We cloned from mouse hepatoma cells a cDNA which encodes the Ah receptor nuclear translocator (Arnt). Sequence comparisons reveal 89% nucleotide and 92% amino acid identity between mouse and human Arnt. Transfection of the cDNA into Arnt-defective mouse hepatoma cells fully restores their responsiveness to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), indicating that the cDNA encodes a functional Arnt protein. Transfection of the cDNA into wild type mouse hepatoma cells increases the magnitude, but not the sensitivity, of the transcriptional response to TCDD. Analyses of mutants indicate that Arnt has a modular organization. The unit that mediates both heterodimerization with the liganded Ah receptor and DNA recognition is functionally distinct from the unit that mediates transcriptional activation. A 96-amino acid, C-terminal domain of Arnt, which includes a glutamine-rich region, confers transcriptional activation capability upon the protein.

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, \(^{1}\) dioxin) is the prototype for a class of potentially toxic halogenated aromatic hydrocarbons, which share a similar mechanism of action but differ in potency. In animals, TCDD produces a spectrum of adverse metabolic, reproductive, immunologic, and neoplastic effects (Poland and Knutson, 1982; Safe, 1986). TCDD’s risk to human health is uncertain; its potential reproductive and carcinogenic effects are of particular concern (Bailar, 1981; Johnson, 1992; Peterson et al., 1993).

TCDD-responsive cells contain an intracellular protein, designated as the aromatic hydrocarbon (Ah) receptor, which binds the compound saturably and with high affinity (Poland et al., 1982). Biochemical and genetic studies implicate the Ah receptor (AhR) in TCDD’s biological effects (Poland and Knutson, 1982; Okey et al., 1993; Swanson and Bradfield, 1993). Mechanistic studies, using the induction of cytochrome P4501A1 (CYP1A1) as a model response, indicate the liganded AhR activates gene transcription (Whitlock, 1993). Cloning of its cDNA reveals that the AhR appears to be a basic helix-loop-helix (bHLH) type of transcription factor (Burbach et al., 1992; Ema et al., 1992).

Genetic analyses of mouse hepatoma cells that respond poorly to TCDD implicate a second protein in dioxin action (Hankinson, 1983; Miller et al., 1983). This second protein has been termed the Ah receptor nuclear translocator (Arnt); cloning of human Arnt cDNA reveals that it contains a bHLH motif (Hoffman et al., 1991). Neither the liganded AhR nor Arnt binds to DNA by itself (Reyes et al., 1992; Whitelaw et al., 1993; Matsushita et al., 1993). In vitro studies imply that the liganded AhR and Arnt heterodimerize via their bHLH domains to generate a DNA-binding species (Whitelaw et al., 1993). The AhR/Arnt heteromer activates transcription by binding to specific recognition sites designated as dioxin-responsive elements (DRE), which are located within an enhancer upstream of the CYP1A1 gene (Denison et al., 1989; Fisher et al., 1990).

AhR and Arnt contain regions with sequence similarity to the Drosophila Per and Sim proteins; by analogy with findings for Per (Huang et al., 1993), these “PAS” domains may contribute to protein-protein interactions between AhR and Arnt. In addition, AhR and Arnt contain regions with multiple glutamine residues; by analogy with findings for the transcription factor Sp1 (Courey and Tjian, 1988), these glutamine-rich regions might represent transcription activation domains.

To analyze the functional organization of Arnt in greater detail, we have isolated mouse Arnt cDNA, determined its nucleotide and deduced amino acid sequences, and verified by transfection that it encodes a functional Arnt protein. We demonstrate that Arnt has a modular organization; the 96 amino acids comprising the C terminus of Arnt confers transcription activation capability upon the protein and functions independently of the domains responsible for heterodimerization and DNA binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and other DNA modifying enzymes (Klenow, T4 DNA ligase, polynucleotide kinase, and Vent polymerase) were purchased from Life Technologies, Inc. and New England Biolabs (Beverly, MA). Radioactive compounds (\(^{32}\)P)ATP, \(^{35}\)S]ATP, \(^{32}\)P]chloramphenicol, \(^{35}\)S]methionine) were from Amer sham Corp. Guanidinium isothiocyanate and cesium chloride were from Sigma. PolyATtract mRNA Isolation System was from Promega (Madison, WI). ZAP-cDNA Synthesis Kit, ZAP Express\(^{\circledR}\) EcoRI/xotl vector, Gigapack\(^{\circledR}\)II Packaging Extract, the ExAssist\(^{\circledast}\)SOLR\(^{\circledast}\) System, and Prime-H\(^{\circledast}\)II Random Primer Kit were from Stratagene (La Jolla, CA). Nitrocellulose membranes were from Millipore (Bedford, MA). Se- quenase 2.0 Kit was from United States Biochemical Corp. (Cleveland, OH). Reagents for chloramphenicol acetyltransferase and \(\beta\)-galactosidase assays, as well as in vitro transcription/translation kit, were from Promega (Madison, WI). The reagent for measuring protein concentration was from Bio-Rad. Cell culture materials were from Life Technologies Inc. 2,3,7,8-tetrachlorodibenzo-p-dioxin was from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Polybrene (hexadimethrine bromide) was from Aldrich.

