MOLECULAR AND CELLULAR BIOLOGY, Sept. 1996, p. 4604–4613 Vol. 16, No. 9
0270-7306/96/$04.00+0

Activation of Vascular Endothelial Growth Factor Gene Transcription by Hypoxia-Inducible Factor 1

JO A. FORSYTHE,[1] BING-HUA JIANG,[2] NARAYAN V. IYER,[2] FATON AGANI,[2] SANDRA W. LEUNG,[2] ROBERT D. KOOS,[1] AND GREGG L. SEMENZA[2]*

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and Center for Medical Genetics, Departments of Pediatrics and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287-3914[2]

Received 4 January 1996/Returned for modification 27 February 1996/Accepted 4 June 1996

Expression of vascular endothelial growth factor (VEGF) is induced in cells exposed to hypoxia or ischemia. Neovascularization stimulated by VEGF occurs in several important clinical contexts, including myocardial ischemia, retinal disease, and tumor growth. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic helix-loop-helix protein that activates transcription of the human erythropoietin gene in hypoxic cells. Here we demonstrate the involvement of HIF-1 in the activation of *VEGF* transcription. *VEGF* 5′-flanking sequences mediated transcriptional activation of reporter gene expression in hypoxic Hep3B cells. A 47-bp sequence located 985 to 939 bp 5′ to the *VEGF* transcription initiation site mediated hypoxia-inducible reporter gene expression directed by a simian virus 40 promoter element that was otherwise minimally responsive to hypoxia. When reporters containing *VEGF* sequences, in the context of the native *VEGF* or heterologous simian virus 40 promoter, were cotransfected with expression vectors encoding HIF-1α and HIF-1β (ARNT [aryl hydrocarbon receptor nuclear translocator]), reporter gene transcription was much greater in both hypoxic and nonhypoxic cells than in cells transfected with the reporter alone. A HIF-1 binding site was demonstrated in the 47-bp hypoxia response element, and a 3-bp substitution eliminated the ability of the element to bind HIF-1 and to activate transcription in response to hypoxia and/or recombinant HIF-1. Cotransfection of cells with an expression vector encoding a dominant negative form of HIF-1α inhibited the activation of reporter transcription in hypoxic cells in a dose-dependent manner. VEGF mRNA was not induced by hypoxia in mutant cells that do not express the HIF-1β (ARNT) subunit. These findings implicate HIF-1 in the activation of *VEGF* transcription in hypoxic cells.

Under normal physiologic conditions, each of the approximately 10^{14} cells in the adult human body is provided with an adequate supply of O_2 to meet its metabolic demands through the concerted function of the pulmonary, hematopoietic, and cardiovascular systems. O_2 is transported through the circulation by erythrocytes, the production of which is controlled by the glycoprotein hormone/growth factor erythropoietin (EPO) (reviewed in references 20, 23, and 43). Cells in the liver and kidney that produce EPO are able to sense O_2 concentration and respond to systemic hypoxia with increased *EPO* gene transcription (8, 15, 46). A hypoxia-inducible enhancer element was identified in the 3′-flanking region of the human and mouse *EPO* genes (2, 3, 33, 42, 48, 50). Hypoxia-inducible factor 1 (HIF-1) was detected in nuclear extracts of hypoxic Hep3B cells (exposed to 1% O_2 for 4 h) and was undetectable in extracts from nonhypoxic cells (maintained at 20% O_2). HIF-1 bound to the *EPO* enhancer, and mutations that eliminated HIF-1 binding also eliminated enhancer function (50). Exposure of hypoxic cells to inhibitors of protein synthesis (cycloheximide) or phosphorylation (2-aminopurine) inhibited the induction of both EPO mRNA and HIF-1 DNA-binding activity, and other inducers of *EPO* expression ($CoCl_2$ and desferrioxamine) also induced HIF-1 activity (50, 58, 60). Methylation interference analysis revealed that HIF-1 bound to the *EPO* enhancer sequence 5′-TACGTGCT-3′ by making major groove contacts with both guanine residues on each strand (59).

Protein purification indicated that HIF-1 was a heterodimeric protein (61). Peptide and nucleic acid sequence analysis demonstrated that both subunits were basic helix-loop-helix (bHLH) proteins (57). HIF-1α was a novel 826-amino-acid polypeptide, whereas HIF-1β was identical to the 774- and 789-amino-acid products of the *ARNT* (aryl hydrocarbon receptor nuclear translocator) gene previously shown to heterodimerize with the aryl hydrocarbon receptor (AHR) (57). HIF-1α, HIF-1β (ARNT), and AHR are all members of a subfamily of bHLH proteins that contain a conserved PAS domain following the bHLH motif (4, 18, 57). In all three polypeptides, the basic domain is required for DNA binding following heterodimerization mediated by the HLH and PAS domains, and the C terminus contains one or more transactivation domains (6, 21, 29, 32, 44, 63). Forced expression of HIF-1α and HIF-1β (ARNT) in cultured cells transfected with a reporter plasmid containing the *EPO* enhancer resulted in significantly higher levels of transcription, both at 1% and at 20% O_2, than in cells transfected with the reporter plasmid alone, demonstrating that transcriptional activation via the *EPO* enhancer is mediated by HIF-1 (21).

In contrast to systemic hypoxia, which elicits increased EPO synthesis, hypoxia can also be restricted to cells within a localized region of a specific organ, usually as a result of insufficient perfusion, as in the case of myocardial ischemia secondary to coronary artery disease. Vascular endothelial growth factor (VEGF) plays a central role in angiogenesis and neovascularization (reviewed in references 13 and 22). VEGF has been implicated in the neovascularization associated with chronic

* Corresponding author. Mailing address: The Johns Hopkins Hospital, CMSC-1004, 600 N. Wolfe St., Baltimore, MD 21287-3914. Phone: (410) 955-1619. Fax: (410) 955-0484. Electronic mail address: gsemenza@welchlink.welch.jhu.edu.

myocardial ischemia (1), retinal ischemia (36, 39), and tumor progression (40, 41, 51). Just as EPO stimulates the survival and proliferation of erythroid progenitors, VEGF is a potent mitogen for vascular endothelial cells (9, 10, 17, 26). In contrast to the cell-type-restricted pattern of *EPO* expression, many different primary and cultured cell types respond to hypoxia by increasing *VEGF* expression (14, 16, 24, 37, 38, 51), which may provide a genetic mechanism by which cells are assured of receiving adequate perfusion under normal physiological conditions. *VEGF* expression is induced by exposing cultured cells to 1% O_2, $CoCl_2$, or desferrioxamine, and induction can be blocked by treating hypoxic cells with cycloheximide (14, 16, 51). Recent studies have demonstrated that in several different cell types, hypoxia induces both increased transcription and decreased degradation of VEGF mRNA (10a, 19, 28, 30). Given the similarities to *EPO* expression, we tested the hypothesis that HIF-1 was also involved in *VEGF* transcriptional activation in hypoxic cells.

MATERIALS AND METHODS

Reporter plasmid constructs. A lambda phage clone containing DNA extending from the 5′-flanking region through exon 2 of the human *VEGF* gene (56) was provided by E. Tischer and J. Abraham (Scios Nova Inc., Mountain View, Calif.). Phage DNA was isolated and digested with *Eco*RI, and a 4.5-kb fragment containing the 5′-flanking region and part of the coding sequence was isolated, digested with *Kpn*I, and subcloned into pBluescript KS+ (Stratagene, La Jolla, Calif.). A 2.65-kb *Kpn*I-*Bss*HII fragment lacking the translation initiation site was ligated into the *Kpn*I-*Mlu*I sites of pGL2-Basic (Promega, Madison, Wis.) which contains firefly luciferase (luc) coding sequences as well as simian virus 40 (SV40) intron and polyadenylation signals but lacks eukaryotic promoter or enhancer elements. The final full-length reporter construct (*VEGF-Kpn*I) contained the *VEGF* sequence from −2274 to +379 relative to the transcription initiation site (56). A series of deletions of the 5′-flanking region was prepared by restriction endonuclease digestion and religation using T4 DNA polymerase and T4 DNA ligase (New England Biolabs).

In subsequent experiments, a series of plasmid constructs was prepared by using PCR to amplify specific segments of the *VEGF* 5′-flanking region. Each primer contained the sequence 5′-CAT-3′ followed by a 6-nucleotide (nt) restriction enzyme recognition site and 17 to 18 nt of *VEGF* sequence (full primer sequences available upon request). PCR was performed with 15 ng of *VEGF-Kpn*I plasmid DNA, 1 μM each primer, 25 μM deoxyribonucleotides, and 1.25 U of *Pfu* polymerase (Stratagene) under the following conditions: 1.5 min at 94°C; 0.5 min at 92°C, 1 min at 50°C, and 1.5 min at 72°C for 20 cycles; and 5 min at 72°C. PCR products for *VEGF*-P1, -P7, -P8, and -P9 were digested with *Kpn*I and *Sac*II and ligated into *Kpn*I-*Sac*II-digested *VEGF-Kpn*I. PCR products for P10 to P12 were digested with *Kpn*I and *Mlu*I and ligated into pGL2-Promoter (Promega), which contains the SV40 basal promoter upstream of luc coding sequences, SV40 intron, and polyadenylation signal. P11m was prepared as for P11w except for the presence of a 3-nt substitution in the forward primer.

