3. MECHANISMS AND MODE OF DIOXIN ACTION

Mechanistic studies can reveal the biochemical pathways and types of biological and molecular events that contribute to dioxin's adverse effects (See Part II, Chapter 2, for a detailed discussion). For example, much evidence indicates that TCDD acts via an intracellular protein (the AhR), which functions as a ligand-dependent transcription factor in partnership with a second protein (Arnt). Therefore, from a mechanistic standpoint, TCDD's adverse effects appear likely to reflect alterations in gene expression that occur at an inappropriate time and/or for an inappropriately long time. Mechanistic studies also indicate that several other proteins contribute to TCDD's gene regulatory effects and that the response to TCDD probably involves a relatively complex interplay between multiple genetic and environmental factors. If TCDD operates through such a mechanism, as all evidence indicates, then there are certain constraints on the possible models that can plausibly account for TCDD's biological effects and, therefore, on the assumptions used during the risk assessment process (e.g., Poland, 1996; Limbird and Taylor, 1998).

Mechanistic knowledge of dioxin action may also be useful in other ways. For example, a further understanding of the ligand specificity and structure of the AhR will likely assist in the identification of other chemicals to which humans are exposed that may add to, synergize, or block the toxicity of TCDD. Knowledge of genetic polymorphisms that influence TCDD responsiveness may also allow the identification of individuals at greater risk from exposure to dioxin. In addition, knowledge of the biochemical pathways that are altered by TCDD may help identify novel targets for the development of drugs that can antagonize dioxin's adverse effects.

As described below, biochemical and genetic analyses of the mechanisms by which dioxin may modulate particular genes have revealed the outline of a novel regulatory system whereby a chemical signal can alter cellular regulatory processes. Future studies of dioxin action have the potential to provide additional insights into mechanisms of mammalian gene regulation that are of a broader interest. Additional perspectives on dioxin action can be found in several reviews (Birnbaum, 1994a, b; Schecter, 1994; Hankinson, 1995; Schmidt and Bradfield, 1996; Gasiewicz, 1997; Rowlands and Gustafsson, 1997; Denison et al., 1998; Hahn, 1998; Wilson and Safe, 1998; Schecter and Gasiewicz, 2003; Matsumura, 2003; Carlson and Perdew, 2002).

Knowledge of the mode(s) of action by which the broad class of chemicals known as dioxins act may facilitate the risk assessment process by contributing to the weight of the evidence for hazard characterization and by imposing bounds on the models used to describe possible responses of humans resulting from exposure to mixtures of these chemicals (see

Sections 2 and 5 of this document). The relatively extensive database on TCDD, as well as the more limited database on related compounds, has been reviewed, with emphasis on the role of the specific cellular receptor for TCDD and related compounds—the AhR—in the postulated mode(s) of action. This discussion focuses on summarizing the elements of the mode(s) of dioxin action that are relevant for understanding and characterizing dioxin risk for humans. These elements include:

- Similarities between humans and other animals with regard to receptor structure and function;

- The relationship between receptor binding and toxic effects; and

- The extent to which the purported mechanism(s) or mode(s) of action might contribute to the diversity of biological responses seen in animals and, to some extent, in humans.

In addition, this section identifies important and relevant knowledge gaps and uncertainties in the understanding of the mechanism(s) of dioxin action and indicates how these may affect the approach to risk characterization.

3.1. MODE VERSUS MECHANISM OF ACTION

In the context of revising its carcinogen risk assessment guidelines, EPA has proposed giving greater emphasis to use of all of the data in hazard characterization, dose-response characterization, exposure characterization, and risk characterization (U.S. EPA, 1996, 1999, 2003). One aid to the use of more information in risk assessment has been the definition of mode versus mechanism of action. Mechanism of action is defined as the detailed molecular description of key events in the induction of cancer or other health endpoints. Mode of action refers to the description of key events and processes, starting with interaction of an agent with the cell through functional and anatomical changes, resulting in cancer or other health endpoints.