**Cell Culture**—Wild type (Hepa 1c1c7), Arnt-defective (BPc1), and AhR-defective (TAOclBPc1) mouse hepatoma cells were cultured in a minimal essential medium containing 10% fetal calf serum, as described previously (Miller et al., 1983).
FIG. 1. Structure of mouse Arnt cDNA and protein. Panel A, nucleotide sequence of cDNA and deduced amino acid sequence of mouse Arnt (mArnt). The deduced amino acid sequence of human Arnt (hArnt) is shown for comparison; dots indicate positions of amino acid identity. Panel B, structural motifs in the Arnt protein. bHLH, basic helix-loop-helix domain; PAS, domains that exhibit homology with Per, receptor, and Sim; 2-rich, glutamine-rich domain.
Activation of Transcription by Mouse Arnt

**Construction of a cDNA Library—Poly(A) + mRNA was prepared after lysis of wild type mouse hepatoma cells in guanidinium isothiocyanate. A cDNA library was synthesized using a ZAP-cDNA Synthesis Kit and linker-primer and reverse transcribed with Maloney murine leukemia virus reverse transcriptase in the presence of 5-methyl dCTP. The RNA-DNA hybrid was digested with RNase H, and double-stranded cDNA was synthesized using DNA polymerase I, using recommended conditions (Stratagene). EcoRI adaptors were attached using oligo(dT) primers based upon the human 5'-CCA AAT TCA CAG ATT TCA CAC TCC AAC CCT GCC CAG GGA TCA GCG CCG ACC TGG ACC TCT AGC TCC CGC CCA GGC TTT GCC 1905

**Table 1—continued**

| 1  | bHLH | PAS I | PAS II | Q-rich 776 |
|----|------|-------|--------|-----------|

**Construction of the cDNA Library**—A cDNA corresponding to a portion of Arnt was synthesized from wild type mouse hepatoma cells total RNA using an RT-PCR kit (Perkin-Elmer Cetus) and degenerate oligonucleotide primers based upon the Arnt sequence (Hoffman et al., 1991). The primers used were: GTCAGGATCTTGCACTAAGAGTCGACGTGAAGATAACCC (nucleotides 276-294) and CTGATCTAGAAGCCATCTTCATGAGTTCA (nucleotides 468-482) containing BamHI and XbaI sites, respectively, which are underlined. The RT reaction was carried out in a thermal cycler in 20 μL containing 1 μg of total RNA and 2.5 μl oligo(dT) sub primer, using conditions suggested by the manufacturer. PCR was performed in 100 μL containing 200 pmol of each primer, 20 pmol of dNTPs, and 2.5 units of Taq polymerase, under the following conditions: 94 °C, 2 min; 55 °C, 0.5 min; 72 °C, 1 min; 1 cycle and then 94 °C, 1 min; 55 °C, 0.5 min; 72 °C, 1 min plus 5 additional seconds in each subsequent cycle; 34 cycles.

Labeled hybridization probes were generated using [α-32P]dATP, random primers, and exonuclease-deficient Klenow DNA polymerase and were used to screen 1 x 10^9 plaque forming units, using recommended conditions (Strategene). Positive clones, contained within the pBluescript phagemid (a eukaryotic expression vector), were excised in the EcoRI/BamHI Site of ZAP Express Vector, and packaged using Gigapack II gold extract.

