J. Biochem. **132**, 37–44 (2002)

Human Hypoxic Signal Transduction through a Signature Motif in Hepatocyte Nuclear Factor 4[1]

Terumasa Tsuchiya,*[2] Yoshihiko Kominato,† and Masatsugu Ueda‡

**Department of Molecular Medicine, Research Center for Molecular Medical Science, 35-1, Shimo, Fussa, Tokyo 197-0023; †Department of Forensic Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-0194; and ‡YS New Technology Institute, Tochigi 329-0512*

Received April 17, 2002; accepted April 19, 2002

We identified a human hypoxic signal transduction pathway acting through a signature motif in the carboxyl terminal of hepatocyte nuclear factor 4 (HNF-4), by functional comparison of the transcriptional and protein–protein interaction activities of the wild type and mutants. It was previously shown that HNF-4 functions as a tissue-specific and hypoxia-activated transcription factor for the erythropoietin (Epo) gene. Human HNF-4 (465 amino acid residues) has DNA-binding, ligand-binding, and transactivation domains. The deletion mutant without the carboxyl terminal transactivation domain (amino acids 369–465) has been shown to be a dominant-negative mutant that repressed Epo transcriptional activity in hypoxia. Further characterization of the hypoxia-responsive domain by site-directed mutagenesis indicated that a TKQE motif of the carboxyl terminal (amino acids 460–463) in HNF-4 was essential for hypoxia-inducible Epo gene expression. We also found, by means of immunoprecipitation and a mammalian two-hybrid system, direct interactions between HNF-4 and hypoxia-inducible factor 1 (HIF-1), a heterodimer composed of α and β subunits. HNF-4 was observed to interact with HIF-1α and HIF-1β (arylhydrocarbon receptor nuclear translocator, ARNT) during hypoxia. In addition, the TKQE motif of HNF-4 was essential for protein–protein interactions with HIF-1α and ARNT. These results indicate that the human hypoxic signal of HIF-1 is transduced through interactions with the signature TKQE motif of the carboxyl terminal of HNF-4, resulting in Epo gene expression as a response to hypoxia.

Key words: erythropoietin, HIF-1, HNF-4, hypoxia.

Oxidative phosphorylation is an essential process for human life. Equally important is the body's ability to sense and respond to hypoxic stress, in order that oxygen homeostasis is accurately maintained. Central to maintenance of this homeostasis is the control of the red blood cell level in the body, as these cells transport oxygen from the lungs to peripheral tissues. Erythropoiesis is regulated by erythropoietin (Epo), a glycoprotein with a molecular mass of about 34 kDa (*1*). Epo gene expression is observed in both the kidneys and liver in response to hypoxia (*2–4*). Hypoxia also activates expression of the Epo gene in human hepatoma-derived cell lines Hep3B and HepG2 (*5*). However, embryonic carcinoma P19 cells produce Epo constitutively but express lactate dehydrogenase in an oxygen-dependent manner (*6*). The molecular basis for oxygen sensing and the role of Epo gene expression in this process are complicated and have not yet been clarified.

The human and mouse Epo genes have been cloned and sequenced (*7–11*), which revealed highly conserved coding regions. The 0.2 kb 5′-flanking region, upstream of the transcription initiation site, is also highly homologous (*10, 11*). This region is the Epo promoter that is repressed during normoxia and activated during hypoxia (*12, 13*). Several kinds of Epo promoter-binding proteins have been previously analyzed (*14–16*). Among them was the highly characterized transcription factor hepatocyte nuclear factor 4 (HNF-4) (*16*). HNF-4 is a liver- and kidney-specific transcription factor (*17–21*), which binds (T/C)GACCC motifs in the Epo promoter (*16*).

The 0.2 kb 3′-flanking region downstream of the poly(A) addition site is also highly homologous. This region is the Epo enhancer that is activated during hypoxia (*16, 22*). The *cis*-regulatory elements in the Epo enhancer are the TACGTG motif, for binding of hypoxia-inducible factor 1 (HIF-1) (*23*), and the (T/C)GACCT motifs for HNF-4 binding (*16*).

Transcription factor HIF-1 is a heterodimer composed of α and β subunits. HIF-1β is also known as arylhydrocarbon receptor (AhR) nuclear translocator (ARNT) (*24*). Both HIF-1α and ARNT have basic-helix-loop-helix and PAS (PER-AhR/ARNT-SIM) domains. Transcription factor HNF-

[1] This work was supported by grants to T. T. from the Ministry of Education, Science, and Culture of Japan. It was also carried out as a part of the "Ground-based Research Announcement for Space Utilization" promoted by the Japan Space Forum.