Despite a desire to construct detailed biologically based toxicokinetic and toxicodynamic models to reduce uncertainty in characterizing risk, few examples have emerged. Use of a mode of action approach recognizes that, although all of the details may not have been worked out, prevailing scientific thought supports moving forward using a hypothesized mode of action supported by data. This approach is consistent with advice offered by the National Academy of

Sciences' National Research Council in its report entitled *Science and Judgment in Risk Assessment* (NAS/NRC, 1994).

Mode of action discussions help to provide answers to the questions: How does the chemical produce its effect? Are there mechanistic data to support this hypothesis? Have other modes of action been considered and rejected? In order to demonstrate that a particular mode of action is operative, it is generally necessary to outline the hypothesized sequence of events leading to effects, to identify key events that can be measured, to outline the information that is available to support the hypothesis, and to discuss those data that are inconsistent with the hypothesis or support an alternative hypothesis. Following this, the information is weighed to determine whether there is a causal relationship between key precursor events associated with the mode of action and cancer or other toxicological endpoint in animals, and ultimately whether this inference can be extended to humans.

3.2. GENERALIZED MODEL FOR DIOXIN ACTION

Dioxin and related compounds are generally recognized to be receptor-mediated toxicants. The generalized model has evolved over the years to appear as illustrated in Table 3-1 and Figure 2-1.

3.2.1. The Receptor Concept

One of the fundamental concepts that influences our approach to risk assessment of dioxin and related compounds is the receptor concept. The idea that a drug, hormone, neurotransmitter, or other chemical produces a physiological response by interacting with a specific cellular target molecule, that is, a "receptor," evolved from several observations. First, many chemicals elicit responses that are restricted to specific tissues. This observation implies that the responsive tissue (e.g., the adrenal cortex) contains a "receptive" component whose presence is required for the physiologic effect (e.g., cortisol secretion). Second, many chemicals are quite potent. For example, picomolar to nanomolar concentrations of numerous hormones and growth factors elicit biological effects. This observation suggests that the target cell contains a site(s) to which the particular chemical binds with high affinity. Third, stereoisomers of some chemicals (e.g., catecholamines, opioids) differ by orders of magnitude in their ability to produce the same biological response. This observation indicates that the molecular shape of the chemical strongly influences its biological activity. This, in turn, implies that the binding site on or in the target cell also has a specific, three-dimensional configuration. Together, these types of observations support the prediction that the biological responses to some chemicals involve

stereospecific, high-affinity binding of the chemicals to specific receptor sites located on or in the target cell. Many of these characteristics have been noted for TCDD and related compounds.

The availability of compounds of high specific radioactivity has permitted quantitative analyses of their binding to cellular components in vitro. To qualify as a potential receptor, a binding site for a given chemical must satisfy several criteria: (1) the binding site must be saturable, that is, the number of binding sites per cell should be limited; (2) the binding should be reversible; (3) the binding affinity measured in vitro should be consistent with the potency of the chemical observed in vivo; (4) if the biological response exhibits stereospecificity, so should the in vitro binding; (5) for a series of structurally related chemicals, the rank order for binding affinity should correlate with the rank order for biological potency; and (6) tissues that respond to the chemical should contain binding sites with the appropriate properties.

The binding of a chemical ("ligand") to its specific receptor is assumed to obey the law of mass action; that is, it is a bimolecular, reversible interaction. The concentration of the liganded, or occupied, receptor [RL] is a function of both the ligand concentration [L] and the receptor concentration [R] as shown in equation 3-1:

$$[L] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RL] \qquad (3\text{-}1)$$

Inherent in this relationship is the fact that the fractional occupancy (i.e., $[RL]/[R_t]$) is a function of ligand concentration [L] and the apparent equilibrium dissociation constant K_D, which is a measure of the binding affinity of the ligand for the receptor, that is, $[RL]/[R_t] = [L]/(K_D + [L])$, where $K_D = [L]\,[R_t]/[LR] = k_2/k_1$. Therefore, the relationship between receptor occupancy and ligand concentration is hyperbolic. At low ligand concentrations (where $[L] << K_D$), a small increase in [L] produces an approximately linear increase in fractional receptor occupancy. At high ligand concentration (where $[L] >> K_D$), the fractional occupancy of the receptor is already very close to 1, that is, almost all receptor sites are occupied. Therefore, a small increase in [L] is likely to produce only a slight increase in receptor occupancy. These issues are discussed in regard to TCDD binding to the AhR and dose-response in Part II, Chapter 8.

