containing AHRC in the nuclear fraction after cells are treated with ligand and have suggested that AHR is translocated into the nucleus in the form of the 280-kDa complex (83, 84). If this is true, HSP90 may play an active role in transporting AHR into the nucleus, as has been suggested to be the case for steroid hormone receptors (reviewed in 85).

In contrast to the situation in Hepa-1 cells, AHR was localized to both cytoplasm and nuclei of liver cells of untreated mice (86). AHR translocation in whole liver could occur as the result of binding of an endogenous ligand or via ligand-independent nuclear targeting.

DNA Binding and Transcriptional Activation

Short segments were identified in the upstream region of the mouse and rat *CYP1A1* genes, which when linked to a heterologous promoter and the chloramphenicol acetyl transferase (CAT) reporter gene, conferred TCDD or PAH inducibility of CAT activity when the construct was transfected into Hepa-1 cells. Induction was shown to depend on the transformed AHRC, since it did not occur in ARNT-deficient mutants of Hepa-1 (87, 88). A consensus sequence identified in these segments has been called the xenobiotic-, dioxin-, or Ah-responsive element (XRE, DRE, or AhRE). Five functional XREs lie within the 1218 bp 5′ to the cap site of the mouse *CYP1A1* gene (89). A "functional XRE consensus sequence" required for ligand- and AHRC-dependent inducibility has been identified by analyzing naturally occurring XREs and mutant forms of the XRE sequence (89, 90) (Table 2).

Concurrently with functional analysis of the XRE, investigations were performed with the gel mobility shift assay to define a consensus XRE sequence for binding the transformed AHRC (Table 2). Substitutions in the central core, 5′-CGTG-3′, eliminated binding of the transformed AHRC, while single substitutions in the other positions only reduced binding affinity up to about eightfold (89–91). The XRE for binding in vitro appears to be less restricted than that for functionality as determined by transient transfection assays, since, for example, the presence of certain nucleotides at positions 1, 3, and 10 completely eliminate functionality but have little or no effect on binding the AHRC (89, 90). Methylation interference and methylation protection analysis demonstrated that all guanine residues in the 4-bp core of the XRE contact the transformed AHRC (92–94), while guanine at position 3 (present in all XREs examined) probably contacts the AHRC less closely (92, 93). All mammalian species probably share the same or a very similar XRE sequence, as assessed by AHRC binding (95).

The above in vitro assays are potentially subject to many artifacts and also detect binding of the AHRC to naked DNA rather than chromatin. The ligation-mediated polymerase chain reaction (LMPCR) in vivo footprinting tech-

Table 2 XRE sequences for functionality and AHRC binding

	1	2	3	4	5	6	7	8	9	10		
5'-	T/G	N	G	C	G	T	G	A/C	G/C	A	-3'	Functional consensus
	N	N	N	C	G	T	G	(A/C)[†]	G/C/T	(A/T)		Binding consensus
			G(*)	C	G*	T	G*	—	G[‡]			Methylation protection
			C	G*	C	A	C		C			or interference
	□		▼		●		●		□			LMPCR in vivo
	G		G	C	G	T	G		G			footprinting
	G	G	C	G	C	A	C	G	G			
	□	□		●				°	△			

[†] Parentheses indicate that individual substitutions at these positions only reduce binding affinity in vitro by up to about eightfold.
* Active
(*) Partially active in these assays
[‡] Inactive
● Protected from methylation in vivo
▼ Enhanced methylation [probably reflecting protein (i.e. AHRC) binding]
° Protected in all XREs where it occurs
□ Not protected in XREs where it occurs
△ Protected in one study (97) but not in two others (96, 98)

nique was used to identify guanine residues in the upstream region of the *CYP1A1* gene that are contacted by protein in the intact cell. Such contacts were identified in six XREs in the upstream region of the mouse *CYP1A1* gene (96, 97) and in three XREs in the rat gene (98). The three guanine residues in the core region of all XREs were protected from dimethylsulfate attack in a ligand- and ARNT-dependent manner (Table 2). The results of the in vivo footprinting assay, like those of the gel mobility shift assay, indicate that certain nucleotides neighboring the 4-bp core of the XRE make a significant contribution to XRE binding. This is also supported by AHRC-XRE UV cross-linking studies of the type described below (89).

As discussed below, the differences between the consensus sequence required for functionality and that required for AHRC binding in vitro may reflect a genuine phenomenon. The observed differences may also be artifacts of the assays used to determine them. Whereas use of LMPCR analysis to derive a consensus XRE sequence for AHRC binding in vivo could potentially improve this situation, the transfection assay for functionality remains an imperfect model for transcriptional activation of the endogenous *CYP1A1* gene.