[2] To whom correspondence should be addressed. Tel: +81-42-539-2630, Fax: +81-42-539-2660, E-mail: rcmms@v004.vaio.ne.jp

Abbreviations: Epo, erythropoietin; HIF-1, hypoxia-inducible factor 1; ARNT, arylhydrocarbon receptor (AhR) nuclear translocator; HNF-4, hepatocyte nuclear factor 4; PAS, PER-AhR/ARNT-SIM; PCR, polymerase chain reaction; D-MEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; RLUs, relative light units; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

4 is a homodimer (*17*).

There are nineteen bases between the binding sites for HIF-1 and HNF-4, suggesting interactions between HIF-1 and HNF-4. Indeed, double disruption of these binding sites resulted in little response to hypoxia (*25*). This strongly suggested that HIF-1 and HNF-4 play a synergistic role in Epo gene expression during hypoxia. It had also been found that HNF-4 interacts with ARNT during both normoxia and hypoxia, and with HIF-1α during hypoxia. Analysis of a series of deletion mutants indicated that the carboxyl terminal transactivation domain of HNF-4 interacts with the PAS domains of HIF-1α and ARNT (*25*).

We had shown that HNF-4 functions as a tissue-specific and hypoxia-activated transcription factor for Epo gene expression (*16, 25*). The deletion mutant without the carboxyl terminal transactivation domain was a dominant-negative mutant that repressed Epo transcriptional activity. In this study, further characterization of the hypoxia-responsive domain of HNF-4 by site-directed mutagenesis reveals a signature motif of HNF-4. We also show direct interactions of the signature motif of HNF-4 with HIF-1α and ARNT. These interactions are shown to be essential for oxygen-dependent Epo gene expression.

METERIALS AND METHODS

Plasmid Construction—The human Epo promoter region (*Apa*I–*Eco*52I, 0.2 kb) was cloned from genomic DNA (*9*) into pUC19. The Epo promoter region was inserted upstream (*Hin*dIII site) of the luciferase cDNA (*26*) and the resultant plasmid was named pEpoPA-Luc (*13*). The human Epo enhancer region (*Apa*I–*Pvu*II, 0.12 kb) is located downstream of the poly(A) addition site (*16, 22*). The Epo enhancer was inserted downstream (*Apa*I site) of the poly(A) addition site of pEpoPA-Luc and the resultant plasmid was named pEpoPLE. Reporter plasmid pEpoPLE can express the luciferase cDNA under the control of the Epo 5′-promoter and 3′-enhancer in a physiological manner.

The human HNF-4α2 cDNA (*19*) fragment was used as a template. The wild-type HNF-4 cDNA (465 amino acid residues) was amplified by polymerase chain reaction (PCR). Mutations were also introduced by PCR. The TCC codon for serine at the 369th amino acid residue (S369) was replaced by the stop codon (TAA). This deletion mutant without the carboxyl terminal transactivation domain (amino acids 369–465) was named mt-del(1–368). Similarly, point mutations were introduced by PCR and amino acids were replaced, as shown in Figs. 2A and 3A. The resultant mutants were named mt(375–388), mt(460), mt(461), mt(462), mt(463), and mt(460–463). All of the sequences of the wild type and mutants were confirmed with a DNA autosequencer, ABI Prism 377 (Perkin Elmer).

A mammalian expression vector, pRC/CMV2 (Invitrogen), was used as an affector plasmid. The wild-type and mutant human HNF-4 cDNAs were cloned into the *Hin*dIII–*Not*I site of pRC/CMV2. The resultant expression vectors were named pRC-hHNF-4-WT, pRC-mt-del(1–368), pRC-mt(375–388), pRC-mt(460), pRC-mt(461), pRC-mt(462), pRC-mt(463), and pRC-mt(460–463).

To produce recombinant proteins, pET-15b (Novagen) was chosen. The human HIF-1α cDNA (*23*) was cloned into the *Nco*I–*Bam*HI site of pET-15b and the resultant plasmid was named pET-hHIF-1α. Similarly, pET-hARNT was constructed by insertion of the human ARNT cDNA (*24*) into the *Nco*I–*Xho*I site of pET-15b. By using the glutathione S-transferase (GST) gene fusion system (Amersham Pharmacia Biotech), GST-fusion protein expression vectors were constructed. The human HNF-4 cDNAs of the wild type and mt(460–463) mutant were cloned into the *Xho*I site of pGEX-5X-1. The resultant expression vectors were named pGEX-hHNF-4-WT and pGEX-hHNF-4-mt(460–463), respectively.