Screening of the cDNA Library—A cDNA corresponding to a portion of Arnt was synthesized from wild type mouse hepatoma cells total RNA using an RT-PCR kit (Perkin-Elmer Cetus) and degenerate oligonucleotide primers based upon the Arnt sequence (Hoffman et al., 1991). The primers used were: GTCAGGATCTTGCACTAAGAGTCGACGTGAAGATAACCC (nucleotides 276-294) and CTGATCTAGAAGCCATCTTCATGAGTTCA (nucleotides 468-482) containing BamHI and XbaI sites, respectively, which are underlined. The RT reaction was carried out in a thermal cycler in 20 μL containing 1 μg of total RNA and 2.5 μl oligo(dT) sub primer, using conditions suggested by the manufacturer. PCR was performed in 100 μL containing 200 pmol of each primer, 20 pmol of dNTPs, and 2.5 units of Taq polymerase, under the following conditions: 94 °C, 2 min; 55 °C, 0.5 min; 72 °C, 1 min; 1 cycle and then 94 °C, 1 min; 55 °C, 0.5 min; 72 °C, 1 min plus 5 additional seconds in each subsequent cycle; 34 cycles.

Labeled hybridization probes were generated using [α-32P]dATP, random primers, and exonuclease-deficient Klenow DNA polymerase and were used to screen 1 x 10^9 plaque forming units, using recommended conditions (Strategene). Positive clones, contained within the pBluescript phagemid (a eukaryotic expression vector), were excised in the EcoRI/BamHI Site of ZAP Express Vector, and packaged using Gigapack II gold extract.

**Generation of Deletion Mutants—**To generate deletion mutants, we used a PCR-based method (Higuchi et al., 1988). The same 5'-primer was used in each case, containing a BamHI restriction site and nucleotides 1–17 of mouse Arnt (underlined): 5'-CCCGGCGATCTTCATG-GCGGCGGACTACAGCG-3'.
BanHI and XbaI sites of the pBK-CMV expression vector. -3’, which contains a XbaI restriction site and nucleotides 1-15 of ACCGGGCTACTCGAGTTCGGAL~AAGGGGGGA~”~’, which contains a XbaI restriction site and nucleotides 2311-2328 of mouse Arnt (underlined).

To generate the mutant designated “-Q” (see Fig. 4A), the 3’-primer was 5’-AATCTAGACCGCGGCCGGCAGCAGTACCAGATGAGGC-3’, which contains a XbaI restriction site and nucleotides 1423-1443 of mouse Arnt (underlined); 5’-ATACCCGGGCTACTCGACCTCCCAGGAAAAAGGGGGAAAA-3’, which contains an ApaI restriction site and nucleotides 2311-2328 of mouse Arnt (underlined). Q: 5’-GTAGGATCCAAGCmCTAGAATGGCCTACCCTGCTAC-3’, which contains a XbaI restriction site and nucleotides 1390-1410 of mouse Arnt (underlined).

Construction of GAL4-Arnt Fusion cDNAs—A GAL4 DNA-binding domain (amino acids 1-147, see Carey et al., 1990) was generated by PCR, digested with HindIII and XbaI, and ligated between the HindIII and XbaI sites of the pBK-CMV expression vector.

To generate the mutant designated “bHLH” (see Fig. 4A), the 3’-primer was 5’-AATCTAGACCGCGGCCGGCAGCAGTACCAGATGAGGC-3’, which contains a XbaI restriction site and nucleotides 430-450 of mouse Arnt (underlined).

The PCR reaction contained (in 100 µl): 50 pmol of primers, 4 fmol of Arnt template, 25 nmol of dNTPs, and 4 units of Vent polymerase (New England Biolabs). The following PCR conditions were used: 94 °C, 5 min; 1 cycle, then 94 °C, 1 min, 55 °C, 1 min, 72 °C, 2 min; 35 cycles, then 94 °C, 1 min, 55 °C, 1 min, 72 °C, 4 min; 1 cycle. The PCR products were digested with BamHI and XbaI and were ligated between the BamHI and XbaI sites of the pBK-CMV expression vector.

Assay—Transfection was carried out using Polybren, as described previously ( Fisher et al., 1990 ). The cloned mouse Arnt cDNA or its mutants were cotransfected with a eukaryotic β-galactosidase expression vector, pCH110 (Promega), and a reporter gene, either pMCat5.9, which contains a CAT gene downstream of five copies of the GAL4 DNA-binding site, or pG5ETCAT, which contains a CAT gene downstream of five copies of the GAL4 DNA-binding site (Carey et al., 1990). The transfected cells were treated with 1 μM TCDD as indicated, for 18 h prior to harvest. 48 h after transfection, cells were harvested in Reporter Lysis Buffer (Promega). CAT activity was measured using a differential extraction/liquid scintillation assay, according to the manufacturer’s suggested protocol (Promega). β-Galactosidase activity was measured to control for differences in transfection efficiency.