When the AHRC in rat hepatic cytosol was transformed by TCDD in vitro and covalently cross-linked to an XRE-containing double-stranded oligonucleotide in which thymines at positions 1, 2, and 6 (see Table 2) in the upper strand of the XRE were substituted with bromouracil, three XRE-protein complexes were observed by Elferink and coworkers (99). One complex, of about 100 kDa, was identified as AHR; another was an apparently novel protein of about 110 kDa; and the third appeared to represent a complex containing both of the above proteins (99). Similar experiments using mouse and human hepatoma cells, transformed in vivo or in vitro, also demonstrated cross-linking of two ligand-dependent proteins of about 95 kDa, which were identified as AHR and ARNT (74). Using rat (and guinea pig) hepatic extracts, Denison and coworkers detected TCDD-dependent cross-linking to the XRE of three proteins of about 100 kDa, in contrast to the earlier studies (100). One protein was identified as AHR (100). The other two proteins may be distinct from each other, as suggested, or both products of the *ARNT* gene, since this gene is differentially spliced in human cells (12). Consistent with the former possibility, Elferink & Whitlock demonstrated that when ligand-treated rat liver cytosol was applied to an XRE-containing DNA-affinity column, several proteins were markedly enriched; that one of the proteins was AHR; that another was ARNT; and that another, of about 110 kDa, appeared to be novel (101). The 110-kDa protein need not necessarily be a DNA-binding protein. Further experiments will be required to confirm or refute whether an additional protein(s) is associated with the transformed AHRC as it binds the XRE.

Since the bromine atom of bromouracil and the N7 of guanine (which is the site of methylation in the in vitro methylation and LMPCR techniques) both

project into the major groove of DNA, the AHRC lies primarily or exclusively in the major groove and thus behaves similarly to other bHLH-containing transcription factors (102–105). Deletion analysis indicates that the basic subdomains of the bHLH domains of both ARNT and AHR constitute the XRE-binding regions (18; B Fukunaga, MR Probst, S Reisz-Porszasz & O Hankinson, unpublished data).

The XRE sequence is not symmetrical, strongly suggesting that AHR and ARNT each bind to different parts of the sequence. The great majority of other bHLH-containing transcription factors, whether they bind as homodimers or heterodimers, bind the E-box sequence 5′-CANNTG-3′. The basic region of ARNT contains a particular arginine residue that, in other bHLH proteins, dictates binding to the E-box subclass 5′-CACGTG3-′ (102, 103, 106). Depending upon its exact boundaries, the basic region of AHR may also contain the appropriate arginine residue. The four 3′-most nucleotides of this latter E-box sequence are identical to the four core nucleotides of the XRE. Whereas the basic region of ARNT conforms well to the consensus for other bHLH proteins, the basic region of AHR conforms poorly. In particular, the latter lacks a glutamic acid residue that is conserved in bHLH proteins and that is known, from X-ray crystallographic analysis of other bHLH-containing proteins, to contact the CA nucleotides at each end of the E-box (102, 104, 105). These observations suggest that ARNT binds to the "E-box side" of the XRE (5′-GTG-3′) while AHR binds 5′ to these nucleotides. Indeed, this has been found to be the orientation of ARNT and AHR on the XRE in the case of the transformed AHRC of mouse Hepa-1 cells (S Bacsi, S Reisz-Porszasz & O Hankinson, unpublished data). Surprisingly, however, in the transformed AHRC from guinea pig, the subunit that binds to the E-box side appeared to be AHR (100).

The mechanism of induction by ligands for AHR has been most thoroughly studied for the *CYP1A1* gene. Induction of the *CYP1A1* gene results principally, or even exclusively, from an increase in the rate of transcriptional initiation of the gene (107–109). Each of the several XREs in the upstream region of *CYP1A1* contributes towards induction (110) (Figure 3). Besides the XREs, LMPCR analysis detected TCDD- and ARNT-dependent in vivo protein binding to certain other sites in the –400 to –1300 upstream enhancer region of the gene, including a guanine-rich sequence (GRB) adjacent to one of the XREs (96–98). Binding of the AHRC to the nearby XREs may lead to disruption of the nucleosomal structure of chromatin at these other sites, thereby allowing access to their cognate binding factors. Indeed, Morgan & Whitlock (111) and Gradin et al (112) have shown that TCDD produces changes in chromatin structure at the enhancer region. The GRB and another non-XRE site footprinted in vivo both augment ligand-dependent activation of transcription by neighboring XREs in transient transfection assays (98, 110).

