The Role of the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) in Hypoxic Induction of Gene Expression

STUDIES IN ARNT-DEFICIENT CELLS*

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Hypoxia-inducible factor-1 (HIF-1), a DNA-binding complex implicated in the regulation of gene expression by oxygen, has been shown to consist of a heterodimer of two basic helix-loop-helix Per-AHR-ARNT-Sim (PAS) proteins, HIF-1α, and HIF-1β. One partner, HIF-1β, had been recognized previously as the aryl hydrocarbon receptor nuclear translocator (ARNT), an essential component of the xenobioreceptor. In the present work, ARNT-deficient mutant cells, originally derived from the mouse cell line Hepa1c1c7, have been used to analyze the role of ARNT/HIF-1β in oxygen-regulated gene expression. Two stimuli were examined: hypoxia itself and desferrioxamine, an iron-chelating agent that also activates HIF-1. Induction of the DNA binding and transcriptional activity of HIF-1 was absent in the mutant cells, indicating an essential role for ARNT/HIF-1β. Analysis of deleted ARNT/HIF-1β genes indicated that the basic, helix-loop-helix, and PAS domains, but not the amino or carboxyl termini, were necessary for function in the response to hypoxia. Comparison of gene expression in wild type and mutant cells demonstrated the critical importance of ARNT/HIF-1β in the hypoxic induction of a wide variety of genes. Nevertheless, for some genes a reduced response to hypoxia and desferrioxamine persisted in these mutant cells, clearly distinguishing ARNT/HIF-1β-dependent and ARNT/HIF-1β-independent mechanisms of gene activation by both these stimuli.

Oxygen is an important regulator of gene expression in many organisms. In mammalian cells a number of different transcription factors can be induced by hypoxia or related metabolic changes, and may play a role in oxygen-regulated gene expression. One system that has been identified through studies of the regulation of erythropoietin, a hormone that controls red cell production in accordance with blood oxygen availability (1). The oxygen-regulated function of the erythropoietin 3’ enhancer in hepatoma cells was found to be dependent on binding of an inducible factor termed hypoxia-inducible factor-1 (HIF-1) (2). Subsequently, transient transfection studies have demonstrated this activity in non-erythropoietin producing cells (3-5) and have now implicated HIF-1 binding sites in the regulation of a number of hypoxically inducible genes. Examples include genes encoding glycolytic enzymes (6-8), glucose transporters (9), vascular endothelial growth factor (10), tyrosine hydroxylase, a key enzyme in catecholamine biosynthesis (11), and nitric oxide synthase (12). These hypoxic responses can be mimicked by particular transition metals including cobalt (13), and iron-chelating agents such as desferrioxamine (14, 15), findings that have led to the proposal that these agents interact closely with the mechanism of oxygen sensing.

Following affinity purification, HIF-1 has recently been shown to consist of a heterodimer of two basic helix-loop-helix PAS proteins termed HIF-1α and HIF-1β (16, 17). HIF-1β was identical to the previously identified aryl hydrocarbon receptor nuclear translocator (ARNT), a molecule essential for the transcriptional response to certain environmental hydrocarbons known as the xenobiotic response (for recent review, see Ref. 18). In this capacity ARNT/HIF-1β forms a heterodimeric complex with another basic helix-loop-helix PAS protein, the ligand-binding subunit termed the aryl hydrocarbon receptor (AHR) (19, 20). This complex binds the xenobioreceptor-responsive element (21, 22), a control sequence for genes such as the CYP1A1 gene, encoding a cytochrome P450 with aryl hydrocarbon hydroxylase activity that can convert aryl hydrocarbons to toxic or carcinogenic metabolites. An important step in the analysis of this mechanism of gene regulation was the derivation of mutant cells selected by survival in the presence of benzo(a)pyrene (23). One group of mutants was shown to be defective in the nuclear translocation of the ligand-binding complex, as assessed by conventional subcellular fractionation procedures (24), and provided the basis for cloning of the gene encoding ARNT by complementation (25). These ARNT-deficient (ARNT-) cells lack ARNT/HIF-1β protein expression and function (26) and provide an opportunity to determine the importance of ARNT/HIF-1β in other aspects of gene regulation.