In Vivo Transcription/Translation—Full-length and mutant mouse Arnt cDNAs in the pBK-CMV vector were used for expression from the T3 promoter. Full-length, functional mouse AhR cDNA was cloned using PCR and was inserted into the pIC-CMV vector ( Invitrogen, San Diego, CA).
Activation of Transcription by Mouse Arnt

FIG. 4. Analysis of Arnt by deletion mutation. Panel A, structure of the deletion mutants. Panel B, functional analysis. Full-length (FL) and mutant cDNAs were transfected into Arnt-defective mouse hepatoma cells and analyzed as described in the legend to Fig. 2. The data represent the mean of duplicate measurements within a single experiment; brackets indicate the range of values. Similar results were obtained in three additional experiments.

Cloning of Mouse Arnt cDNA—We generated a cDNA fragment of mouse Arnt using RT-PCR and primers based upon the human cDNA sequence. The fragment was used to isolate a 2537-base pair cDNA from a mouse hepatoma cell library. Comparison of its deduced amino acid sequence with that of human Arnt reveals that the cDNA encodes the shorter form of Arnt, expressed proteins were analyzed using an electrophoretic mobility shift assay, as described below.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSA was performed as described previously, using as a DNA probe the recognition sequence for the AhR/Arnt heteromer designated DRE D (Lusaka et al., 1993). Preparation of hepatoma cell nuclear extract, 32P-labeling of DNA using T4 polynucleotide kinase, electrophoresis, and autoradiography were as described previously (Denison et al., 1989). The in vitro translated proteins were incubated with poly(dIdC) for 15 min at room temperature (~23°C), followed by the addition of radiolabeled probe, further incubation for 20 min, and electrophoresis.

RESULTS

Cloning of Mouse Arnt cDNA—We used transient transfection to analyze the function of the cloned cDNA. The plasmid pMcat5.9, which contains the chloramphenicol acetyltransferase gene under the control of a TCDD-responsive, AhR-dependent, Arnt-dependent transcriptional enhancer, was cotransfected as a reporter system. Transfections into Arnt-defective cells produced no change in their responsiveness to TCDD, complementing the defect in these cells; these findings indicate that the cloned cDNA restores responsiveness to TCDD, complementing the defect in these cells; these findings indicate that the cloned cDNA encodes a functional Arnt protein (Fig. 2). In contrast, transfection of the cDNA into Ah receptor-defective cells produces no change in their responsiveness to TCDD (data not shown). Thus, the complementation is specific for the Arnt-defective cells.

Transfection of the cDNA into wild type cells increases the maximal level of TCDD-inducible CAT expression 2-3-fold (Fig. 2). Thus, an increase in the intracellular Arnt concentration is associated with an increase in the extent to which a target gene responds to TCDD. This finding implies that the concentration of Arnt limits the magnitude of TCDD-induced gene expression in this cell system. This observation led us to ask whether the Arnt concentration also influences the sensitivity of a target gene to TCDD. Dose-response experiments (Fig. 3) indicate that increased Arnt expression in wild type cells does not alter

CA) for expression from the T7 promoter; a synthetic Kozak consensus sequence (Kozak, 1987) was inserted immediately upstream of the initiation methionine codon of the AhR.

In vitro transcription and translation were performed at 30°C for 90 min, using TnT*-coupled reticulocyte lysate (Promega), as recommended by the manufacturer. Expression of the cDNAs was verified by including [35S]methionine in the incubations and analyzing the proteins by SDS-polyacrylamide gel electrophoresis and autoradiography. The expressed proteins were analyzed using an electrophoretic mobility shift assay, as described below.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSA was performed as described previously, using as a DNA probe the recognition sequence for the AhR/Arnt heteromer designated DRE D (Lusaka et al., 1993). Preparation of hepatoma cell nuclear extract. 32P-labeling of DNA using T4 polynucleotide kinase, electrophoresis, and autoradiography were as described previously (Denison et al., 1989). The in vitro translated proteins were incubated with poly(dIdC) for 15 min at room temperature (~23°C), followed by the addition of radiolabeled probe, further incubation for 20 min, and electrophoresis.

bHLH

Panel A, structure of the deletion mutants. Panel B, functional analysis. Full-length (FL) and mutant cDNAs were transfected into Arnt-defective mouse hepatoma cells and analyzed as described in the legend to Fig. 2. The data represent the mean of duplicate measurements within a single experiment; brackets indicate the range of values. Similar results were obtained in three additional experiments.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSA was performed as described previously, using as a DNA probe the recognition sequence for the AhR/Arnt heteromer designated DRE D (Lusaka et al., 1993). Preparation of hepatoma cell nuclear extract, 32P-labeling of DNA using T4 polynucleotide kinase, electrophoresis, and autoradiography were as described previously (Denison et al., 1989). The in vitro translated proteins were incubated with poly(dIdC) for 15 min at room temperature (~23°C), followed by the addition of radiolabeled probe, further incubation for 20 min, and electrophoresis.

