To identify new dimerization partners for the aromatic hydrocarbon receptor nuclear translocator (Arnt), we used its N-terminal region (amino acids 1-470) as a target in a two-hybrid screening procedure, and we cloned the murine form of hypoxia-inducible factor 1α (HIF1α). Sequence comparisons reveal substantial identity between mouse and human HIF1α. Hypoxia induces a 10-fold accumulation of phosphoglycerate kinase 1 mRNA in wild type mouse hepatoma (Hepa 1c1c7) cells; the induction mechanism is Arnt dependent because induction does not occur in Arnt-defective cells. Furthermore, induction of phosphoglycerate kinase 1 mRNA requires Arnt's N-terminal region, which mediates DNA binding and heterodimerization; in contrast, induction does not require Arnt's C-terminal region, which mediates transactivation. We also show that a GAL4-HIF1α fusion protein transactivates a GAL4-dependent gene in the absence of Arnt, that HIF1α's transactivation capability is inducible by hypoxia, and that both hypoxia responsiveness and transactivation capability reside within the C-terminal 83 amino acids of HIF1α. Our findings generate new insights into the mechanism by which Arnt and HIF1α induce transcription in response to hypoxia.

The aromatic hydrocarbon receptor nuclear translocator (Arnt)1 was first identified and characterized as a component of factor 1 and we cloned the murine form of hypoxia-inducible factor 1α (HIF1α). Sequence comparisons reveal substantial identity between mouse and human HIF1α. Hypoxia induces a 10-fold accumulation of phosphoglycerate kinase 1 mRNA in wild type mouse hepatoma (Hepa 1c1c7) cells; the induction mechanism is Arnt dependent because induction does not occur in Arnt-defective cells. Furthermore, induction of phosphoglycerate kinase 1 mRNA requires Arnt's N-terminal region, which mediates DNA binding and heterodimerization; in contrast, induction does not require Arnt's C-terminal region, which mediates transactivation. We also show that a GAL4-HIF1α fusion protein transactivates a GAL4-dependent gene in the absence of Arnt, that HIF1α's transactivation capability is inducible by hypoxia, and that both hypoxia responsiveness and transactivation capability reside within the C-terminal 83 amino acids of HIF1α. Our findings generate new insights into the mechanism by which Arnt and HIF1α induce transcription in response to hypoxia.

1 The abbreviations used are: Arnt, AhR nuclear translocator; AhR, aromatic hydrocarbon receptor; HIF1α, hypoxia-inducible factor 1α; CAT, chloramphenicol acetyltransferase; PGK1, phosphoglycerate kinase 1; PCR, polymerase chain reaction; kb, kilobase; bHLH, basic helix-loop-helix.

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** The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U59496.
RESULTS

Cloning of a Dimerization Partner for Arnt—Previous observations indicate that Arnt forms heterodimers with AhR and Sim, both of which contain bHLH and PAS domains (37). Such findings suggested the existence of additional bHLH/PAS partners for Arnt. We tested this hypothesis by using a yeast two-hybrid system to screen a mouse liver cdNA library; this approach identifies potential partners by virtue of their ability to interact directly with the target protein (38). As the target, we used the N-terminal region of Arnt (amino acids 1-470) because it contains the HLH and PAS domains, which are primarily responsible for Arnt’s interactions with AhR and, we presumed, for Arnt’s interactions with other proteins. In addition, Arnt’s C terminus had to be deleted from the target because the C terminus contains a transactivation function that introduces false-positive artifacts into the two-hybrid screening procedure.

Using two-hybrid screening, we isolated a 1.2-kb cdNA fragment, whose nucleotide sequence indicated that it was homologous to HIF1α, previously identified in human cells (27). We used this 1.2-kb fragment as a hybridization probe to isolate full-length HIF1α cdNA from a mouse hepatoma (Hepa 1c1c7) cdNA library. Fig. 1 reveals the nucleotide and deduced amino acid sequence of mouse HIF1α. The mouse protein exhibits 90% amino acid identity (89% at the nucleotide level) with the human protein. The N-terminal region (amino acids 1-330) is somewhat more conserved and exhibits 96% amino acid identity (91% at the nucleotide level) between the species; the C-terminal region (amino acids 331-822) exhibits 86% identity at both the amino acid and nucleotide levels between the mouse and human proteins. Like the human protein, murine HIF1α is a bHLH/PAS protein that exhibits similarities to Per, AhR, Arnt, and Sim (27).

Role of Arnt in the Response to Hypoxia—The identification of HIF1α as a potential heterodimerization partner for Arnt raised the possibility that Arnt may participate in cellular responses to hypoxia. To test this hypothesis, we first identified a hypoxia-responsive gene suitable for analysis in mouse hepatoma cells. PGK1 is a glycolytic enzyme that is induced by hypoxia in other cell systems (30, 39). We observed that exposure of wild-type mouse hepatoma cells to hypoxic conditions induced the accumulation of PGK1 mRNA by about 10-fold (Fig. 2). In contrast, hypoxia failed to induce PGK1 mRNA in Arnt-defective cells; however, reconstitution of Arnt-defective cells with full-length Arnt cdNA restored the response of the PGK1 gene to hypoxia (Fig. 2). These findings demonstrate that Arnt is required for the induction of PGK1 mRNA by hypoxia in this cell system.