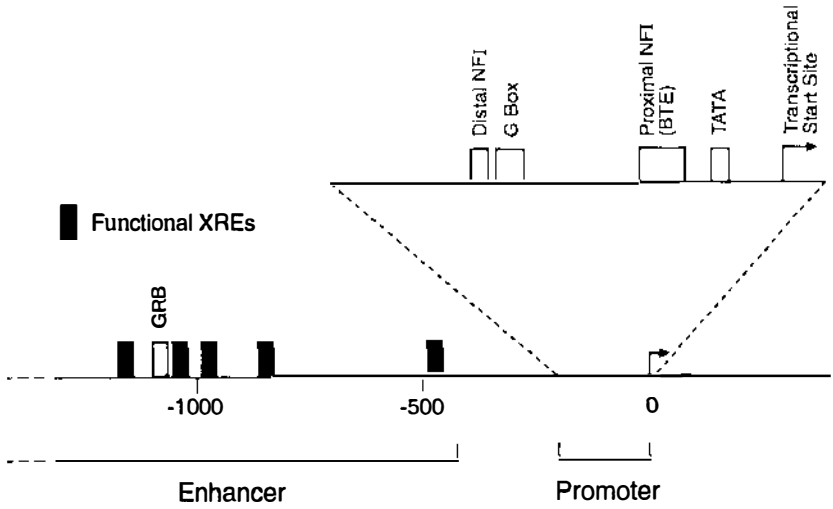

Figure 3 Regulatory and potential regulatory domains 5′ to the transcriptional start site of the mouse *CYP1A1* gene.

The pattern of dimethylsulfate cleavage over the enhancer region was the same in uninduced Hepa-1 and rat hepatocyte-derived LCS7 cells analyzed with LMPCR as in naked DNA methylated in vitro (96–98). Thus no proteins were detectable in the major groove of the DNA in the enhancer region, prior to TCDD treatment. [Nucleosomal proteins do not affect susceptibility to methylation by dimethylsulfate (113).] Since it is improbable that sequence-specific repressor proteins would be located exclusively in the minor groove (because of the limited specificity of amino acid–nucleotide interactions of proteins located in this groove), LMPCR analysis indicates that the AHRC does not activate transcription via displacement of specific repressor proteins at the XREs. However, an analysis of DNase I hypersensitive sites provides some evidence against this conclusion. Such sites were detected at the approximate locations of two XREs in the *CYP1A1* gene in nuclei from rat liver. Sensitivity at these sites was unaffected by exposure of the animal to a ligand for AHR. One possible explanation for this observation is that the transformed AHRC displaces a repressor protein located at the XREs (114). The apparently conflicting results obtained in these experiments may be due to the different biological materials that were analyzed. Further experiments are required to resolve this issue.

Besides the XREs, other DNA segments that are located within 200 nucleotides upstream of the transcriptional initiation site are required for inducibility by AHR ligands (Figure 3). This ~200-bp promoter region has very little or

perhaps no ability to support transcription in the absence of the upstream XRE sequences. Mutations in the TATA box decreased both the magnitude and fidelity of transcription (115). Mutation or deletion of an ~20-bp sequence [named the Basal Transcriptional Enhancer (BTE) in the rat and the proximal nuclear factor 1 (NF1) site in the mouse] located at about position –40 reduced both TCDD-induced and constitutive activity. This segment also contains a consensus Sp1 binding site. A novel protein, the basic transcription element binding protein (BTEB), was cloned that binds this segment (116). However, the roles, if any, of the NF1, Sp1, and BTEB proteins in transcription of *CYP1A1* have not been established. Mutation analysis identified another segment (encompassing nucleotides –110 to –130 in the mouse gene), containing a stretch of guanine residues (G box) and a second NF1-like recognition site, which apparently only has a moderate stimulatory effect on transcription. Proteins capable of binding the BTE (proximal NF1 site) and G box are present in uninduced cells, but they appear to actually bind these regions only after TCDD treatment (115, 117, 118). This suggests that the transformed AHRC somehow modifies chromatin in the promoter region to allow access of the regulatory proteins that bind these sites. Consistent with this idea, TCDD treatment causes disruption of two nucleosomes that appear to be located in fixed positions over the promoter region of the *CYP1A1* gene in uninduced cells. Disruption is independent of active transcription (111). Therefore, binding of transformed AHRCs at XREs located a considerable distance removed results in effects on chromatin structure at the promoter. Because binding of the AHRC at an XRE causes DNA bending at or near the XRE (119), stretches of DNA between the XREs and the promoter may loop out and bring the bound AHRC in direct contact with the promoter. The AHRC may remain associated with the promoter even after nucleosomal disruption and may play an active role in recruitment of the above proteins.