RESULTS

Cloning of Mouse Arnt cDNA—We generated a cDNA fragment of mouse Arnt using RT-PCR and primers based upon the human cDNA sequence. The fragment was used to isolate a 2537-base pair cDNA from a mouse hepatoma cell library. Comparison of its deduced amino acid sequence with that of human Arnt reveals that the cDNA encodes the shorter form of Arnt, expressed proteins were analyzed using an electrophoretic mobility shift assay, as described below.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSA was performed as described previously, using as a DNA probe the recognition sequence for the AhR/Arnt heteromer designated DRE D (Lusaka et al., 1993). Preparation of hepatoma cell nuclear extract, 32P-labeling of DNA using T4 polynucleotide kinase, electrophoresis, and autoradiography were as described previously (Denison et al., 1989). The in vitro translated proteins were incubated with poly(dIdC) for 15 min at room temperature (~23°C), followed by the addition of radiolabeled probe, further incubation for 20 min, and electrophoresis.

bHLH

Panel A, structure of the deletion mutants. Panel B, functional analysis. Full-length (FL) and mutant cDNAs were transfected into Arnt-defective mouse hepatoma cells and analyzed as described in the legend to Fig. 2. The data represent the mean of duplicate measurements within a single experiment; brackets indicate the range of values. Similar results were obtained in three additional experiments.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSA was performed as described previously, using as a DNA probe the recognition sequence for the AhR/Arnt heteromer designated DRE D (Lusaka et al., 1993). Preparation of hepatoma cell nuclear extract, 32P-labeling of DNA using T4 polynucleotide kinase, electrophoresis, and autoradiography were as described previously (Denison et al., 1989). The in vitro translated proteins were incubated with poly(dIdC) for 15 min at room temperature (~23°C), followed by the addition of radiolabeled probe, further incubation for 20 min, and electrophoresis.

bHLH

Panel A, structure of the deletion mutants. Panel B, functional analysis. Full-length (FL) and mutant cDNAs were transfected into Arnt-defective mouse hepatoma cells and analyzed as described in the legend to Fig. 2. The data represent the mean of duplicate measurements within a single experiment; brackets indicate the range of values. Similar results were obtained in three additional experiments.

Electrophoretic Mobility Shift Assay (EMSA)—The EMSA was performed as described previously, using as a DNA probe the recognition sequence for the AhR/Arnt heteromer designated DRE D (Lusaka et al., 1993). Preparation of hepatoma cell nuclear extract, 32P-labeling of DNA using T4 polynucleotide kinase, electrophoresis, and autoradiography were as described previously (Denison et al., 1989). The in vitro translated proteins were incubated with poly(dIdC) for 15 min at room temperature (~23°C), followed by the addition of radiolabeled probe, further incubation for 20 min, and electrophoresis.

bHLH

Panel A, structure of the deletion mutants. Panel B, functional analysis. Full-length (FL) and mutant cDNAs were transfected into Arnt-defective mouse hepatoma cells and analyzed as described in the legend to Fig. 2. The data represent the mean of duplicate measurements within a single experiment; brackets indicate the range of values. Similar results were obtained in three additional experiments.
A labeled wild type with TCDD (20 nM, 2 h) as indicated and were analyzed by EMSA, using Mouse Arnt cDNA and mouse for comparison. The bHLH domain of Arnt appears to mediate heterodimerization and induces the concentration of TCDD at which induction of CAT activity is half-maximal. Thus, Arnt influences the magnitude of the induction response but not the sensitivity of the response to the inducer.

**Transcription Activation Function of Arnt**—Transcription factors are often modular and contain domains that are functionally distinct (Ptashne, 1988; Tjian and Maniatis, 1993). The bHLH domain of Arnt appears to mediate heterodimerization and DNA recognition (Whitelaw, 1993). Here, we have asked whether Arnt also has a transcriptional activation domain and, if so, whether it is functionally distinct from the domain that mediates heterodimerization and DNA binding.