To delineate in greater detail Arnt’s role in regulating the response to hypoxia, we analyzed the induction of PGK1 mRNA in Arnt-defective cells that had been reconstituted with Arnt mutants (Fig. 3A). In these studies, the high efficiency of retroviral gene transfer allows us to analyze the response of the target PGK1 gene in its native chromosomal setting, thus avoiding potential artifacts associated with the use of an episomal reporter gene. Our results (Fig. 3B) reveal that hypoxia fails to induce PGK1 mRNA in cells reconstituted with ArntΔHLH and ArntΔNT, mutants which lack Arnt’s bHLH domain and N-terminal domain, respectively. These observations imply that Arnt’s DNA-binding and heterodimerization functions are required for the induction of PGK1 gene transcription. We envision that heterodimerization generates an HIF1α/Arnt complex that induces transcription by binding to its cognate DNA recognition motif in the vicinity of the PGK1 gene (30). In contrast, substantial induction of PGK1 mRNA...
occurs in cells reconstituted with ArntΔC1 and ArntΔC2, mutants which lack Arnt's transactivation domain (Fig. 3B). These findings imply that the response of the PGK1 gene to hypoxia does not require transactivation by Arnt.

Transactivation Function of HIF1α—Because the induction of PGK1 gene expression by hypoxia does not require Arnt's transactivation capability, we hypothesized that HIF1α must itself contain a transactivation function; we tested this idea by measuring the ability of GAL4-HIF1α fusion proteins (Fig. 4A) to activate the expression of a GAL4-dependent reporter gene. We performed these studies in Arnt-defective cells to ensure that our findings reflect the transactivation capability of HIF1α and not the HIF1α/Arnt heterodimer. Our results (Fig. 4B) reveal that HIF1α exhibits constitutive (basal) transacti-

Fig. 1. Structure of mouse HIF1α cDNA and protein. The nucleotide sequence of the mouse cDNA (top line) and the deduced amino acid sequence of the mouse protein (second line) are shown. The deduced amino acid sequence of human HIF1α (third line) is shown for comparison. Dots indicate positions of amino acid identity.

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vation capability that is about 5-fold higher than background; in addition, we find that its transactivation function is inducible about 5-fold by hypoxia. Analyses of deletion mutants reveal that HIF1α’s inducible transactivation function is located within the C-terminal 491 amino acids of the protein and not within its N-terminal portion, which contains its bHLH and PAS domains. These observations indicate that HIF1α contains a hypoxia-inducible transactivation capability that does not require Arnt for expression. Also, HIF1α’s transactivation domain functions independently of its (presumed) DNA-binding and heterodimerization domains; thus, like other transcription factors, HIF1α appears to have a modular organization (40).

The inducible nature of its transactivation function raised the possibility that HIF1α contained a hypoxia-responsive region that was distinct from its transactivation domain. For example, Arnt’s other dimerization partner, AhR, has a dioxygen-responsive domain that is separate from its transactivation domain (22, 24, 26). To determine whether hypoxia responsiveness and transactivation were separable functions, we assayed progressively smaller fragments of HIF1α’s C-terminal region for hypoxia-inducible transactivation. Our findings (Fig. 5) reveal that HIF1α’s constitutive transactivation capability maps to an 83-amino acid segment at the protein’s C terminus; furthermore, this segment also exhibits the ability to respond to hypoxia. Thus, the domains responsible for transactivation and hypoxia responsiveness occupy the same small region of HIF1α, implying that they overlap or are congruent.

**FIG. 2.** Induction by hypoxia of PGK1 mRNA in mouse hepatoma cells. Cells were exposed to hypoxic conditions for 18 h. 10 μg of total RNA was analyzed by 1% agarose gel electrophoresis, transfer to nitrocellulose, hybridization with 32P-labeled PGK1 cDNA, and autoradiography. Lane 1, wild-type cells; lane 2, Arnt-defective cells; lane 3, Arnt-defective cells reconstituted with full-length Arnt cDNA.

