Dimerization, DNA Binding, and Transactivation Properties of Hypoxia-inducible Factor 1*

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Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic helix-loop-helix transcription factor that regulates hypoxia-inducible genes including the human erythropoietin (EPO) gene. In this study, we report structural features of the HIF-1α subunit that are required for heterodimerization, DNA binding, and transactivation. The HIF-1α and HIF-1β (ARNT; aryl hydrocarbon receptor nuclear translocator) subunits were coimmunoprecipitated from nuclear extracts, indicating that these proteins heterodimerize in the absence of DNA. In vitro-translated HIF-1α and HIF-1β generated a HIF-1/DNA complex with similar electrophoretic mobility and sequence specificity as HIF-1 present in nuclear extracts from hypoxic cells. Compared to 826-amino acid, full-length HIF-1α, amino acids 1–166 mediated heterodimerization with HIF-1β (ARNT), but amino acids 1–390 were required for optimal DNA binding. A deletion involving the basic domain of HIF-1α eliminated DNA binding without affecting heterodimerization. In cotransfection assays, forced expression of recombinant HIF-1α and HIF-1β (ARNT) activated transcription of reporter genes containing EPO enhancer sequences with intact, but not mutant, HIF-1 binding sites. Deletion of the carboxy terminus of HIF-1α (amino acids 391–826) markedly decreased the ability of recombinant HIF-1α to activate transcription. Overexpression of a HIF-1α construct with deletions of the basic domain and carboxy terminus blocked reporter gene activation by endogenous HIF-1 in hypoxic cells.

Multiple developmental and physiological mechanisms exist to provide each cell in the human body with sufficient O2 to meet its metabolic demands. Essential to the maintenance of O2 homeostasis is the production of adequate numbers of erythrocytes to transport O2 from the lungs to peripheral tissues. Reduced O2 delivery, whether due to anemia, acute hemorrhage, decreased ambient O2 tension, or decreased O2-hemoglobin dissociation, is sensed by cells in the liver and kidney, which increase their synthesis of erythropoietin (EPO), the glycoprotein hormone/growth factor that stimulates the survival, proliferation, and differentiation of bone marrow erythroid progenitor cells (reviewed in Refs. 1–3).

Analysis of EPO expression in the human hepatoblastoma line Hep3B has demonstrated that in cells subjected to hypoxia by incubation in 1% O2, EPO transcription is increased relative to nonhypoxic cells cultured in 20% O2 (4, 5). DNA sequences in the human EPO gene 3′-flanking region functioned as a hypoxia-inducible enhancer in transient expression assays (reviewed in Ref. 6). A 50-bp 3′-flanking sequence mediated a 7-fold higher level of reporter gene expression in cells cultured at 1% compared to 20% O2 (7). Mutational analysis indicated that the 50-bp enhancer was functionally tripartite (7). Mutations at site 1 or site 2 eliminated enhancer function (7, 8). The first 33 bp of the enhancer (containing sites 1 and 2 only) functioned at one-half the level of the 50-bp element, but full activity could be restored by the presence of two copies of the 33-bp element, indicating that factors binding at site 3 amplified the induction signal but were not absolutely required for transcriptional activation (7). The orphan receptor hepatocyte nuclear factor 4 may bind at site 3 (9), the factor binding at site 2 is uncharacterized, and site 1 is bound by hypoxia-inducible factor 1 (HIF-1) (7).

Several lines of evidence indicate that HIF-1 plays a key role in EPO gene transcriptional activation in hypoxic cells. (a) A 3′-bp substitution at site 1 eliminated enhancer activity and binding of HIF-1 (7). (b) Exposure of cells to 1% O2, cobalt chloride, or desferrioxamine induced both EPO expression and HIF-1 activity with similar kinetics (10–12). (c) Treatment of hypoxic cells with the protein kinase inhibitor 2-aminopurine or the protein synthesis inhibitor cycloheximide blocked induction of EPO RNA and HIF-1 activity (7, 10). In a variety of non-EPO-producing lines, including Chinese hamster ovary and human embryonic kidney 293 cells, HIF-1 was induced by hypoxia and EPO 3′-flanking sequences functioned as hypoxia-inducible enhancers (11, 13). Expression of genes encoding vascular endothelial growth factor and glycolytic enzymes was induced by exposure of EPO-producing and nonproducing cells to 1% O2, cobalt chloride, or desferrioxamine, and these genes contained HIF-1 binding sites within sequences mediating transcriptional activation in hypoxic cells (14–19). These results indicate a general role for HIF-1 in O2 homeostasis.