Ligand binding constitutes only one aspect of the receptor concept. By definition, a receptor mediates a response, and the functional consequences of the ligand-receptor binding represent an essential aspect of the receptor concept. Receptor theory attempts to quantitatively relate ligand binding to biological responses. The classic "occupancy" model of Clark (1933)

postulated that (1) the magnitude of the biological response is directly proportional to the fraction of receptors occupied, and (2) the response is maximal when all receptors are occupied. However, analyses of numerous receptor-mediated effects indicate that the relationship between receptor occupancy and biological effect is not as straightforward as Clark envisioned.

In certain cases, no response occurs even when there is some receptor occupancy. This suggests that there may be a threshold phenomenon that reflects the biological "inertia" of the response (Ariens et al., 1960). In other cases, a maximal response occurs well before all receptors are occupied, a phenomenon that reflects receptor "reserve" (Stephenson, 1956). Therefore, one cannot simply assume that the relationship between fractional receptor occupancy and biological response is linear. Furthermore, for a ligand (such as TCDD) that elicits multiple receptor-mediated effects, one cannot assume that the binding-response relationship for a simple effect (such as enzyme induction) will necessarily be identical to that for a different and more complex effect (such as cancer).

The cascades of events leading to different complex responses (e.g., altered immune response to pathogens or development of cancer) are likely to be different, and other rate-limiting events likely influence the final biological outcome, resulting in different dose-response curves. Thus, even though ligand binding to the same receptor is the initial event leading to a spectrum of biological responses, ligand-binding data may not always mimic the dose-effect relationship observed for particular responses.

Another level of complexity is added when one considers different chemical ligands that bind to the same receptor. Relative potencies are determined by two properties of the ligand: affinity for the receptor and capacity to confer a particular response in the receptor (e.g., a particular conformational change), also called efficacy (Stephenson, 1956). Ligands with different affinities and the same degree of efficacy would be expected to produce parallel dose-response curves with the same maximal response within a particular model system. However, ligands of the same affinity with different efficacies may result in dose-response curves that are not parallel or that differ in maximal response. These issues relate particularly to Ah receptor ligands that are not "dioxins," where different efficacies or an inability to elicit the suite of dioxin-like responses compound differences in binding affinity for the Ah receptor. This complicates the use of the toxic equivalency approach, particularly for extrapolation purposes beyond the closely related congener groups. As described previously, this argues strongly for the use of all available information in setting TEFs and highlights the important role that scientific judgment plays in addressing uncertainty in the face of incomplete mechanistic understanding.

3.2.2. A Framework to Evaluate Mode of Action

In its revised proposed guidelines for carcinogen risk assessment (U.S. EPA, 1999, 2003), EPA recommends the use of a structured approach to evaluating mode of action. This approach is similar to and builds upon an approach developed within the WHO/IPCS Harmonization Project (WHO, 2000). Fundamentally, the approach uses a modification of the "Hill Criteria" (Hill, 1965), which have been used in the field of epidemiology for many years to examine causality between associations of exposures and effects. The framework calls for a summary description of the postulated mode of action, followed by the identification of key events that are thought to be part of the mode of action. These key events are then evaluated as to strength, consistency, and specificity of association with the endpoint under discussion. Dose-response relationships between the precursor key events are evaluated and temporal relationships are examined to be sure that "precursor" events actually precede the induction of the endpoint. Finally, biological plausibility and coherence of the data with the biology are examined and discussed. All of these "criteria" are evaluated and conclusions are drawn with regard to postulated mode of action.

In the case of dioxin and related compounds, elements of such an approach are found for a number of effects, including cancer, in Part II. Application of the framework to dioxin and related compounds may now proceed in a step-wise fashion to evaluate the association between the chemical or complex mixture and clearly adverse effects. The approach can be applied sequentially to early events, for example, receptor binding and intermediate events such as enzyme induction or endocrine impacts. Additional data will be required to extend the framework to most effects, but several have data that would support a framework analysis, a number of which are discussed below.