Expression vector constructs. The human HIF-1α expression vector pCEP4/HIF-1α3.2T7 was constructed by subcloning the 3.4-kb *Kpn*I-*Not*I fragment from pBluescriptSK/HIF-1α3.2T7 (57) into pCEP4 (Invitrogen) downstream of the cytomegalovirus promoter. The human HIF-1β (ARNT) expression vector pBM5neo/M1-1 (18) was provided by O. Hankinson (University of California at Los Angeles). The plasmid expressing the dominant negative form of HIF-1α, pCEP4/HIF-1αDN, was constructed as follows: DNA sequences encoding the basic domain of HIF-1α were deleted by replacement of an *Nco*I-*Bgl*II fragment in pBluescriptSK/HIF-1α3.2T7 with a double-stranded oligonucleotide (5′-CAT GGAGGGGATCGATG-3′ and 5′-GATCCATCGATCCCCTC-3′) to generate HIF-1αΔNB. pCEP4/HIF-1αDN was created by transfer of the *Kpn*I-*Not*I fragment containing HIF-1αΔNB cDNA sequences to pCEP4 and deletion of an *Afl*II-*Bam*HI fragment, encoding the carboxyl terminus of HIF-1α, by digestion and religation.

Transient expression assays. Hep3B cells were maintained in culture as previously described (50). Plasmid DNA was prepared by using commercial kits (Qiagen) and transfected into cells by electroporation with a Gene Pulser (Bio-Rad) at 260 V and 960 μF. Duplicate electroporations were pooled and split on to six tissue culture dishes (60 by 15 mm; Corning) containing 2.2 ml of medium. Cells were allowed to recover for 24 h in a 5% CO_2–95% air incubator at 37°C. The cells were given fresh medium, and three plates from each set were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, Calif.), which was flushed with 1% O_2–5% CO_2–94% N_2, sealed, and placed at 37°C. Cells were harvested 48 or 72 h after transfection. Cell pellets were resuspended in 0.25 M Tris HCl (pH 8.0), and extracts were prepared by four freeze-thaw cycles. Protein concentrations were determined by a commercial kit (Bio-Rad), using bovine serum albumin as the standard. β-Galactosidase (β-Gal) activity was determined by the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (Promega), using 25 μg of extract at 37°C for 1 h, as measured by the A_{420}. Luc activity was determined by using 20 μg of cell extract and 100 μl of luc assay reagent (Promega), which were mixed briefly and placed in a luminometer (Tropix). Light production was measured for 15 s, and results were expressed as relative light units (RLU). Each extract was assayed twice, and the mean RLU was corrected by values obtained from an extract prepared from nontransfected cells. The relative luc activity (mean ± standard error of the mean [SEM]) was calculated as luc (RLU)/β-Gal (A_{420} per milligram of protein per hour).

EMSA. Nuclear extracts were prepared from Hep3B and Hepa1 cells as described previously (50, 61). Electrophoretic mobility shift assay (EMSA) was performed by incubating 5 μg of nuclear extract with 10^4 cpm (~0.2 ng) of ^{32}P-labeled, double-stranded oligonucleotide probe in 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, 1.2 mM sodium vanadate) as described previously (50, 61). Competition experiments were performed with 3 to 50 ng of unlabeled double-stranded oligonucleotide (1 ng represents a fivefold molar excess relative to probe). HIF-1α and HIF-1β (ARNT) proteins were transcribed and translated in vitro from pBluescriptSK/HIF-1α3.2T7 and pBM5neo/M1-1 DNA templates, using the TNT coupled reticulocyte lysate system (Promega). EMSA using in vitro-translated proteins was performed as for nuclear extracts except that the binding reaction mixtures contained equal volumes of in vitro translation reaction mixtures and 2× buffer Z+ (50 mM Tris-HCl [pH 7.6], 40% glycerol, 200 mM KCl, 0.4 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.2 mM sodium vanadate) and 100 ng of calf thymus DNA in a final volume of 40 μl. Competition experiments using in vitro-translated proteins were performed with 100 ng of unlabeled oligonucleotides. For supershift analysis (57), 1 μl of preimmune serum or antiserum specific for HIF-1α or HIF-1β (ARNT), diluted 1:3 or 1:10, respectively, was added to the binding reaction mixture, which was incubated for 30 min on ice.

RNA blot hybridization. Total RNA was isolated by the acid-guanidinium-phenol-chloroform method (5). Fifteen-microgram aliquots of RNA were fractionated by 1.4% agarose–2.2 M formaldehyde gel electrophoresis at 30 V overnight and transferred to a nylon membrane (Schleicher & Schuell) for hybridization with a 0.5-kb rat VEGF cDNA (62) (provided by R. Wenger and M. Gassmann, University of Zurich), which was ^{32}P-labeled by using a random primer-labeling kit (BRL GIBCO), in Quik-Hyb (Stratagene) at 67°C. Blots were washed in 15 mM NaCl–1.5 mM sodium citrate–0.1% sodium dodecyl sulfate (SDS) at 50°C. The 18S rRNA oligonucleotide, 5′-ACGGTATCTGATCGTCT TCGAACC-3′ (provided by A. Choi, Johns Hopkins University), was 5′-end labeled with T4 DNA kinase (BRL GIBCO), hybridized at 65°C, and washed at 45°C as described above.

Immunoblot assay. Fifteen-microgram aliquots of nuclear extracts were fractionated by SDS–7% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, incubated with affinity-purified anti-HIF-1β antibodies followed by horseradish peroxidase anti-immunoglobulin conjugate, and developed with ECL reagents (Amersham) as previously described (57).

RESULTS

Functional analysis of *VEGF* 5′-flanking sequences. To determine whether the 5′-flanking region of the *VEGF* gene could mediate transcriptional responses to cellular hypoxia, we constructed reporter plasmids in which *VEGF* 5′-flanking sequences were fused to luc coding sequences (Fig. 1A). This series of 5′ deletion mutants was transfected into Hep3B human hepatoblastoma cells, which have previously been shown to inducibly express *VEGF* in response to hypoxia (16). The transfected cells were split on to two plates and, after 24 h at 20% O_2, were incubated at 1 or 20% O_2 for 48 h. To correct for variable transfection efficiency, cells were cotransfected with a pSVβgal plasmid containing *Escherichia coli* β-Gal coding sequences under the control of the SV40 promoter and enhancer. Cell extracts were assayed for luc and β-Gal activity, and the luc/β-Gal ratio was determined. Each reporter plasmid was assayed in at least four independent transfection experiments, and the mean luc/β-Gal value was normalized to the results obtained with the *VEGF-Kpn*I reporter in cells at 20% O_2 to generate the relative luc activity.

The results shown in Fig. 1B demonstrate that the full-length *VEGF-Kpn*I reporter, containing 2,274 nt of 5′-flanking DNA, mediated a 8.9-fold-greater level of luc expression in cells exposed to 1% O_2 compared with cells exposed to 20% O_2. The deletion of sequences between −2274 and −131 relative to the transcription initiation site had no significant negative effect on reporter gene expression in cells at 20% O_2. In con-

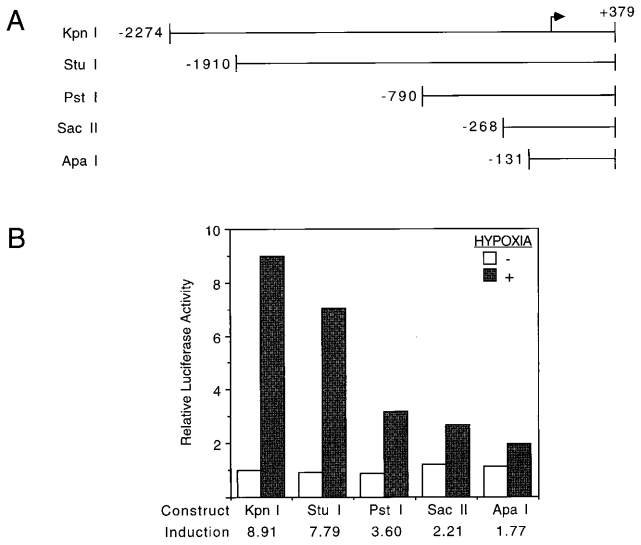

FIG. 1. Functional analysis of human *VEGF* 5′-flanking sequences in transient expression assays. (A) *VEGF*-luc reporter constructs. Each deletion construct is designated by the restriction enzyme used to generate the 5′ end, the coordinate of which is indicated relative to the transcription initiation site (bent arrow). The 3′ end of each construct (+379) was fused to luc coding sequences. (B) Expression in Hep3B cells. *VEGF*-luc and pSVβgal plasmids were cotransfected into Hep3B cells, which were split on to two plates that were incubated at 20% O_2 (open boxes) or 1% O_2 (closed boxes) for 48 h. Relative luc activity represents the mean luc/β-Gal ratio from four to nine independent transfection experiments. The SEM was ≤15% for all values shown.

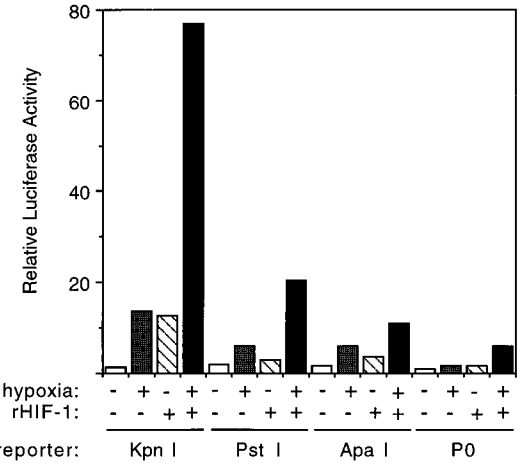

FIG. 2. Cotransfection of *VEGF*-luc reporter and HIF-1 expression vectors. *VEGF*-luc and pSVβgal plasmids were cotransfected into Hep3B cells with or without rHIF-1 (HIF-1α and HIF-1β expression vectors), incubated for 24 h at 20% O_2, and then incubated at 20 or 1% O_2 for an additional 24 h. Mean values (SEM ≤25%) from three transfections are shown.

trast, the ability of the reporter to respond to hypoxia decreased markedly as 5′-flanking sequences were deleted. The most significant effect was associated with the deletion of sequences between the *Stu*I site at −1910 and the *Pst*I site at −790. An additional but less marked loss of induction occurred with the deletion of sequences between the *Pst*I site at −790 and the *Sac*II site at −268, although in this case the loss of induction was due to both increased expression at 20% O_2 and decreased expression at 1% O_2. These results demonstrate that (i) *VEGF* 5′-flanking sequences mediate transcriptional responses to hypoxia in Hep3B hepatoma cells as previously reported for C6 glioma (19), HeLa cervical carcinoma (37), PC12 pheochromocytoma (28), and pulmonary artery endothelial (30) cells; (ii) one or more important *cis*-acting regulatory elements are located between −1910 and −268; and (iii) these regulatory sequences are specifically involved in mediating hypoxia-inducible, as opposed to basal, transcription.