Purification of HIF-1 by DNA affinity chromatography (20) and characterization of amino acid and cDNA sequences revealed that HIF-1 was a heterodimeric transcription factor of the bHLH-PAS family (21). The bHLH domain, present in a large number of transcription factors, mediates DNA binding and protein dimerization (reviewed in Refs. 22 and 23). The PAS (PER-ARNT-SIM) domain was described previously in the amide gel electrophoresis; IP, immunoprecipitation; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; FL, full length.
PER and SIM proteins of Drosophilae melanogaster (24, 25) and the AHR and ARNT proteins, which constitute the mammalian dioxin receptor (26–28). PAS domains contain two internal homology units, the A and B repeats, and are implicated in the AHR and ARNT proteins, which constitute the mammalian

***MATERIALS AND METHODS***

Production of Glutathione S-Transferase (GST) Fusion Proteins—Recombinant plasmids containing a HIF-1α cDNA fragment (encoding amino acids 329–531) cloned into pGEX-3X and a HIF-1α (ARNT) cDNA fragment (encoding amino acids 406–789) cloned into pGEX-2T were constructed as described previously (21). Transformed Escherichia coli DH5α cells were cultured in 50 ml of LB medium supplemented with 50 μg/ml ampicillin at 37 °C for 200 rpm overnight, inoculated into 1 liter of LB medium supplemented with 50 μg/ml ampicillin, and cultured at 37 °C for 200 rpm until A600 = 1.0. GST/HIF-1α fusion protein synthesis was induced by adding isopropyl-1-thio-galactopyranoside to 0.1 mM and shaking at 200 rpm for 30 min, incubated with 2× SDS sample buffer and boiled. Purified GST/HIF-1α fusion protein (0.82 mg) was coupled to 1 ml of resin (0.3 g of freeze-dried powder), and purified GST protein (2.5 mg) was coupled to 2.5 ml of resin (0.71 g of freeze-dried powder). After washing with 1 ml of Tris-HCl, pH 8.0, and washing with 1 ml of sodium acetate, pH 4.0, 0.5 ml NaCl, followed by 1 ml of Tris-HCl, pH 8.0, and 0.5 ml NaCl, the gel was suspended in storage buffer (0.5 M sodium acetate, pH 4.5, 25% glycerol) and stored at -20°C. Purified fusion proteins were used as immunogens.

**Fusion Protein-Resin Preparation—** Purified fusion protein was coupled to hydrated cyanogen bromide-activated Sepharose 4B resin (Pharmacia Biotech Inc.) in 0.1 M NaHCO3 (pH 9.0) and 0.5 M NaCl for 1 h at room temperature, according to the manufacturer's instructions. The coupling efficiency was greater than 95%, as determined by analyzing the unbound protein in the supernatant. Purified HIF-1α fusion protein (4.5 mg) was coupled to 2.5 ml of resin (0.71 g of freeze-dried powder), purified HIF-1α fusion protein (0.82 mg) was coupled to 1 ml of resin (0.3 g of freeze-dried powder), and purified GST protein (2.5 mg) was coupled to 2.5 ml of resin (0.71 g of freeze-dried powder). After blocking with 1 ml of Tris-HCl, pH 8.0, and washing with 1 ml of sodium acetate, pH 4.0, 0.5 ml NaCl, followed by 1 ml of Tris-HCl, pH 8.0, and 0.5 ml NaCl, the gel was suspended in storage buffer and stored at -20°C. Purified fusion proteins were used as immunogens.

**Antibody Purification—** A 1:1:10 mixture (by volume) of anti-GST/HIF-1α antisemur/TBS/DH5α cell lysate containing GST/HIF-1α (with a total protein concentration of 8.9 μg/ml) was incubated at room temperature for 1 h with agitation. A volume of GST-coupled Sepharose 4B equal to the volume of antiserum was added and incubated at 4°C for 4 h. After centrifugation at 1500 × g for 3 min, the supernatant was combined with a volume of GST/HIF-1α-coupled Sepharose 4B equal to the volume of antiserum and incubated at 4°C for 4 h with agitation. The resin was washed twice with 10 volumes of 50 mM sodium phosphate, pH 7.6, and 0.1% Triton X-100, then washed twice with 10 volumes of 10 mM sodium phosphate, pH 7.6. The adsorbed protein was eluted with 0.2 M glycine-HCl, pH 2.0, 0.1 M NaCl, and 0.1% Triton X-100 at room temperature for 15 min with agitation and collected in a tube containing 0.1 volume of 1 M Tris-HCl, pH 8.0, then dialyzed twice at 4°C in 100 volumes of TBS for a total of 4 h.

**Immunoaffinity Purification—** Antiserum against HIF-1α antisemur (1.2 ml) was incubated with 10.8 ml of TBS and 1.2 ml of GST-coupled Sepharose 4B for 4 h at 4°C. After centrifugation at 1500 × g for 3 min, the supernatant was incubated with 1 ml of GST/HIF-1α-coupled Sepharose 4B for 4 h at 4°C. The resin was washed twice with 12 ml of 50 mM sodium phosphate, pH 7.6, and 1% Triton X-100; once with 12 ml of 10 mM sodium phosphate, pH 7.6, and 0.1% Triton; and once with 12 ml of 10 mM sodium phosphate, pH 7.6. The adsorbed protein was eluted and dialyzed as described above.

