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Identification of Hypoxia-inducible Factor 1 Ancillary Sequence and Its Function in Vascular Endothelial Growth Factor Gene Induction by Hypoxia and Nitric Oxide*

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Transcription of hypoxia-inducible genes is regulated by hypoxia response elements (HREs) located in either the promoter or enhancer regions. Analysis of these elements reveals the presence of one or more binding sites for hypoxia-inducible factor 1 (HIF-1). Hypoxia-inducible genes include vascular endothelial growth factor (VEGF), erythropoietin, and glycolytic enzyme genes. Site-directed mutational analysis of the VEGF gene promoter revealed that an HIF-1 binding site (HBS) and its downstream HIF-1 ancillary sequence (HAS) within the HRE are required as cis-elements for the transcriptional activation of VEGF by either hypoxia or nitric oxide (NO). The core sequences of the HBS and the HAS were determined as TACGTG and CAGGT, respectively. These elements form an imperfect inverted repeat, and the spacing between these motifs is crucial for activity of the promoter. Gel shift assays demonstrate that as yet unknown protein complexes constitutively bind to the HAS regardless of the presence of these stimuli in several cell lines, in contrast with hypoxia- or NO-induced activation of HIF-1 binding to the HBS. A common structure of the HRE, which consists of the HBS and the HAS, is seen among several hypoxia-inducible genes, suggesting the presence of a novel mechanism mediated by the HAS for the regulation of these genes.

Most higher eucaryotes require oxygen to meet essential metabolic demands including oxidative phosphorylation, in which oxygen serves as the terminal electron acceptor in mitochondria. Low cellular oxygen tension is seen in physiological conditions, such as high altitude and physical exercise, and in pathological conditions including ischemia, inflammation, and neoplasm. A variety of systemic and cellular responses for homeostatic adaptations are provoked in these hypoxic conditions, including erythropoiesis, vasodilatation, angiogenesis, and glycolysis. Hypoxia activates the transcription of genes whose products mediate these responses (1). Hypoxia-inducible genes within these respective categories include erythropoietin (EPO)[1] (2), vascular endothelial growth factor (VEGF) (3, 4), inducible nitric-oxide synthase (5), heme oxygenase 1 (6, 7), aldolase A (8, 9), enolase 1 (9), glucose transporter 1, lactate dehydrogenase A (LDHA) (8–11), and phosphoglycerate kinase 1 (8, 10).

The hypoxia response elements (HREs) of these genes have in common one or more binding sites for hypoxia-inducible factor 1 (HIF-1). HIF-1 was originally reported by Semenza and Wang (2) as a nuclear factor that was induced by hypoxia and bound to the HRE in the EPO gene. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β (aryl hydrocarbon nuclear translocator, ARNT) subunits, both of which belong to the basic helix-loop-helix-*per-arnt-sim* family, and its activity is tightly regulated by cellular oxygen tension (12).

VEGF is a hypoxia-inducible gene whose regulation and function have been studied extensively. VEGF plays a key role in physiological angiogenesis, as observed in tissue regeneration, and in pathophysiological angiogenesis, as observed in wound healing, tumor growth, metastasis, psoriasis, and diabetic retinopathy (13). VEGF expression is regulated by a variety of stimuli including hypoxia, cobaltous ion, nitric oxide (NO), growth factors, and cytokines (14). Although hypoxia is regarded as the most potent regulator of this gene, NO has drawn a great deal of attention recently as a regulator of the VEGF gene. However, the role of NO in VEGF expression remains inconclusive. There are some observations that NO down-regulates the expression of VEGF in vascular smooth muscle cells and hepatoma cells (15–17). In contrast, our previous studies report that NO induces VEGF gene transcription in glioblastoma and hepatoma cells (18, 19). Recently, Dulak *et al.* (20) demonstrated that endogenous NO enhances VEGF synthesis in rat vascular smooth muscle cells.

Analysis of the VEGF promoter has uncovered that one HIF-1 binding site (HBS) in its 5′-flanking region functions as a cis-element regulating the hypoxic induction of VEGF. Liu *et al.* (3) suggest that not only this HBS, but also an adjacent sequence located immediately downstream within the HRE, is essential for the hypoxic activation of this promoter. We demonstrated that both elements are indispensable to the transcriptional activation of the VEGF gene by NO and hypoxia (19). However, no further structural or functional analyses of this downstream sequence have been performed.

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[1] The abbreviations used are: EPO, erythropoietin; VEGF, vascular endothelial growth factor; LDHA, lactate dehydrogenase A; HRE, hypoxia response element; HIF-1, hypoxia-inducible factor 1; ARNT, aryl hydrocarbon nuclear translocator; HBS, HIF-1 binding site; HAS, HIF-1 ancillary sequence; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; EMSA, electrophoretic mobility shift assay; wt, wild-type; nt, nucleotide(s); HAF, HIF-1 ancillary factor.

In this study, we demonstrate that this adjacent HIF-1 ancillary sequence (HAS) is a novel cis-element for VEGF gene induction by NO and hypoxia and that protein complexes constitutively bind to the HAS in several cell lines. In addition, we show that a common structure of the HRE, consisting of the HBS and the HAS, is widely seen among hypoxia-inducible genes including VEGF and EPO genes and some glycolytic enzyme genes.

EXPERIMENTAL PROCEDURES

Plasmids and Transient Transfection—The sequence of pHRE contains the 5′-flanking sequence of the human VEGF gene between positions −1014 and −903 relative to the transcription start site. This segment, which contains the HRE, was prepared by polymerase chain reaction amplification with primers 5′-CGTGGATCCAGCTGCCTCCCCCTTTG-3′ (sense strand) and 5′-GCCTCGAGGAGAACGGGAAGCTGTGTGG-3′ (antisense strand) and inserted between *Bam*HI and *Xho*I of pT81luc0 (19). The reporter pT81luc0 contains the herpes simplex virus thymidine kinase promoter, upstream of the luciferase-coding sequence. A series of pHRE-related mutants were prepared by cloning polymerase chain reaction-amplified segments, which contained substituted, deleted, or inserted sequences within the HRE, into the same site of pT81luc0. A SV40-driven plasmid pSV-nlsLacZ, containing the β-galactosidase gene, was utilized for normalization of luciferase activity of the reporter plasmids (19). A reporter plasmid pHREepo and its related mutants were prepared by cloning a segment of the 3′-flanking sequence of the human EPO gene (positions 3061 and 3116), including either the wild-type HRE or a mutated HBS (TACGTG→TACtgt) or a mutated HAS (ACACAG→AacaAG), into the same site of pT81luc0 as pHRE.