The mammalian two-hybrid system (Promega) was used to assay *in vivo* protein–protein interactions. As a bait, the wild-type and mt(460–463) HNF-4 cDNAs were cloned into the *Bam*HI–*Not*I site of pBIND (GAL4). The resultant vectors were named pBIND-hHNF-4-WT and pBIND-hHNF-4-mt(460–463), respectively. As a prey, the human HIF-1α cDNA (*23*) was cloned into the *Bam*HI–*Not*I site of pACT (VP16) and the resultant plasmid was named pACT-hHIF-1α. Similarly, pACT-hARNT was constructed by insertion of the human ARNT cDNA (*24*) into the *Bam*HI–*Not*I site of pACT.

Transactivation Assay—Hep3B cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% calf serum (HyClone) under 95% air and 5% CO_2 at 37°C. The cells were seeded at 1×10^6 cells/60 mm plate and cultured overnight. The plates were washed twice with serum-free OPTI-MEM I (Life Technologies). The reporter plasmid of pEpoPLE (0.1 μg) and the affector plasmid of the pRC series (0.2–1.6 μg) were transfected with pRSV-β-Gal (0.1 μg) (*27*) using lipofectin (5 μl) and OPTI-MEM I (1 ml) for 6 h according to the instruction manual (Life Technologies). After transfection, 4 ml of D-MEM supplemented with 10% calf serum was added. The cells were cultured under 95% air and 5% CO_2 (normoxia) at 37°C overnight. The culture was continued under normoxic conditions, or the cells were exposed to 2% O_2, 93% N_2, and 5% CO_2 (hypoxia) at 37°C for 24 h. The cells were washed with phosphate-buffered saline (PBS) twice and then cell lysis buffer (Pica Gene, Toyo Ink) was added (500 μl/plate). The luciferase activity of 100 μl of cell lysate was assayed with a luciferase assay system (Pica Gene, Toyo Ink) and a luminometer (Lumat LB 9501, Berthold) for 1 min. Relative light units (RLUs) were recorded and the raw data were corrected by subtraction of the background. Gene transfer efficiency was normalized with respect to the β-galactosidase activity of cotransfected pRSV-β-Gal as an internal control (*27*). Relative activity was calculated by dividing the corrected transcriptional activity of each sample by the average of the controls.

Immunoprecipitation and Western Blotting—Rabbit antiserum against human HNF-4α2 (TT971106) was raised by immunization with a synthetic peptide (PAYTTLEFENVQVLTMGNDT). Mouse monoclonal antibodies against human HIF-1α (H72320) and ARNT (A78420) were purchased (Transduction Laboratories). These monoclonal antibodies are not cross-reactive.

Subconfluent Hep3B cells were cultured under hypoxic conditions for 6 h, washed with PBS twice, and then suspended in PBS by scraping. The suspended Hep3B cells (1×10^8 cells/ml) were centrifuged at 2,000 rpm for 5 min at 4°C. The cell pellet was resuspended in PBS containing 1 mM PMSF and 1% Triton X-100. Sonication was performed and the sample was then centrifuged at 15,000 rpm for 10 min at 4°C. Immunoprecipitation of the hypoxic Hep3B cell

extract (1×10^8 cells) was performed using 100 μl of Protein A Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) immobilized with rabbit serum (preimmune serum or anti–hHNF-4 antiserum). The pellets were washed with PBS five times. The precipitated samples were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The proteins were detected using anti–hHIF-1α and anti-hARNT monoclonal antibodies (Transduction Laboratories) in an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

Binding Assay with GST-Fusion Proteins—Radio-labeled recombinant human HIF-1α and ARNT were synthesized using the ^{35}S-methionine and DNA templates of pET-hHIF-1α and pET-hARNT in a T7 coupled reticulocyte lysate system (Promega). *In vitro* transcription and translation were performed in 50 μl of the reaction mixture for 90 min at 30°C, as set out in the instruction manual. GST-fusion proteins were expressed in *Escherichia coli* strain BL21(DE3) harboring chaperone GroESL (*28*). GST, GST-hHNF-4(WT), and GST-hHNF-4(mt) were produced in *E. coli* carrying pGEX-5X-1, pGEX-hHNF-4-WT, and pGEX-hHNF-4-mt-(460–463), respectively. The recombinant products were immobilized with Glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

^{35}S-Methionine–labeled recombinant human HIF-1α and ARNT (50 μl of the reaction mixture) were bound to 100 μl of Glutathione-Sepharose 4B immobilized with GST, GST-hHNF-4(WT), and GST-hHNF-4(mt). The pellets were washed with PBS five times. Samples were analyzed by 12% SDS-PAGE, the gel was dried, and then autoradiography was performed.