**Electrophoretic Mobility Shift Assay (EMSA)—** EMSA using Hep3B nuclear extracts was performed with oligonucleotide probe W18 (21, 22). Unlabeled HIF-1α, its mutant derivatives, and HIF-1α (ARNT) were synthesized in vitro as described above. EMSA using proteins was performed as for nuclear extracts, except that binding reactions contained equal volumes of in vitro translation reactions and 2× buffer Z (50 mM Tris-HCl (pH 7.5), 40% glycerol, 200 mM KCl, 0.4 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.2 mM sodium vanadate). 100 ng of calf thymus DNA, and 1× buffer Z (25 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.2 mM sodium vanadate) added to a final volume of 39 μl. After preincubation for 5 min at room temperature, 1 μl of labeled probe W18 (107 cpm) was added and incubated on ice for 15 min. Oligonucleotide competition experiments were performed with 1 ng or 10 ng of unlabeled competitor DNA. The sense and antisense strand sequences of the 5′-GATGAGG-GTACGATG-3′ and 5′-GATCCATGCACTCCTC-3′, respectively, the basic domain of HIF-1α was deleted by the replacement of an NcoI/BglII fragment in pBluescript SK/HIF-1α-3 with a double-stranded digonucleotide (sense and antisense strand sequences, 5′-CATCAGGCGAGTACGATG-3′ and 5′-GATCCATGCACTCCTC-3′, respectively) to generate HIF-1αNABN. The KpnI/NotI fragment encompassing the complete coding sequence of HIF-1α DNA or its derivative was ligated into KpnI/NotI-digested pCEP4 (Invitrogen) using T4 DNA ligase (Strategene). HIF-1α/NABNAB was created by transfer of the HIF-1α/NABN sequence to pCEP4 and deletion of an AscI-BamHI fragment. In Vitro Transcription and Translation—** The HIF-1α DNA and its mutant derivatives in pBluescript SK contained either the T7 or T3 polymerase promoter in the appropriate orientation for in vitro expression. pBMS/NeoM1-1 (27, 34), a plasmid containing the T7 polymerase promoter for in vitro expression. In vitro transcription and translation was carried out using the TNT T7 or T3 coupled reticulocyte lysate system (Promega) in the presence or absence of [35S]methionine (Amersham Corp.) according to the manufacturer's instructions.

**Transcriptional Analysis—** Transcriptional analysis was carried out using the TNT T7 or T3 coupled reticulocyte lysate system (Promega) in the presence or absence of [35S]methionine (Amersham Corp.) according to the manufacturer's instructions.

**Electrophoretic Mobility Shift Assay (EMSA)—** EMSA using Hep3B nuclear extracts was performed with oligonucleotide probe W18 (21, 22). Unlabeled HIF-1α, its mutant derivatives, and HIF-1α (ARNT) were synthesized in vitro as described above. EMSA using proteins was performed as for nuclear extracts, except that binding reactions contained equal volumes of in vitro translation reactions and 2× buffer Z (50 mM Tris-HCl (pH 7.5), 40% glycerol, 200 mM KCl, 0.4 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.2 mM sodium vanadate). 100 ng of calf thymus DNA, and 1× buffer Z (25 mM Tris-HCl (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.2 mM sodium vanadate) added to a final volume of 39 μl. After preincubation for 5 min at room temperature, 1 μl of labeled probe W18 (107 cpm) was added and incubated on ice for 15 min. Oligonucleotide competition experiments were performed with 1 ng or 10 ng of unlabeled competitor DNA. The sense and antisense strand sequences of the double-stranded W18 and M18 oligonucleotides are 5′-GCGCTAGCGTTGCTGCTCA-3′ and 5′-GGCCCTAAAGCGTGCTCTCA-3′, respectively. Supershift analysis was performed with the incubation of 1 μl of pre-immune serum or antisemur (21) against HIF-1α at 1:3 dilution or HIF-1α at 1:6 dilution for 20 min on ice after probe addition.

**Functional Analysis of HIF-1α**—Antiserum against HIF-1α and its mutant derivatives were transcribed and translated in vitro in the presence of [35S]methionine (Amersham). Aliquots (13 μl) of labeled in vitro translation reactions of HIF-1α or its derivatives were mixed with 13 μl of unlabeled in vitro translation reactions programmed with pBS/M/K-mat-1 and 26 μl buffer Z and incubated at 30°C for 15 min. At the end of the incubation, the reaction mixture was placed on ice for 30 min, incubated with 2 μl of affinity-purified HIF-1α antibodies for 2 h at 4°C, and then incubated with 13 μl of 50% protein A-Sepharose 4B in IP buffer for 1 h. The pellets were washed five times with IP buffer,
RESULTS

Communoprecipitation of HIF-1α and HIF-1β from Crude Nuclear Extracts—We have demonstrated previously that antisera raised against recombinant HIF-1α or HIF-1β (ARNT) could supershift the HIF-1/αDNA complex present in nuclear extracts of hypoxic Hep3B cells, suggesting that these proteins bind to DNA as a heterodimer (21). To demonstrate that HIF-1α and HIF-1β associate in the absence of DNA, crude nuclear extracts from Hep3B cells were tested for communoprecipitation of HIF-1α and HIF-1β. Nuclear extracts were prepared from hypoxic and nonhypoxic Hep3B cells and incubated with affinity-purified antibodies against HIF-1α or HIF-1β (ARNT). HIF-1-antibody complexes were precipitated with protein A-Sepharose 4B and fractionated by SDS-PAGE; HIF-1α or HIF-1β protein was identified by immunoblot assay. As shown in Fig. 1 (top panels), HIF-1α was immunoprecipitated from hypoxic nuclear extracts using antibodies raised against either HIF-1α (Fig. 1, lane 4) or HIF-1β (Fig. 1, lane 8). HIF-1α was detected as a series of isoforms as described previously (21) with an apparent molecular mass of approximately 113–131 kDa. HIF-1α was undetectable by this assay when extracts from nonhypoxic cells were immunoprecipitated with HIF-1α (Fig. 1, lane 2) or HIF-1β (Fig. 1, lane 6) antibodies, consistent with the extremely low levels of HIF-1α detected in nonhypoxic Hep3B cells by direct immunoblot assay (21). No HIF-1α was precipitated from hypoxic (Fig. 1, lanes 3 and 7) or nonhypoxic (Fig. 1, lanes 1 and 5) extracts using preimmune serum from the rabbits used for preparation of HIF-1α- and HIF-1β-antibodies, demonstrating that the communoprecipitation of HIF-1α by HIF-1β antibodies was specific.