3.2.3. Mechanistic Information and Mode of Action—Implications for Risk Assessment

A substantial body of evidence from investigations using experimental animals indicates that the AhR mediates the biological effects of TCDD. The key role of the AhR in the effects of dioxin and related compounds is substantiated by four lines of research: (1) structure/activity relationships, (2) responsive versus nonresponsive mouse strains, (3) mutant cell lines, and (4) the development of transgenic mice in which the gene for the AhR has been "knocked out" (Birnbaum, 1994a; Fernandez-Salguero et al., 1996; Lahvis and Bradfield, 1998). Dioxin appears not to cause effects in the AhR knockout mouse (Fernandez-Salguero et al., 1996; Lahvis and Bradfield, 1998; Peters et al., 1999).

It is clear that the AhR is necessary, but not sufficient, for essentially all of the well-studied responses to dioxin. The AhR functions as a ligand-activated transcription factor, controlling the expression of specific genes via interaction with defined nucleotide sequences in the promoter regions. In order to control transcription, the TCDD-AhR complex interacts with another protein, Arnt, to bind to the dioxin response element. This complex is also bound by other nuclear coactivators and/or corepressors to bind to the transcriptional complex and initiate transcription (Gu et al., 2000). However, Arnt has many other partners that control hypoxia response, neuronal differentiation, morphological branching, etc. (Gu et al., 2000).

It is possible that there are other mechanisms that impact how dioxin initiates its toxic effects, apart from its direct transcriptional activation of drug metabolizing genes. It may be that the adverse effects of dioxin may result from competition of the ligand-activated AhR with other Arnt partners (Gradin et al., 1996). The AhR, Arnt, and Arnt partners are all members of the Per-Arnt-Sim (PAS) family of basic helix-loop-helix proteins that function as nuclear regulatory proteins (Gu et al., 2000). The PAS proteins are highly conserved, with homologous proteins being present in prokaryotes. They play key roles in circadian rhythms and development. The embryolethality of Arnt knockout mice, as well as the reduced fertility and viability of the AhR knockout mice (Abbott et al., 1999), point to a key role of these proteins in normal physiology.

Another potential mechanism by which TCDD can cause effects involves the protein/protein interactions of the AhR. When not bound to a ligand, the AhR exists in a multimeric protein complex that involves two molecules of heat shock protein 90 as well as other proteins, including AIP/XAP2/ara9, ara3, ara6, src, rel, and Rb (Carver et al., 1998; Enan and Matsumura, 1996; Puga et al., 2000b). AIP/XAP2/ara9 is a 37 kilodalton protein that is related to known immunophilins and is involved in the control of signal transduction processes. C-src has been shown to be associated with the AhR in several tissues and is a tyrosine kinase (Enan and Matsumura, 1996). Dioxin has been shown to cause a rapid increase in phosphorylation upon exposure. Recent studies have shown that rel, which is a key component of the NF-kappaB complex that controls apoptosis, binds to the AhR complex (Tian et al., 1999; Puga et al., 2000c). Similarly, several investigators have demonstrated an association between the AhR and the retinoblastoma protein; this has been shown to affect cell cycling (Puga et al., 2000b).

Thus, the AhR may act as a negative regulator of key regulator molecules involved in phosphorylation, cell cycling, and apoptosis in its unliganded state. Upon binding of TCDD, these other proteins are now able to exert their effects. In addition, dioxin may act by competing for Arnt, thus blocking key roles of other PAS regulatory proteins. Both of these mechanisms for the effects of dioxin are in addition to the direct role of the ligand-bound form of the receptor in

control of transcription via the well-studied mechanism of binding to a dioxin-response element in DNA.

Although studies using human tissues are much less extensive, it appears reasonable to assume that dioxin's mode of action to produce effects in humans includes receptor-mediated key events. Studies using human organs and cells in culture are consistent with this hypothesis. A receptor-based mode of action would predict that, except in cases where the concentration of TCDD is already high (i.e., [TCDD]~K_D), incremental exposure to TCDD will lead to some increase in the fraction of AhRs occupied. However, it cannot be assumed that an increase in receptor occupancy will necessarily elicit a proportional increase in all biological response(s), because numerous molecular events (e.g., cofactors, other transcription factors, genes) that contribute to the biological endpoint are integrated into the overall response. That is, the final biological response should be considered as an integration of a series of dose-response curves, with each curve dependent on the molecular dosimetry for each particular step.

