Abnormal Liver Development and Resistance to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin Toxicity in Mice Carrying a Mutation in the DNA-Binding Domain of the Aryl Hydrocarbon Receptor

Maureen K. Bunger,* Edward Glover,* Susan M. Moran,* Jacqueline A. Walisser,* Garet P. Lahvis,*†‡ Erin L. Hsu,*† and Christopher A. Bradfield*†‡

*McArdle Laboratory for Cancer Research; †Molecular and Environmental Toxicology Center; and ‡Departments of Surgery and Psychology, University of Wisconsin School of Medicine and Public Health, Wisconsin 53706

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The aryl hydrocarbon receptor (AHR) is known for its role in the adaptive and toxic responses to a large number of environmental contaminants, as well as its role in hepatovascular development. The classical AHR pathway involves ligand binding, nuclear translocation, heterodimerization with the AHR nuclear translocator (ARNT), and binding of the heterodimer to dioxin response elements (DREs), thereby modulating the transcription of an array of genes. The AHR has also been implicated in signaling events independent of nuclear localization and DNA binding, and it has been suggested that such pathways may play important roles in the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Here, we report the generation of a mouse model that expresses an AHR protein capable of ligand binding, interactions with chaperone proteins, functional heterodimerization with ARNT, and nuclear translocation, but is unable to bind DREs. Using this model, we provide evidence that DNA binding is required AHR-mediated liver development, as Ahr<sup>dbd/dbd</sup> mice exhibit a patent ductus venosus, similar to what is seen in Ahr<sup>-/-</sup> mice. Furthermore, Ahr<sup>dbd/dbd</sup> mice are resistant to TCDD-induced toxicity for all endpoints tested. These data suggest that DNA binding is necessary for AHR-mediated developmental and toxic signaling.

Key Words: aryl hydrocarbon receptor; dioxin; TCDD; ductus venosus.

The aryl hydrocarbon receptor (AHR)<sup>1</sup> is a basic helix-loop-helix (bHLH)-per-ARNT-sim (PAS) protein that mediates the toxic response to an array of lipophilic environmental toxicants, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Those responses include thymic involution, liver hypertrophy, tumor promotion, epithelial hyperplasia, and teratogenesis (Poland and Knutson, 1982). The generation of an Ahr-null allele in mice has also provided evidence that the receptor plays an important role in mammalian development (Gonzalez and Fernandez-Salgueiro, 1998; Schmidt et al., 1996). Characterization of the Ahr-null mouse revealed a transient microvesicular steatosis in perinatal hepatocytes, prolonged extramedullary hematopoiesis, and a reduced relative liver size throughout life. Recent evidence has shown that Ahr-null mice fail to resolve a fetal vascular structure, the ductus venosus (DV), which may be the underlying cause of liver atrophy. These outcomes are suggestive of a role for the AHR in vascular biology or hematopoiesis during mammalian development (Lahvis et al., 2000; Walisser et al., 2005).

In response to xenobiotic agonists, the AHR functions as a ligand-activated transcription factor. Upon binding agonists such as TCDD, the AHR translocates from the cytoplasm to the nucleus, where it dimerizes with another bHLH-PAS protein known as the AHR nuclear translocator (ARNT) (Hankinson, 1995). This heterodimeric complex recognizes dioxin response elements (DREs), which regulate the transcription of a battery of genes encoding xenobiotic metabolizing enzymes (XMEs). These XMEs include Phase I enzymes such as Cytochromes P450 1a1, 1a2, and 1b1, as well as the Phase II enzymes, GST-Ya, and UDPGT (reviewed in Hankinson, 1995; Schmidt et al., 1996).

Although the mechanism for AHR-mediated transcriptional activation of XMEs is well-established, it has been difficult to link specific target genes to most TCDD-induced toxic responses. Similarly, null alleles of three known transcriptional targets of AHR, Cytochromes P450 1a1, 1a2, and 1b1, have not been reported to possess any of the same phenotypes of Ahr<sup>-/-</sup> mice (Buters et al., 1999; Liang et al., 1996; Pineau et al., 1995). This latter observation suggests that none of the most responsive AHR target genes play an individual role in the developmental signaling of the AHR.