Human glioblastoma A172, hepatoma Hep3B, cervical carcinoma Hela, and green monkey kidney COS-1 cells were incubated in Dulbecco's modified Eagle's medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum, at 37 °C in humidified incubators. Five μg of reporter plasmid and 1 μg of pSV-nlsLacZ were transfected into A172 cells in 10-cm dishes using 20 μl of Lipofectin (Life Technologies, Inc.) in serum-free Opti-MEM (Life Technologies, Inc.). After incubation for 15 h, the medium was replaced with the regular culture medium. The cells were harvested 36 h after medium replacement and dissolved in 0.25 M Tris-Cl, pH 7.5. Prior to harvest, the cells were exposed either to normoxia (21% O_2) or hypoxia (1% O_2) or to 0.5 mM *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) (dissolved in 0.1% Me_2SO) or 0.1% Me_2SO for 12 h. Cell lysis was performed by four freeze-thaw cycles. Luciferase and β-galactosidase activities were assayed as described before (19). The relative luciferase activity was calculated as luciferase activity divided by β-galactosidase activity. Fold induction was expressed as ratios of relative luciferase activity of either SNAP/Me_2SO or hypoxia/normoxia cells. Protein concentration was determined by Bio-Rad protein assays (Bio-Rad).

Electrophoretic Mobility Shift Assays (EMSAs)—The sense strands of oligonucleotides used in EMSA are as follows: wt HBS, 5′-CAGTGCATACGTGGGCTCCA-3′; wt HBS + HAS, 5′- CAGTGCATACGTGGGCTCCAACAGGTCCTCTTCC-3′; wt HAS, 5′-GCTCCAACAGGTCCTCTTCC-3′; mutant HAS, 5′-GCTCCAAactcaCCTCTTCC-3′. Nuclear extracts were prepared as described previously (21). In brief, A172, Hela, and COS-1 cell extracts were harvested either 3 h after Me_2SO (0.1%) or SNAP (0.5 mM in 0.1% Me_2SO) exposure or 8 h after normoxic (21% O_2) or hypoxic (1% O_2) exposure. Hep3B cell extracts were harvested either 8 h after Me_2SO or SNAP exposure or 12 h after normoxic or hypoxic exposure. The collected cells were centrifuged at 270 × *g* for 10 min at 4 °C. The pellet was resuspended in buffer A (10mM Tris-HCl (pH 7.6), 1.5 mM $MgCl_2$ 10 mM KCl, 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 2 μg/ml each of pepstatin A, leupeptin, and antipain) and kept on ice for 10 min. The cell solution was then homogenized by pipetting more than five times with a syringe, followed by centrifugation at 12,000 × *g* for 2 min at 4 °C. The pellet was resuspended in ice-cold buffer C (0.42 M KCl, 20 mM Tris-HCl (pH 7.6), 20% glycerol, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 2 μg/ml each of pepstatin A, leupeptin, and antipain) and rotated slowly at 4 °C for 30 min. After centrifugation at 12,000 × *g* for 10 min, the supernatant was stored at −70 °C for EMSA.

Nuclear protein (5 μg) was incubated with 3×10^4 cpm of ^{32}P-labeled double-stranded probes and 0.1 μg of calf thymus DNA in modified buffer Z+ (25 mM Tris-HCl (pH 7.6), 80 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.2 mM Na_3VO_4, and 3 μg/ml each of pepstatin A, leupeptin, and antipain) for 30 min at room temperature (22). Electrophoresis was performed on 5% nondenaturing acrylamide gels at 25 mA at 4 °C. The gels were dried, and the radioactivity was localized with a Bioimage Analyzer (Fuji Film, Tokyo, Japan). In competition assays, excessive amounts of unlabeled competitors were added 5 min prior to addition of the labeled probe.

Sequence Analysis with GenBank™ Data—With the aid of GenBank™, we obtained the sequences of the HRE in hypoxia-inducible genes and compared these to the core sequences of the HBS and their ancillary sequences.

Statistical Analysis—The results are shown as the means ± S.E. The statistical significance was assumed at a value of $p < 0.05$ by the use of the unpaired Student's *t* test.

RESULTS

Mutational Analysis of the HIF-1 Binding Site and Its Ancillary Sequence in the VEGF Gene—The protein complexes containing a heterodimer of HIF-1α and HIF-1β were found to bind to the HBS in the VEGF promoter. The HBSs are located in the promoter or enhancer regions of several hypoxia-inducible genes, and the consensus sequence was described as either 5′-(G/C/T)ACGTGC(G/C)-3′ (3) or 5′-RCGTG-3′ (23). To determine the exact extent of the HBS in the human VEGF gene, a series of pHRE mutants with 1–3 nucleotide (nt) exchanges (pHREm1a–pHREm1k) were synthesized, and their reporter expression was compared with that of the wild type (pHRE) after NO and hypoxic treatments. The pHRE mutants pHREm1b, 1c, 1e, 1f, and 1g, which encompass the substitution of a 6-nt sequence, lost their response to both 0.5 mM SNAP and hypoxia (1% O_2) (Fig. 1, *A* and *B*). Therefore, the 6-nt sequence, TACGTG, contains the core of the HBS. In addition, a mutation at the last G (pHREm1i) of this sequence eliminated its response to both stimuli, whereas a mutation at T, the first nucleotide of TACGTG (pHREm1h), partially attenuated the activity of the luciferase reporter (Fig. 1*B*). Thus, at least, the sequence ACGTG is required for the HBS to function. To clarify the importance of the initial T within the sequence, TACGTG, we substituted the T with an A, C, or G and tested the reporter activity of the resultant constructs. Fig. 1*C* shows that the promoter responded better to NO and hypoxia if the first nucleotide was a T or G rather than an A or C ($p < 0.05$). A compilation of HBSs (24) showed that the HRE contains a sequence (T/G)ACGTG as a functional HBS in many hypoxia-inducible genes, whereas A or C in the first nucleotide of this sequence is found in only a few genes. This finding is consistent with our result that the sequence (A/C)ACGTG was less functional (Fig. 1*C*). Together, our findings suggest that a 6-nt sequence, TACGTG, is the core motif of the HBS in the VEGF promoter.