Dose-response relationships that will be specific for each endpoint must be considered when using mathematical models to estimate the risk associated with exposure to TCDD. It remains a challenge to develop models that incorporate all the complexities associated with each biological response. Furthermore, the parameters for each mathematical model may apply only to a single biological response within a given tissue and species.

Given TCDD's widespread distribution, its persistence, and its accumulation within the food chain, it is likely that most humans are exposed to some level of dioxin; thus, the population at potential risk is large and genetically heterogeneous. By analogy with the findings in inbred mice, polymorphisms in the AhR probably exist in humans. Therefore, a concentration of TCDD that elicits a particular response in one individual may not do so in another. For example, studies of humans exposed to dioxin following an industrial accident at Seveso, Italy, failed to reveal a simple and direct relationship between blood TCDD levels and the development of chloracne (Mocarelli et al., 1991). These differences in responsiveness to TCDD may reflect genetic variation either in the AhR or in some other component of the dioxin-responsive pathway. Therefore, analyses of human polymorphisms in the AhR and Arnt genes have the potential to identify genotypes associated with higher (or lower) sensitivities to dioxin-related effects. Such molecular genetic information may be useful in the future for accurately predicting the health risks posed by dioxin to humans.

Complex responses (such as cancer) probably involve multiple events and multiple genes. For example, a homozygous recessive mutation at the *hr* (hairless) locus is required for TCDD's action as a chloracnegen and tumor promoter in mouse skin (Poland et al., 1982). Thus, the *hr*

locus influences the susceptibility of a particular tissue (in this case, skin) to a specific effect of dioxin (tumor promotion). An analogous relationship may exist for the effects of TCDD in other tissues. For example, TCDD may produce porphyria cutanea tarda only in individuals who have inherited uroporphyrinogen decarboxylase deficiency (Doss et al., 1984). Such findings suggest that, for some adverse effects of TCDD, the population at risk may be limited to individuals who have a particular genetic predisposition.

Other factors can influence an organism's susceptibility to TCDD. For example, female rats are more prone to TCDD-induced liver neoplasms than are males; this phenomenon is related to the hormonal status of the animals (Lucier et al., 1991). In addition, hydrocortisone and TCDD synergize in producing cleft palate in mice (Abbott et al., 1992). Retinoic acid and TCDD produce a similar synergistic teratogenic effect (Couture et al., 1990). These findings indicate that, in some cases, TCDD acts in combination with hormones or other chemicals to produce adverse effects. Such phenomena might also occur in humans. If so, the difficulty in assessing risk is increased, given the diversity among humans in hormonal status, lifestyle (e.g., smoking, diet), and chemical exposure.

Dioxin's action as a tumor promoter and developmental toxicant presumably reflects its ability to alter cell proliferation and differentiation processes. There are several plausible mechanisms by which this could occur. First, TCDD might activate a gene (or genes) that is directly involved in tissue proliferation. Second, TCDD-induced changes in hormone metabolism may lead to tissue proliferation (or lack thereof) and altered differentiation secondary to altered secretion of a trophic hormone. Third, TCDD-induced changes in the expression of growth factor or hormone receptors may alter the sensitivity of a tissue to proliferative stimuli. Fourth, TCDD-induced toxicity may lead to cell death, followed by regenerative proliferation. These mechanisms likely differ among tissues and period of development, and they may be modulated by different genetic and environmental factors.