Cotransfection of *VEGF* reporter plasmids with HIF-1 expression vectors. While this study was in progress, cDNA sequences encoding HIF-1 were isolated (57). To determine whether the *VEGF* 5′-flanking sequences that mediated hypoxia-inducible transcription could functionally interact with HIF-1, we cotransfected Hep3B cells with *VEGF* reporter and pSVβgal control plasmids in the presence or absence of HIF-1α and HIF-1β (ARNT) expression vectors. Cells were incubated at 1 or 20% O_2 for 24 h (rather than 48 h as in the previous experiment), and the luc/β-Gal values were normalized to the results from cells transfected with *VEGF-Kpn*I and incubated at 20% O_2. Three *VEGF* reporter constructs (*Kpn*I, *Pst*I, and *Apa*I) were tested, as was a reporter (P0) in which luc coding sequences were under control of the SV40 promoter. Cotransfected cells received 10 μg of reporter and 1 μg of each expression vector.

In cells cotransfected with HIF-1α and HIF-1β (ARNT) expression vectors, there was a dramatic activation of *VEGF-Kpn*I reporter expression both at 1 and 20% O_2 (Fig. 2). In cells at 20% O_2, luc expression was 10.6-fold greater in the presence (lane 3) than in the absence (lane 1) of HIF-1 expression vectors (recombinant HIF-1 [rHIF-1]) and approached the level of the endogenous response to hypoxia (lane 2). In cells at 1% O_2, reporter gene expression was 5.6-fold greater in the presence (lane 4) than in the absence (lane 2) of rHIF-1 and 77-fold greater in hypoxic cells cotransfected with rHIF-1 (lane 4) than in nonhypoxic cells that were not cotransfected with rHIF-1 (lane 1). Reporter gene expression increased in a dose-dependent manner over the range of 0 to 10 μg of each expression vector (data not shown). The effect of forced HIF-1 expression on each of the other reporter plasmids diminished in parallel with the response of the reporter to hypoxia in the absence of rHIF-1. These results suggest that HIF-1 was capable of activating transcription selectively through DNA sequences required for hypoxia-inducible transcription.

Localization of sequences required for transcriptional response to hypoxia and cotransfected HIF-1 expression vectors. Inspection of *VEGF* 5′-flanking sequences revealed two potential HIF-1 binding sites which differed by no more than one nucleotide from the consensus binding-site sequence 5′-BAC GTGCK-3′ that was derived from bona fide HIF-1 binding sites previously identified in genes encoding EPO and glycolytic enzymes (49). The *VEGF* sequence 5′-TACGTGGG-3′ was located between −975 and −968 in the same orientation as the transcriptional unit, whereas the sequence 5′-TACGT GCG-3′, located between −306 and −313, was in the opposite orientation (Fig. 3A). Reporter constructs containing both the −975 and −306 sites (*VEGF*-P7), −306 site only (*VEGF*-P1), −975 site only (*VEGF*-P8), or neither site (*VEGF*-P9) were transfected into Hep3B cells in the presence or absence of rHIF-1 and incubated at 1 or 20% O_2 for 24 h. The P0 reporter was also tested as a negative control, and the luc/β-Gal ratios were normalized to the results obtained from cells transfected with P0 and incubated at 20% O_2.

In cells transfected with the *VEGF*-P7 vector, which contained both potential HIF-1 sites, luc expression at 20% O_2 (Fig. 3B, lane 5) was twofold higher than in P0-transfected cells

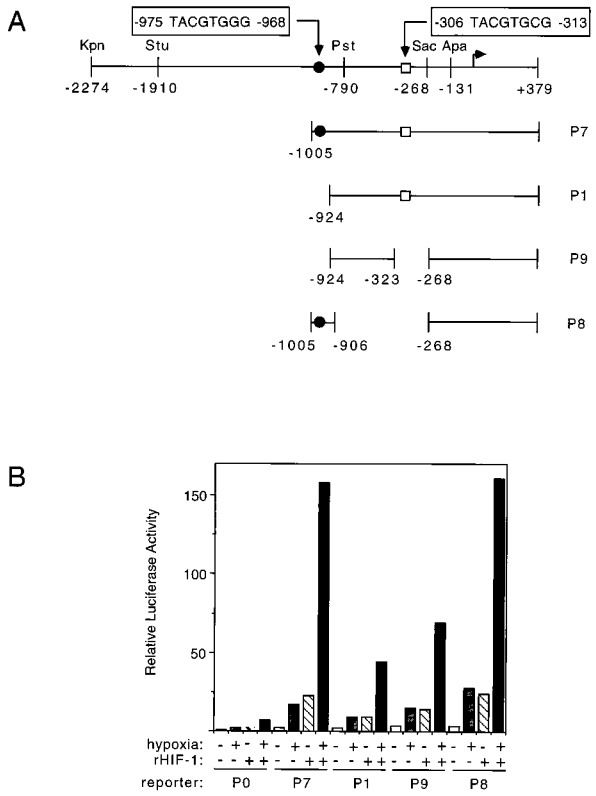

FIG. 3. Localization of *VEGF* 5′-flanking sequences that mediate transcriptional responses to 1% O_2 and rHIF-1. (A) *VEGF* sequences present within constructs. (B) Cotransfection assays. Mean values (SEM ≤20%) from three transfections are shown.

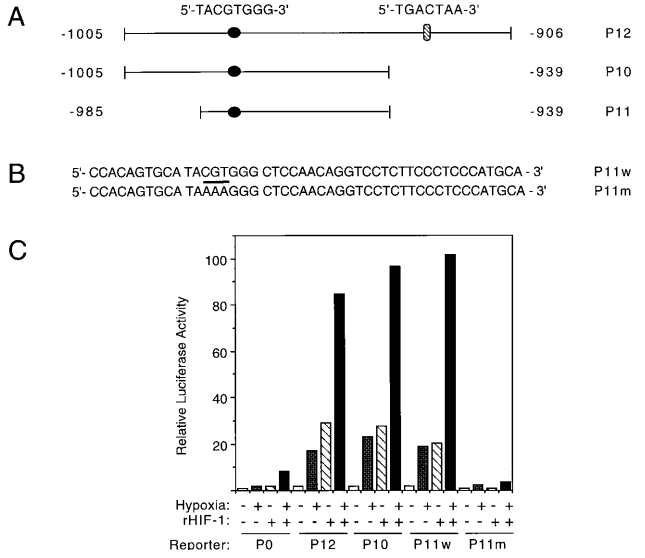

FIG. 4. Identification of a hypoxia response element in the *VEGF* 5′-flanking region. (A) *VEGF* sequences contained within constructs. Indicated *VEGF* sequences were cloned 5′ to an SV40 promoter-luc transcription unit. (B) The 47-bp hypoxia response element. Wild-type (P11w) and mutant (P11m) sequences are shown, with the site of the 3-bp substitution in P11m indicated (bar). (C) Cotransfection assays. Mean values (SEM ≤20%) from three transfections are shown.

(lane 1). *VEGF*-P7 expression was 8.4-fold higher in cells at 1% O_2 (lane 6) than in cells at 20% O_2 (lane 5) in the absence of rHIF-1. Compared with cells transfected with reporter only, cotransfection of rHIF-1 increased *VEGF*-P7 expression 11.0-fold in cells at 20% O_2 (lane 7) to levels exceeding those in cells at 1% O_2 in the absence of rHIF-1 (lane 6). In hypoxic cells transfected with rHIF-1, there was a 9.1-fold increase in *VEGF*-P7 expression (lane 8) compared with that in hypoxic cells transfected with reporter only (lane 6) and a 76-fold increase compared with levels in nonhypoxic cells without rHIF-1 (lane 5).

Cells transfected with *VEGF*-P1 (Fig. 3B, lanes 9 to 12) or *VEGF*-P9 (lanes 13 to 16) had similar levels of expression that were less than those of *VEGF*-P7-transfected cells but significantly higher than those of P0-transfected cells, both in the presence and in the absence of rHIF-1. *VEGF*-P8-transfected cells (lanes 17 to 20) showed a pattern of high-level expression that was identical to that of *VEGF*-P7-transfected cells (lanes 4 to 8), both in the presence and in the absence of rHIF-1. These results indicate that (i) when joined to the −268 to +379 (*VEGF-Sac*II) fragment, sequences between −1005 and −906 mediate the same magnitude of response to hypoxia and/or rHIF-1 as the full-length *VEGF* 5′-flanking sequence, implicating the −975 putative HIF-1 binding site in transcriptional induction; (ii) the potential HIF-1 site at −306 appears to play no role in mediating transcriptional activation of reporter genes in response to hypoxia and/or rHIF-1; and (iii) sequences located between −924 and +379 can also mediate modest transcriptional responses to hypoxia and/or rHIF-1.