Previously, we demonstrated that not only the HBS, but also its downstream HAS, is essential for NO and hypoxic induction of the VEGF reporter gene (19). To identify the extent of the HAS, located downstream of the HBS, we tested the response of pHRE and its related mutants (pHREm2a–pHREm2k) to NO and hypoxia. Fig. 2, *A* and *B*, illustrates the results of the mutation analysis of the HAS. Because pHREm2b, 2c, and 2d lost NO- and hypoxia-induced luciferase activity (Fig. 2*A*), a 9-nt sequence, AACAGGTCC, was found to contain the core of the HAS. Further analysis of the sequence requirement (Fig. 2*B*) revealed that a 2-base pair mutation within ACAGGT (pHREm2g, 2h, and 2i) resulted in the loss of response, but a mutation at the first A (pHREm2k) did not abrogate luciferase activity. A mutation at TCC (pHREm2d), but not at CC (pHREm2j), within the sequence ACAGGTCC attenuated the reporter activity. Thus, any substitution within CAGGT eliminated the promoter response to either stimulus. This result suggests that these 5 nt constitute the HAS, and the strict sequence requirement might be indicative of the binding of

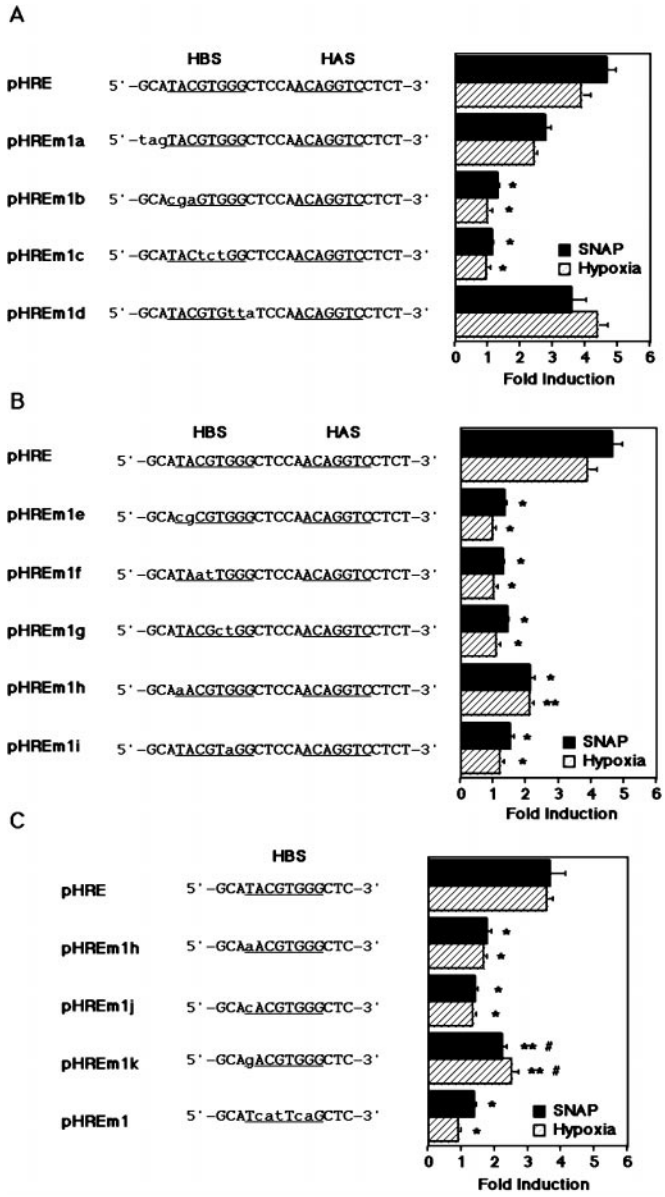

FIG. 1. **The HIF-1 binding site of the VEGF gene is determined as TACGTG.** A172 cells transiently transfected with reporter plasmids and pSV-nlsLacZ were harvested after a 12-h exposure to normoxia (21% O_2), hypoxia (1% O_2), Me_2SO (0.1%), or SNAP (0.5 mM in 0.1% Me_2SO). Results were expressed as the mean ± S.E. of six independent experiments. Nucleotides of putative HBS and HAS are *underlined*, and substituted bases are shown in *lowercase letters*. *A*, a series of pHRE-related mutants with 3-nt substitutions within the HRE were prepared, and their responses to SNAP and hypoxia were tested. *, $p < 0.01$ *versus* pHREm1a and pHREm1d. *B*, a series of pHRE-related mutants with 1- or 2-nt substitutions within the HRE were prepared, and their responses to both stimuli were tested. *, $p < 0.01$; **, $p < 0.05$ *versus* pHRE. *C*, a series of pHRE-related mutants with substitution of the first nucleotide of TACGTG were prepared, and their responses to both stimuli were tested. *, $p < 0.01$; **, $p < 0.05$ *versus* pHRE; #, $p < 0.05$ *versus* pHREm1h and pHREm1j.