Disruption of the nucleosomes at the promoter and subsequent binding of the appropriate proteins may lead to some activation of transcription, but analogy with many other inducible genes (120) suggests that full transcriptional activation undoubtedly requires an additional activity of the transformed AHRC. This supposition is supported by the observation that XREs activate transcription in a TCDD- and ARNT-dependent fashion in transient transfection assays, under which conditions DNA does not appear to be arranged in nucleosomes (121). The transformed AHRC most probably has a transcriptional activation domain (or domains) that interacts directly or indirectly with proteins at the promoter, including components of the general transcription machinery, to stimulate transcription. A candidate for a transcriptional activation domain is the carboxy-terminal region of AHR, which is rich in glutamine residues [a characteristic of transcriptional activation domains of certain other proteins (reviewed in 122)], although a carboxy-terminal glutamine-rich seg-

ment of ARNT can be deleted with only a moderate effect on biological activity (12, 18). Interestingly, Lusska et al (123) and Reick et al (124) have obtained some evidence for a rapidly turning over protein that inhibits the transcriptional activation function of the transformed AHRC without affecting its binding to the XRE.

Within the nucleus, the transformed AHRC fulfills a number of functions during activation of transcription. The multiple roles of the transformed AHRC may explain why the sequence required for a functional XRE sequence is more restricted than that required for binding of the transformed AHRC. Thus certain XRE sequences that can bind the transformed AHRC may not allow sufficient bending of the DNA or permit the AHRC to acquire a confirmation required for a subsequent step in transcriptional activation, such as a productive interaction with the general transcription machinery (89).

Role of Phosphorylation in AHRC Functioning

Prolonged treatment of Hepa-1 cells, human keratinocytes, or the whole mouse with tetradecanoyl phorbol acetate (TPA) inhibits induction of CYP1A1 by ligands of AHR (125–127). Since TPA has been shown to down-regulate protein kinase C (PKC), these findings indicate that PKC-dependent phosphorylation is required for activity of the AHRC. This conclusion is supported by observations that induction is also inhibited by protein kinase inhibitors, including calphostin C, which is relatively specific for PKC (125, 127). Treatment of the transformed AHRC with phosphatase inhibited its binding to the XRE (125, 127, 128), while binding of ligand by the untransformed AHRC was unaffected (128, 129). Although the latter result suggests that ligand binding does not depend upon phosphorylation, the relevant phosphorylation sites may be shielded from attack by phosphatase. Transformation of the AHRC to the XRE-binding state was achieved in vitro in cytosolic extracts lacking PKC activity and supplemented with calphostin C and other more general protein kinase inhibitors (130). Thus the relevant sites appear to be phosphorylated prior to ligand treatment. ARNT synthesized in a reticulocyte lysate translation system was mixed with a crude cytosolic extract containing AHR, and the mixture was treated with TCDD. ARNT and AHR dimerized under these conditions and the dimer could bind the XRE. If ARNT was pretreated with phosphatase, dimerization did not occur. Pretreatment of the crude cytosol did not prevent dimerization, but prevented subsequent XRE binding (127). Thus phosphorylation of ARNT appears to be required for dimerization (XRE binding cannot be tested independently of dimerization), whereas phosphorylation of AHR appears to be required for XRE binding but not dimerization (see Figure 2). Interestingly, however, three of the five potential PKC phosphorylation sites in both human and mouse AHR are located

in the basic region of the bHLH motif (20), where phosphorylation might be expected to inhibit, rather than promote, DNA binding.

Although the studies described above certainly indicate a role for phosphorylation in AHRC activity, the exact nature of this role remains unknown. For example, PKC may not directly phosphorylate any component of the AHRC but may rather participate as one step in a kinase cascade in which the final phosphorylation step is accomplished by a different protein kinase. More definitive information will undoubtedly be forthcoming from additional, more direct experiments.