Our inability to link DRE-regulated genes to most aspects of TCDD toxicity or AHR developmental biology has led to the development of a number of models which propose that the AHR takes part in important signaling events that are independent of DRE binding or even ARNT dimerization. Included in this list of models is the hypothesis that the ligand-activated AHR...
signals through direct interactions with cellular proteins such as cSrc kinase, the retinoblastoma protein (Rb), and RelA (Blankenship and Matsumura, 1997; Enan and Matsumura, 1996; Ge and Elferink, 1998; Puga et al., 2000). Similarly, it has been proposed that TCDD toxicity may occur as a result of the activated AHR sequestering available ARNT in the cell. In in vitro and cell culture model systems, the capacity of an activated AHR to reduce ARNT participation in hypoxia signal transduction has been demonstrated and has also been challenged (Berghard et al., 1993; Chan et al., 1999; Grdin et al., 1996; Pollenz et al., 1999).

The various proposals suggesting that AHR may be involved in cellular signal transduction mechanisms independent of interactions with ARNT or DREs has led to a complicated picture of the mechanism of AHR-mediated development and TCDD toxicity. In an effort to test the role of DRE-independent signaling by the AHR in these processes, we have developed mouse models with deficiencies in specific signaling steps. We have shown previously that nuclear translocation of the AHR is required for normal liver development and TCDD-induced toxicity in mice (Bunger et al., 2003). Through the use of a similar gene-targeting approach, we have now generated a mouse line that expresses a mutant Ahr that is unable to bind DREs. We present evidence which suggests that the binding of AHR to DREs is required for developmental processes as well as AHR-mediated toxicity in vivo.

MATERIALS AND METHODS

Oligonucleotides and expression constructs. Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and are designated as follows:

- OL65: 5'-ATCCGACAGGTTCATAGGTTCTGC-3'
- OL941: 5'-CTGAGGGGAGCTGTTATG-3'
- OL942: 5'-AACATTTCAGCCTAGATGAG-3'
- OL1352: 5'-GCTGTCTCGTATGACTGTTG-3'
- OL1353: 5'-GCTGACCGATGTTGTTAGATG-3'
- OL1354: 5'-GCTGACCGATGTTGTTAGATG-3'
- OL1355: 5'-GCTGACCGATGTTGTTAGATG-3'
- OL1403: 5'-GGACATTTCAGGCGCGGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGCGCGCGCGGTCGC
Madison, WI), was used as a reporter and contains five Gal upstream activation sequences (UAS) upstream of an SV40 minimal promoter and the luciferase gene. A green fluorescence protein expression construct (Clontech, Mountain View, CA) was used as a control for transfection efficiency. Briefly, equal amounts of plasmids pTgTAHRdbd (PL1548) or pTgTAHRs (PL1550) were cotransfected with PL283 and the pG5L5 reporter. These cells were treated with InM TCDD and luciferase assays were performed using The Luciferase Assay kit (Promega) and read on a luminometer.

Generation of Ahrdbd/dbd mice. A 15-kb region of homology surrounding exon 2 of Ahr was isolated from a 129SvJ genomic library (Genome Systems) as described (Schmidt et al., 1993, 1996). A six nucleotide insertion (GAATTC) was introduced into exon 2 by megaprimer PCR using OL1793 and OL942. This product was used as a reverse megaprimer for PCR with OL659. An SphiI fragment from this PCR product was used to replace exon 2 in an 8-kb BamHI genomic fragment. A 5.5-kb region containing the mutated exon 2 was amplified with OL1352 and OL1353 and cloned into the KpnI site of ploxPNT (Tybulewicz et al., 1991). A 7-kb SphiI fragment from the 5′ region of exon 2 was cloned into the NotI/NotI site of this construct to generate the final targeting construct, designated PL1238.