The parallels between animal and human data relating to dioxin's tumor-promotion potential can assist in informing determinations of human risk, recognizing that the complexity of these intracellular processes limits our current mechanistic understanding. Using a weight-of-evidence approach, the Agency considers the cancer promotion data from in vitro and in vivo animal studies to be relevant and informative to humans. Although the specific mechanism(s) by which dioxin causes cancer remains to be established (as, indeed, for cancer in general), the intracellular factors and mechanistic pathways involved in dioxin's cancer-promotion mode of action all have parallels between animals and humans. No qualitative differences have been

reported to indicate that humans should be considered fundamentally different from the multiple animal species in which bioassays have demonstrated dioxin-induced neoplasia. Notably:

- the intracellular molecular protein, DNA, and RNA factors and mechanisms postulated in dioxin cancer promotion are common to animals and humans, reflecting intracellular functions that have been preserved phylogenetically over millions of years. These factors include the AhR, Arnt heterodimerization, cellular growth and differentiation functions, dioxin responsive elements, DNA transcription mechanisms, and oxidative enzyme induction; and,

- similar dioxin-induced toxic outcomes are evident between animals and humans across a variety of endpoints, progressing from enzyme induction, altered intracellular regulatory proteins, dermal lesions, and liver function and porphyria through to in vitro neoplastic cell promotion and clonal expansion following viral or chemical induction (in addition to the epidemiological cancer results following occupational exposures).

As detailed in Part II, Chapter 2 (mechanism of action), the mode of action parallels between humans and animals can be traced through dioxin's impacts at the subcellular level, as follows:

AhR binding: The AhR has been phylogenetically retained over hundreds of millions of years of evolution in humans and animals (Hahn, 1998) and is highly expressed in developing tissues (Abbott et al., 1995), pointing to a fundamental role in cellular growth, differentiation and/or endogenous/xenobiotic metabolism. Species-specific AhR molecular structures reveal them to be members of a family of transcription-activating proteins that exhibit a basic helix-loop-helix (bHLH) DNA binding motif, PAS domain for dimerization and ligand binding, and a C-terminal transactivation domain related to transcription induction and associated with a variety of toxic endpoints.

Notable similarities exist in the AhR across animal taxa, particularly at the bHLH and PAS sites (Fujii-Kuriyama et al., 1995), with human AhR being structurally most closely related to that of the guinea pig (75% base homology) and other sensitive animal strains (Korkalainen et al., 2001). Dioxin-resistant strains of rats and hamsters exhibit mutations in the AhR and/or increased homology differences, particularly in the C-terminal transactivation domain and Q-rich

subdomain (Korkalainen et al., 2001). Human AhR binding affinities vary ~20-fold (Kd ~ 0.3–38.8 nM) (Okey et al. 1997), encompassing the range from sensitive C57BL/6 mice (0.27 nM) to relatively resistant DBA/2 mice (1.5 nM) (Ema et al., 1994). Evidence suggests that within species, the AhR binding affinity correlates with biochemical effects and toxicity (Birnbaum et al., 1990, Poland and Glover, 1980), whereas between species, relative AhR binding affinities do not determine dioxin sensitivity because multiple downstream events intercede (DeVito and Birnbaum, 1995). Differences in conformational changes in the AhR following ligand binding are also likely to impact toxicity (Henry and Gasiewicz, 2003).

TCDD-AhR binding to Arnt: Following ligand binding, the TCDD-Arnt complex translocates to the nucleus, where it heterodimerizes (joins) with the bHLH-PAS transcription partner protein, Arnt. Arnt has been phylogenetically retained over evolutionary time in both humans and animals in several related forms and is essential for fetal survival. Arnt molecular weights vary across species from 85 kDa for the mouse, 87 kDa for humans, and 88 kDa for the rat (Pohjanvirta et al., 1999). The Arnt protein also dimerizes with other receptor/transcription pathways in the cell nucleus, indicating its importance and fundamental role in regulating DNA transcription (Schmidt and Bradfield, 1996; Zaher et al., 1998; Ge and Elferink, 1998; Tian et al., 1999).