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Here we describe the effects of this phenotype on hypoxic gene regulation. The ARNT- and wild type Hepa-1 cells were compared with respect to the induction of HIF-1 DNA binding activity, expression of transfected plasmids bearing oxygen-regulated cis-acting elements, and the induction of endogenous genes by hypoxia. Deletion mutants of ARNT/HIF-1β were analyzed to determine the domains that are critical for the functional activity of HIF-1.

**EXPERIMENTAL PROCEDURES**

Cell Lines and Culture—The murine hepatoma line Hepa-1 clone Hepa1c1c7 (hereafter referred to as Hepa-1) and derivatives, c4, and c4, Vt (V) were used in the experiments (23, 25, 27, 28). Briefly, the c4 mutant clone was obtained from a mutagenized culture of Hepa-1 cells that was selected for loss of argi hydrocarbon hydroxylase activity in a single-step maneuver by survival in the presence of benz(a)pyrene (23). c4 lacks ARNT/HIF-1β function (29) and also fails to express the ARNT/HIF-1β protein, as assessed by Western blot analysis using an antibody to the carboxyl-terminal half of the protein (26). c3 is a similarly but independently derived clone from the same complementation group. Both c4 and c4 express ARNT mRNA of normal size (25). Vt (V) cells are c4 (a hypoxaenine phosphoglycerate kinase-1 mutant) derivatives of c4.) Stable transfected with pBM5/Neo-M1–1 containing a 2.5-kilobase-pair insert of ARNT/HIF-1β cDNA and then selected for reacquired argi hydrocarbon hydroxylase activity (25). c4 is a revertant line, derived from c4 by the same "reverse selection" procedure, which possesses wild type levels of ARNT activity (27). c31 is a mutant selected in the same way as c4 and c4, which possesses prominently acting repressor preventing transcriptional activation of the xenobioc reaction (28).

All cells were grown in minimal essential medium without nucleosides (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Globepharm), 100 mM glutamine (2 mM), penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml). For Vt (V) cells, 400 μg/ml G418 (Life Technologies, Inc.) was added to the medium. In all experiments, on day 3 of culture, cells were stimulated with 100 mM DFO, and incubated with 1% desferrioxamine mesylate (Sigma). Hypoxic conditions were generated in a Napco 7001 incubator (Precision Scientific, Chicago, IL) with 1% modified air with 5% CO2 (normoxia), or exposed to hypoxia or 100 mM desferrioxamine mesylate (Sigma). Hypoxic conditions were generated in a Napco 7001 incubator (Precision Scientific, Chicago, IL) with 1% modified air with 5% CO2 (normoxia), or exposed to hypoxia or 100 mM desferrioxamine mesylate (Sigma).

**RNA Analysis—**RNA was extracted by a modified acid/guanidinium thiocyanate/phenol/chloroform method (RNAzol B, Cinna/Biotech Laboratories, Houston), dissolved in hybridization buffer (80% formamide, 20 mM PIPES, 400 mM sodium chloride, and 1 mM EDTA, pH 8) and analyzed by RNase protection assay. Riboprobes used are described in Table 1. Riboprobes were transcribed using SP6 RNA polymerase. For endogenous genes, the quantity of RNA analyzed was as follows: β-actin, 0.5–1 μg; LDH-A and Glut-1, 1–10 μg each; PGK-1 and VEGF, 30 μg each; HIF-1α, HIF-1β, and PDGF-B, 50 μg each. Quantitation of the protected species was performed on a flat-bed scintillation counter (model 1205 Betaplate, Pharmacia-Wallac OY), after excision from the dried gel.

**Transient Transfection—**All transfections were performed by electroporation using 1-millifarad capacitor array charged at 350 V. To assay for HIF-1-dependent transcriptional responses, one of two plasmids, pEpol-25, SV40GH (50 μg) or pPGK24, TKGH (50 μg), was transiently transfected with plasmids pBr322 (125 μg) and pBS (3). Cells from each transfection were split for parallel incubation under normoxic conditions (21%), or stimulated conditions; hypoxia (1%), or desferrioxamine (100 μM). To test for the ability of wild type or mutated ARNT/HIF-1β genes to reactivate the hypoxically inducible response in ARNT- cells, c4 cells were co-transfected with pEpol-25, SV40GH (30 μg), pBr322 (125 μg), and the mouse ARNT expression plasmid, pcDNA1/Neo/ARNT, or mutations thereof (50 μg). Transfected cells (approximately 1 × 10⁶) were split equally, incubated in normoxic conditions for 24 h to allow for expression of ARNT/HIF-1β, and then grown in either normoxia or in hypoxia (1% O₂) for another 16 h before harvesting.