Functional dissection of a hypoxia response element from the *VEGF* 5′-flanking region. The results shown in Fig. 3 implicated sequences between −1005 and −906 in the transcriptional response to hypoxia and rHIF-1, but the interpretation of these data was complicated by the fact that constructs lacking these sequences retained some inducibility. To analyze these sequences in isolation from any other *VEGF* 5′-flanking DNA, the −1005 to −906 sequence was cloned 5′ to the SV40 promoter in the P0 reporter to generate P12 (Fig. 4A). Expression of P12 was 8.5-fold higher in cells at 1% than in cells at 20% O_2 in the absence of rHIF-1 (Fig. 4C, lanes 5 and 6), similar to the maximal level of hypoxic induction mediated by the *VEGF*-promoter reporter genes (Fig. 1 to 3). In the presence of rHIF-1, P12 expression increased 14.4-fold in cells at 20% O_2 (Fig. 4C, lane 7) to levels exceeding those in cells at 1% O_2 without rHIF-1 (lane 6). Compared with P0, P12 mediated 8.8- and 10.5-fold-higher levels of expression at 1% O_2 in the absence and presence of rHIF-1, respectively (compare lanes 7 and 3 and lanes 8 and 4).

In addition to the putative HIF-1 site at −975, the −1005 to −906 sequence contained a potential AP-1 binding site, 5′-TGACTAA-3′. To analyze the potential involvement of this site in transcriptional regulation, we constructed reporter P10, which contained sequences from −1005 to −939 that did not include the potential AP-1 site (Fig. 4A). The expression pattern of P10 was essentially identical to that of P12 (Fig. 4C; compare lanes 5 to 8 with lanes 9 to 12), indicating that the potential AP-1 site plays no role in the transcriptional response to hypoxia, which is consistent with a previous report (10a). To further localize the hypoxia response element, we constructed reporter P11, which contained only 47 bp of *VEGF* 5′-flanking sequence between −985 and −939 (Fig. 4A). P11 also showed a pattern of expression that was not significantly different from that of P12 or P10 (Fig. 4C, lanes 13 to 16, labeled P11w). A 3-bp substitution was introduced into P11w to generate P11m (Fig. 4B). This mutation (underscored) altered the putative

HIF-1 binding site from 5′-TACGTGGG-3′ to 5′-TAAAAGGG-3′. An analogous mutation in the *EPO* enhancer, 5′-TACGTGCT-3′ to 5′-TAAAAGCT-3′, completely eliminated hypoxia-inducible reporter gene expression (50). Expression of reporter P11m was not significantly induced by hypoxia and/or HIF-1 (Fig. 4C, lanes 17 to 20). These results indicate that (i) a 47-bp sequence from the *VEGF* 5′-flanking region is sufficient to mediate hypoxia-inducible transcription and (ii) a 3-bp substitution within a putative HIF-1 binding site eliminates hypoxia-inducible transcription mediated by this element.

Inhibition of *VEGF* reporter gene expression in hypoxic cells by a dominant negative form of HIF-1α. To further evaluate the involvement of HIF-1 in mediating transcriptional activation of reporter genes containing the *VEGF* hypoxia response element, we used a dominant negative form of HIF-1α. The pCEP/HIF-1αDN construct encodes a form of HIF-1α lacking the amino-terminal basic domain required for DNA binding and the carboxy-terminal transactivation domain. HIF-1αDN can heterodimerize with HIF-1β and inhibit the activation of reporter genes containing the *EPO* enhancer in hypoxic Hep3B cells (21). Hep3B cells were cotransfected with the P11w reporter plasmid and increasing amounts of the pCEP/HIF-1αDN expression vector and incubated at 1% O_2. The total amount of expression vector was held constant by cotransfecting the parental vector pCEP4 to a total of 40 μg (Fig. 5A). Luc/β-Gal ratios from hypoxic cells were normalized to the result obtained from cells transfected with pCEP4 only (lane 1). There was a progressive decrease in reporter gene expression in cells transfected with increasing amounts of pCEP/HIF-1αDN (lanes 2 to 7). In cells transfected with 40 μg of pCEP/HIF-1αDN (lane 7), expression was 20-fold less than in cells transfected with 40 μg of pCEP4 (lane 1). These results suggest that in hypoxic cells, reporter gene activation is mediated by HIF-1.

We also determined the effect of pCEP/HIF-1αDN cotransfection on the expression of reporter plasmids containing *VEGF* promoter sequences (Fig. 3) that either contained (*VEGF*-P7) or lacked (*VEGF*-P1) the hypoxia response element present in P11w. A submaximal amount (10 μg) of pCEP/HIF-1αDN was used to guard against any potential nonspecific transcriptional effects due to large amounts of transfected expression vector (Fig. 5B). Although *VEGF*-P7 expression was greater than *VEGF*-P1 expression in hypoxic cells as previously demonstrated (Fig. 3), expression of *VEGF*-P7 and that of *VEGF*-P1 were repressed to similar degrees in the presence of pCEP/HIF-1αDN (Fig. 5B). These data complement the results obtained by cotransfection of wild-type HIF-1α and HIF-1β expression vectors (Fig. 3), and together these experiments provide evidence that the hypoxia-inducible expression of both reporter plasmids is mediated by HIF-1.

Binding of HIF-1 to *VEGF* sequences. When an 18-bp oligonucleotide probe containing the HIF-1 binding site from the *EPO* enhancer (WT EPO [Fig. 6A]) was incubated with nuclear extracts from hypoxic Hep3B cells, complexes containing HIF-1 and constitutively expressed factor(s) were detected (Fig. 6B, lane 1) as previously demonstrated (50). An excess of unlabeled oligonucleotide WT EPO competed with the probe for binding of HIF-1 (lane 3), whereas an oligonucleotide containing a 3-bp substitution in the HIF-1 binding site (MUT EPO [Fig. 6A]) did not compete for binding (Fig. 6B, lane 4). We also synthesized a 21-bp oligonucleotide spanning nucleotides −979 to −959 of the *VEGF* 5′-flanking sequence (WT VEGF [Fig. 6A]). An excess of unlabeled WT VEGF also competed with the WT EPO probe for binding to HIF-1 (Fig. 6B, lane 2). Oligonucleotide WT VEGF was also used as the probe and showed a pattern of binding (lane 5) which was identical to that of the WT EPO probe (lane 1), as both HIF-1 and a constitutive DNA-binding activity were detected by the probe. The binding of HIF-1 to the WT VEGF probe could be competed for by an excess of unlabeled WT VEGF (lane 6) or WT EPO (lane 7) but not by MUT EPO (lane 8).

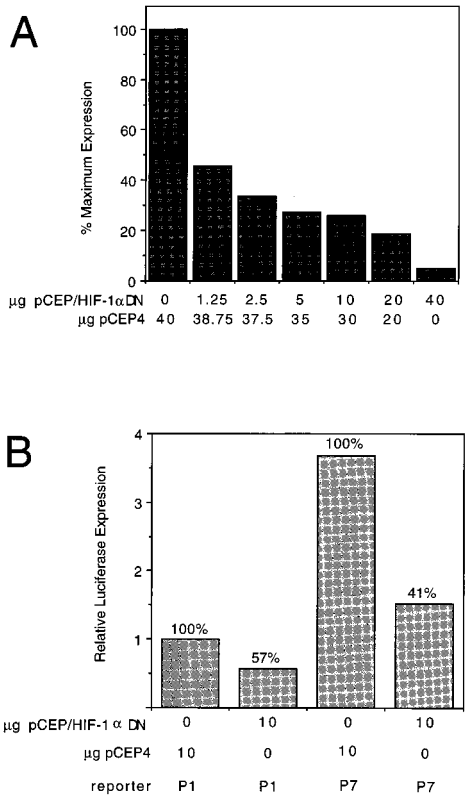

FIG. 5. Effect of a dominant negative form of HIF-1α on the transcriptional response to hypoxia. (A) Expression of the P11w reporter plasmid. Hep3B cells were cotransfected with the P11w reporter and increasing amounts of the expression vector pCEP/HIF-1αDN. Relative luc activity is expressed as percentage of activity in cells transfected with the parental expression vector pCEP4 only (lane 1). Mean values (SEM ≤25%) from three transfections are shown. (B) Expression of P1 and P7 reporter plasmids. Hep3B cells were cotransfected with either P1 or P7 in the presence of 10 μg of either pCEP4 or pCEP/HIF-1αDN. Transfected cells were exposed to 1% O_2 for 48 h prior to harvesting for luc and β-Gal assays. The mean luc/β-Gal ratios from three transfection experiments were normalized to the result obtained for P1 cotransfected with pCEP4. For P1 and P7, the relative expression in the presence of pCEP4 (100%) and pCEP/HIF-1αDN is also indicated.

Polyclonal antisera raised against rHIF-1α or HIF-1β (ARNT) disrupted formation of the WT EPO probe/HIF-1 complex (reference 57 and Fig. 8B). Binding of HIF-1 to WT VEGF was also disrupted by antiserum raised against HIF-1α (Fig. 6B, lane 9) or HIF-1β (lane 11) but not by the corresponding preimmune serum (lanes 10 and 12). This finding formally demonstrates that the nuclear protein which formed a complex with WT VEGF of the same mobility and sequence specificity as the protein which complexed with WT EPO was in fact HIF-1.