Regulation of Other Proteins by the AHRC

A number of proteins, or activities, are known to be induced (or in a few instances repressed) by ligands for AHR (Table 3). These include phase I (CYP1A1, CYP1A2, and CYP1B1) and phase II [NADP(H):oxidoreductase (the *NQO1* gene product in the rat), the glutathione *S*-transferase Ya subunit (the *GST-Ya* gene product in the rat), and UDP-glucuronosyltransferase (the Ugt1*06 gene product in mouse)] enzymes of xenobiotic metabolism, several proteins involved in the control of cell growth [plasminogen activator inhibitor (PAI-2), transforming growth factors-α and -β_2 (TGF-α and TGF-β_2), c-fos, Jun-B, c-jun, and Jun-D)], aldehyde dehydrogenase (*ALDH3* gene product in rat and *ALDH3c* gene product in mouse), and δ-aminolevulinic acid synthetase (ALAS). In several instances regulation has been shown to be dependent upon the AHRC, as evidenced by one or more of the following: (*a*) Responsiveness segregates with the Ah^{b-1} allele in the mouse, while non- or reduced responsiveness segregates with the Ah^{d} allele. (*b*) The response does not occur in AHR- or ARNT-deficient mutants of Hepa-1 cells. [However it is conceivable that AHR may mediate some effects without involvement of ARNT and without binding to the XRE (see below).] (*c*) Within a particular class of AHR ligands, the activity of individual isomers or congeners for stimulating the response is closely related to the affinity with which the compound binds to AHR. (*d*) One of the known partial antagonists of AHR reduces the effect of TCDD on the parameter in question. (This is less satisfactory evidence, since such compounds may have multiple effects on the organism or cell.)

Certain phase II enzymes [and perhaps CYP1A2 (131)] are induced by PAHs by two separate mechanisms. The 5′ flanking regions of the glutathione *S*-transferase Ya and NAD(P)H:quinone oxidoreductase genes each contain an XRE sequence through which TCDD and PAHs activate transcription via the AHRC. The genes also contain a distinct Antioxidant Response Element (ARE). Induction through this element by PAHs requires their metabolism to electrophilic derivatives which probably function by modifying an ARE binding protein. [Some, but not all, evidence indicates that this may be the AP-1 transcription factor (132).] It is possible that some genes will prove to have

Table 3 Gene products regulated by ligands of the AHRC

	AHR and/or ARNT dependence	XRE dependence	Primary response	Increase in specific mRNA	Increase in rate of transcription	References
CYP1A1	+	+	+	+	+	49, 107, 108, 182
CYP1A2	+			+	+	126, 183
CYP1B1			+	+	+	137, 184
NQO_1	+	+		+	+	42, 185 186
GST-Ya	+	+		+	+	187
Ugt 1*06 gene	+			+		42
PAI-2				+	+	137, 188
TGF-β_2			−	+	+	142
TGF-α				+	−	142
c-fos	−		+	+		143
Jun-B	−		+	+		143
c-jun			+	+		143
Jun-D			+	+		143
ALAS	+		−			189
ALDH 3	+		+	+	+	43, 190, 191

functional AREs and no functional XREs. Induction of such genes by PAHs could occur completely independently of the AHRC if the tissue in question expresses constitutive cytochrome P450s with PAH-metabolizing activity. Since TCDD is refractory to metabolism, it is a better ligand than PAHs for identifying AHRC-dependent processes. Proteins that have been shown to be induced or repressed only by PAH ligands for AHR are not included in Table 3. Some effects have been shown to occur in the presence of a protein synthesis inhibitor, indicating that they represent primary responses to ligand.

A number of other proteins or mRNAs not listed in Table 3 have also been shown to be modulated by TCDD in particular tissues or cells in certain animals. These include ornithine decarboxylase (133), a 60-kDa microsomal esterase (134), malic enzyme activity (135), transforming growth factor–β1 protein and mRNA (136), aldehyde dehydrogenase isozyme ALD3m (43), interleukin 1β mRNA (137), epidermal transglutaminase (138), protein kinase C activity (139, 140), c-erb-A messenger RNA levels, phosphorylation of $pp60^{src}$, and Ras-associated GTP binding (141). None of the above effects has been shown to be a primary response or has been proven to be AHRC dependent.