**RESULTS**

**HIF-1 DNA Binding Activity—**To determine whether ARNT/HIF-1β was necessary for some of the HIF-1 DNA-binding reactions, we compared HIF-1 activity in wild-type Hepa1c1c7 and the ARNT- derivative, c4. In the wild type cells, the inducible species, HIF-1, was clearly observed in cells after exposure to hypoxia (1% oxygen), whereas it was undetectable in hypoxic c4 cells. No new species were observed in the c4 cells, and no consistent changes were observed in the constitutive

### Table 1

| Gene Accession no. | Riboprobe template | Protected fragment | Downloaded from |
|------------------|--------------------|--------------------|-----------------|
| β-Actin  | V01217  | 184–443 (g)  | 75  |
| α-Globin  | V00488  | 38–260 (g)  | 133  |
| Glut-1   | M23384  | 1077–1407 (c) | 120  |
| GH      | M14398  | 1–192 (c)  | 192  |
| HIF-1α   | X95002  | 32–130 (c)  | 99  |
| HIF-1β   | U14333  | 932–1164 (g) | 46  |
| PDG-F    | M648478 | 106(ωvα)–171(ωvβ) | 147  |
| PGK-1    | M18735  | 417–900 (g) | 47  |
| VEGF     | M95200  | 177–345 (c) | 169  |
Two plasmids were tested: p(Epo1–25)3SV40GH and plasmids bearing such elements linked to reporter genes. cis-9 from c4 cells exposed to DFO. Similar results were obtained but had slightly greater mobility than the complex induced by HIF-1 by DFO. In wild type cells, HIF-1 was induced by DFO species which bind at this site. We also tested induction of HIF-1 by DFO. In wild type cells, HIF-1 was induced by DFO but had a slightly greater mobility than the complex induced by hypoxia. Again, this complex was absent in nuclear extract from c4 cells exposed to DFO. Similar results were obtained using different HIF-1-binding oligonucleotides, derived from the mouse erythropoietin 3’ enhancer (E24) and the mouse phosphoglycerate kinase-1 5’ enhancer (P24) (Fig. 1).

Regulation of Hypoxia-responsive Elements—To analyze the role of ARNT/HIF-1β in the function of hypoxically responsive cis-acting elements, wild type and c4 cells were transfected with plasmids bearing such elements linked to reporter genes. Two plasmids were tested: p(Epo1–25)3SV40GH and p(PGK24)TKGH, which contain three copies of the HIF-1 site from the mouse erythropoietin 3’ enhancer in c4 cells. The mouse phosphoglycerate kinase-1 5’ enhancer, respectively. Results are summarized in Table II. In normoxic Hepa-1 and c4 cells, the reporter gene expression was similar. In wild type Hepa-1 cells, each of the plasmids supported an approximately 6-fold induction of reporter gene by hypoxia. In the c4 cells, induction by hypoxia was virtually absent, although a low level of inducibility was observed occasionally using each of the reporter plasmids.

Further transfections were performed to compare the inducible responses to hypoxia and to DFO in wild type and mutant cells. Cells in these experiments were split into three aliquots after transfection with p(Epo1–25)3SV40GH; one aliquot was maintained in normoxia, one in hypoxia, and the third exposed to DFO (100 μM). Results are shown in Table II. Wild type cells showed similar responses to hypoxia and DFO. In c4 cells, responses to both stimuli were severely reduced. The small and inconsistent response to DFO was similar to that observed with hypoxia.