As shown in Fig. 1 (bottom panels), HIF-1β was also immunoprecipitated from hypoxic nuclear extracts using either HIF-1α (Fig. 1, lane 4) or HIF-1β (Fig. 1, lane 8) antibodies. HIF-1β was detected as isoforms with apparent molecular masses of approximately 100–107 kDa. After immunoprecipitation with HIF-1α antibodies, HIF-1β was detected in hypoxic (Fig. 1, lane 4) but not in nonhypoxic (Fig. 1, lane 2) extracts. Preimmune serum precipitated small amounts of a protein from nonhypoxic (Fig. 1, lane 1) and hypoxic (Fig. 1, lane 3) extracts that was of similar mobility to HIF-1β and which cross-reacted with the anti-immunoglobulin conjugate used for detection of antibody-antigen complexes as it was visualized, even when the HIF-1β antibody was omitted from the immunoblot reaction (data not shown). HIF-1β was more easily detected when HIF-1α antibodies were used for immunoprecipitation (Fig. 1, lane 8) and a small amount of HIF-1β could be detected in immunoprecipitates from nonhypoxic extracts (Fig. 1, lane 6), consistent with previous direct immunoblot assays (21). HIF-1β was not immunoprecipitated by the preimmune serum from nonhypoxic (Fig. 1, lane 5) or hypoxic (Fig. 1, lane 7) nuclear extracts. Taken together, the results of these immunoprecipitation experiments indicate that HIF-1α and HIF-1β are present as a heterodimer in nuclear extracts from hypoxic cells and suggest that not all HIF-1β exists as a heterodimer with HIF-1α, especially in nonhypoxic nuclear extracts.

Reconstitution of HIF-1 DNA Binding Activity by In Vitro-Translated HIF-1α and HIF-1β—We next tested whether in vitro translation of HIF-1α and HIF-1β proteins could reconstitute HIF-1 DNA binding activity. We performed EMSA using as probe a double-stranded oligonucleotide (W18) containing the HIF-1 binding site from the EPO enhancer (7). When unprogrammed reticulocyte lysates were assayed, a nonspecific DNA binding activity was detected by the probe (Fig. 2, lane 1). A similar pattern was seen when lysates were programmed with cDNA encoding HIF-1α (Fig. 2, lane 2) or HIF-1β (Fig. 2, lane 3). However, when lysates were programmed with both HIF-1α and HIF-1β (ARNT) cDNA (Fig. 2, lane 4), a new DNA binding activity was detected with mobility similar to that of HIF-1 present in nuclear extracts from hypoxic Hep3B cells (Fig. 2, lane 14). The recombinant HIF-1-αDNA complex coimerced with the slower-migrating endogenous HIF-1-αDNA complex. Gel retardation sedimentation analysis suggested that the faster- and slower-migrating complexes contain HIF-1α/HIF-1β heterodimers and heterotetramers, respectively (20). As previously demonstrated for endogenous HIF-1 (7), binding of recombinant HIF-1 to the probe could be competed by increasing amounts of unlabeled W18 oligonucleotide (Fig. 2, lanes 5 and 6) but not by unlabeled M18 oligonucleotide containing a 3-bp substitution within the HIF-1 binding site (Fig. 2, lanes 7 and 8). The addition of antisera raised against HIF-1α (Fig. 2, lane 10) or HIF-1β (Fig. 2, lane 12) to binding reactions containing in vitro-translated HIF-1 resulted in disruption of HIF-1-αDNA complexes, whereas the respective preimmune serum had no effect (Fig. 2, lanes 9 and 11). Thus, antisera specifically disrupted complexes containing recombinant HIF-1 rather than resulting in the supershift previously seen when crude nuclear extracts were analyzed (21). This difference may reflect the much lower protein concentrations in binding reactions containing in vitro-translated proteins rather than crude nuclear extracts.
The demonstration that recombinant HIF-1α and HIF-1β can reconstitute HIF-1 DNA binding activity in vitro, although the presence of a required cofactor in reticulocyte lysates cannot be excluded.