There are a few apparent exceptions to the paradigm that induction of primary response genes by TCDD and other "pure" AHR ligands occurs via binding of the transformed AHRC to XRE(s), resulting in an increase in the rate of synthesis of the corresponding mRNA. Induction of TGF-α in human keratinocytes is apparently caused by stabilization of its mRNA rather than stimulation of mRNA

synthesis (142). Most interestingly, induction of the c-*fos* and *Jun-B* protooncogenes appears to occur through an ARNT- and AHR-independent mechanism (143). TCDD was found to cause a rapid twofold increase in phosphorylation of proteins in an extranuclear fraction of adipose tissue in vitro, and it also increased protein kinase C activity in a cytosolic extract of liver (144, 144a). These findings are exciting because they indicate the existence of a novel pathway for TCDD action that is independent of the XRE and ARNT. Omeprazole, benzo(e)pyrene, and mevinolin induce CYP1A1, and isosafrole induces CYP1A2, but none of them have been reported to be ligands for AHR, suggesting that in each case induction occurs by a novel mechanism (145–147a). However, mevinolin does not induce CYP1A1 in AHR- and ARNT-deficient mutants of Hepa-1 (147), and omeprazole does trigger translocation of AHR to the nucleus (148). Thus, for these two compounds, a metabolite is the true inducer, AHR is activated indirectly, or the AHR assay was not sufficiently sensitive to detect their binding.

TCDD has also been shown to modulate a number of more complex biochemical parameters. Many of these modulations probably constitute secondary or higher-order responses to TCDD. In vivo TCDD treatment leads to autophosphorylation and internalization of the hepatic EGF receptor (141, 149). This may result from induction of TGF-α, which is a ligand for the EGF receptor. TCDD modulates a broad range of estrogen responses, both in the whole organism and in cells in culture. In MCF-7 human breast cancer cells and/or Hepa-1 cells, it reduced the level of binding of estradiol to the estrogen receptor, the amount of estrogen receptor protein, the level of binding of the estrogen receptor to the estrogen response element, and transcriptional activation of estrogen responsive genes. However, neither estrogen receptor mRNA levels nor the rate of estrogen receptor gene transcription were affected. These effects are AHR and ARNT dependent but do not constitute a primary response (150–153). TCDD considerably enhanced the endotoxin-stimulated increase in the serum concentration of tumor necrosis factor–α (TNF-α, or cachexin) in mice in a process dependent on AHR (154). TCDD also increased tyrosine phosphorylation of certain liver proteins, including the cyclin-dependent kinases, p34cdc2 and p33cdk2 (155, 156). TCDD reduced glucose-transporting activity in the plasma membrane of adipose tissue of guinea pig pancreas (157).

TCDD produces many different pathological effects (reviewed in 7, 9, 11). The motive for studying many of the biochemical parameters described above was to identify changes that might be involved in particular pathways of TCDD pathogenesis. Correlations between changes in these biochemical parameters and particular pathological effects of TCDD have been observed, and in certain cases, as is discussed below, perturbations of particular pathological effects of TCDD have been achieved experimentally and have provided some insights into the mechanisms responsible for toxicity.

TCDD has been shown to be a teratogen and/or to cause fetal toxicity in a variety of species, and these effects are AHR dependent in the mouse (158; reviewed in 11, 159). Teratogenesis caused by TCDD in mice was enhanced by coadministration of retinoic acid, thyroid hormones, or glucocorticoids (159). A characteristic acute response in all animals studied is progressive weight loss followed by delayed lethality (i.e. wasting syndrome). This response is AHR dependent in mice. As stated above, one effect of TCDD is to increase the endotoxin-induced serum concentration of TNF-α in the mouse. Interestingly, treatment with antibodies to TNF-α reduced the TCDD-induced weight loss and lethality in mice, while the glucocorticoid dexamethasone, which inhibits transcription of TNF-α, nearly completely blocked both responses to TCDD (160). Thyroidectomy also protects rats against TCDD-induced death (161). TCDD is toxic to the immune system, suppressing both T cell– and B cell–dependent parameters in an AHR-dependent process (see 11). In all species studied, TCDD causes atrophy of the thymus. Quercetin, an inhibitor of certain protein tyrosine kinases, blocks this effect of TCDD in mice (141). TCDD produces a hyperplastic response in the skin of some mammals, including humans, where it causes a characteristic syndrome termed chloracne. A similar response in the mouse is dependent on AHR and occurs in mice that are homozygous for a recessive allele, *hr*, at the hairless locus but not in *hr*/+ mice (162). TCDD also decreases both male and female reproductive performance in rodents (reviewed in 163).

TCDD is a multisite carcinogen in all mammalian species tested. It is controversial whether TCDD is a carcinogen in humans at the levels that have occurred after accidental exposures (reviewed in 164, 165). TCDD appears to act as a nongenotoxic carcinogen and is extremely potent when tested in tumor promotion protocols. Although nongenotoxic, TCDD can also act as a complete carcinogen in experimental animals (reviewed in 164, 166). Mutations arising spontaneously or from the effects of endogenous promutagens, mutagens, or such factors present in food may constitute the initiating (genotoxic) events. TCDD promotes tumors in the livers of female rats but not of male or of ovariectomized female rats. Moreover, TCDD caused a loss of plasma membrane EGF receptor in the livers of intact but not of ovariectomized rats (167). These results indicate a role for ovarian hormones, and perhaps the EGF receptor, in TCDD promotion of tumors in rat liver.