Restoration of Hypoxia-inducible Responses by Transfection with ARNT Expression Plasmids—In order to determine whether the ARNT c4 cells could regain inducible responses to hypoxia following expression of a functional ARNT/HIF-1β gene, cells were co-transfected with the mouse ARNT/HIF-1β expression plasmid pcDNA1/Neo/mARNT and the hypoxically inducible reporter plasmid p(Epo1–25)3SV40GH. After electroporation, cells were divided into two aliquots, which were incubated in normoxia for 24 h to allow expression of ARNT/HIF-1β before one of the aliquots was exposed to hypoxia for 16 h. Cells were harvested a total of 40 h after transfection. Each experiment was controlled positively (wild type cells transfected with p(Epo1–25)3SV40GH alone) and negatively (c4 mutant cells transfected with p(Epo1–25)3SV40GH and the empty vector pcDNA1/Neo). In cells transfected with the ARNT/HIF-1β expression plasmid, a substantial inducible response to hypoxia was restored, which was not seen in cells co-transfected with pcDNA1/Neo; the inducible response in the complemented c4 cells was not quite as great as in wild type Hepa-1 cells, being 2.5-fold versus 4.2-fold in the parallel experiments (Fig. 2). However, under these experimental conditions, the normoxic level of reporter gene expression was slightly higher than in the wild type cells.

To determine which domains of ARNT/HIF-1β were required for functional restoration of the hypoxically inducible response, a series of deletional mutants of ARNT/HIF-1β was tested in this co-transfection system. Transfection of plasmids lacking either the basic region (Δb), the helix-loop-helix domain (ΔHLH), or the PAS domain (ΔAB) all failed to restore function. In contrast, a plasmid containing only the basic, helix-loop-helix, and PAS domains but lacking the carboxyl and amino termini (ΔHLHAB) restored hypoxic inducibility almost as effectively as the full coding sequence of ARNT/HIF-1β (Fig. 2).

Regulation of Endogenous Gene Expression by Hypoxia and DFO—Since ARNT/HIF-1β was necessary for the functional responses to hypoxia and DFO conveyed by transfected cis-acting sequences which contained HIF-1 binding sites, we compared hypoxically inducible endogenous gene expression in the wild type Hepa-1 cells and the ARNT c4 mutant cells using RNase protection assays for a variety of mouse genes. The results are summarized in Table II. Normoxic and hypoxic Hepa-1 cells were assayed for erythropoietin mRNA expression, but no signal was detectable from 100 μg of RNA.

Exposure of wild type cells to hypoxia (1% O2 for 16 h) led to induction of mRNA for phosphoglycerate kinase-1 (PGK-1) and lactate dehydrogenase-A (LDH-A) by 4-fold and 5-fold, respectively. This response was virtually abolished in the ARNT c4 cells, being 2.5-fold versus 0.2 for p(Epo1–25)3SV40GH in Hepa-1 and c4 cells, respectively. The inducible response is the ratio of stimulated to normoxic reporter gene expression. Values are mean ± S.D.; the number of experiments is shown in parentheses. * indicates p < 0.05 (statistical analysis of differences in inducible responses between the two cell types by Student’s t test). Both the Epo and PGK-1 elements conveyed inducible responses in wild type cells, but this response was markedly diminished in ARNT c4 cells. The inducible responses to hypoxia and DFO were of similar magnitude in wild type cells and were similarly abrogated in ARNT c4 cells.

| Plasmid                  | Stimulus | Hepa-1 | c4 |
|--------------------------|----------|--------|----|
| p(Epo1–25)3SV40GH        | Hypoxia  | 5.3 ± 1.3 | 1.3 ± 0.7* |
| pPGK24)TKGH              | Hypoxia  | 5.7 ± 2.4 | 1.3 ± 0.3* |

**TABLE II** Regulation of hypoxia-inducible elements from the mouse Epo and PGK-1 genes by hypoxia and comparison of induction by hypoxia and desferrioxamine in wild type Hepa-1 and ARNT c4 cells.