Ten micrograms of the targeting construct was electroporated into GS1 ES cells (Genome Systems) and selection was performed using 200 μg/ml G418 and 1 mM Ganciclovir. Clones were screened by Southern blot on BamHI-digested genomic DNA using a probe 3′ to the end of the targeting construct (PL311). Correctly targeted clones were injected into 3.5-day postcoital C57BL/6J blastocysts, and the resulting chimeras were backcrossed to C57BL/6J in accordance with the known size of the wild-type protein. Photoaffinity labeling experiments indicated that the AHRdbd protein bound ligand with a capacity and affinity that was similar to its wild-type counterpart (data not shown). The DRE binding properties of AHRdbd were analyzed by a gel-shift protocol. Neither AHR nor AHRdbd proteins interacted significantly with a 32P-labeled DRE oligonucleotide in the absence of ARNT or in the presence of ARNT when an agonist was not present. The addition of the agonist, β-naphthoflavone (BNF), induced formation of the AHR/ARNT/DRE complex, but not the AHRdbd/ARNT/DRE complex (Fig. 2A).

Co-IP experiments were utilized to determine whether the AHRdbd protein retains the ability to interact with HSP90 and ARA9. An HSP90-specific antibody was capable of precipitating both 35S-labeled AHR and AHRdbd, and to the same degree (Fig. 2B). Furthermore, T7-peptide antibody-coupled agarose beads equally precipitated 35S-labeled ARA9 when incubated in the presence of T7-tagged AHR and T7-tagged AHRdbd (Fig. 2C). Together, these results indicate that AHRdbd is capable of interacting with ligand, HSP90, and ARA9 in a manner similar to the wild-type AHR, but is not capable of interacting with DREs.

Characterization of AHRdbd Signaling in Ahr−/− Fibroblasts

To determine whether the AHRdbd could signal effectively in cell culture, we performed transient transfections of AHR or AHRdbd with a DRE-driven luciferase reporter in immortalized Ahr−/− 3T3 fibroblasts. Upon transfection of wild-type AHR cDNA, luciferase activity increased relative to cells transfected with reporter alone. This response was enhanced 2.5-fold by exposure of the cells to 1nM TCDD. In comparison, luciferase activity in cells transfected with AHRdbd did not increase upon exposure of cells to TCDD (Fig. 3A).

Indirect immunofluorescence analysis was used to determine the subcellular localization of AHRdbd. Ahr−/− 3T3 fibroblasts were transiently transfected with AHR or AHRdbd cDNAs and

**RESULTS**

**Characterization of the AHRdbd Protein In Vitro**

A mutant AHR cDNA, designated “AHRdbd,” was generated by the insertion of nucleotides GGTACC, coding for amino acids glycine and serine, between arginine-39 (R39) and aspartate-40 (D40) of the wild-type AHR cDNA (Fig. 1).

![FIG. 1.](image-url)
ARNT-as "fish." Cotransfection of wild-type AHR with the Gal4-ARNT-
ARNT, we performed a mammalian two-hybrid analysis using
AHRdbd Interacts with ARNT in a Mammalian 2-Hybrid
Assay
To determine whether AHRdbd functionally interacts with
ARNT, we performed a mammalian two-hybrid analysis using
Gal4-ARNT-ΔTAD as "bait" and the full-length AHRdbd as "fish." Cotransfection of wild-type AHR with the Gal4-
ARNT-ΔTAD along with a Gal4UAS-luciferase reporter
showed a slight increase in RLU over reporter alone. Exposure
to 1nM TCDD increased luciferase activity threefold. Cotrans-
fection of the AHRdbd construct with the Gal4-ARNT-ΔTAD
and reporter also slightly increased luciferase activity over cells
with reporter alone and showed a 10-fold increase in luciferase
activity after TCDD treatment (Fig. 3C).