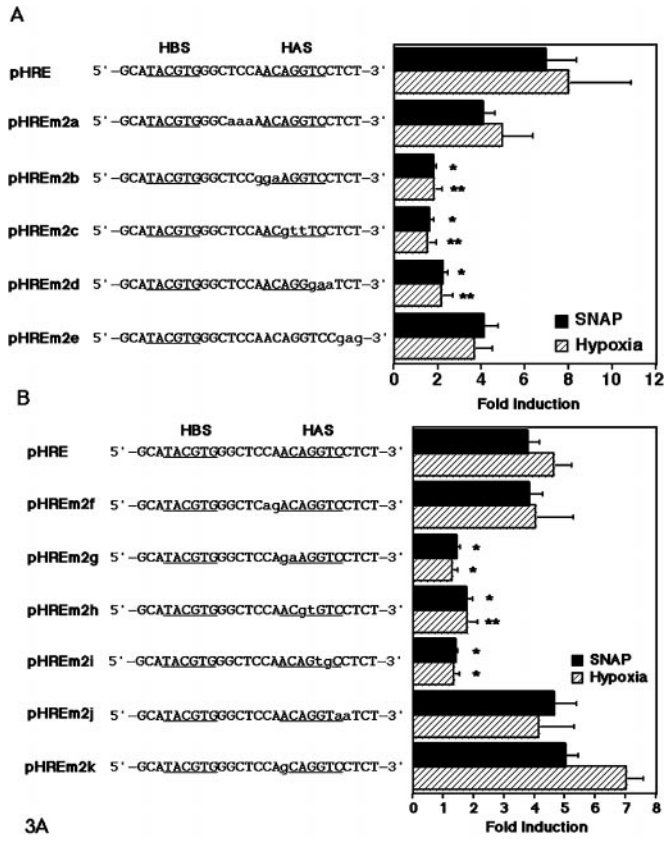

FIG. 2. **The HIF-1 ancillary sequence of the VEGF gene is determined as CAGGT.** A172 cells transiently transfected with reporter plasmids and pSV-nlsLacZ were harvested under the same conditions as in Fig. 1. Results were expressed as the mean ± S.E. of six independent experiments. *, $p < 0.01$; **, $p < 0.05$ *versus* pHRE. Nucleotides of putative HBS and HAS are *underlined*, and substituted bases are shown in *lowercase letters*. *A*, a series of pHRE-related mutants with 3-nt substitutions within the HRE were prepared, and their responses to SNAP and hypoxia were tested. *B*, a series of pHRE-related mutants with 1- or 2-nt substitutions within the HRE were prepared, and their responses to both stimuli were tested.

some factor to the HAS. These experiments indicate that NO and hypoxia similarly enhance the VEGF promoter activity, and both the HBS and the HAS are required as cis-elements for the activation of VEGF by these stimuli.

Spatial Alignment of the HBS and the HAS—The sequences ACGTG, within the HBS, and CAGGT, the core sequence of the HAS, form an imperfect inverted repeat. This raises a question whether a secondary or tertiary structure formed by the above two elements is critical. Therefore we prepared mutants containing either an inverted (pHREm3a) or direct (pHREm3b) repeat of the HBS of the VEGF gene, whose spacer is identical to that of the wild type (Fig. 3*A*). A pHRE mutant, with a perfect inverted repeat of the HBS (pHREm3a), had a 2-fold greater activity than that of the wild type (pHRE) ($p < 0.01$). In contrast, a direct repeat of the HBS (pHREm3b) lost its responsiveness to both stimuli ($p < 0.01$). The same experiments were performed using mutants containing either an inverted (pHREm3c) or direct (pHREm3d) repeat of the HAS of the VEGF gene. Both mutants were found to be unresponsive to either stimulus. These results suggest that a secondary structure of the HRE may be critical for the promoter activity and that the HAS cannot compensate for the HBS.

The HBS and the HAS are located adjacent to each other. To investigate whether these two elements function interdependently, we constructed pHRE mutants, with either a 2-nt deletion within (pHREm3e), or a 5-nt insertion (pHREm3f) into, the spacer and tested the reporter activity after NO and hypoxic treatments. As shown in Fig. 3*B*, either mutation resulted in a loss of reporter activation, suggesting that the spacing between these motifs is crucial for the promoter activity. This result raises the possibility that some putative factor, what we call an HIF-1 ancillary factor (HAF), may bind to the HAS and interact with HIF-1 for VEGF gene induction.

Characterization of Nuclear Proteins That Bind to the HAS of the Human VEGF Promoter—Hypoxia-induced HIF-1 activity mediates transcriptional activation of the VEGF gene. HIF-1

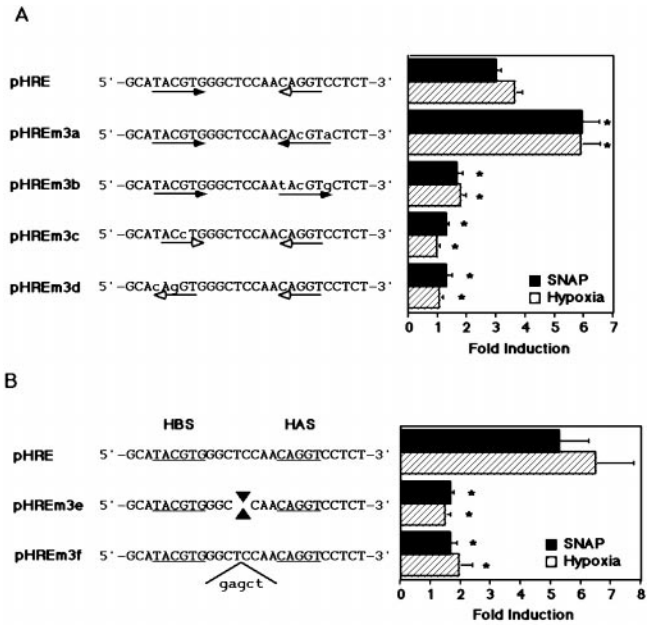

FIG. 3. **The spatial alignment of the HBS and the HAS is crucial for NO and hypoxic induction of the VEGF gene.** A172 cells transiently transfected with reporter plasmids and pSV-nlsLacZ were harvested under the same conditions as in Fig. 1. Results were expressed as the mean ± S.E. of six independent experiments. *, $p < 0.01$ *versus* pHRE. Substituted or inserted bases are shown in *lowercase letters*. *A*, pHRE-related mutants with an inverted or direct repeat of the HBS or the HAS were prepared, and their responses to SNAP and hypoxia were tested. The *closed* and *open arrows* indicate the HBS and the HAS, respectively. The *arrowheads* arbitrarily indicate the orientation of the half-site. *B*, pHRE-related mutants with either a 2-nt deletion or a 5-nt insertion within the spacer between the HBS and the HAS were prepared, and their responses to both stimuli were tested. Nucleotides of putative HBS and HAS are *underlined*.

forms DNA-binding complexes containing the p300/ cAMP-response element-binding protein when bound to its target HBS under hypoxic conditions (25). However, no previous study has described binding factor(s) to the adjacent HAS. To identify proteins that bind to the HAS of the VEGF gene, we analyzed, *in vitro*, the binding of nuclear proteins to three kinds of ^{32}P-labeled oligonucleotides corresponding to the HBS (wt HBS), the HAS (wt HAS), and both elements (wt HBS + HAS) in NO- or hypoxia-treated cells. Nuclear proteins were extracted from A172 cells cultured under 1% O_2 for 8 h or 0.5 mM SNAP for 3 h or respective control cells. The extracts were incubated with labeled probes for 30 min at room temperature in the binding buffer Z+, and the mixtures were electrophoresed on nondenaturing acrylamide gels. As shown in Fig. 4*A*, EMSA revealed that DNA-protein complexes (C1) were always present when either wt HBS or wt HBS + HAS was used as a probe. These bands were observed with probes reproducing the HBS of VEGF (3, 22) and represent constitutive binding protein complexes. Doublet bands of less mobility (Fig. 4*A*, H1 and H2) appeared only when nuclear extracts from the NO- or hypoxia-treated cells were used. These bands include an HIF-1 heterodimer, which was assessed by supershift assays using antiserum against either HIF-1α or HIF1β (ARNT) (19). Similar patterns of H1 and H2 bands were observed when nuclear extracts from the NO- or hypoxia-treated cells were used with wt HBS and wt HBS + HAS probes.