Tissue Distribution and Regulation of AHR and ARNT Expression

TCDD or PAH inducibility of CYP1A1, AHRC ligand binding activity, AHR protein, AHR mRNA, and ARNT mRNA occur in most examined rodent and human tissues, although the degree of expression of each varies considerably between the different tissues (16, 20, 168, 169, 169a; reviewed in 10). Little

systematic analysis has been undertaken to assess the degree to which the above parameters are coordinately expressed in particular tissues.

Treatment with the non-AHR ligand phenobarbital over several days increased AHR ligand–binding levels moderately in the rat, although this increase is not associated with an increase in the degree of inducibility of CYP1A1 (170). The phenobarbital-induced form has some kinetic properties that are different from those of the constitutive receptor (171). TCDD and certain aromatic amines also moderately increased AHR ligand–binding activity in rat liver after several days (172–174). In contrast, a four-day exposure of the mouse fetus to TCDD caused a slight but statistically significant decrease in AHR protein levels and a more marked decrease in AHR mRNA levels in the craniofacial region (86). Treatment of rat and mouse hepatoma cells and rat hepatocyte-derived cells with TCDD causes rapid nuclear translocation, followed by a rapid decline in nuclear receptor activity (124, 174, 175). Down-regulation was prevented by inhibitors of protein or RNA synthesis, indicating that a labile protein is involved. It was suggested that the labile protein either directly or indirectly degrades the transformed AHRC or converts it to an inactive form (124). Interestingly, cigarette smoking (which generates ligands for AHR, including PAHs) appears to induce both AHR and ARNT mRNA levels in blood cells of the human (169). Future experiments will undoubtedly clarify these potentially important regulatory mechanisms and provide insights into their biological significance.

Future Directions For Research

Identification of the putative endogenous ligand (or ligands) for the AHR would be a major breakthrough in the field. Promising places to look for such a ligand are fetal and adult tissues in which immunostaining shows AHR to be located in the nucleus, even in the untreated animal (e.g. 86). The fact that TCDD is a powerful teratogen and can also affect the state of differentiation of keratinocytes (reviewed in 159, 176) suggests that the AHRC plays a direct role in developmental processes, perhaps by binding to the putative endogenous ligand(s). This possibility can be investigated by generating homozygous knockout mice for the *AHR* and *ARNT* loci via homologous recombination in mouse embryonal stem cells. It should be noted that although mice that are homozygous for the Ah^d allele are viable, the protein encoded by this allele is not completely inactive and may be sufficiently active for the putative developmental role. If either knockout mouse is viable, it could be used in experiments to investigate what role the corresponding protein plays in determining susceptibility to the toxic effects of ligands for AHR, including carcinogens.

Determining the temporal and spatial expression of AHR and ARNT proteins would provide further insight into the potential roles of the proteins in development and would illuminate the relationship between tissue distribution

of expression and toxic responses to AHR ligands. Further analysis of the 5′ upstream regulatory region of the *AHR* gene (27) and isolation and analysis of the corresponding regions of the *ARNT* gene should provide useful information concerning potential developmental, tissue-specific, and ligand-dependent transcriptional regulation of these genes.

Analysis of nuclear translocation could be pursued via in vitro mutagenesis of the *ARNT* and *AHR* (and perhaps HSP90) cDNAs. Potential nuclear translocation signals (20, 45) are obvious targets for mutagenesis. Potential signal sequences could also be fused to a reporter protein for nuclear translocation in order to directly assess their function. The role of phosphorylation in the functioning of the AHRC will undoubtedly be further illuminated by additional experiments that directly analyze the proteins involved. These experiments could include immunoprecipitation of AHR, ARNT, and HSP90 proteins from cells labeled with $^{32}PO_4^{3-}$ in vivo; functional analysis of these proteins after site-directed mutagenesis of putative phosphorylation sites; utilization of appropriate protein kinases; and eventual reconstruction of ligand binding, dimerization, and XRE binding with purified proteins in vitro. Cloning or identification of the B and D genes (at least one of which probably does not correspond to the *AHR* gene) and the dominant gene affecting functioning of the AHRC in Hepa-1 cells are also important research goals.