Generation and Characterization of Ahr<sup>dbd/dbd</sup> Mice
We used a megaprimer PCR approach to insert a GGATCC
sequence (encoding Proline-Arginine) immediately down-
stream of the basic region of exon 2 in a 15-kb region of
homologous genomic DNA derived from the Ahr locus
(Fig. 4A). The final targeting construct, ploxPNT/AHR<sup>dbd</sup>,
was electroporated into GS-1 ES cells (Genome Systems) and
selected in both G418 and Ganciclovir (Roche). Double-
selected clones (150 total) were screen by Southern blot and
five correctly-targeted clones were identified. One clone gave
rise to a chimera that transmitted the Ahr<sup>dbd</sup> allele to the
germline. The resulting Ahr<sup>dbd/dbd</sup> Targeted allele mice were
genotyped by PCR and restriction digest (Fig. 4B). To generate the
Floxed allele mice, the neomycin cassette was excised by
breeding to CMV-Cre mice, and the resulting offspring were
genotyped by PCR. All Cre-positive mice were negative for
neo. The N3F1 mice were born at the expected frequency
(Fig. 4C) and were fertile. Western blot analysis of liver protein
extracts from Ahr<sup>dbd/dbd</sup> (Targeted and Floxed) mice and wild-
type littermates was used to quantify the relative in vivo
expression levels of the AHRdbd protein, and showed that
although the Targeted allele produced hypomorphic expression
of the Ahr<sup>dbd</sup> protein as compared with wild-type littermates,
the amount of protein produced in the Floxed mice was
equivalent to wild-type littermates (Fig. 4D).

Characterization of AHRdbd Signaling In Vivo
To determine whether AHRdbd signals effectively in vivo,
Ahr<sup>dbd/dbd</sup> (Floxed) and wild-type mice were injected in-
traperitoneally with 100 μg/kg TCDD. After 6 days, liver
microsomes were isolated and analyzed for EROD activity.
Microsomes from wild-type mice showed a low basal EROD
activity that was induced 10-fold by TCDD. In contrast,
microsomes from Ahr<sup>dbd/dbd</sup> mice showed extremely low basal
EROD activity, which was unaltered by TCDD treatment,
indicating that AHRdbd lacks the ability to activate gene
transcription from DRE elements in vivo (Fig. 5).

Ahr<sup>dbd/dbd</sup> (Floxed) Mice Exhibit Developmental Defects
Similar to Ahr<sup>−/−</sup> Mice
Wild-type and Ahr<sup>dbd/dbd</sup> mice were examined for the
developmental phenotypes found previously in Ahr<sup>−/−</sup> mice
(Fernandez-Salguero et al., 1996, 1997; Gonzalez and
Fernandez-Salguero, 1998; McDonnell et al., 1996; Schmidt
et al., 1996; Lahvis and Bradfield, 1998; Lahvis et al., 2000;
Peters et al., 1999; Zaher et al., 1998). Tissue wet weights were
determined for liver, spleen, heart, thymus, and testis of
8-week-old male Ahr<sup>dbd/dbd</sup> and wild-type littermates. Similar
to Ahr-null mice, the Ahr<sup>dbd/dbd</sup> mice were found to exhibit

visualized using a BEAR3 primary antibody and a FITC-
conjugated secondary antibody. The AHR was found to
localize to the cytosol in untreated fibroblasts, but localized
to the nucleus within 2 h of treatment with 1nM TCDD.
Interestingly, the AHRdbd protein is constitutively nuclear in
the absence of ligand, and localization was not altered by
treatment with 1nM TCDD (Fig. 3B).
25% smaller livers than wild-type littermate controls. Conversely, the hearts and spleens were 25 and 58% larger, respectively, in these animals (p < 0.005, Fig. 6A). Histopathological analysis of livers taken from Ahr^d/dbd^ mice at postnatal days (PND) 7, 14, and 21 revealed a transient microvesicular steatosis around PND 7, which resolved by PND 14, and appeared identical to livers from age-matched Ahr^-/- mice (Fig. 6B and data not shown). Histopathological analyses were also performed on adult spleen, heart, thymus, testis, lung, colon, kidney, eye, and brain, but revealed no significant differences between Ahr^d/dbd^ and wild-type mice (data not shown). These findings are consistent with those reported in our previous work characterizing the Ahr^-/- mouse (Schmidt et al., 1996).