To correlate the transcriptional and DNA-binding assays, we synthesized an oligonucleotide (MUT VEGF [Fig. 6A]) which contained the same 3-bp substitution that resulted in the loss of function of the P11m reporter (Fig. 5). When WT VEGF was used as the probe, HIF-1 could be detected in nuclear extracts prepared from hypoxic Hep3B cells (Fig. 6C, lane 2), but there was little HIF-1 activity in nonhypoxic Hep3B cells (lane 1) as

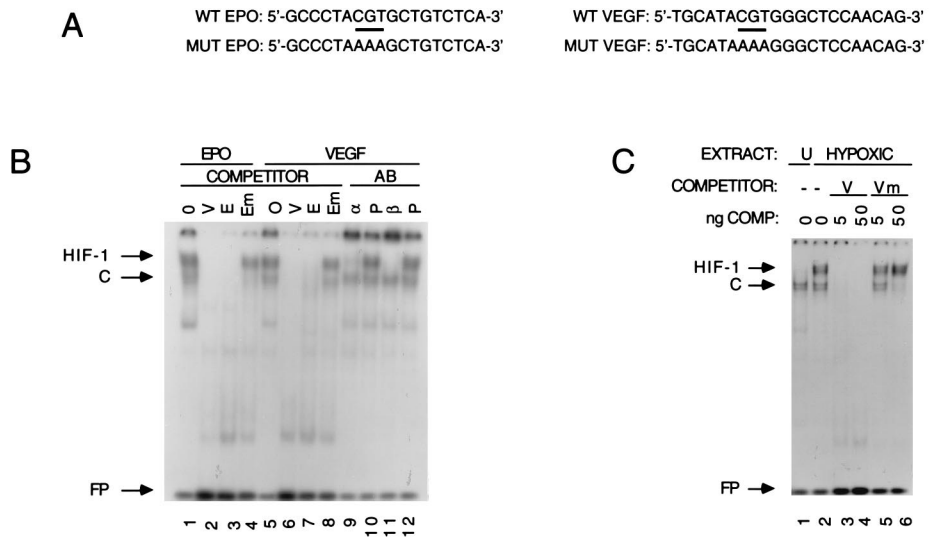

FIG. 6. Gel shift assays of Hep3B nuclear extracts for binding of HIF-1 to *EPO* and *VEGF* sequences. (A) Oligonucleotide sequences. The coding-strand sequence of each double-stranded oligonucleotide is shown. Sites of 3-bp substitutions in oligonucleotides MUT VEGF and MUT EPO are indicated by bars. (B) Comparison of DNA-binding activities recognized by EPO and VEGF probes. Oligonucleotide WT EPO (lanes 1 to 4) or WT VEGF (lanes 5 to 12) was ^{32}P labeled and incubated with 5-μg aliquots of nuclear extract from hypoxic Hep3B cells. For competition assays (lanes 1 to 8), 50 ng of unlabeled oligonucleotide was included in the binding reaction mixture as indicated: 0, no competitor (lanes 1 and 5); V, WT VEGF (lanes 2 and 6); E, WT EPO (lanes 3 and 7); Em, MUT EPO (lanes 4 and 8). For supershift assays (lanes 9 to 12), antiserum (AB) raised against recombinant HIF-1α (lane 9) or HIF-1β (lane 11) or the respective preimmune serum (lanes 10 and 12) was added to the binding reaction mixture. Complexes containing HIF-1 or the constitutive binding activity (C) and the location of free probe (FP) are indicated at left. (C) Specificity of HIF-1 binding to the VEGF probe. Aliquots (5 μg) of nuclear extract from untreated (U; lane 1) or hypoxic (lanes 2 to 6) Hep3B cells were incubated with the WT VEGF probe in the presence or absence of unlabeled oligonucleotide WT VEGF (V) or MUT VEGF (Vm). COMP, competitor.

previously demonstrated for the WT EPO probe (reference 50 and Fig. 8A). Five or 50 ng of unlabeled WT VEGF completely eliminated binding of HIF-1 to the probe (Fig. 6C, lanes 3 and 4), whereas 5 ng of unlabeled MUT VEGF had no effect (lane 5) and 50 ng partially eliminated binding of the constitutive factor(s) but had no effect on HIF-1 binding (lane 6).

To provide further evidence that HIF-1 binds to WT VEGF, HIF-1α and HIF-1β were transcribed in vitro and translated in rabbit reticulocyte lysates. Neither HIF-1α nor HIF-1β alone showed binding to the WT VEGF probe, as the nonspecific DNA-binding activity was present in unprogrammed lysates (Fig. 7, lanes 1 and 2, and data not shown). In the presence of both HIF-1α and HIF-1β, a probe-protein complex formed (lane 3) that was competed for by excess unlabeled WT VEGF (lane 4) or WT EPO (lane 5) but not by MUT EPO (lane 6). The binding of in vitro-translated HIF-1 to the WT VEGF probe was also disrupted by incubation with anti-HIF-1α (lane 7) or anti-HIF-1β (lane 9) antiserum but not by the corresponding preimmune serum (lanes 8 and 10).

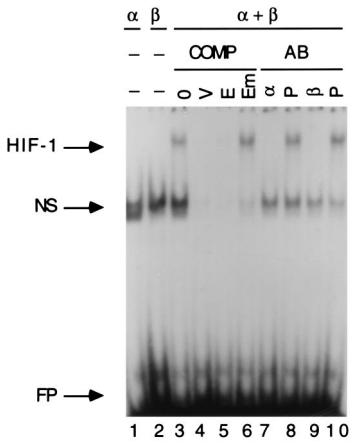

FIG. 7. Binding of in vitro-translated HIF-1 to the VEGF probe. HIF-1α and HIF-1β were translated individually (lanes 1 and 2) or cotranslated (lanes 3 to 10) in reticulocyte lysates and incubated with the WT VEGF probe in the absence (lanes 1 to 3) or presence of the indicated unlabeled oligonucleotide competitor (COMP; lanes 4 to 6) or antiserum (AB; lanes 7 to 10). NS, nonspecific DNA-binding activity present in reticulocyte lysates.

Taken together, these results demonstrate that (i) HIF-1 binds to the WT VEGF sequence by all criteria established for the binding of HIF-1 to WT EPO (50, 57) and (ii) as in the case of the *EPO* enhancer (21, 50), a mutation in the *VEGF* hypoxia response element which disrupted HIF-1 binding also eliminated transcriptional activation in response to hypoxia and/or rHIF-1. Thus the effect of mutations in the *cis*-acting element (P11m) and *trans*-acting factor (pCEP/HIF-1αDN) establish a strong link between HIF-1 binding and *VEGF* response element function.

Expression of HIF-1 and *VEGF* in wild-type and HIF-1β (ARNT)-deficient Hepa1 cells. To further explore the role of HIF-1 in the regulation of *VEGF* expression, we analyzed four clones of Hepa1 mouse hepatoma cells which differed with respect to their expression of HIF-1β (ARNT). Clones Hepa1 c1c7 and c4 are wild type and mutant, respectively, for *ARNT* gene expression, VT2 is a c4 line transfected with a plasmid expressing HIF-1β (ARNT), and RB13 is a c4 revertant cell line (18). We initially demonstrated that HIF-1 was induced by hypoxia in wild-type Hepa1 cells (Fig. 8A). The binding of HIF-1, present in hypoxic Hep3B (lane 2) and Hepa1 (lane 8) nuclear extracts, to the WT EPO probe was competed for by increasing amounts of unlabeled oligonucleotide WT EPO (lanes 3 and 4 for Hep3B; lanes 9 and 10 for Hepa1) but not by oligonucleotide MUT EPO (lanes 5 and 6 for Hep3B; lanes 11 and 12 for Hepa1). The HIF-1–DNA complex was specifically supershifted by anti-HIF-1α (Fig. 8B; lane 4 for Hep3B and

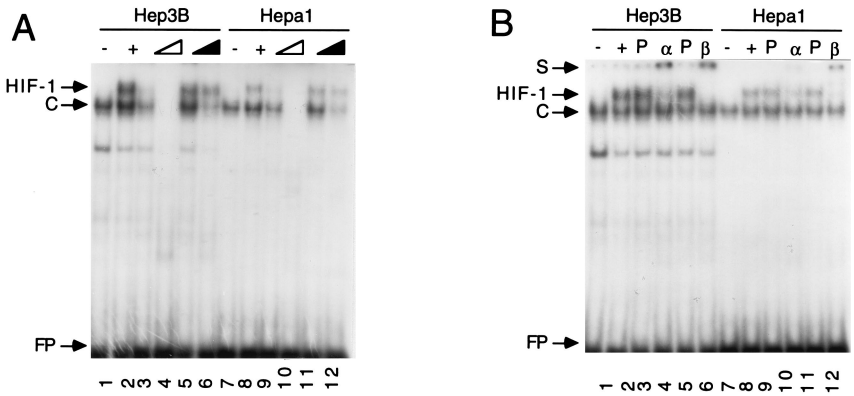

FIG. 8. Analysis of Hepa1 cells. (A) Comparison of HIF-1 DNA-binding activity in Hep3B and wild-type Hepa1 cells by oligonucleotide competition assay. Nuclear extracts from nonhypoxic (lanes 1 and 7) and hypoxic (lanes 2 to 6 and 8 to 12) Hep3B cells (lanes 1 to 6) and Hepa1 cells (lanes 7 to 12) were analyzed for HIF-1 DNA-binding activity, using the WT EPO probe in the presence of no competitor (lanes 1, 2, 7, and 8) or 3 ng (lanes 3, 5, 9, and 11) or 30 ng (lanes 4, 6, 10, and 12) of unlabeled oligonucleotide WT EPO (open triangles; lanes 3, 4, 9, and 10) or MUT EPO (closed triangles; lanes 5, 6, 11, and 12). (B) Comparison of HIF-1 DNA-binding activity in Hep3B and wild-type Hepa1 cells by supershift assay. Nuclear extracts from nonhypoxic (lanes 1 and 7) or hypoxic (lanes 2 to 6 and 8 to 12) Hep3B cells (lanes 1 to 6) and Hepa1 cells (lanes 7 to 12) were incubated with the WT EPO probe in the absence (lanes 1, 2, 7, and 8) or presence of anti-HIF-1α (lanes 4 and 10), anti-HIF-1β (lanes 6 and 12), or the respective preimmune serum (lanes 3, 5, 9, and 11). S, supershifted complex; FP, free probe.

lane 10 for Hepa1) or anti-HIF-1β (lane 6 for Hep3B and lane 12 for Hepa1) antiserum. Thus, wild-type Hepa1 cells express HIF-1 in response to hypoxia, as demonstrated by competition and supershift assays.