To address this issue, we first constructed several mutants, which contained progressively larger deletions from the C terminus of Arnt (Fig. 4A). We tested the mutants for function by cotransfection into Arnt-defective cells, together with the CAT reporter plasmid. Our findings (Fig. 4B) reveal that deletion of 96 amino acids from the C terminus (generating the mutant designated as -Q, because the deleted segment contains a glutamine-rich region) is associated with a 70–80% decrease in TCDD-inducible, Arnt-dependent CAT activity. This observation indicates that the deleted region contains a domain(s) that makes a substantial contribution to Arnt function and TCDD responsiveness. Deletion of an additional 210 amino acids from the C terminus (generating the mutant designated as -CT) produces no further loss of TCDD-inducible, Arnt-dependent CAT expression. Therefore, this region makes no obvious contribution to Arnt function. Deletion of still another 320 amino acids (generating the mutant designated as bHLH) results in the loss of essentially all Arnt-dependent CAT activity. This observation implies that Arnt contains a second functional domain, which is located within the deleted region. Notably, the deleted region contains the two PAS homologies.

We performed several additional analyses to characterize the deletion mutants in greater detail. First, we used an in vitro transcription/translation system, combined with an electrophoretic mobility shift assay, to determine whether the mutant Arnt proteins interact with AhR to form a complex with the correct DNA recognition characteristics. Control experiments (Fig. 5A) indicate that both Arnt and AhR are required to generate a TCDD-inducible protein-DNA complex at a dioxin-responsive element. Thus, this assay displays the Arnt dependence, AhR dependence, TCDD dependence, and DNA sequence dependence that is characteristic of the binding of the AhR/Arnt heteromer to DNA.

Analyses of the full-length and mutant Arnt cDNAs reveal that removal of 96 amino acids from the C-terminal of Arnt does not impair the ability of the truncated protein to participate in the formation of a TCDD-inducible protein-DNA complex with the DRE (Fig. 5B). This observation implies that the mutant protein retains its capacity both to heterodimerize with the liganded AhR and to specifically recognize the DRE. Therefore, the failure of the mutant protein to fully complement the Arnt-defective cells (Fig. 4B) must reflect the loss of some other function. Deletion of an additional 210 amino acids generates a noticeably smaller protein-DNA complex in the EMSA; however, the deletion has no apparent effect on the ability of Arnt to heterodimerize or to bind DNA (Fig. 5B). This observation is consistent with the transfection studies, which revealed no further loss of Arnt function associated with this deletion (Fig. 4B). The deletion of still another 320 amino acids abolishes the ability of Arnt to form a TCDD-inducible protein-DNA complex (Fig. 5B). This finding suggests that the deleted region is required for heterodimerization and/or DNA binding. Loss of either capability can account for the absence of function in the transfection experiments (Fig. 4B).

These findings led us to hypothesize that the 96 amino acid segment comprising the C terminus of Arnt contains a transcriptional activation domain. To test this idea directly, we analyzed several fragments of Arnt, using an approach designed to measure its transcriptional activation function independent of its DNA binding function. Our findings (Fig. 6) reveal that the full-length Arnt protein exhibits substantial activity as a transcriptional activator; the activity is independent of TCDD, implying that it is inherent to Arnt and does not involve the AhR. Deletion of the C-terminal 96 amino acids

---

**Fig. 5. Heterodimerization and DNA recognition capabilities of full-length Arnt and mutants.** Panel A, control experiments. Mouse Arnt cDNA and mouse AhR cDNA were transcribed/translated in vitro in approximately equimolar ratios. The proteins were incubated with TCDD (20 nM, 2 h) as indicated and were analyzed by EMSA, using a 32P-labeled wild type DRE as the DNA recognition sequence. Unlabeled wild type (WT) or mutant (M) competitor DNA was added in 100-fold molar excess, as indicated. Nuclear extracts (NE) from uninduced and TCDD-induced (1 nM, 2 h) wild type cells were also analyzed, for comparison. The arrows indicate the position of the TCDD-inducible, Arnt-dependent, AhR-dependent protein-DNA complex. Panel B, analysis of full-length and mutant Arnt cDNAs. The cDNAs designated as FL, -Q, -CT, and bHLH are diagrammed in Fig. 4A. They were analyzed as described in panel A above.
Activation of Transcription by Mouse Arnt

Fig. 6. Transcriptional activation function of Arnt. Panel A, structure of fusion proteins. Panel B, functional analyses. The GAL4-Arnt fusion cDNAs were transfected into wild type cells together with the reporter plasmid pG5ETCAT and were analyzed as described in the legend to Fig. 2. The data represent the mean of duplicate measurements within a single experiment; brackets indicate the range of values. Similar results were obtained in two additional experiments.