Localization of HIF-1α Dimerization and DNA Binding Domains—The demonstration that in vitro-translated HIF-1α and HIF-1β could reconstitute HIF-1 DNA binding activity indicated that the recombinant proteins could heterodimerize in vitro. To demonstrate in vitro heterodimerization in the absence of DNA and to identify the HIF-1α sequences required for dimerization and DNA binding, mutant derivatives were constructed. HIF-1αΔStul, ΔHindIII, ΔAcc, ΔAfII, and full-length (FL) HIF-1α contained the aminoterminal 56, 166, 245, 390, and 826 amino acids of HIF-1α, respectively (Fig. 3A). HIF-1αΔNB contained a deletion of amino acids 4–27, which overlap the basic domain that encompasses amino acids 17–30 (21). HIF-1α and deletion mutants were translated in vitro in the presence of [35S]methionine. The six proteins were synthesized in equal amounts, except for HIF-1αΔNB, which was synthesized with a higher relative efficiency (data not shown). The [35S]-labeled proteins were each mixed with full-length unlabeled HIF-1β (ARNT). IP of labeled HIF-1α by HIF-1β antibodies (pellet) indicated heterodimerization, whereas detection of labeled protein only in the supernatant indicated a lack of heterodimerization (Fig. 3B). HIF-1αΔNB dimerized with HIF-1β (Fig. 3B, lane 4), indicating that the basic domain is not required for heterodimerization. HIF-1αΔAfII (Fig. 3B, lane 5), ΔAcc (Fig. 3B, lane 6), and ΔHindIII (Fig. 3B, lane 7) were also able to heterodimerize with HIF-1β, indicating that the HLH/PAS-A regions of HIF-1α are sufficient for heterodimerization. HIF-1αΔStul, which contained only a truncated bHLH region, did not dimerize with HIF-1β (Fig. 3B, lane 8).

After dimerization of unlabeled HIF-1β (ARNT) with unlabeled HIF-1α or deletion mutant, the reactions were used to assay binding to the labeled W18 probe (Fig. 3C). As previously demonstrated, HIF-1β (Fig. 3C, lane 1) or HIF-1α (Fig. 3C, lane 2) alone did not bind to W18, whereas DNA binding activity was detected in the presence of both full-length proteins (Fig. 3C, lane 3). In the presence of full-length HIF-1β, HIF-1αΔNB (Fig. 3C, lane 4), ΔHindIII (Fig. 3C, lane 7), and ΔStul (Fig. 3C, lane 8) did not generate DNA binding activity, whereas a HIF-1αDNA complex was formed in the presence of ΔAf II (Fig. 3C, lane 5) or ΔAcc (Fig. 3C, lane 6), which generated protein-DNA complexes of increased mobility due to the truncation of HIF-1α. These HIF-1αDNA complexes were competed by an excess of unlabeled oligonucleotide and were disrupted by HIF-1β antiserum (data not shown). These results indicate that DNA binding required the heterodimerization of HIF-1β with HIF-1α containing an intact basic domain. The reduced and absent DNA binding associated with the ΔAcc and ΔHindIII mutants, respectively, suggest that an intact PAS domain is required for optimal binding of the HIF-1α-HIF-1β heterodimer to DNA.

Transcriptional Activation by Recombinant HIF-1α—To determine whether forced expression of HIF-1α and HIF-1β (ARNT) could activate transcription, HIF-1α and ARNT expression vectors were used to cotransfect cells with reporter plasmids containing EPO 3′-flanking sequences shown previously to function as a hypoxia-inducible enhancer (7, 11). SV40 promoter–CAT reporter plasmids (Fig. 4A) contained the wild-type 50-bp EPO enhancer (WT50) or a mutant enhancer (MUT50) containing a 3-bp substitution that was previously shown to eliminate enhancer function and that, when present in oligonucleotide M18, prevented HIF-1 binding (7). Reporter plasmids were cotransfected into Hep3B cells with an SV40 promoter–βgal plasmid (pSVβgal) in the presence or absence of HIF-1α and HIF-1β (ARNT) expression vectors. Transfected cells were split onto two plates, incubated at 20% O2 for 12 h, and then incubated at 1 or 20% O2 for 24 h. CAT:βgal ratios were normalized to those for the WT50 reporter in the absence of expression plasmids at 20% O2. WT50 expression was induced 13-fold by hypoxia in the absence of expression vector (Fig. 4B). In the presence of 0.5 and 5 μg of expression vectors, there was a dose-dependent increase in CAT expression at both 20 and 1% O2. In contrast, MUT50 reporter gene expression was not induced significantly by hypoxia in the absence or presence of expression vectors. These results indicate that recombinant HIF-1α can activate transcription of reporter genes containing an EPO enhancer with an intact HIF-1 binding site.

We also analyzed the effect of increasing amounts of HIF-1α and HIF-1β (ARNT) expression vectors on transcription of the 2xWT33 reporter plasmid (containing two copies of the first 33 bp of the EPO enhancer) (Fig. 4A). For these experiments, we used 293 cells which, in contrast to Hep3B cells, do not express the EPO gene. In addition, we have shown previously that the response of reporter genes to hypoxia is more modest in 293 cells compared to Hep3B cells (11). Thus, these experiments provide analysis of a different reporter plasmid in a different cellular milieu. The CAT:βgal ratios were normalized to the result obtained in cells at 20% O2 in the absence of expression vectors. There was a 2-fold increase in reporter gene expression in response to hypoxia in the absence of expression vectors (Fig. 4C). Relative CAT activity increased with the increasing amount of expression vectors used over the range of 0–8 μg under both hypoxic and nonhypoxic conditions. The relative CAT activity was 7- and 21-fold higher in nonhypoxic and hypoxic cells transfected with 8 μg of expression vectors, respectively, compared to cells transfected without the expression vectors. The large difference in CAT activity in 293 cells transfected with 4 and 8 μg of expression vectors was not seen in Hep3B cells, where the effect of 4 μg was intermediate between that of 2 and 8 μg (data not shown). Taken together,
the results in Fig. 4 indicate that recombinant HIF-1α can mediate sequence-specific and concentration-dependent transcriptional activation in both EPO-producing and -nonproducing cells.