We next analyzed HIF-1α and HIF-1β protein levels by immunoblot assay using affinity-purified antibodies and HIF-1 DNA-binding activity by EMSA in the four hepatoma cell lines (Fig. 9A). HIF-1β expression (middle panel) was induced by hypoxia in wild-type Hepa1 cells (lane 2) but not in c4 cells (lane 4), confirming that the latter cells lack HIF-1β (ARNT). Compared with wild-type cells, HIF-1β was overexpressed in nonhypoxic (lane 5) and hypoxic (lane 6) VT2 cells. HIF-1β expression was also induced by hypoxia in revertant RB13 cells (lane 8), although the levels were not as high as in wild-type Hepa1 cells (lane 2). HIF-1α (top panel) was also induced by hypoxia in wild-type Hepa1 cells, but HIF-1α did not accumulate in c4 cells lacking HIF-1β. HIF-1α levels in VT2 and RB13 cells also paralleled the HIF-1β levels. These results suggest that HIF-1α may not be stable in the absence of HIF-1β. EMSA results (bottom panel) paralleled the results of the immunoblot assays under all conditions, demonstrating that HIF-1 DNA-binding activity is determined by the levels of HIF-1α and HIF-1β proteins.

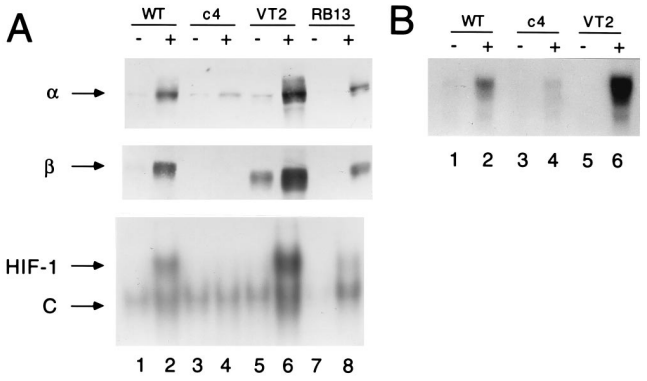

FIG. 9. Analysis of HIF-1 and *VEGF* expression in wild-type, mutant, and derivative Hepa1 cells. (A) Expression of HIF-1. Nuclear extracts were prepared from the following nonhypoxic (−) or hypoxic (+) cells: WT, wild-type Hepa1 cells; c4, mutant cells that do not express HIF-1β (ARNT); VT2, c4 cells transfected with a plasmid expressing HIF-1β (ARNT); and RB13, revertant c4 cells. Nuclear extracts were assayed for HIF-1α (top panel) and HIF-1β protein (middle panel) by immunoblot assay using affinity-purified antibodies and for HIF-1 DNA-binding activity by EMSA (bottom panel). (B) Expression of VEGF mRNA. Total RNA was isolated from wild-type Hepa1 (lanes 1 and 2), mutant c4 (lanes 3 and 4), or corrected VT2 (lanes 5 and 6) cells incubated for 16 h under nonhypoxic (−) or hypoxic (+) conditions. Blot hybridization analysis of 15-μg aliquots was performed with a rat VEGF cDNA probe.

To determine the effect of the absence of HIF-1 activity on *VEGF* expression, total RNA was isolated from wild-type Hepa1, mutant c4, and transfected VT2 cells after 16 h at 20 or 1% O_2. Compared with Hepa1 cells (Fig. 9B, lane 2), VEGF mRNA levels were markedly reduced in hypoxic c4 cells (lane 4), whereas VEGF mRNA was superinduced in VT2 cells (lane 6) which overexpress HIF-1. Hybridization of the same blot with an oligonucleotide complementary to 18S rRNA demonstrated that there were no significant differences in RNA loading or transfer between lanes (data not shown). The relative VEGF mRNA levels in the different cell lines were therefore in complete agreement with the HIF-1 immunoblot assay and EMSA results. These data provide further evidence for the essential role of HIF-1 in the activation of *VEGF* expression in hypoxic cells.

DISCUSSION

Complexity of *VEGF* regulation. *VEGF* expression can be induced by subjecting cells to hypoxia or hypoglycemia (53). In contrast, the only known physiologic stimulus for *EPO* expression is hypoxia. Neither *EPO* expression in isolated, perfused kidneys (55) nor *EPO* enhancer activity in reporter gene assays (42) responded to compounds that affect cellular energy metabolism such as cyanide and 2-deoxyglucose, whereas inhibition of oxidative phosphorylation by amobarbital induced *VEGF* expression (27). These results are consistent with the distinct physiologic roles played by EPO and VEGF. EPO stimulates erythropoiesis, which increases O_2 delivery, whereas VEGF stimulates angiogenesis, which increases delivery of both O_2 and energy substrates such as glucose. The complexity of *VEGF* regulation is further illustrated by the finding that in hypoxic C6 and PC12 cells, the rate of VEGF RNA transcription is increased and the rate of VEGF mRNA degradation is

decreased (19, 26a, 28, 53). Hypoglycemia and hypoxia each affect VEGF RNA transcription and stability (53). The *cis*-acting DNA sequences that mediate the *VEGF* response to hypoglycemia have not been identified. In contrast, studies reported here and others published while this work was in progress (19, 28, 30, 37) have identified 5′-flanking sequences required for the *VEGF* transcriptional response to hypoxia.

***VEGF* 5′-flanking sequences mediate transcriptional responses to hypoxia.** Deletion analysis of the human *VEGF* 5′-flanking region in the context of the native promoter indicated the presence of multiple *cis*-acting elements in the region between −2274 and +379 that affect the transcriptional response to hypoxia in Hep3B cells, as judged from the finding that there was a gradual loss of hypoxia-induced reporter expression whereas basal expression was unaffected by the deletion of 5′-flanking sequences (Fig. 1 to 3). Similar results were reported on the basis of a deletion analysis of rat *VEGF* 5′-flanking sequences in PC12 cells (28). In contrast, studies of human *VEGF* reporters in bovine pulmonary artery endothelial cells (30) and rat C6 glioma cells (19) revealed an abrupt and complete loss of hypoxia-induced expression associated with loss of sequences 5′ to −795 and −888, respectively. Taken together, these results suggest that certain *cis*-acting elements may function in a cell-type-specific manner.

Despite minor differences in the analysis of 5′-deletion mutants, the results reported here and in another recent study (30) identified identical overlapping sequences that mediated hypoxia-inducible transcription via a heterologous promoter. Our studies showed that 47 bp between −985 and −939 were sufficient for transcriptional activation of the SV40 basal promoter in hypoxic Hep3B cells (Fig. 4). A 35-bp sequence (−985 to −951) was sufficient for a maximal transcriptional response of the thymidine kinase promoter in bovine pulmonary artery endothelial cells (30). A homologous 28-bp sequence from the rat *VEGF* gene was also shown to increase expression from the thymidine kinase promoter in hypoxic PC12 cells (28). The human *VEGF* element was shown to function when cloned in either orientation 5′ or 3′ to the reporter gene, thus satisfying the criteria for an enhancer element (30).

Transcriptional activation via the *VEGF* hypoxia-inducible enhancer is mediated by HIF-1. The isolation of cDNA encoding both subunits of HIF-1 (57) enabled us to demonstrate for the first time that HIF-1 and *VEGF* 5′-flanking sequences interact to mediate transcriptional activation in hypoxic cells, as evidenced by the following data: (i) forced expression of HIF-1α and HIF-1β (rHIF-1) increased *VEGF* reporter expression in cells exposed to 1 or 20% O_2 in a dose-dependent manner (Fig. 1 to 4 and data not shown); (ii) deletions of *VEGF* 5′-flanking sequences resulted in a progressive, parallel decrease in the magnitude of the transcriptional response to hypoxia and rHIF-1 (Fig. 2 and 3); (iii) a HIF-1 binding site was demonstrated within the *VEGF* enhancer on the basis of sequence-specific DNA binding by native HIF-1 present in nuclear extracts from hypoxic Hep3B cells and in vitro-translated HIF-1 present in reticulocyte lysates, as identified by using antisera specifically raised against HIF-1α and HIF-1β (Fig. 6); (iv) a 3-bp substitution that eliminated transcriptional responses to hypoxia and rHIF-1 (Fig. 4) also eliminated binding of HIF-1 (Fig. 6); and (v) overexpression of a dominant negative form of HIF-1 inhibited the transcriptional response to hypoxia mediated by the *VEGF* enhancer in a dose-dependent manner (Fig. 5A). Identical criteria have been met for establishing the role of HIF-1 in mediating transcriptional activation via the *EPO* enhancer (21, 50). Comparison of the sequence of the human *VEGF* hypoxia response element with the corresponding mouse and rat sequences (Fig. 10) reveals a very high degree of conservation, especially over the first 30 bp containing the HIF-1 site and flanking (A/C)ACAG repeats (see below).

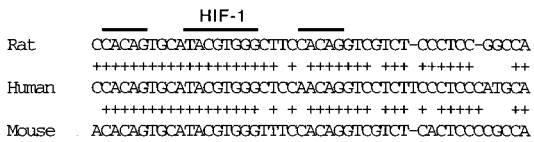

FIG. 10. Conservation of the hypoxia response element in mammalian *VEGF* genes. The nucleotide sequence (56) of the 47-bp human *VEGF* hypoxia response element is compared with the corresponding sequences in the mouse (50a) and rat (28) genes. Crosses mark sequence identity. The HIF-1 site and (A/C)ACAG sequences are overlined.