In competition assays, using wt HBS as a labeled probe, HIF-1 bands induced by SNAP (Fig. 4*B*, H1 and H2) were displaced by excessive unlabeled wt HBS oligonucleotides but not by wt HAS. This result suggests that these bands are specific for the HBS, but the HAS is not a candidate for the binding site of HIF-1. The same result was obtained with hypoxia-treated extracts (data not shown).

Fig. 4*A* also reveals the presence of constitutive bands (C2) distinct from C1 when wt HBS + HAS or wt HAS was used as a probe. Another EMSA (Fig. 4*C*) shows that mutated HAS did not form C2 protein bands, and these bands were displaced only by excessive unlabeled wt HAS oligonucleotides. These results demonstrate that protein complexes that represent C2 bands specifically bind to the HAS of the human VEGF gene. Furthermore, this C2 band was not supershifted by antibodies against HIF-1α, HIF-1β, or CBP/p300 (data not shown), suggesting that the C2 band is irrelevant to these transcriptional factors.

Our mutational analysis of the VEGF promoter suggests a possible interaction of HIF-1 and the HAS-binding factor for the VEGF gene induction. To test if this HAS factor is present in cell lines where HIF-1 is activated under hypoxic conditions, we performed EMSA by using nuclear extract from A172, Hep3B, Hela, and COS-1 cells after normoxic or hypoxic exposure. Fig. 4, *A* and *D* shows that the HIF-1 band is induced only in NO or hypoxic nuclear extracts, whereas the HAS-binding complex is always visible and inhibited in part by hypoxia and NO in all analyzed cell lines.

To analyze the influence of the HAS on HIF-1 binding activity to the HBS, we performed another EMSA by using wt HBS as a labeled probe and either wt HBS or wt HBS + HAS as competitors. As shown in Fig. 5, excessive unlabeled wt HBS and wt HBS + HAS similarly displaced NO-induced HIF-1 bands (H1 and H2) and constitutive bands (C1), suggesting that the HAS does not significantly influence the binding affinity of HIF-1 to the HBS. This is also the case when using nuclear extracts from the hypoxia-treated cells (data not shown).

Structural Conservation of HREs in Hypoxia-inducible Genes—Hypoxia induces a number of genes whose promoter or enhancer region contains one or more HBSs. They include aldolase A, enolase 1, glucose transporter 1, LDHA, phosphofructokinase L, inducible nitric-oxide synthase, phosphoglycerate kinase 1, heme oxygenase 1, EPO, transferrin, and VEGF genes. Most of them have (T/G)ACGTG as a consensus sequence for the HBS, although HBSs in glucose transporter 1 (GGCGTG) and enolase 1 (TGCGTG) do not meet this consensus sequence perfectly (24).

We have determined the exact extents of the HBS and the HAS in VEGF and found that both elements form an imperfect inverted repeat. Moreover, the spacing of 8 nt in the VEGF gene is crucial. Surprisingly, analysis of the HREs of the above genes revealed that the HREs in 7 of 11 hypoxia-inducible genes form an imperfect inverted repeat and that the spacing is 8 nt in 6 genes and 9 nt in the rest (Fig. 6). These data suggest that a novel common mechanism may exist, where a putative HAF has a pivotal role for induction of these genes.

Responses of the Human EPO Enhancer to NO and Hypoxia—We have demonstrated that the HAS is essential for VEGF gene induction and that it is present in several hypoxia-inducible genes. To test whether NO up-regulates the promoter function of other hypoxia-inducible genes and whether the HAS functions as a cis-element in these genes, we prepared reporter plasmids containing a wild-type HRE (pHREepo), a mutated HBS (pHREepom1), and a mutated HAS (pHREepom2) of the EPO gene upstream of the herpes simplex virus thymidine kinase promoter. These constructs were transfected into A172 cells, and the luciferase activity of the extracts was assayed. After treatments of either SNAP or hypoxia for 12 h, pHREepo was induced 10- and ~40-fold, respectively, when compared with their respective controls. However, a mutation in either the HBS or the HAS almost completely abolished the response