Cells were transfected with p(Epo1–25)3SV40GH or p(PGK24)3TKGH. Expression of the non-inducible plasmid pBS5a was used to correct for transfection efficiency. Reporter gene expression in normoxia was similar in the two cell types (being 1.5 and 1.6 for p(Epo1–25)3SV40GH and 0.2 and 0.3 for p(PGK24)3TKGH in Hepa-1 and c4 cells, respectively). The inducible response is the ratio of stimulated to normoxic reporter gene expression. Values are mean ± S.D.; the number of experiments is shown in parentheses. * indicates p < 0.05 (statistical analysis of differences in inducible responses between the two cell types by Student’s t test). Both the Epo and PGK-1 elements conveyed inducible responses in wild type cells, but this response was markedly diminished in ARNT c4 cells. The inducible responses to hypoxia and DFO were of similar magnitude in wild type cells and were similarly abrogated in ARNT c4 cells.
was reduced to approximately 2-fold. Of PDGF-B mRNA was observed; the induction of VEGF mRNA increased approximately 7-fold for VEGF and approximately 10-fold by hypoxia in wild type cells. This response was substantially reduced. Surprisingly, however, the response to DFO in the c4 cells was better preserved than the response to hypoxia. This difference was observed in each experiment for all of the genes assayed, but the difference was most striking for PGK-1 and LDH-A. In c4 cells, these genes were virtually unresponsive to hypoxia, whereas the response to DFO persisted at approximately 50% of that seen in wild type cells.

To determine whether induction by DFO and hypoxia were altered in a similar manner in the c4 mutant cells, responses were compared directly in equal aliquots of cells divided for parallel incubations in normoxia, hypoxia, and DFO (100 μM). Four genes were studied: LDH-A, PGK-1, VEGF, and Glut-1 (Table I). In wild type cells, these genes were induced to a similar extent by both stimuli. In the c4 mutants, both inducible responses were substantially reduced. Surprisingly, however, the response to DFO in the c4 cells was better preserved than the response to hypoxia. This difference was observed in each experiment for all of the genes assayed, but the difference was most striking for PGK-1 and LDH-A. In c4 cells, these genes were virtually unresponsive to hypoxia, whereas the response to DFO persisted at approximately 50% of that seen in wild type cells.

To ascertain that the altered pattern of hypoxically inducible endogenous gene expression resulted from a defective ARNT/HIF-1β gene product, rather than some other difference in the c4 cell line, or some indirect consequence of the defective xenobiotic response itself, we tested hypoxically inducible gene expression in a series of other cell lines derived from Hepa-1. Cells were incubated in parallel either in normoxia or exposed to hypoxia for 2 h (2 h) or for 16 h (16 h). Hypoxic induction is the ratio of gene expression in hypoxia to that in normoxia. The value given for normoxic expression is in each case related to the abundance of β-actin mRNA, which was arbitrarily assigned a value of 1000 in wild type cells. Each bar represents the mean ± S.D. of at least four experiments. * indicates p < 0.05 (statistical analysis of differences in inducible responses between the two cell types by Student’s t test). □, wild type Hepa-1 cells; □, ARNT− c4 cells.

Fig. 3. Hypoxic induction of endogenous gene expression in wild type Hepa-1 and ARNT− c4 cells. Cells were incubated in parallel either in normoxia or exposed to hypoxia for 2 h (2 h) or for 16 h (16 h). Hypoxic induction is the ratio of gene expression in hypoxia to that in normoxia. The value given for normoxic expression is in each case related to the abundance of β-actin mRNA, which was arbitrarily assigned a value of 1000 in wild type cells. Each bar represents the mean ± S.D. of at least four experiments. * indicates p < 0.05 (statistical analysis of differences in inducible responses between the two cell types by Student’s t test). □, wild type Hepa-1 cells; □, ARNT− c4 cells.
The Role of ARNT in Hypoxic Gene Regulation

Transient transfection assays do not always correlate with the activity of the xenobiotic response. Responses of LDH-A and Glut-1 mRNAs were analyzed. Results are shown in Fig. 4. c39 cells behaved very similarly to c4 cells, whereas both vT(2) cells and Rc4 showed restored hypoxic responsiveness. Rc4 cells showed a higher normoxic expression of Glut-1 and vT(2) cells a higher normoxic expression of LDH-A than Hepa-1 cells. However, HIF-1 activity in these lines was comparable to that in Hepa-1 and was not increased in either normoxia or hypoxia (data not shown). The dominant dioxin-resistant c31 cells showed normal hypoxic gene regulation.