**FIG. 3.** Induction by hypoxia of PGK1 mRNA in Arnt-defective cells reconstituted with full-length or mutant Arnt cDNAs. Panel A, structure of full-length and mutant Arnt cDNAs. Panel B, reconstitution experiments. Arnt-defective cells were reconstituted with the indicated cDNAs by retroviral infection. Cells were exposed to hypoxic conditions for 18 h. 10 μg of total RNA was analyzed by 1% agarose gel electrophoresis, transfer to nitrocellulose, hybridization to 32P-labeled PGK1 cDNA, and autoradiography. Lane 1, full-length Arnt; lane 2, ArntΔC1; lane 3, ArntΔC2; lane 4, ArntΔbHLH; lane 5, ArntΔNT.
DISCUSSION

We have used a yeast two-hybrid system to clone and identify mouse HIF1α as a dimerization partner for mouse Arnt. The functional importance of this protein-protein interaction is revealed by our observation that the PGK1 gene fails to respond to hypoxia in Arnt-defective cells. Our findings complement and extend those of Wang et al. (27) and Wang and Semenza (41), who identified HIF1α and Arnt as components of a DNA-binding protein complex that mediates cellular responses to hypoxia in human cells. The high amino acid sequence homology between mouse and human HIF1α suggests that the regulatory mechanism by which cells adapt to low oxygen tension is similar for the two species.

Two observations imply that Arnt's HLH domain is important for its interaction with HIF1α. First, successful cloning relied upon a protein-protein interaction between HIF1α and a fragment of Arnt that contains its bHLH and PAS domains, which have been implicated in Arnt's heterodimerization capability. Second, deletion of Arnt's bHLH domain abrogates the response of the PGK1 gene to oxygen deprivation. The simplest explanation for this finding is that ArntΔbHLH fails to heterodimerize with HIF1α.

Our findings indicate that Arnt's transactivation domain is dispensable for the induction of PGK1 gene expression by hypoxia. Similarly, we reported previously that Arnt's transactivation capability is not required for the induction of CYP1A1 transcription by dioxin (12). Therefore, in both the hypoxia-responsive and dioxin-responsive systems, Arnt appears to serve as a dimerization partner that confers DNA recognition capability upon HIF1α/Arnt and AhR/Arnt, respectively.

Studies of dioxin-inducible transcription reveal that AhR/Arnt binds to the consensus DNA sequence 5'-TNNGCGTG-3' in
the CYP1A1 enhancer (42, 43). Cross-linking studies and binding site selection experiments suggest that Arnt’s basic domain binds to the 5′-GTA-3′ component of the CYP1A1 consensus sequence (37, 44). By comparison, studies of hypoxia-responsive genes imply that HIF1α/Arnt recognizes the consensus DNA sequence 5′-(G/T)ACGTGC(G/T)-3′ upstream of PGK1 (30). We note that this nucleotide sequence also contains a 5′-GTA-3′ motif; we speculate that it interacts with Arnt’s basic domain during the induction of PGK1 transcription by hypoxia. We also note that AhR/Arnt’s recognition sequence and HIF1α/Arnt’s recognition sequence both contain a CpG dinucleotide, a motif that may undergo cytosine methylation. Cytosine methylation blocks the binding of AhR/Arnt to its recognition sequence and the response to dioxin (45). By analogy, we envision that cytosine methylation may also block DNA binding by HIF1α/Arnt and the response to hypoxia. Thus, we hypothesize that DNA methylation has the potential to inhibit some cellular responses to decreased oxygen tension, possibly in tissue-specific fashion.

Our findings indicate that HIF1α and AhR differ with respect to their transactivation mechanisms. In particular, we show that HIF1α’s transactivation function is expressed independently of Arnt; however, in the dioxin-responsive system, transactivation by AhR requires heterodimerization with Arnt. We envision that heterodimerization triggers a conformational change in AhR, which allows its latent transactivation function to be expressed (12, 24). Transactivation by HIF1α must involve a different mechanism because it does not require Arnt for expression.

In several other receptor-dependent regulatory systems (i.e. those that respond to glucocorticoid hormones, heat shock, or dioxin), the receptor domain that mediates inducibility is distinct from the domain that mediates transactivation (24, 46, 47). Thus, the observation that hypoxia responsiveness and transactivation capability map to the same 83-amino acid region of HIF1α is unusual because it suggests that the functional domains are congruent. Its amino acid composition and primary sequence reveal that HIF1α’s C-terminal 83-amino acid segment is not rich in acidic residues, glutamine, or proline; thus, it does not resemble certain transactivation domains described previously (40). The segment is enriched in leucine and isoleucine residues (−20%), as well as in serine residues (−11%), and it exhibits clusters of hydrophilic and hydrophobic amino acids. These typical properties may reflect the fact that the segment mediates hypoxia responsiveness as well as transactivation and, therefore, that its structure reflects a combination of these two functions.

The fact that Arnt is a component of more than one regulatory pathway is interesting for several reasons. First, it increases the plausibility that Arnt mediates additional adaptive responses to environmental stimuli and that other Arnt-dependent signaling systems exist within the cell. Second, it raises the possibility that “cross-talk” exists between Arnt-dependent regulatory systems and that activation of one pathway (e.g. by dioxin) may affect the cell’s ability to respond to a second stimulus (e.g. hypoxia). These appear to be potentially interesting issues for future research.

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Induction of Phosphoglycerate Kinase 1 Gene Expression by Hypoxia: ROLES OF ARNT AND HIF1α
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