Analysis of Transcriptional Activation Mediated by HIF-1α Deletion Mutants—Expression vectors containing HIF-1α mutant derivatives were created as illustrated in Fig. 5A. A translation stop codon was introduced at the Pst I and Afl II sites of HIF-1α cDNA to generate HIF-1αDPst I and DAfl II, respectively. HIF-1αNB contained deletions of both the basic region (amino acids 4–27) and the carboxy terminus (amino acids 390–826) encoded by sequences distal to the Afl II site in HIF-1α cDNA. Each mutant HIF-1α expression vector was cotransfected into 293 cells with HIF-1β (ARNT) expression vector, 2xWT33 reporter, and pSVβgal. CAT/βgal activity obtained for each HIF-1α mutant construct was normalized to the values obtained in the absence of expression vectors at 20% O2. Expression of full-length HIF-1α resulted in 7- and 29-fold higher levels of relative CAT activity at 20 and 1% O2, respectively, than in the absence of expression vectors (Fig. 5B). HIF-1αΔPst I (amino acids 1–813), which lacked the last 13 amino acids at the carboxy terminus of HIF-1α, activated significantly lower levels of CAT expression than full-length HIF-1α with approximately 5- and 17-fold increases over control levels at 20 and 1% O2, respectively. HIF-1αΔAfl II (amino acids 1–390) mediated extremely reduced levels of reporter gene transactivation, with only 4- and 6-fold increases over control levels at 20 and 1% O2, respectively.

The reduced transactivation mediated by HIF-1αΔPst I and ΔAfl II could be an indirect effect due to changes in protein expression, dimerization, or DNA binding activity. We, therefore, performed an EMSA using the W18 probe and nuclear extracts prepared from cells transfected with full-length HIF-1α (ARNT) expression vector and either vector only, full-length HIF-1α, or one of the deletion mutants (Fig. 5C). Autoradiographic signals were quantitated by laser densitometry. In cells transfected with full-length HIF-1α (Fig. 5C, lanes 3 and 4), there was 4-fold increased HIF-1 DNA binding activity.

Fig. 3. Dimerization and DNA binding analysis of HIF-1α deletion mutants. A, structure of HIF-1α constructs and summary of results. Box, full-length HIF-1α polypeptide with arrows indicating the first and last amino acid of bHLH domain and A and B internal homology units within the PAS domain (hatched); thin line, amino acid sequences of HIF-1α constructs, with carboxyl-terminal amino acid residue indicated. Ability of each polypeptide to heterodimerize with full-length HIF-1β and bind to DNA is indicated (right). B, dimerization analysis of HIF-1α. Each IP reaction contained an equal volume of unlabelled in vitro-translated HIF-1β (ARNT) (except in lanes 1 and 2) and 35S-labeled full-length HIF-1α or the indicated mutant derivative (except in lane 1, which contains 35S-labeled in vitro-translated HIF-1β only). HIF-1β antibodies were used for IP. Pellet and supernatant were subjected to 15% SDS-PAGE and autoradiography. Migration of protein standards (mass in kDa) is indicated (left). C, DNA binding analysis of HIF-1α. EMSA was performed using 6 μl each of in vitro-translated HIF-1β and HIF-1α or indicated mutant derivative, except in lanes 1 and 2, where 12 μl of HIF-1β or HIF-1α alone were used, respectively. Arrow, complex containing the full-length (HIF-1) or truncated (HIF-1') form of HIF-1α, nonspecific DNA binding activity (NS), or free probe (FP).
at 20 and 1% O2 compared to vector-transfected cells (Fig. 5C, lanes 1 and 2). Compared to control cells, HIF-1 DNA binding activity was 5-fold increased in cells transfected with HIF-1α ΔPstI (Fig. 5C, lanes 5 and 6). In cells transfected with HIF-1α ΔAfII, a new DNA binding activity with increased electrophoretic mobility was detected (Fig. 5C, lanes 7 and 8) which, as in the case of the in vitro-translated protein (Fig. 3C), represented probe complexes containing HIF-1β and the trun-
Functional Analysis of HIF-1α

Dimerization and DNA Binding Properties of Endogenous and Recombinant HIF-1α—We showed previously that the HIF-1α/DNA complex generated by incubation of nuclear extracts from hypoxic Hep3B cells with W18 probe contained proteins encoded by the cloned HIF-1α and HIF-1β (ARNT) cDNA sequences (21). This finding did not rule out the possibility that HIF-1α- and HIF-1β-bound DNA independently or heterodimerized only in the presence of DNA. We have now demonstrated by communoprecipitation that HIF-1α and HIF-1β (ARNT) exist as a heterodimer in the absence of DNA, as proposed previously based upon the results of glycerol gradient sedimentation analysis (20). We have also demonstrated that in vitro-translated HIF-1α and HIF-1β (ARNT) can heterodimerize and reconstitute DNA binding activity with electrophoretic mobility, sequence specificity, and molecular composition similar to that of HIF-1 present in nuclear extracts of hypoxic Hep3B cells.