Cross-talk among intracellular regulatory proteins: As noted, cancer is inherently a loss of the regulation of normal cell growth, differentiation, and death (apoptosis) that is locked into the genetic coding through clonal expansion. Central to the control of cell cycling and programmed cell death are numerous regulatory proteins (e.g., EGF, HIF-1α, TNF-α, TGF-β_1, NF-κB, RB), whose functional roles, although being rapidly elucidated, remain uncertain. These regulatory proteins are expressed in humans and animals and can be impacted by dioxin exposure, as in the role of EGF in dioxin-induced cleft palate in mice (Bryant et al., 2001). The Arnt protein is a common co-transcription factor for many bHLH-PAS regulatory proteins in addition to its role in the TCDD-AhR transcription pathway. The potential exists, therefore, for prolonged, inappropriate TCDD-AhR induction to impact multiple Arnt-related functions in the nucleus, thereby altering other regulatory pathways.

Competition for the Arnt protein has been demonstrated regarding the hypoxia inducible factor 1 (HIF-1α) pathway following dioxin administration and Arnt cross-talk (Gradin et al., 1996; Nie et al., 2001). In addition, dioxin-induced clonal expansion in human and animal cell cultures has resulted in fixed changes to the intranuclear expression of plasminogen activation

inhibitor (PAI-2), tumor necrosis factor alpha (TNF-α), and transforming growth factor β_1 (TGF-β_1), although it remains to be determined whether these changes were cause or effect of the dioxin-promoted clonal expansion (Yang et al., 1999).

Dioxin response elements (DREs): In the well-studied pathway of cytochrome mixed function oxidase induction (e.g., CYP1A1, 1A2), the ligand-AhR-Arnt heterodimer binds 1:1 to DREs upstream of the DNA gene battery transcription site (Denison et al., 1989). This mechanism is common to the mouse (six DREs) (Lusska et al. 1993), the rat (three DREs), and humans (two DREs) (Swanson and Bradfield, 1993), and is based on the 3'A-CGCAC5' DNA sequence. Subsequent to DRE binding, the C-terminal transactivation domain of the AhR alters histone proteins and causes unwinding of the chromatin, exposing the dioxin promoter and aryl hydrocarbon hydroxylase (AHH) gene battery to constitutively expressed DNA transcription proteins (Whitlock et al., 1996).

Enzyme induction: At least seven enzyme genes, and likely more, are included in the AhR-Arnt induced gene battery: three oxidative P450 cytochromes (CYP1A1, 1A2 ,1B1) and four non-P450 enzymes responsive to reactive oxygenated metabolites and oxidative stress (for example, a quinone oxidoreductase, aldehyde dehydrogenase, glucuronosyltransferase, and glutathione transferase [Nebert et al., 2000; Zhang et al., 1998]). These enzymes are expressed in humans and animals. Similar EC_{50}s were reported for CYP1A1 induction in lymphocytes in mice (1.3 nM) and humans (1.8nM) (Clark et al., 1992). However, substantial interspecies differences have been noted between cultured human and mouse embryonic palatal cells regarding CYP1A1 induction and morphological effects. Paralleling a ~200-fold lower sensitivity for morphological and cellular effects on embryonic palatal tissue, human cell cultures expressed ~350-fold fewer receptors and exhibited ~1500-fold lower dioxin-induced CYP1A1 m-RNA induction than mice (Abbott et al., 1999). Notably, though, effects on human and rat embryonic palatal shelf tissue occur at similar in vitro concentrations as compared to the much higher sensitivity shown in mice, suggesting that mice may exhibit a particular sensitivity to effects on palatal differentiation (Abbott and Birnbaum, 1990, 1991; Couture et al., 1990).

For CYP1A2 there is a ~40-fold variability in protein and enzyme activity levels in the human population (Eaton et al., 1995; Nebert et al., 1996). The importance of CYP1A2 to dioxin toxicity in rodents has been demonstrated in knockout mice, where dioxin-induced porphyrin changes did not occur in the absence of CYP1A2, and hepatic toxicity was substantially reduced

(Smith et al., 2001). This is likely due to the lack of hepatic sequestration in the absence of CYP1A2 (Diliberto et al., 1999).