A consistent phenotype found in all Ahr^-/- mice is the presence of a ductous venosus (DV) throughout life (Lahvis et al., 2005). To determine whether Ahr^d/dbd^ mice exhibit a patent DV, the flow of contrast medium through the perfused liver was observed with the use of serial angiograms. In a wild-type littermate, contrast medium flowed into the portal vein and immediately into the portal branches of the liver (Fig. 6C). After filling the major branching veins of the liver, contrast entered the suprahepatic inferior vena cava (IVC) and then flowed retrograde, filling the infrahepatic IVC. However, in the Ahr^d/dbd^ mice, contrast flowed directly from the portal vein to the IVC. The DV in the Ahr^d/dbd^ mouse was clearly visible as a short segment that runs perpendicular to both the portal vein and the IVC. Trypan blue perfusion was also used to score for a patent DV. Whereas 0% of wild-type mice (0/6) showed a patent DV, 100% (6/6) of Ahr^d/dbd^ mice scored positive for this structure, a vascular pattern consistent with the frequency of patent DV seen in Ahr^-/- mice (Fig. 6D) (Lahvis et al., 2000).

Ahr^d/dbd^ Mice are Resistant to TCDD-Induced Toxicity

To determine the importance of DNA binding in the AHR-mediated physiologic response to TCDD, 4-week-old male Ahr^d/dbd^ mice, wild-type littermates, and Ahr^+/- mice were treated with 100 µg/kg TCDD. Mice were sacrificed 6 days later and assayed for hepatomegaly and thymic involution, two classic endpoints associated with TCDD toxicity in these animals. In response to TCDD, the Ahr^+/- mice (n = 10) showed a 23% increase and 59% decrease in liver and thymus weights, respectively (p < 0.001; Figs. 7A and 7B). The Ahr^+/- (n = 11) mice showed a 16% increase in liver weight (p < 0.001).
and a 51% decrease in thymus weight 6 days after TCDD exposure ($p < 0.001$). In contrast, the $Ahr^{dbd/dbd}$ mice ($n = 10$) showed no significant difference in liver or thymus weights ($p = 0.52$, and $p = 0.97$, respectively), mimicking the response seen in $Ahr^{-/-}$ mice (Schmidt et al., 1996).

TCDD is known to cause intrahepatic lipid accumulation in $Ahr$ wild-type but not $Ahr^{-/-}$ mice (Poland and Knutson, 1982; data not shown). We therefore qualitatively examined the presence of lipids in $Ahr^{dbd/dbd}$ mice by Oil Red-O staining. As expected, TCDD caused a significant increase in hepatic lipid content in $Ahr^{+/+}$ mice, and $Ahr^{-/-}$ mice accumulated lipid to the same degree. However, similar to $Ahr^{-/-}$ mice, $Ahr^{dbd/dbd}$ mice were resistant to the effects of TCDD and did not accumulate lipid (data not shown and Fig. 7C).

As TCDD is a potent teratogen, we sought to determine whether $Ahr^{dbd/dbd}$ mice were resistant to TCDD-induced cleft palate and hydronephrosis. Whereas 29% (10/35) of wild-type embryos (ED17–18) exhibited cleft palate upon TCDD treatment, $Ahr^{dbd/dbd}$ embryos were completely resistant (0/52). Similarly, 100% (35/35) of wild-type mice exhibited TCDD-induced hydronephrosis, but $Ahr^{dbd/dbd}$ mice were entirely resistant (0/52; Table 1).