The results presented in this study allow localization of HIF-1α sequences required for dimerization and DNA binding. As demonstrated previously for ARNT (34) and other bHLH proteins, we have shown that disruption of the HIF-1α basic domain eliminates DNA binding without affecting heterodimerization. Previous analysis of ARNT deletion mutants also demonstrated that an intact HLH domain was necessary but not sufficient for dimerization with its alternative partner, AHR (34). A truncated ARNT protein consisting of the complete bHLH and PAS domains heterodimerized with AHR and recognized an AHR/ARNT binding site with high efficiency, whereas a truncated protein consisting of the bHLH and PAS-A domains showed reduced heterodimerization with AHR and greatly reduced DNA binding activity (34). Our analysis of HIF-1α indicated that whereas amino acids 1-166, encompassing the bHLH and PAS-A domain, were sufficient for heterodimerization, optimal DNA binding of the HIF-1α/HIF-1β heterodimer required the presence of HIF-1α aa 1-390, encompassing complete bHLH and PAS domains. These results suggest that the presence of an intact PAS domain may be necessary to allow the basic domain of HIF-1α (and perhaps HIF-1β) to assume a proper conformation for DNA binding. It should be noted that the efficiency of heterodimerization and DNA bind-

![Fig. 6. Effect of a dominant-negative form of HIF-1α on transcriptional activation in hypoxic cells.](image)

**Fig. 6. Effect of a dominant-negative form of HIF-1α on transcriptional activation in hypoxic cells.** Hep3B cells were cotransfected with 2xWT33-luciferase reporter, pSVβgal, and increasing amounts of the expression vector pCEP4/HIF-1αΔNBΔAB. Relative luciferase activity is normalized as a percentage of activity in cells transfected with pCEP4 vector only (column 1). Mean values are from three transfections; bars, S.E.

HIF-1αΔAFIII protein. Remarkably, the DNA binding activity was much greater than that seen with any other construct, and equivalent levels of activity were seen in hypoxic and nonhypoxic cells, with 11-fold higher levels of DNA binding activity than in control hypoxic cells. These results indicate that the decreased transactivation mediated by HIF-1αΔPSTL and ΔAFIII was not due to reduced DNA binding activity and must, therefore, represent a specific loss of transactivation function.

We also analyzed HIF-1α and HIF-1β (ARNT) protein expression in the same nuclear extracts by immunoblot assay (Fig. 5D) and quantitation by laser densitometry. Transfection of full-length expression vectors resulted in increased levels of HIF-1α protein at 20% O2 (Fig. 5D, top panel, lane 3) and 1% O2 (Fig. 5D, top panel, lane 4) compared to mock-transfected control cells (Fig. 5D, top panel, lanes 1 and 2). Increased levels of HIF-1α protein were also detected in cells transfected with HIF-1αΔPSTL (Fig. 5D, top panel, lanes 5 and 6), and deletion of the last 13 amino acids did not appear to alter the electrophoretic mobility of HIF-1αΔPSTL relative to HIF-1αFL. In hypoxic cells overexpressing HIF-1αFL or HIF-1αΔPSTL, there were 20- and 29-fold higher levels of HIF-1α than in control hypoxic cells. The HIF-1α antibodies were raised against amino acids 329-531 of HIF-1α, whereas HIF-1αΔAFIII included only amino acids 1-390. Immunoblot analysis of transfected cells thus revealed endogenous HIF-1α (Fig. 5D, top panel, lane 7 and 8) but not HIF-1αΔAFIII (data not shown) due to insufficient cross-reactivity.

HIF-1β (ARNT) protein levels were also increased in cells transfected with full-length HIF-1β (ARNT) and HIF-1αFL (Fig. 5D, bottom panel, lanes 3 and 4) or HIF-1αΔPSTL (Fig. 5D, bottom panel, lanes 5 and 6). As in the case of HIF-1α, there was a greater increase in HIF-1β levels in transfected cells exposed to 1% O2 (Fig. 5D, bottom panel, lanes 4 and 6) than in cells exposed to 20% O2 (Fig. 5D, bottom panel, lanes 3 and 5), even when the induction of endogenous protein at 1% O2 was taken into account. In cells transfected with HIF-1β (ARNT) and HIF-1αΔAFIII expression vectors, there was a dramatic increase in HIF-1β protein levels at both 20% O2 (Fig. 5D, bottom panel, lane 8) and 1% O2 (Fig. 5D, bottom panel, lane 7) to levels 21-fold higher than in hypoxic control cells. The analysis of HIF-1α and HIF-1β protein expression thus paralleled the results obtained by EMSA (Fig. 5C), indicating that the constitutively increased DNA binding activity in cells expressing HIF-1αΔAFIII and HIF-1β was due to constitutively increased levels of HIF-1β and, presumably, HIF-1αΔAFIII protein.