**DISCUSSION**

The hypoxically inducible transcriptional factor, HIF-1, was first recognized as a nuclear factor binding to a site that was critical for the function of the erythropoietin 3′ enhancer in transiently transfected cells. The recent purification and molecular cloning of HIF-1 have demonstrated that it is a heterodimer of two basic helix-loop-helix PAS proteins. HIF-1α, a newly described member of this family, and HIF-1β, a transcription factor previously recognized as the ARNT (25), of the ARNT− mutant clones has now provided the opportunity to analyze further the importance of ARNT/HIF-1β in responses to hypoxia.

The ARNT− c4 cells were first tested for HIF-1 activity. They failed to show inducible HIF-1-DNA binding and were unable to support the hypoxically inducible responses usually conveyed by multimerized HIF-1 binding sites from the mouse Epo and PGK-1 enhancers. The defective response was observed despite the presence of an increased level of HIF-1α mRNA in c4 cells and indicates that ARNT/HIF-1β is an essential component of the complex that recognizes HIF-1 binding sites. Although the responses to hypoxia conveyed by the HIF-1-binding elements were usually absent in c4 cells, a very small response was occasionally seen. The mutation responsible for lack of ARNT function is unknown. It is possible that, in contrast with the xenobiotic response, some function in the hypoxic response is retained. Another explanation would be that HIF-1α has other dimerization partners, which can interact weakly with the HIF-1 binding sequence.

Regulation by hypoxia has been reported for a number of widely expressed transcription factors including NF-κB (33), some members of the Fos/Jun family (34), and p53 (35), in addition to HIF-1, implying that a large number of transcriptional activation mechanisms may contribute to the response to hypoxia. Somewhat surprisingly, transient transfection studies of cis-acting sequences of several different genes have implicated binding sites for HIF-1 (6–12), rather than those for other transcription factors, in hypoxic induction. However, results of transient transfection assays do not always correlate well with other analyses of gene regulation. The ARNT− cells therefore provided an important opportunity to test the role of ARNT/HIF-1β in the induction of endogenous genes by hypoxia. For PGK-1 and LDH-A, transient transfection studies have implicated HIF-1 binding sites in the hypoxic response. Induction of these genes was essentially absent in c4 cells, indicating a crucial role for ARNT/HIF-1β. The sequences conveying hypoxically inducible responses on the PDGF-B chain gene have not been analyzed. Nevertheless, the loss of regulation in ARNT− cells also indicates a critical role for ARNT/HIF-1β in this response. In the case of VEGF, both transcriptional and post-transcriptional mechanisms have been implicated in the regulation of mRNA levels by hypoxia (10). Studies of cis-acting elements by transient transfection have given contradictory results. Minchenko et al. reported that...
hypochemically inducible responses were conveyed by sequences lying both 5’ and 3’ to the human gene, of which only the 3’ region contained a HIF-1 site (36). Goldberg and colleagues did not find these regions to be active, but defined a conserved HIF-1 site lying further 5’ to the rat gene (10), as did Kourimbanas and colleagues working on the human gene (37). In c4 cells the alteration in induction of VEGF mRNA was different from that observed for PGK-1 or LDH-A mRNA; although a consistent and substantial reduction in the level of induction was observed, an inducible response clearly persisted. A very similar pattern of altered expression was observed for Glut-1 mRNA. This indicates that both ARNT/HIF-1β-dependent and ARNT/HIF-1β-independent mechanisms of hypoxic induction are operating on the regulation of Glut-1 and VEGF mRNAs.

Security in assigning these altered responses to the defect in ARNT/HIF-1β was provided by the analysis of several related cell lines, in which hypoxic induction was clearly correlated with the presence or absence of a functional ARNT/HIF-1β gene product. Responses were reduced in both of the independently derived ARNT− lines c4 and c39, whereas in the ARNT-expressing stably transfected line Vt (2) the revertant Rc4, and line c31, they were essentially similar to those observed in wild-type cells. The results in c31 cells were interesting. These cells possess a dominantly acting repressor, which interferes with both the transcriptional response to dioxin and the DNA-binding proteins responsible for in vivo footprints at the xenobiotic-responsive element (XRE), but does not impair in vitro DNA binding of the activated receptor complex to the XRE (28). The preserved responses to hypoxia in these cells indicate that the repressor does not interfere with the function of HIF-1.