In addition to the HIF-1 binding site at −975, we identified a second HIF-1 site at −306 which matched the HIF-1 binding site consensus sequence (49). However, deletion of the −306 site had no effect on the transcriptional response to hypoxia or rHIF-1 (Fig. 3). We have recently identified HIF-1 binding sites in genes encoding aldolase A and enolase 1, deletion of which has no effect on the transcriptional response to hypoxia (47). Previous analysis of the *EPO* enhancer demonstrated that the sequence 5′-CACAG-3′ located 5 bp downstream of the HIF-1 site was essential for enhancer activity (3, 50). Substitution of sequences 5′ or 3′ to the HIF-1 site in the *VEGF* enhancer reduced or eliminated activity (30). Remarkably, the sequences 5′-CACAG-3′ and 5′-AACAG-3′ are located 5 bp upstream and downstream, respectively, of the HIF-1 binding site in the human *VEGF* gene (Fig. 4B). On the basis of these observations, we conclude that (i) a HIF-1 binding site is necessary but not sufficient for hypoxia response element activity; (ii) a similar accessory factor(s) may bind to the *EPO* and *VEGF* enhancers; and (iii) the absence of a binding site(s) for an accessory factor(s) may explain the inactivity of the −306 site in transcription assays. Inclusion of the −975 HIF-1 site, 5′-TACGTGGG-3′, with the previously established HIF-1 binding-site sequences (49) broadens the consensus sequence to 5′-BACGTGSK-3′. Inspection of the *VEGF* gene sequence did not reveal any additional sequences matching the revised consensus. However, the full range of sequences recognized by HIF-1 has not been established, and the presence of other functional HIF-1 sites in the *VEGF* 5′-flanking sequence cannot be excluded (see below).

The parallel response of 5′-flanking sequences to 1% O_2 or rHIF-1 suggests that transcriptional activation mediated by sequences 3′ to −924 in response to hypoxia (Fig. 3) may also involve HIF-1. This conclusion was also supported by the demonstration that a dominant negative form of HIF-1α repressed the expression of the −924 promoter construct (*VEGF*-P1) in hypoxic cells (Fig. 5B). Several possible mechanisms could explain these results: (i) HIF-1 may directly bind to other DNA sequences in the 5′-flanking region; (ii) HIF-1 may bind indirectly via protein-protein interactions with other transcription factors bound to these sequences; and (iii) HIF-1 may induce expression of other factors that stimulate *VEGF* transcription. The sequence 5′-GACGTGTG-3′, located at −510, is a potential HIF-1 binding site which differs from the revised consensus sequence only at the seventh position. Of note is the fact that the sequence GAGAG is located 5 bp upstream of the potential HIF-1 site. Additional transient expression assays will be required to determine whether this sequence represents a functional HIF-1 binding site.

Hypoxia and rHIF-1 activate reporter gene transcription synergistically. When Hep3B cells were transfected with reporters that contained the *VEGF* enhancer (*VEGF-Kpn*I, *VEGF*-P7, *VEGF*-P8, P12, P10, or P11w), transcription was activated by exposing the cells to 1% O_2 or by cotransfection with rHIF-1. In cells cotransfected with rHIF-1 and exposed to 1% O_2, a synergistic response was observed. If the only effect of hypoxia was to increase the synthesis of HIF-1α and HIF-1β and such synthesis was necessary and sufficient for a maximal transcriptional response to hypoxia, then the effects of rHIF-1 and 1% O_2 should be additive at best. The observed synergism suggests that additional events occur which are required for a maximal transcriptional response in hypoxic cells. For example, HIF-1 dimerization, DNA binding, transactivation, or protein stability may be increased by the synthesis of a cofactor(s) and/or the posttranslational modification of HIF-1α or HIF-1β.

***VEGF* expression is not induced by hypoxia in cells lacking HIF-1.** The increase in steady-state levels of VEGF RNA in hypoxic C6 and PC12 cells has been shown to result from both increased transcription and increased RNA stability (19, 26a, 28, 53). In the ARNT (HIF-1β)-deficient c4 clone of Hepa1 cells, which lack HIF-1 DNA-binding activity, VEGF mRNA levels increased only slightly in response to hypoxia, whereas VEGF mRNA was markedly induced in the parental Hepa1 and corrected VT2 cell lines which expressed HIF-1. Two conclusions can be drawn from these data. First, there is a very strict correlation between HIF-1 activity and *VEGF* expression in hepatoma cells that we have analyzed. Second, the decreased steady-state VEGF mRNA levels in hypoxic c4 cells indicate that either the rate of VEGF transcription or the stability of VEGF mRNA is decreased in c4 cells. Preliminary analysis of VEGF mRNA stability indicates that the half-life of VEGF mRNA is not decreased in hypoxic c4 cells (19a), which is consistent with the hypothesis that the absence of HIF-1 results in reduced transcriptional activation. *ARNT* gene products also heterodimerize with AHR (4, 6, 45) as well as undergoing homodimerization (52, 54), and it therefore remains a formal possibility that the lack of *VEGF* expression in hypoxic c4 cells is due to the absence of ARNT homodimers, AHR-ARNT heterodimers, or other uncharacterized ARNT-containing heterodimers. However, the most parsimonious explanation of these data, when taken together with the results of transfection experiments with Hep3B cells, is that HIF-1 plays an essential role in the activation of *VEGF* expression in hypoxic cells. To complement our studies of *VEGF* transcriptional regulation in cultured cells, we have recently demonstrated that in fetal sheep subjected to anemia, both VEGF and HIF-1α protein levels are increased in the heart (34).

HIF-1 mediates homeostatic responses to hypoxia. EPO and VEGF play critical roles in adaptive responses to systemic and local hypoxia, respectively. Our data indicate that transcription of the *EPO* and *VEGF* genes is activated in hypoxic cells via remarkably similar molecular mechanisms. In each case, a *cis*-acting enhancer element of 50 bp or less has been characterized and shown to contain a HIF-1 binding site that is necessary but not sufficient for enhancer activity. The presence of HIF-1 DNA-binding activity in hypoxic cells is necessary for transcriptional activation and forced expression of HIF-1 in nonhypoxic cells also results in transcriptional activation. Critical *cis*-acting DNA sequences that contain essential HIF-1 binding sites have been identified in other hypoxia-inducible genes, including those encoding heme oxygenase 1 (25), inducible nitric oxide synthase (31, 35), glucose transporter 1 (7), and the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, and phosphoglycerate kinase 1 (11, 12, 47, 49). The protein products of these genes are also likely to be involved in adaptive responses to hypoxia. Although none of these genes has been subjected to the rigorous analysis that has confirmed the role of HIF-1 in *EPO* and *VEGF* transcriptional activation, it is likely that they are regulated in similar manners. These results and recent data indicating that HIF-1α and HIF-1β (ARNT) mRNAs are expressed in all human, mouse, and rat organs assayed (64) provide support for the hypothesis that HIF-1 coordinates homeostatic transcriptional responses to hypoxia. The utilization of knockout and transgenic techniques to alter HIF-1 activity in vivo will allow more definitive tests of this hypothesis to be performed.

ACKNOWLEDGMENTS

We acknowledge the generosity of J. Abraham, E. Tischer, A. Choi, R. Wenger, and M. Gassmann for providing oligonucleotides and plasmids. We are extremely grateful to O. Hankinson and E. Minehart for providing plasmids and cells. We thank A. Choi for comments on the manuscript.

G.L.S. is an Established Investigator of the American Heart Association. This work was supported by grants from the American Heart Association and Lucille P. Markey Charitable Trust to G.L.S. and by grants DK39869 (G.L.S.) and CA45055 (R.D.K.) and training grant HD07170 (J.A.F.) from the National Institutes of Health.

REFERENCES

1. **Banai, S., D. Shweiki, A. Pinson, M. Chandra, G. Lazarovici, and E. Keshet.** 1994. Upregulation of vascular endothelial growth factor expression induced by myocardial ischemia: implications for coronary angiogenesis. Cardiovasc. Res. **28:**1176–1179.
2. **Beck, I., S. Ramirez, R. Weinmann, and J. Caro.** 1991. Enhancer element at the 3′-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. J. Biol. Chem. **266:**15563–15566.
3. **Beck, I., R. Weinmann, and J. Caro.** 1993. Characterization of hypoxia-responsive enhancer in the human erythropoietin gene shows presence of hypoxia-inducible 120-kd nuclear DNA-binding protein in erythropoietin-producing and nonproducing cells. Blood **82:**704–711.
4. **Burbach, K. M., A. Poland, and C. A. Bradfield.** 1992. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc. Natl. Acad. Sci. USA **89:**8185–8189.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Dolwick, K. M., H. I. Swanson, and C. A. Bradfield.** 1993. In vitro analysis of Ah receptor domains involved in ligand-activated transcription. Proc. Natl. Acad. Sci. USA **90:**8566–8570.
7. **Ebert, B. L., J. D. Firth, and P. J. Ratcliffe.** 1995. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct cis-acting sequences. J. Biol. Chem. **270:**29083–29089.
8. **Fandrey, J., and H. F. Bunn.** 1993. In vivo and in vitro regulation of erythropoietin mRNA: measurement by competitive polymerase chain reaction. Blood **81:**617–623.
9. **Ferrara, N., and W. J. Henzel.** 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem. Biophys. Res. Commun. **161:**851–858.
10. **Ferrara, N., K. Houck, L. Jakman, and D. W. Leung.** 1992. Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrinol. Rev. **13:**18–32.

10a. **Finkenzeller, G., A. Technau, and D. Marme.** 1995. Hypoxia-induced transcription of the vascular endothelial growth factor gene is independent of functional AP-1 transcription factor. Biochem. Biophys. Res. Commun. **208:**432–439.