Expression of a Dominant-negative Form of HIF-1α—We next investigated the effect of overexpressing HIF-1αΔNBΔAB (Fig. 5A), which contains the basic domain deletion that affects DNA binding (Fig. 3C), and the carboxyl-terminal truncation that affects transactivation (Fig. 5B). Based upon the results shown in Fig. 3, we hypothesized that this deletion mutant could heterodimerize with endogenous HIF-1α, generating biologically inactive heterodimers that would be unable to bind DNA and activate reporter gene transcription, thus competing with endogenous HIF-1α for heterodimerization with HIF-1β. Hep3B cells were cotransfected with a constant amount of 2xWT33 reporter plasmid and pSVβgal and increasing amounts of HIF-1αΔNBΔAB expression vector along with the parental pCEP4 vector such that all cells received a total of 40 μg of expression vector. In hypoxic cells, the activation of reporter gene expression by endogenous HIF-1 was inhibited by HIF-1αΔNBΔAB in a concentration-dependent manner such that reporter gene expression in the presence of 40 μg of HIF-1αΔNBΔAB expression vector was reduced to 6% of the levels seen in cells transfected with 40 μg of the parental pCEP4 vector (Fig. 6). These results provide further evidence that hypoxia-induced transcriptional activation of reporter genes containing the EPO enhancer is mediated by HIF-1.
ing by in vitro-translated HIF-1α and HIF-1β was relatively modest, suggesting that posttranslational modification of one or both subunits, which occurs in vivo but not in reticulocyte lysates, is necessary for optimal heterodimerization and/or DNA binding. Alternatively, cofactor(s) present in vivo but not in reticulocyte lysates may be required for optimal activity.

HIF-1 is a Transcriptional Activator—Previous studies indicating that mutations which disrupted HIF-1 binding eliminated enhancer function (7) provided indirect evidence that HIF-1 was a transcriptional activator. In this study, we provide direct evidence from cotransfection assays that forced expression of HIF-1α and HIF-1β (ARNT) is sufficient to activate transcription of reporter genes containing EPO enhancer elements with intact HIF-1 binding sites. In addition to binding site specificity, expression of reporter genes showed a dose-response relationship with respect to the amount of HIF-1α and HIF-1β (ARNT) expression vectors that were cotransfected. Previous studies have identified transactivation domains at the carboxy terminus of AHR and ARNT (36–38). Cotransfection of full-length HIF-1α (ARNT) with truncation mutants of HIF-1α suggest that a transactivation domain is located in the carboxyl-half of HIF-1α. The deletion of amino acids 391–826 in HIF-1αΔAflI decreased reporter gene activation to 13% of that observed with full-length HIF-1α in cells at 1% O2, whereas at 20% O2 HIF-1αΔAflI retained 57% of the activity of full-length HIF-1α. These results suggest that transactivation in cells at 1% O2 is mediated primarily by the HIF-1α carboxyl domain, whereas in cells at 20% O2 another domain, such as the ARNT transactivation domain, plays an important role. As further evidence for a transactivation domain in HIF-1α, we have recently demonstrated that a fusion protein consisting of the GAL4 DNA binding domain and the HIF-1α carboxy-terminal domain strongly transactivates reporter genes containing GAL4 binding sites.

At all levels of expression vectors tested (including both wild-type and deletion mutants of HIF-1α), reporter gene transcription was greater in cells at 1% than at 20% O2. Although these results may be explained in part by the greater expression of endogenous HIF-1 in cells at 1% O2, they also suggest that, in addition to the synthesis of HIF-1α and HIF-1β protein, other hypoxia-induced events occur that are required for maximal transactivation by HIF-1. We have shown previously that HIF-1α and HIF-1β mRNA and protein are extremely unstable in posthypoxic cells (21). Increased reporter gene expression in cotransfected cells cultured at 1% O2 may, therefore, be due to stabilization of HIF-1 mRNA and/or protein in hypoxic cells. This conclusion is supported by the analysis of HIF-1 DNA binding activity and protein levels in transfected cells. In particular, the constitutively increased levels of DNA binding activity in transfected cells expressing HIF-1αΔAflI should be noted. This result implied high levels of HIF-1αΔAflI and HIF-1β protein in these cells. Although we could not determine HIF-1αΔAflI protein levels directly, there was a dramatic increase in HIF-1β levels that, in nonhypoxic cells transfected with HIF-1αΔAflI, were 22-fold higher than in cells transfected with HIF-1αFL. Deletion of the carboxy terminus of HIF-1α may increase its stability, similar to the effect of deleting the amino terminus of c-Jun (39). The carboxy terminus of HIF-1α may target both HIF-1α and HIF-1β for degradation, similar to the manner in which c-Jun targets both itself and its heterodimeric partner c-FOS for proteolysis (40). Pulse-chase experiments in cells expressing epitope-tagged proteins will be required to determine whether increased expression of HIF-1αΔAflI and HIF-1β protein is due to increased synthesis or decreased degradation.

We also demonstrated that a dominant-negative mutant, HIF-1αΔNΔAB, which lacks both the basic DNA binding domain and carboxyl-terminal transactivation domain, could block transactivation of reporter genes containing the EPO enhancer in hypoxic cells, presumably by competing with endogenous HIF-1α for heterodimerization with endogenous HIF-1β. Heterodimers of HIF-1αΔNΔAB and HIF-1β are biologically inactive due to loss of DNA binding activity, as demonstrated in vitro for HIF-1αΔNΔB. These results provide further evidence that the cloned HIF-1 subunits are involved in transactivation via the EPO enhancer and also provide an experimental paradigm through which it may be possible to analyze the biological effects of inactivating HIF-1 function in vivo.

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