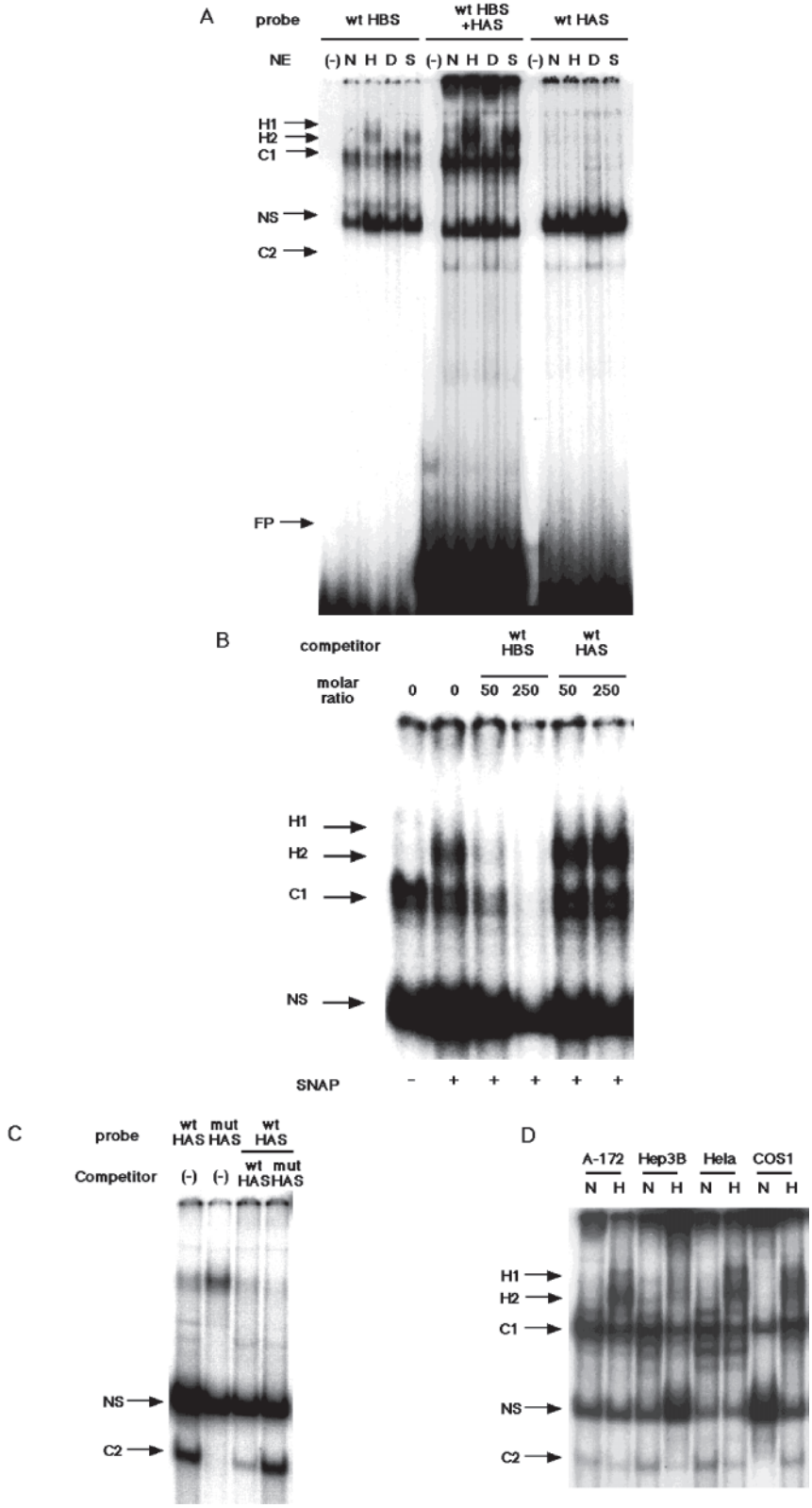

FIG. 4. **The HAS is not a binding site for HIF-1, but specific protein complexes bind to the HAS of VEGF in several cell lines.** Oligonucleotides for EMSA were as follows: wt HBS, which contains the wild-type HBS, but not the HAS; wt HBS + HAS, which contains the wild-type HBS and HAS; and wt HAS, which contains the wild-type HAS but not the HBS. Cells were exposed either to 0.1% Me_2SO or 0.5 mM SNAP (in 0.1% Me_2SO) or to normoxia (21% O_2) or hypoxia (1% O_2). Nuclear extracts (5 μg per lane) from these cells were incubated with ^{32}P-labeled probes in the presence or absence of competitors prior to electrophoresis. DNA-protein complexes are indicated by *arrows*. Hypoxia- or NO-induced complexes with slower (*H1*) and faster (*H2*) mobility, constitutively binding complexes to the HBS (*C1*) or HAS (*C2*), and nonspecific bands (NS) are indicated. *(−)*, no serum; *N*, normoxia; *H*, hypoxia; *D*, Me_2SO; *S*, SNAP. *A*, comparison of DNA binding activities recognized by wt HBS, wt HBS + HAS, and wt HAS in A172 cells. *B*, competition assays with either Me_2SO- or SNAP-treated nuclear extracts from A172 cells using wt HBS as a labeled probe in the presence of a 0-, 50-, or 250-fold molar excess of competitors. *C*, binding specificity of the C2 band to its target HAS in A172 cells. Competition assays were performed in the presence of a 250-fold molar excess of unlabeled oligonucleotides. *mut*, mutant. *D*, comparison of DNA binding activities recognized by wt HBS + HAS in normoxic (*N*) or hypoxic (*H*) extracts from A172, Hep3B, Hela, and COS-1 cells.

to either stimulus (Fig. 7), indicating that NO up-regulates EPO transcription and that the HAS functions as a cis-element in the EPO gene.

DISCUSSION

Positive Cooperativity of the HBS and the HAS in Human VEGF Gene Induction—Previously, we demonstrated that NO and hypoxia up-regulate transcription of the VEGF gene by enhancing HIF-1 binding activity (19). This observation suggests that the mechanisms of VEGF gene induction by these stimuli share common features and that HIF-1 has a central role in the transcriptional activation. Analysis of the VEGF promoter reveals that deletion of the HRE completely abolishes VEGF induction by NO and hypoxia. A further analysis of the HRE shows that not only the HBS, but also its downstream HAS, is essential for induction by these stimuli and that the

AP-1 site is required for its optimal response (19). Similar cooperativity among several domains within the HRE was also reported in the EPO and LDHA genes.

In the case of the human EPO gene, the HBS, its adjacent sequence CACAG, and the binding site for hepatic nuclear factor 4 are crucial for the enhancer activity, and a mutation of either site abolished its hypoxic response (2, 26). The promoter analysis of the human LDHA gene revealed that a mutation in the HBS entirely abrogated the response to hypoxia, and mutation in either its upstream ACGT or its downstream cyclic AMP response element significantly, but not completely, reduced the promoter activity (11). These data indicate that multiple factors mediate transcriptional regulation of these genes through a complex interaction among these factors.

An inverted repeat of half-sites spaced by some nucleotides is recognized as a common structure of the response elements for nuclear receptors. In principle, nuclear receptors dimerize in solution and bind to their response elements as dimers, although they can interact with and bind to the half-sites independently as monomers (27). The dimerization often enhances the binding of receptors to their response elements by stabilization of the receptor-DNA complex rather than by an increase in the association rate (28). In these cases, a spacing between the half-sites does not usually affect its function (28, 29). Our EMSA result revealed that HIF-1 can bind to its response element in the absence of the HAS. In addition, unlike nuclear receptors, positive cooperativity in HIF-1 binding between the HBS and the HAS was not observed.

Protein Complexes That Specifically Bind to the HAS of the Human VEGF Gene—Our mutational analysis of the VEGF promoter revealed that the core sequences of the HBS and the HAS form an imperfect inverted repeat with a spacing of 8 nt.