Recent human epidemiological data have reported long-term hepatic enzyme and porphyrin ratio changes many years after industrial dioxin exposure (Neuberger et al., 1999). The prolonged up-regulation of mixed-function oxidase (MFO) enzymes has been postulated to impact the carcinogenic potential of xenobiotics that are metabolically activated, such as the PAHs. Indeed, carcinogenicity from PAHs is absent in AhR-knockout mice, presumably from lack of induction of the mixed-function oxidases. In a related mechanistic postulate, emphasis has been placed on the existence of both MFOs (CYP1A1, 1A2) and detoxifying/scavenging phase II transferase enzymes in the dioxin-induced gene battery, suggesting an evolutionary mechanism that creates reactive oxidative products through the MFOs (possibly as a result of endogenous ligand metabolism) yet provides a protective mechanism for mitigating the resulting oxidative stress through the phase II transferase enzymes. Abnormal regulation of this mechanism could cause oxidative stress that is related both to DNA damage and cell cycling/apoptosis regulation (Nebert et al., 2000).

Toxic effects and clonal proliferation: A spectrum of toxic effects has been demonstrated in both animals and humans following dioxin exposure, including developmental impacts, hormonal changes, skin lesions, and liver damage (DeVito et al., 1995). Dioxin has also been demonstrated to promote neoplastic changes and clonal expansion in human and animal cell cultures following viral induction. Exposure of normal human keratinocytes in vitro leads to accelerated differentiation, increased cell proliferation, and decreased senescence in differentiating cells (Ray and Swanson, 2003). These changes were accompanied by decreased levels of a number of cell regulatory proteins, including p53, supporting the concept that dioxin may exert its tumor promoting effects, in part, through this mechanism.

In Yang et al. (1992), human epidermal keratinocytes immortalized by adenovirus 12 - simian virus 40 exposure (SV40) underwent neoplastic transformation after 2 weeks of dioxin exposure in vitro at $\geq$ 0.1 nM, exhibiting increased saturation density, colony formation on soft agar, and squamous cell carcinoma when inoculated into athymic nude mice. These phenomena did not occur in the absence of SV40 virus induction or in control cell lines, including the immortalized cell culture. Both the neoplastic cell transformation and AHH induction in the untransformed cells were dose dependent. Follow-up analyses demonstrated alterations in growth regulatory gene expression (PAI-2, TNF-α, and TGF-β_1) that became fixed in the genome following successive division in TCDD-damaged cells (Yang et al., 1999).

Conversely, under certain circumstances, exposure to TCDD may elicit beneficial effects in selected tissue or cells. For example, TCDD protects against the subsequent carcinogenic effects of PAHs in mouse skin, possibly reflecting induction of detoxifying enzymes (Cohen et al., 1979; DiGiovanni et al., 1980). In other situations, TCDD-induced changes in estrogen metabolism may alter the growth of hormone-dependent tumor cells, producing a potential anticarcinogenic effect (Spink et al., 1990; Gierthy et al., 1993). However, several recent studies in mice indicate that the AhR has an important role in the genetic damage and carcinogenesis caused by components in tobacco smoke, such as BaP, through its ability to regulate CYP1A1 gene induction (Dertinger et al., 1998; Shimizu et al., 2000). TCDD's biological effects likely reflect a complicated interplay between genetic and environmental factors. These issues complicate the risk assessment process for dioxin.

Thus, it is clear that the robust database on mode(s) of dioxin action related to biochemical effects and to clearly adverse effects supports an understanding of dioxin's impact on biological and cellular processes. This database is among the best available for xenobiotic chemicals. The short-comings described above will stimulate additional research to further elucidate details in this understanding of the impact of dioxins, but they should not detract from the recognition that, among the data available to aid hazard characterization and risk assessment, these are remarkably consistent and useful findings.

Table 3-1. Early molecular events in response to dioxin[a]

Diffusion into the cell
Binding to the AhR protein
Impacts on cytoplasmic phosphorylation
Dissociation from hsp90
Active translocation from cytoplasm to nucleus
Association with Arnt protein
Competition for Arnt with other nuclear cofactors
Conversion of liganded receptor to the DNA-binding form
Binding of liganded receptor heteromer to enhancer DNA
Enhancer activation
Altered DNA configuration
Histone modification
Recruitment of additional proteins
Nucleosome disruption
Increased accessibility of transcriptional promoter
Binding of transcription factors to promoter
Enhanced mRNA and protein synthesis

[a] These events are discussed in detail in Part II, Chapter 2.