from Arnt is associated with the loss of 90–95% of the transcriptional activation function (Fig. 6). Furthermore, the 96 amino acid fragment by itself (designated as Q) exhibits activity similar to that of full-length Arnt. A larger C-terminal fragment of Arnt (designated as CT) has activity comparable to that of the 96 amino acid fragment. Again, these activities are independent of TCDD and, therefore, presumably do not involve the AhR. These findings indicate that Arnt has a transcriptional activation domain, which is located within 96 amino acids of its C terminus. Furthermore, the transcriptional activation domain is functionally distinct from the domain(s) that mediates heterodimerization and DNA binding.

DISCUSSION

We have cloned Arnt cDNA from mouse hepatoma cells. Sequence comparisons imply that the mouse and human Arnt proteins are very similar in structure. In particular, the bHLH and PAS domains of Arnt are essentially identical in mouse and human. Their conservation of primary structure among species implies that these domains are functionally important. This conclusion is consistent with the finding that deletion of the bHLH domain of Arnt is associated with loss of responsiveness to TCDD, probably because the mutant cannot heterodimerize with the liganded AhR (Whitelaw et al., 1993). Here, we find that a C-terminal deletion that removes the PAS domains of Arnt abolishes the TCDD-inducible signal in the EMSA. Thus, the deletion affects Arnt's heterodimerization and/or DNA binding capability. Because PAS domains appear to participate in protein-protein interactions (Huang et al., 1993), our observation suggests that the PAS domains of Arnt are required for heterodimerization with AhR. However, this hypothesis requires additional study.

Expression of Arnt cDNA in Arnt-defective cells restores their responsiveness to TCDD, indicating that the cDNA encodes a functional protein. Expression of Arnt cDNA in wild type cells increases the magnitude of the response to TCDD. This finding implies that the intracellular concentration of Arnt is a variable that can limit the extent to which a target gene responds to TCDD. Because Arnt functions in partnership with AhR, we infer that the concentration of Arnt in wild type cells does not vastly exceed that of AhR and that AhR-Arnt heterodimerization obeys the law of mass action. Our findings also imply that conditions which alter the effective Arnt concentration have the potential to affect the magnitude of the response to TCDD. For example, the level of Arnt gene expression or the presence of dominant inhibitory protein partners for Arnt would influence its effective concentration within the cell. Differences among tissues in such parameters may contribute to tissue-specific variation in the responses that TCDD elicits. Our analyses imply that Arnt, like other transcription factors, has a modular structure and is organized into distinct units, which can function independently of each other. For example, our analyses of transcriptional activation indicate that a 96-amino-acid, C-terminal segment of Arnt functions as an independent unit and exhibits full activity even when separated from the heterodimerization/DNA-binding segment(s) of the protein. We speculate that additional proteins may use these same independent functional units in different combina-
tions and in other transcriptional regulatory contexts.

The relationship between protein structure and transcriptional activation is poorly understood; several different protein motifs are capable of activating transcription (Hahn, 1993). The 96 amino acid C terminus of Arnt contains a glutamine-rich region and, in this respect, resembles the transcriptional activation domain of Sp1 (Courey and Tjian, 1988). However, the contribution(s) that the glutamine residues make to transcriptional activation is unclear, and it is possible that other amino acids play more important roles in the process (Gill et al., 1994). Deletion of 96 amino acids from the C terminus of Arnt decreases its transcriptional activation capability by >70–80%. Therefore, we hypothesize that the AhR contains a domain(s) that can partially compensate for the effect of the Arnt deletion.

Transcriptional activation presumably reflects a protein-protein interaction between Arnt and another component of the transcriptional apparatus. However, the protein(s) that Arnt contacts is unknown. By analogy with findings for Sp1 (Emili et al., 1994), Arnt may touch the TATA-binding protein directly and stabilize its binding to the CYP1A1 promoter. A second possibility (again, by analogy with findings for Sp1 (Pugh and Tjian, 1990)) is that Arnt contacts a coactivator protein that functions as an intermediary between Arnt and TATA-binding protein. With respect to the latter possibility, we note that DNA-protein cross-linking studies reveal a 110-kDa protein associated with the DNA-bound AhR/Arnt heteromer (Elferink et al., 1990); furthermore, a 110-kDa protein copurifies with AhR and Arnt during DNA recognition site chromatography (Elferink and Whitlock, 1994). We hypothesize that this 110-kDa protein is a coactivator, which Arnt contacts in the process of activating transcription.