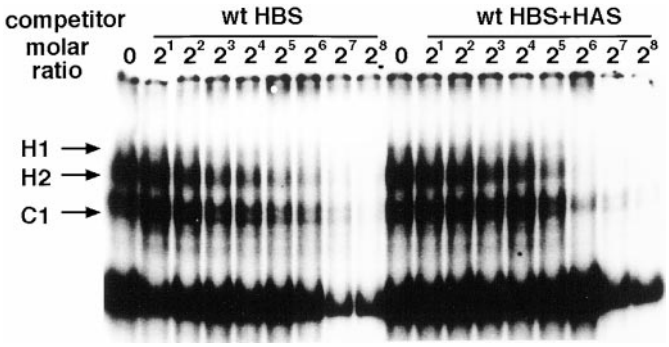

FIG. 5. **The HAS has no effect on HIF-1 binding affinity to the HBS.** Competition assays, with SNAP-treated nuclear extracts using wt HBS as a labeled probe, were performed in the presence of a 0- to 2^8-fold molar excess of competitors. Nitric oxide-induced complexes with slower (*H1*) and faster (*H2*) mobility and constitutively binding complexes to the HBS (*C1*) are indicated.

This observation suggested that HAF, a putative protein that binds to the HAS, might be identical or similar to HIF-1. Although mutations that increase stability of an inverted repeat enhance the reporter activity (Fig. 3*A*), changes in spacing between half-sites abolished the activity (Fig. 3*B*). Thus, the geometry of these motifs might be more important than the secondary structure. A strict requirement of the HAS sequence and precise spacing between the two motifs indicate that a putative HAF might interact with the HIF-1 heterodimer as a novel transcriptional factor in NO- and hypoxia-induced VEGF expression.

We demonstrated specific protein binding to the HAS of the VEGF gene in EMSA for the first time. These specific DNA-protein complexes are present in either normoxic or hypoxic nuclear extracts from A172, Hep3B, Hela, and COS-1 cells, but they are inhibited in part by hypoxia and NO (Fig. 4, *A* and *D*). It may be that part of the HAS-binding factors form protein complexes with HIF-1 under NO and hypoxic conditions, whereas the rest constitute attenuated C2 bands. The HAS-specific C2 complexes do not contain HIF-1 or CBP/p300, both of which are known to be essential for VEGF gene induction. These findings suggest that an unknown HAS-binding protein may function as a basic transcriptional factor in a wide range of tissues and organs, in contrast with tissue-specific regulation of hepatic nuclear factor 4 in the Epo gene (26). Although we have no information concerning the identity of the HAS-bind-

Genes	HRE	Spacing (nt)	HAS
VEGF	5'-CATACGTGGGCTCCAACAGGTCCT-3'	8	CAGGT
EPO	5'-CCTACGTGCTGTCTCACACAGCCT-3'	8	CACAG
ALDA	5'-GGGATGTGGTCCGAGTCACGTCCG-3'	8	CACAT
ENO-1	5'-CGCACGTGGCCCCGGACACGCAGC-3'	8	CACGC
LDHA	5'-CACACGTGGGTTCCCGCACGTCCG-3'	8	CACGT
GLUT-1	5'-CAGGCGTGCCGTCTGACACGCATC-3'	8	CACGC
HO-1	5'-CGGACGTGCTGGCGTGGCACGTCCT-3'	9	CACGT

FIG. 6. **The HREs of several hypoxia-inducible genes contain a common structure consisting of the HBS and the HAS.** The sequences that contain the HBS and the HAS of several hypoxia-inducible genes are shown. Note that these two motifs are usually spaced by 8 nt, and all HASs, except for VEGF, contain CACG(T/C) or CACA(G/T). The *closed* and *open arrows* indicate the HBS and the HAS, respectively. The *arrowheads* arbitrarily indicate the orientation of the half-site. *Underlined nucleotides* match the corresponding nucleotides in the remaining half-site as an inverted repeat.

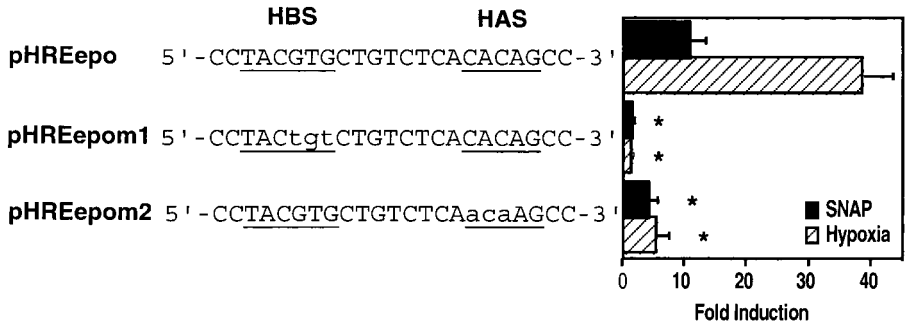

FIG. 7. **Nitric oxide up-regulates the EPO reporter activity via the HBS and the HAS.** A172 cells transiently transfected with reporter plasmids and pSV-nlsLacZ were harvested under the same conditions as in Fig. 1. The reporters pHREepo, pHREepom1, and pHREepom2 contain the wild-type HRE, a mutated HBS, and a mutated HAS of the human EPO gene, respectively. Results were expressed as the mean ± S.E. of six independent experiments. *, $p < 0.01$ *versus* pHREepo. Nucleotides of putative HBS and HAS are *underlined*, and substituted bases are shown in *lowercase letters*.

ing factor in the DNA-protein complex, its characterization is now in progress.

The HAS-specific complex is more visible the lower the concentration of potassium in the binding reaction in A172 cells.[2] Even 250 times excess of unlabeled wt HAS could not displace this band completely (Fig. 4*C*). Therefore, it is possible that the HAS-specific complex may bind to its target DNA with relatively lower affinity, and the amount of these proteins may be abundant, as compared with competitors.

Common Structures of HRE in Hypoxia-inducible Genes—A comparison of sequences of HREs (Fig. 6) shows that a similar structure, where the HBS and the HAS form an imperfect repeat, is seen among several hypoxia-inducible genes and that these motifs are spaced, most commonly, by 8 nt. Given that CACGT instead of CAGGT in the HAS of the VEGF gene functions as a cis-element for the promoter activity, all the HASs can be described as either CACG(T/C) or CACA(G/T) sequences (Fig. 6). In mutational analysis of the HAS of VEGF, pHREm3a, which contains an inverted repeat of the wild-type HBS (CAGGTC′CACGTA), had a 2-fold greater activity as compared with pHRE (Fig. 3*A*), and a pHRE-related mutant containing the HAS of LDHA or heme oxygenase 1 (CAGGT-′CACGT) had an identical response to pHREm3a. In contrast, an exchange of CAGGT (the HAS of VEGF) with CACAG (the HAS of EPO) in the VEGF promoter abrogated induction by NO and hypoxia.[2] These results indicate that the effect on increasing inducibility of the reporter gene by hypoxia and NO is mediated by a gene-specific inverted HBS rather than by a consensus HAS sequence. The C2 complex, as seen in Fig. 4, could not be detected in EMSA by using probes that contained the HAS of LDHA and EPO.[2] These findings suggest that HAS-binding factors of VEGF may be distinct from those of LDHA and EPO.