Mammalian Two-Hybrid System—Hep3B cells were seeded at 1×10^6 cells/60 mm plate and cultured overnight. The plates were washed twice with serum-free OPTI-MEM I (Life Technologies). The plasmid vectors used for the mammalian two-hybrid system (Promega) were as follows. The reporter plasmid (0.1 μg) of pG5luc, bait vectors (0.1 μg) of pBIND, pBIND-hHNF-4-WT, and pBIND-hHNF-4-mt(460–463), prey vectors (0.1 μg) of pACT, pACT-hHIF-1α, and pACT-hARNT, were transfected with pRSV-β-Gal (0.1 μg) (*27*) using lipofectin (5 μl) and OPTI-MEM I (1 ml) for 6 h, as set out in the instruction manual (Life Technologies). After transfection, 4 ml of D-MEM supplemented with 10% calf serum was added. The cells were cultured under normoxic conditions overnight. The culture was continued under normoxic conditions, or the cells were exposed to hypoxic conditions for 24 h. The cells were washed with PBS twice and then cell lysis buffer (Pica Gene, Toyo Ink) was added (500 μl/plate). The luciferase activity of 100 μl of cell lysate was assayed with a luciferase assay system (Pica Gene, Toyo Ink) and a luminometer (Lumat LB 9501, Berthold) for 1 min. RLUs were recorded and the raw data were corrected by subtraction of the background. Gene transfer efficiency was normalized with respect to the β-galactosidase activity of cotransfected pRSV-β-Gal as an internal control (*27*). Relative activity was calculated by dividing the corrected transcriptional activity of each sample by the average of the controls.

RESULTS

Hypoxia-Responsive Transactivation Domain of Human HNF-4—Human HNF-4α2 is a member of a steroid/thyroid/retinoid receptor superfamily and is composed of A/B, C, D, E, and F regions, as shown in Fig. 1A. The DNA-binding domain (amino acids 51–116) consists of zinc finger motifs. The hypoxia-responsive transactivation domain (amino acids 369–465) is located in the carboxyl terminal, as shown below. We had already shown that HNF-4 functions as a tissue-specific and hypoxia-activated transcription factor for Epo gene expression (*16*). The deletion mutant (amino acids 1–354) without the carboxyl terminal domain was a dominant-negative mutant that could repress Epo transcriptional activity. However, the molecular mechanism of the role of the transactivation domain of HNF-4 remained unclear. In order to further characterize the hypoxia-responsive domain of HNF-4, we performed site-directed mutagenesis, as shown below.

A deletion mutant, mt-del(1–368), without the carboxyl terminal (amino acids 369–465) was constructed and its transcriptional activity was assayed. As shown in Fig. 1B, no effect was observed in normoxia. In contrast, transactivation activity for the Epo gene of wild-type HNF-4 (WT) was observed in hypoxia (Fig. 1C). However, the deletion mutant (mt), mt-del(1–368), repressed transcriptional activity, indicating that mt-del(1–368) is a dominant-negative mutant and that the carboxyl terminal (amino acids 369–465) is the hypoxia-responsive transactivation domain for Epo gene regulation.

As shown above, the transactivation domain (amino acids 369–465) of human HNF-4α2 plays a critical role in hypoxia-activated Epo gene expression. To establish the precise motif for activation, we introduced a series of point mutations in this region. We focused on two regions, amino acids 375–388 and 460–463, because these motifs are highly conserved among species (*17–21*). The motif comprising amino acids 375–388 includes histidine residues. The imizadole ring of histidine is well known to be very important for the binding and metabolism of oxygen and iron. We replaced all of the histidine residues with leucine residues, as shown in Fig. 2A. The resultant mutant was named mt(375–388), and its transcriptional activity was assayed. In normoxia, no effect was observed. In hypoxia, the transcriptional activity of the mutant was higher than that of the wild type (Fig. 2B). This indicates that highly conserved histidine residues are not critical for hypoxia-activated Epo gene expression.

We then introduced point mutations into another highly conserved region of HNF-4α2, the TKQE motif (amino acids 460–463), as shown in Fig. 3A. The transcriptional activities of these mutants were assayed. No effect was observed in normoxia. Figure 3B shows that T460 and E463 are critical for Epo gene expression in response to hypoxia. To confirm these results, all of the amino acid residues were mutated. As expected, mutant mt(460–463) shows no transcriptional activation.

We have shown that the deletion mutant without the carboxyl terminal transactivation domain (amino acids 369–465) was a dominant-negative mutant that could repress Epo transcriptional activity in hypoxia. Further characterization of the hypoxia-responsive domain by site-directed mutagenesis indicated that the highly conserved carboxyl terminal (amino acids 460–463, TKQE motif) of HNF-4 was essential for hypoxia-inducible Epo gene expression.