The dioxin-responsive enhancer upstream of the CYP1A1 gene contains multiple binding sites for the AhR/Arnt heteromer. Deletion analyses of the enhancer indicate that the multiplicity of binding sites produces a synergistic effect on TCDD-inducible gene expression (Fisher et al., 1990). We hypothesize that the transcriptional activation function of Arnt accounts for this synergy, in that the binding of multiple AhR/Arnt heteromers to the enhancer could permit several Arnt molecules to simultaneously contact the transcriptional machinery at the CYP1A1 promoter to stabilize it in an active configuration (for discussion, see Lin et al., 1990; Carey et al., 1990). An analogous mechanism may account for the synergy between AhR/Arnt and Sp1 (Fisher et al., 1990). These hypotheses can be tested once the additional protein(s) that Arnt contacts are known.

Acknowledgments—We appreciate the advice of Qiang Ma. We thank Margaret Tuggle for secretarial assistance and Kent Wright for comments on the manuscript.

REFERENCES

Bailar, J. C., III (1991) N. Engl. J. Med. 324, 260–262
Burbach, K. M., Poland, A., and Bradfield, C. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8185–8189
Carey, M., Lin, Y.-S., Green, M. R., and Ptasnke, M. (1990) Nature 345, 361–364
Courey, A. J., and Tjian, R. (1988) Cell 55, 887–898
Dahl, A., Fisher, J. M., and Whitlock, J. P., Jr. (1989) J. Biol. Chem. 264, 16478–16482
Dulan, L., Cheyroux, A., and Algâl, M. (1989) Nucleic Acids Res. 17, 2873
Elferink, C. J., Gesterwickz, T. A., and Whitlock, J. P., Jr. (1990) J. Biol. Chem. 265, 29708–29712
Elferink, C. J., and Whitlock, J. P., Jr. (1994) Receptor 4, 157–173
Fisher, J. M., Jones, R. W., and Whitlock, J. P., Jr. (1990) Mol. Carcinogenesis 1, 216–221
Fisher, J. M., Wu, L., Denison, M. S., and Whitlock, J. P., Jr. (1990) J. Biol. Chem. 265, 3676–3681
Gill, G., Paschal, E., Tseng, Z. H., and Tjian, R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 192–196
Hahn, S. (1993) Cell 72, 481–483
Hankinson, O. (1988) Somatic Cell Genet. 9, 497–514
Higuchi, R., Krummel, B., and Saiki, R. K. (1988) Nucleic Acids Res. 16, 7351–7367
Hoffman, E. C., Reyes, H., Chu, F.-F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. (1991) Science 253, 946–948
Huang, J. Z., Edery, I., and Rebash, M. (1993) Nature 364, 269–272
Johnson, E. S. (1992) Toxicology 1, 451–463
Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8149
Kozak, M. (1989) Nucleic Acids Res. 17, 395–416
Lusska, A., Shen, E., and Whitlock, J. P., Jr. (1993) J. Biol. Chem. 268, 6575–6580
Matsushita, N., Sogawa, K., Ema, M., Yoshid, A., and Fujii-Kuriyama, Y. (1993) J. Biol. Chem. 268, 21092–21096
Miller, A. G., Israel, D. I., and Whitlock, J. P., Jr. (1983) J. Biol. Chem. 258, 3523–3527
Okey, A. B., J. Riddick, D. S., and Harper, P. A. (1993) Trends Pharmacol. Sci. 14, 226–227
Peterson, R. E., Theobald, H. M., and Kimmel, G. L. (1993) CRC Crit. Rev. Toxicol. 23, 283–335
Poland, A., Glover, E., and Kende, A. S. (1976) J. Biol. Chem. 251, 4936–4946
Poland, A., and Knutson, J. C. (1982) Annu. Rev. Pharmacol. Toxicol. 22, 517–554
Puga, B. F., and Tjian, R. (1986) Cell 51, 1187–1197
Reyes, H., Basu-Porszasz, S., and Hankinson, O. (1992) Science 256, 1193–1195
Safe, S. H. (1986) Annu. Rev. Pharmacol. Toxicol. 26, 371–399
Swanson, H. L., and Bradfield, C. A. (1992) Pharmacogenetics 3, 213–220
Tjian, R., and Maniatis, T. (1994) Cell 77, 5–8
Whitlock, J. P., Jr. (1993) Chem. Res. Toxicol. 6, 754–763