Overall, these results indicate that ARNT/HIF-1β has a major role in oxygen-dependent gene regulation. For PGK-1, LDH-A, and PDGF-B, the loss of induction indicates that limited redundancy exists in the mechanisms underlying induction of these genes by hypoxia. We found no evidence that the XRE conveyed a response to hypoxia (data not shown). Nevertheless, ARNT/HIF-1β-dependent responses to hypoxia are not necessarily restricted to HIF-1 itself, since it is conceivable that ARNT/HIF-1β has other undiscovered dimerization partners involved in the response to hypoxia. The results also provide clear evidence for ARNT/HIF-1β-independent mechanisms of hypoxic gene induction; such processes could involve other mechanisms of transcriptional activation or increased mRNA stability.

Since many transcription factors bind cis-acting elements within their own regulatory sequences, and HIF-1α and ARNT/HIF-1β mRNA levels have been reported to be induced markedly by hypoxia (17), these mRNAs were examined in wild type and c4 cells. In wild type Hepa-1 cells, we did not observe a high level of induction of these mRNAs by hypoxia; an increase in mRNA was consistently observed for HIF-1α but was less than 2-fold. Thus, it appears that in Hepa-1 cells mRNA induction is not a major mechanism of HIF-1 activation. However, interesting changes in expression were observed in ARNT− cells. As has been reported previously, ARNT/HIF-1β mRNA levels were near normal (25). However, HIF-1α mRNA was considerably increased; the basal level was 2-fold higher than in wild type cells, and a 4-fold induction was observed after 16 h of hypoxia. This is compatible with the existence of some form of regulatory mechanism operating in compensation for the lack of a functional HIF-1 complex.

Previous studies have shown that HIF-1 is induced by DFO as well as hypoxia (14). The DNA binding and transient transfection studies in c4 cells exposed to DFO showed that, as for hypoxia, ARNT/HIF-1β is essential for the formation of the HIF-1 DNA-binding complex and transcriptional activation.

The analysis of endogenous gene expression in cells exposed to DFO was interesting. Inducible responses to DFO were clearly reduced in c4 cells compared with wild-type cells. However, in c4 cells, an inducible response was retained for Glut-1 and VEGF, which was similar although somewhat greater than the hypoxic responses. Thus, DFO appears able to activate ARNT/HIF-1β-independent mechanisms of gene regulation by hypoxia in addition to those dependent on ARNT/HIF-1β. One surprising finding was that the response to DFO was consistently better preserved in c4 cells than the response to hypoxia, particularly for PGK-1 and LDH-A. This might imply some difference in the mechanisms of gene activation by hypoxia and DFO but precise understanding will require a clearer knowledge of the mechanism of oxygen sensing and how DFO interacts with this process.

Transfection of the c4 cells with ARNT/HIF-1β expression plasmids also provided a means of determining which domains of ARNT/HIF-1β are required for HIF-1-mediated transcriptional responses to hypoxia. Function was lost when any of the basic, helix-loop-helix, or PAS domains were deleted. Assuming that similar levels of protein expression were achieved, these results indicate that all of these regions are functionally critical for the operation of HIF-1. This result is similar to that obtained for functional analysis of ARNT/HIF-1β deletions in the xenobiotic response (32) and suggests that the interaction of ARNT/HIF-1β with HIF-1α is similar to its interaction with AHR. The bHLHAB deletion, which lacked the 5’ and 3’ regions of ARNT/HIF-1β, including the 3’ transactivation domain (38, 39), retained function. Again, this result is similar to that reported for the xenobiotic response and indicates that the 3’ transactivation domain of ARNT/HIF-1β is not necessary for transcriptional response in either system.

In summary, these studies have demonstrated the critical importance of ARNT/HIF-1β in the hypoxic regulation of many different genes. The ARNT− mutant cells are an important tool for analyzing the extent and mechanisms of gene regulation by this system and should also prove useful in understanding its role in cell physiology.

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