All of these HASs share a common sequence, CAC, except for the VEGF gene. CAC in the sense or GTG in the antisense strand is a recognition site of ARNT, as seen in the HBS ((T/G)ACGTG). ARNT is known as the central dimerization partner for basic helix-loop-helix-*per-arnt-sim* family transcription factors including HIF-1α, endothelial *per-arnt-sim* homology domain protein 1, arylhydrocarbon receptor, and Sim (30). It is possible that ARNT dimerizes with a member of the *per-arnt-sim* homology family and binds to its target HAS. Taken together, these results suggest that a common mechanism, other than an HIF-1-mediated pathway, may exist for NO- and hypoxia-induced expression of the hypoxia-inducible genes.

Conclusions and Implications—In summary, a detailed analysis of the VEGF gene promoter reveals that the sequences TACGTG and CAGGT are the cores of the HBS and the HAS, respectively, and that both sites are essential for the up-regulation of VEGF expression by NO and hypoxia. We also show that the constitutive protein complex binds to the HAS of VEGF. A similar structure of the HRE is seen among several hypoxia-inducible genes, indicating that a common mechanism may exist in which an HAF-mediated pathway positively regulates the transcription of these genes. We could not identify HAF in this study, but it may interact with, or rather collaborate with, HIF-1 for the promoter activation. VEGF plays a central role in tumor growth, progression, and metastasis by enhancing its angiogenesis and vascular permeability. If the HAF of VEGF is unique and distinct from that of other genes, inhibition of HAF function would be able to suppress VEGF induction without affecting expression of other hypoxia-inducible genes.

[2] H. Kimura, A. Weisz, T. Ogura, Y. Hitomi, Y. Kurashima, K. Hashimoto, F. D'Acquisto, M. Makuuchi, and H. Esumi, unpublished data.

REFERENCES

1. Guillemin, K., and Krasnow, M. A. (1997) *Cell* **89,** 9–12
2. Semenza, G. L., and Wang, G. L. (1992) *Mol. Cell. Biol.* **12,** 5447–5454
3. Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995) *Circ. Res.* **77,** 638–643
4. Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. (1995) *J. Biol. Chem.* **270,** 13333–13340
5. Melillo, G., Musso, T., Sica, A., Taylor, L. S., Cox, G. W., and Varesio, L. (1995) *J. Exp. Med.* **182,** 1683–1693
6. Lee, P. J., Jiang, B. H., Chin, B. Y., Iyer, N. V., Alam, J., Semenza, G. L., and Choi, A. M. (1997) *J. Biol. Chem.* **272,** 5375–5381
7. Norris, M. L., and Millhorn, D. E. (1995) *J. Biol. Chem.* **270,** 23774–23779
8. Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. (1994) *J. Biol. Chem.* **269,** 23757–23763
9. Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P., and Giallongo, A. (1996) *J. Biol. Chem.* **271,** 32529–32537
10. Firth, J. D., Ebert, B. L., Pugh, C. W., and Ratcliffe, P. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6496–6500
11. Firth, J. D., Ebert, B. L., and Ratcliffe, P. J. (1995) *J. Biol. Chem.* **270,** 21021–21027
12. Wang, G. L., and Semenza, G. L. (1993) *J. Biol. Chem.* **268,** 21513–21518
13. Ferrara, N., and Davis-Smyth, T. (1997) *Endocr. Rev.* **18,** 4–25
14. Klagsbrun, M., and D'Amore, P. A. (1996) *Cytokine Growth Factor Rev* **7,** 259–270
15. Sogawa, K., Numayama-Tsuruta, K., Ema, M., Abe, M., Abe, H., and Fujii-Kuriyama, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7368–7373
16. Liu, Y., Christou, H., Morita, T., Laughner, E., Semenza, G. L., and Kourembanas, S. (1998) *J. Biol. Chem.* **273,** 15257–15262
17. Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A., and Bunn, H. F. (1999) *J. Biol. Chem.* **274,** 9038–9044
18. Chin, K., Kurashima, Y., Ogura, T., Tajiri, H., Yoshida, S., and Esumi, H. (1997) *Oncogene* **15,** 437–442
19. Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M., and Esumi, H. (2000) *Blood* **95,** 189–197
20. Dulak, J., Jozkowicz, A., Dembinska-Kiec, A., Guevara, I., Zdzienicka, A., Zmudzinska-Grochot, D., Florek, I., Wojtowicz, A., Szuba, A., and Cooke, J. P. (2000) *Arterioscler. Thromb. Vasc. Biol.* **20,** 659–666
21. Wang, G. L., and Semenza, G. L. (1995) *J. Biol. Chem.* **270,** 1230–1237
22. Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. (1996) *Mol. Cell. Biol.* **16,** 4604–4613
23. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5510–5514
24. Wenger, R. H., and Gassmann, M. (1997) *Biol. Chem.* **378,** 609–616
25. Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M. A., Bunn, H. F., and Livingston, D. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 12969–12973
26. Galson, D. L., Tsuchiya, T., Tendler, D. S., Huang, L. E., Ren, Y., Ogura, T., and Bunn, H. F. (1995) *Mol. Cell. Biol.* **15,** 2135–2144
27. Glass, C. K. (1994) *Endocr. Rev.* **15,** 391–407
28. Kuntz, M. A., and Shapiro, D. J. (1997) *J. Biol. Chem.* **272,** 27949–27956
29. Kato, S., Sasaki, H., Suzawa, M., Masushige, S., Tora, L., Chambon, P., and Gronemeyer, H. (1995) *Mol. Cell. Biol.* **15,** 5858–5867
30. Long, W. P., Chen, X., and Perdew, G. H. (1999) *J. Biol. Chem.* **274,** 12391–12400

Identification of Hypoxia-inducible Factor 1 Ancillary Sequence and Its Function in Vascular Endothelial Growth Factor Gene Induction by Hypoxia and Nitric Oxide

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