**DISCUSSION**

Several reports have suggested that AHR-DRE binding may not be a requirement for TCDD signaling and toxicity (Blankenship and Matsumura, 1997; Enan and Matsumura, 1996; Ge and Elferink, 1998; Puga et al., 2000). The implication of these models is that the AHR may signal in a toxicologically relevant manner through protein interactions in the cytosolic or nuclear compartment. In this regard, it has been reported that the cytosolic cSrc protein tyrosine kinase becomes activated in response to TCDD in cell-free extracts of guinea pig adipose tissue and mouse NIH3T3 cells (Enan and...
This interaction was shown to be dependent on AHR, as activity was lower in AHR-immunodepleted extracts. An interaction of AHR with the retinoblastoma protein (pRB) has also been proposed. The AHR was shown to be immunoprecipitated in rat hepatoma 5L cells by antibodies to pRB, but in yeast and cell-free interaction analysis, only truncated forms of AHR showed significant interactions. In a second study, this interaction was reported to be important in G1 cell-cycle arrest and occurred only after ligand-bound AHR translocated to the nucleus (Ge and Elferink, 1998; Puga et al., 2000). A third proposed mechanism through which AHR may mediate toxicity is through a repression of NF-κB. Experiments in vitro and in cell culture have shown that AHR-NF-κB interactions may occur through direct binding of AHR and the RelA subunit (Tian et al., 1999). We and others have considered the notion that cross-talk occurs between the AHR and HIF-1α signaling pathways via their common dimerization partner, ARNT, the underlying idea being that TCDD toxicity may be the result of ARNT sequestration rather than AHR-ARNT-DRE interactions (Berghard et al., 1993; Chan et al., 1999; Gradin et al., 1996; Pollenz et al., 1999).

We hypothesized that if any of these models were correct, then related toxic responses to TCDD should occur in animal models harboring a correctly folded AHR protein with a mutation that prevents its binding to the DRE. Such a mutant should be capable of sequestering ARNT, as well as interacting with cSrc, pRB, and NF-κB, and yet be unable to activate transcription of DRE-driven genes. We therefore used homologous recombination to replace the endogenous bHLH region of the Ahr locus with a bHLH region carrying both an I25G mutation (SrfI site) and a GS insertion (BamHI site) at amino acid residue 39. This type of insertion mutation was generated with the idea that it would effectively shift the basic region out of the major groove of DNA without disrupting the dimerization capability of the HLH domain or the overlapping nuclear localization signal (Bacsi and Hankinson, 1996; Ikuta et al., 1998). Upon construction of the corresponding mutant cDNA, we found that the AHRdbd protein does in fact form a robust, ligand-inducible interaction with the ARNT protein in a ligand-dependent manner using mammalian two-hybrid analysis. Moreover, the resulting protein binds ligand and interacts with its known cellular chaperones, Hsp90 and ARA9. In keeping with our predicted impact on function, the AHRdbd protein was incapable of forming an AHRdbd/ARNT/DRE complex in a gel-shift analysis. Surprisingly, we found that although this mutation did not change amino acids thought to be directly involved in nuclear localization, it did appear to target the protein constitutively to the nuclear compartment. Although this finding implicates a disruption in ARA9- and HSP90-AHR interactions, Co-IP experiments demonstrate that these interactions were similar to those seen with wild-type AHR. Moreover, our two-hybrid experiments indicated that the AHRdbd still bound similar amounts of agonist upon exposure in cell culture.

In order to test the ability of Ahrβbd/βbd mice to signal in classical xenobiotic adaptation pathways, we quantified EROD activity following TCDD exposure. We found that Ahrβbd/βbd mice failed to show an increase in EROD activity in response