11. **Firth, J. D., B. L. Ebert, C. W. Pugh, and P. J. Ratcliffe.** 1994. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3′ enhancer. Proc. Natl. Acad. Sci. USA **91:**6496–6500.
12. **Firth, J. D., B. L. Ebert, and P. J. Ratcliffe.** 1995. Hypoxic regulation of lactate dehydrogenase A: interaction between hypoxia-inducible factor 1 and cAMP response elements. J. Biol. Chem. **270:**21021–21027.
13. **Folkman, J., and Y. Shing.** 1992. Angiogenesis. J. Biol. Chem. **267:**10931–10934.
14. **Gleadle, J. M., B. L. Ebert, J. D. Firth, and P. J. Ratcliffe.** 1995. Regulation of angiogenic growth factor expression by hypoxia, transition metals, and chelating agents. Am. J. Physiol. **268:**C1362–C1368.
15. **Goldberg, M. A., C. C. Gaut, and H. F. Bunn.** 1991. Erythropoietin mRNA levels are governed by both the rate of gene transcription and posttranscriptional events. Blood **77:**271–277.

16. **Goldberg, M. A., and T. J. Schneider.** 1994. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J. Biol. Chem. **269:**4355–4359.
17. **Gospodarowicz, D., J. A. Abraham, and J. Schilling.** 1989. Isolation of a vascular endothelial cell mitogen produced by pituitary-derived folliculo-stellate cells. Proc. Natl. Acad. Sci. USA **86:**7311–7315.
18. **Hoffman, E. C., H. Reyes, F.-F. Chu, F. Sander, L. H. Conley, B. A. Brooks, and O. Hankinson.** 1991. Cloning of a factor required for activity of the Ah (dioxin) receptor. Science **252:**954–958.
19. **Ikeda, E., M. G. Achen, G. Breier, and W. Risau.** 1995. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J. Biol. Chem. **270:**19761–19766.
19a. **Iyer, N. V., and G. L. Semenza.** 1996. Unpublished data.
20. **Jelkmann, W.** 1992. Erythropoietin: structure, control of production, and function. Physiol. Rev. **72:**449–489.
21. **Jiang, B.-H., E. Rue, G. L. Wang, R. Roe, and G. L. Semenza.** Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J. Biol. Chem., in press.
22. **Klagsbrun, M., and S. Soker.** 1993. VEGF/VPF: the angiogenesis factor found? Curr. Biol. **3:**699–702.
23. **Krantz, S. B.** 1991. Erythropoietin. Blood **77:**419–434.
24. **Ladoux, A., and C. Frelin.** 1993. Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart. Biochem. Biophys. Res. Commun. **195:**1005–1010.
25. **Lee, P., B.-H. Jiang, G. L. Semenza, J. Alam, and A. Choi.** Unpublished data.
26. **Leung, D. W., G. Cachianes, W. J. Kuang, D. V. Goeddel, and N. Ferrara.** 1989. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science **246:**1306–1309.
26a. **Levy, A. P., N. S. Levy, and M. A. Goldberg.** 1996. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. J. Biol. Chem. **271:**2746–2753.
27. **Levy, A. P., N. S. Levy, J. Loscalzo, A. Calderone, N. Takahashi, K.-T. Yeo, G. Koren, W. S. Colucci, and M. A. Goldberg.** 1995. Regulation of vascular endothelial growth factor in cardiac myocytes. Circ. Res. **76:**758–766.
28. **Levy, A. P., N. S. Levy, S. Wegner, and M. A. Goldberg.** 1995. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J. Biol. Chem. **270:**13333–13340.
29. **Li, H., L. Dong, and J. P. Whitlock, Jr.** 1994. Transcriptional activation function of the mouse Ah receptor nuclear translocator. J. Biol. Chem. **269:**29098–28105.
30. **Liu, Y., S. R. Cox, T. Morita, and S. Kourembanas.** 1995. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Circ. Res. **77:**638–643.
31. **Lowenstein, C., and G. L. Semenza.** Unpublished data.
32. **Ma, Q., L. Dong, and J. P. Whitlock, Jr.** 1995. Transcriptional activation by the mouse Ah receptor: interplay between multiple stimulatory and inhibitory functions. J. Biol. Chem. **270:**12697–12703.
33. **Madan, A., and P. T. Curtin.** 1993. A 24-base-pair sequence 3′ to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. Proc. Natl. Acad. Sci. USA **90:**3928–3932.
34. **Martin, C., A. Yu, B.-H. Jiang, A. R. Hohimer, L. E. Davis, and G. L. Semenza.** Unpublished data.
35. **Melillo, G., T. Musso, A. Sica, L. S. Taylor, G. W. Cox, and L. Varesio.** 1995. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J. Exp. Med. **182:**1683–1693.
36. **Miller, J. W., A. P. Adamis, D. T. Shima, P. A. D'Amore, R. S. Moulton, M. S. O'Reilly, J. Folkman, H. F. Dvorak, L. F. Brown, B. Berse, T.-K. Yeo, and K.-T. Yeo.** 1994. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am. J. Pathol. **145:**574–584.
37. **Minchenko, A., S. Salceda, T. Bauer, and J. Caro.** 1994. Hypoxia regulatory elements of the human vascular endothelial growth factor gene. Cell. Mol. Biol. Res. **40:**35–39.
38. **Nomura, M., S. Yamagishi, S. Harada, Y. Hayashi, T. Yamashima, J. Yamashita, and H. Yamamoto.** 1995. Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes. J. Biol. Chem. **270:**28316–28324.
39. **Pierce, E. A., R. L. Avery, E. D. Foley, L. P. Aiello, and L. E. H. Smith.** 1995. Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. Proc. Natl. Acad. Sci. USA **92:**905–909.
40. **Plate, K. H., G. Breier, B. Millauer, A. Ullrich, and W. Risau.** 1993. Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. Cancer Res. **53:**5822–5827.
41. **Plate, K. H., G. Breier, H. A. Weich, and W. Risau.** 1992. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature (London) **359:**845–848.
42. **Pugh, C. W., C. C. Tan, R. W. Jones, and P. J. Ratcliffe.** 1991. Functional analysis of an oxygen-regulated transcriptional enhancer lying 3′ to the mouse erythropoietin gene. Proc. Natl. Acad. Sci. USA **88:**10553–10557.
43. **Ratcliffe, P. J.** 1994. Molecular biology of erythropoietin. Kidney Int. **44:**887–904.
44. **Reisz-Porszasz, S., M. R. Probst, B. N. Fukunaga, and O. Hankinson.** 1994. Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). Mol. Cell. Biol. **14:**6075–6086.
45. **Reyes, H., S. Reisz-Porszasz, and O. Hankinson.** 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science **256:**1193–1195.
46. **Schuster, S. J., E. V. Badiavas, P. Costa-Giomi, R. Weinmann, A. J. Erslev, and J. Caro.** 1987. Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. Blood **73:**13–16.
47. **Semenza, G. L., S. Leung, B.-H. Jiang, and A. Giallongo.** Unpublished data.
48. **Semenza, G. L., M. K. Nejfelt, S. M. Chi, and S. E. Antonarakis.** 1991. Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. Proc. Natl. Acad. Sci. USA **88:**5680–5684.
49. **Semenza, G. L., P. H. Roth, H.-M. Fang, and G. L. Wang.** 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J. Biol. Chem. **269:**23757–23763.
50. **Semenza, G. L., and G. L. Wang.** 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell. Biol. **12:**5447–5454.
50a. **Shima, D. T., M. Kuroki, U. Deutsch, Y.-S. Ng, A. P. Adamis, and P. A. D'Amore.** 1996. The mouse gene for vascular endothelial growth factor: genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences. J. Biol. Chem. **271:**3877–3883.
51. **Shweiki, D., A. Itin, D. Soffer, and E. Keshet.** 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (London) **359:**843–845.
52. **Sogawa, K. R. Nakano, A. Kobayashi, Y. Kikuchi, N. Ohe, N. Matsushita, and Y. Fujii-Kuriyama.** 1995. Possible function of Ah receptor nuclear translocator (Arnt) homodimer in transcriptional regulation. Proc. Natl. Acad. Sci. USA **92:**1936–1940.
53. **Stein, I., M. Neeman, D. Shweiki, A. Itin, and E. Keshet.** 1995. Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. Mol. Cell. Biol. **15:**5363–5368.
54. **Swanson, H. I., W. K. Chan, and C. A. Bradfield.** 1995. DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. J. Biol. Chem. **270:**26292–26302.
55. **Tan, C. C., and P. J. Ratcliffe.** 1991. Effect of inhibitors of oxidative phosphorylation on erythropoietin mRNA in isolated perfused rat kidneys. Am. J. Physiol. **261:**F982–F987.
56. **Tischer, E., S. R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J. C. Fiddes, and J. A. Abraham.** 1991. The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. J. Biol. Chem. **266:**11947–11954.
57. **Wang, G. L., B.-H. Jiang, E. A. Rue, and G. L. Semenza.** 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc. Natl. Acad. Sci. USA **92:**5510–5514.
58. **Wang, G. L., and G. L. Semenza.** 1993. General involvement of hypoxia-inducible factor in transcriptional response to hypoxia. Proc. Natl. Acad. Sci. USA **90:**4304–4308.
59. **Wang, G. L., and G. L. Semenza.** 1993. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J. Biol. Chem. **268:**21513–21518.
60. **Wang, G. L., and G. L. Semenza.** 1993. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood **82:**3610–3615.
61. **Wang, G. L., and G. L. Semenza.** 1995. Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem. **270:**1230–1237.
62. **Wenger, R. H., A. Rolfs, H. H. Marti, C. Bauer, and M. Gassmann.** 1995. Hypoxia, a novel inducer of acute phase gene expression in a human hepatoma cell line. J. Biol. Chem. **270:**27865–27870.
63. **Whitelaw, M. L., J.-A. Gustafsson, and L. Poellinger.** 1994. Identification of transactivation and repression functions of the dioxin receptor and its basic helix-loop-helix/PAS partner factor Arnt: inducible versus constitutive modes of regulation. Mol. Cell. Biol. **14:**8343–8355.
64. **Wiener, C. M., G. Booth, and G. L. Semenza.** Unpublished data.