X-ray crystallographic analysis and/or nuclear magnetic resonance imaging of purified AHR and ARNT, or of relevant fragments of these proteins, should permit resolution of their structures. Of particular interest would be analysis of the ligand-binding site, since knowledge of its structure may allow for the design of antagonists and may also provide clues about the structure of the putative endogenous ligand. Resolution of the structure of the transformed AHRC bound to the XRE would be particularly challenging, since at least two proteins are involved. Analysis of this last structure requires reconstruction of ligand binding, dimerization, and XRE binding with purified proteins. An important question is whether these activities can be achieved with ARNT and AHR proteins alone or whether other structural proteins are also required.

Our knowledge of the process whereby the activated AHRC stimulates transcription is rudimentary. Progress in this area will depend to a considerable degree on improvements in our understanding of transcriptional activation in general. Development of an AHRC-dependent transcriptional activation system that uses pure components in vitro, particularly in conjunction with chromatin, rather than naked DNA would constitute an important advance. This goal presently appears to be quite distant, although AHRC-dependent transcription has been achieved in vitro using crude nuclear extracts (177).

ARNT and/or AHR may have dimerization partners other than themselves that are additional members of the bHLH PAS family. Such novel heterodimers may bind sequences different from the XRE. The sequences of mouse and

human ARNT are much more similar to each other (92% amino acid identity overall, 98% over the bHLH PAS segment encompassing the dimerization domains) (18) than the mouse and human AHR (74% identity overall, 88% over the bHLH PAS segment) (20). The evolution of the dimerization domains of ARNT therefore appears to have been more constrained than the evolution of the equivalent regions of AHR. This suggests that ARNT is more likely than AHR to have alternative dimerization partners. Such additional dimerization partners for ARNT could be receptors for other xenobiotic compounds. The DNA-binding activity of certain bHLH proteins appears to be negatively regulated in vivo by dimerization with partners lacking a basic region (reviewed in 21). Such dominant negative regulators of ARNT or AHR activity may also exist and await discovery. It will also be of interest to ascertain whether transcription factors from other classes can interact with AHR or ARNT.

Elucidation of the mechanisms involved in the various pathological responses to TCDD (and other agonists of AHR) constitutes an extremely important, but difficult, research objective. Several of these complex responses, including tumor promotion (178, 179) and chloracne (180), can be mimicked in particular cell culture systems. Such cell culture systems provide opportunities for perturbing and thereby analyzing the pathways of pathogenesis, for example by using antisense oligonucleotides to specific mRNAs. The possibility that liganded AHR can affect cellular functions in a DNA- and ARNT-independent manner, perhaps by activating particular protein kinases, is a provocative one, since it indicates a novel pathway for AHR action (discussed in 181). Further experiments will undoubtedly shed light on this matter. Studies on the TCDD-resistant and -sensitive rat strains could lead to the identification of proteins involved in the pathway of TCDD lethality downstream from the steps involving the AHRC and may, therefore, provide important insights into the mechanism of TCDD lethality. Although it has been known for many years that rodent species differ dramatically in their sensitivity to TCDD lethality (reviewed in 3), such differences cannot be exploited so thoroughly as genetic differences within a single species.

Finally, it will be interesting to see whether genetic variation in either the *ARNT* or *AHR* genes is responsible for variation in the degree of inducibility of CYP1A1 and/or susceptibility to the toxic and carcinogenic effects of AHRC ligands in the human population.

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NOTE ADDED IN PROOF

The unoccupied AHRC has now been shown to contain two molecules of HSP90 (192). The carboxy-terminal regions of both AHR and ARNT exhibit transcriptional activation capability when fused to heterologous DNA-binding domains (193–195). However, deletion of the carboxy-terminal region of AHR only moderately reduces its transcriptional activation potential (193). The reduction in transcriptional activation potential of ARNT resulting from deletion of its carboxy-terminal region varies from 50 to 90% in different studies (18, 193, 194). The degrees to which the glutamine-rich domains in the carboxy-terminal regions of AHR and ARNT are involved in transcriptional activation have not therefore been fully resolved. Evidence has been obtained that the activity of AHR requires a tyrosine kinase–dependent pathway in human keratinocytes but not in human hepatoma cells (196). The amino acid differences responsible for the reduced K_d for TCDD of the AHR protein encoded by the Ah^d, compared with AHR encoded by the Ah^{b-1} allele, have been identified. The reduced affinity of the Ah^d protein for TCDD is ascribable to a combination of both the presence of the 43 additional amino acids at its carboxy terminus and an amino acid substitution within the PAS B region (195).

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