**FIG. 6.** Developmental phenotype of Ahrβbd/βbd mice. (A) Relative organ wet weights of Ahrβbd/βbd mice (white bars) and wild-type littermates (black bars) sacrificed at 8 weeks of age (n = 5). *Indicates p < 0.01 by Student’s t-test (wild-type versus Ahrβbd/βbd). (B) Representative H&E sections of livers from 7-day-old wild-type (littermate), Ahrβbd/βbd, and Ahr−/− mice (40× magnification). (C) Time-lapse angiography of wild-type (top row) and Ahrβbd/βbd (bottom row) littersmates. Arrows identify key features as follows: BV, branching vessel; PV, portal vein; shIVC, suprahepatic inferior vena cava; ihIVC, infrahepatic inferior vena cava. Total time elapsed from the first panel to the last is approximately 10 s. (D) Incidence of patent DV in wild-type and Ahrβbd/βbd male mice as measured by trypan blue perfusion.
to TCDD, indicating that DRE-mediated transcriptional events are eliminated in these mice. We also assayed for several of the known developmental defects observed in Ahr$^{-/-}$ mice and found that Ahr$^{dbd/dbd}$ mice are identical to Ahr$^{-/-}$ mice in all of these aspects, including a patent DV. The DV is a portal-systemic shunt that connects the umbilical cord blood with blood from both the portal vein and inferior vena cava (Schermerhorn et al., 1996). This structure normally resolves shortly after birth, yet remains open in Ahr$^{-/-}$ mice. Similar to Ahr$^{-/-}$ mice, Ahr$^{dbd/dbd}$ mice display a patent DV in addition to a transient perinatal microvesicular steatosis in hepatocytes and a 25% reduction in liver weight.

To determine whether Ahr$^{dbd/dbd}$ mice are sensitive to TCDD-induced toxicity, a number of classical toxic responses to TCDD were also quantified. We observed that Ahr$^{dbd/dbd}$ mice failed to exhibit the obvious liver and thymic toxicity normally associated with this dosing regimen. We also found that similar to the Ahr-null, TCDD-induced cleft palate, hydronephrosis, and intrahepatic lipid accumulation were nonexistent in these mice.

Although the AHRdbd protein appears to be constitutively nuclear in the absence of ligand, we showed previously that a mutation introduced at the nuclear localization sequence also causes abnormal liver development and renders mice resistant to TCDD-induced toxicity (Bunger et al., 2003). Therefore, it is unlikely that the cytosolic interactions lost due to localization are important in the developmental or toxic phenotypes of these animals, including those of cSrc, RelA, and pRB. Furthermore, in combination with our previously reported observations on Ahr$^{nls/nls}$ mice, these results convincingly show that nuclear localization alone is necessary but not sufficient for Ahr to function in development and toxicity. Therefore, sequestration of ARNT within the nucleus as a mechanism of toxicity is unlikely. However, we do not rule out the possibility that Ahr$^{dbd/dbd}$ mice may still show responsiveness to TCDD at other endpoints not tested, such as tumor promotion.

We show here that AHR-mediated XME induction may be directly related to a toxic response. Ahr$^{dbd/dbd}$ mice express an AHR that can be ligand-activated to form a heterodimer with the ARNT protein in a similar manner to wild-type AHR, but

![FIG. 7. TCDD-induced phenotypic changes in Ahr$^{dbd/dbd}$ mice. (A) Hepatomegaly (expressed as relative liver weight) and (B) thymic involution (expressed as relative thymus weight) of DMSO- or TCDD-treated Ahr$^{+/+}$ (n = 10), Ahr$^{+/-}$ (n = 11), and Ahr$^{dbd/dbd}$ (n = 10) mice as quantified 6 days after a single i.p. injection of p-dioxane (white bars) or 100 μg/kg TCDD (black bars). *Indicates p < 0.001. (C) Intrahepatic lipid accumulation. Frozen sections from DMSO- or TCDD-treated Ahr$^{+/+}$, Ahr$^{+/-}$, and Ahr$^{dbd/dbd}$ mice were stained with Oil Red-O (lipids, red) and hematoxylin (nuclei, blue).]
cannot bind to DREs and activate XME expression. The fact that these mice fail to exhibit a toxic response suggests that DRE-driven gene expression is indeed upstream of the physiologic effects of TCDD and that ARNT sequestration may in fact not play a significant role in the TCDD-induced toxic endpoints assessed here. Furthermore, a developmental phenotype of the Ahr
\textsuperscript{db/dab} mice consistent with that of Ahr
\textsuperscript{−/−} mice suggests that DRE-driven genes are also involved in early liver development and vascular remodeling.

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