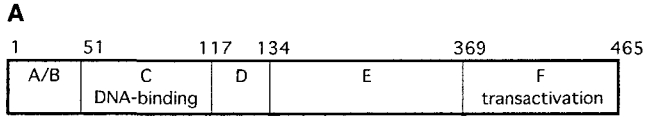
Interactions between Human HIF-1 and HNF-4—The Epo hypoxia-inducible enhancer is located in the 0.2 kb 3′-flanking region downstream of the poly(A) addition site (*16, 22*). The *cis*-regulatory elements in the Epo enhancer are the TACGTG motif, for binding of HIF-1 (*23*), and the (T/C)GACCT motifs for HNF-4 binding (*16*). There are nineteen bases between the HIF-1 and HNF-4 binding sites, suggesting interactions between HIF-1 and HNF-4. Indeed double disruption of these binding sites resulted in little response to hypoxia (*25*). This strongly suggested that HIF-1 and HNF-4 play a synergistic role in Epo gene expression in response to hypoxia. We had also found that HNF-4 interacts with HIF-1α and ARNT using a mammalian two-hybrid system (*25*).

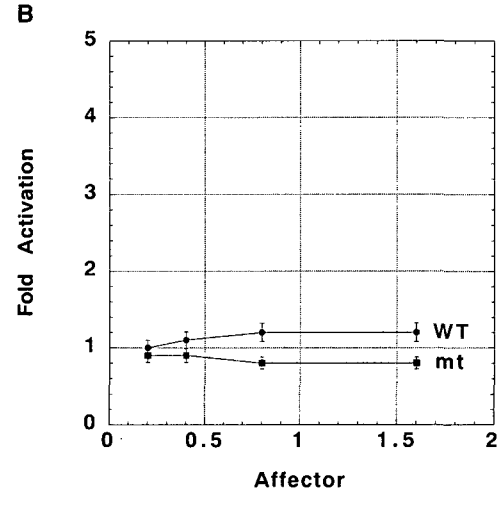
To confirm physiological interactions between native HIF-1 and HNF-4, we performed immunoprecipitation and Western blotting. A total cell extract of hypoxic Hep3B cells was immunoprecipitated with either preimmune serum (Pre) or anti–hHNF-4 antiserum (H4), as shown in Fig. 4. The precipitated samples were assayed using monoclonal antibodies against hHIF-1α and hARNT. Figure 4A indicates that the sample precipitated with anti–hHNF-4 antiserum contains hHIF-1α, while Fig. 4B shows that the same sample contains hARNT. The normoxic experiment gave the same band of hARNT as in Fig. 4B. These results indicate *in vivo* direct or indirect interactions of human HNF-4 with HIF-1α and ARNT.

Interaction Motif of Human HNF-4 for HIF-1α and ARNT—HIF-1 is known to be a heterodimer composed of HIF-1α and ARNT (*24*). To establish whether or not HNF-4 interacts with HIF-1α and ARNT, we performed binding assays on GST-HNF-4 fusion proteins with recombinant HIF-1α and ARNT. The human HNF-4 cDNAs of the wild type and mt(460–463) mutant were cloned into the GST-fusion protein expression vector. The resultant GST-fusion proteins were named GST-hHNF-4(WT) and GST-hHNF-4(mt), respectively.

As shown in Fig. 5A, recombinant HIF-1α bound to GST-hHNF-4(WT) (lane 2) but not to GST (lane 1). The binding activity of recombinant HIF-1α as to the mutant HNF-4, GST-hHNF-4(mt), was decreased to approximately 30% (lane 3). Similarly, Fig. 5B shows that recombinant ARNT specifically bound to GST-hHNF-4(WT) (lane 2) but not to GST (lane 1). The binding activity of recombinant ARNT as to the mutant HNF-4, GST-hHNF-4(mt), was almost completely lost (lane 3). These results strongly suggest that the essential TKQE motif (amino acids 460–463) of the hypoxia-responsive transactivation domain in HNF-4 (Fig. 3) plays a role in the interactions with HIF-1α (Fig. 5A) and ARNT (Fig. 5B). However, *in vitro* pull-down binding assays sometimes detect non-specific protein–protein interactions. This is because the hydrophobic surfaces of naked recombinant products can associate with each other in a non-specific manner.

To confirm the evidence for protein–protein interactions, we then assayed the *in vivo* interactions of HNF-4 with HIF-1α and ARNT using a mammalian two-hybrid system. Figure 6A shows that a prey (pACT-hHIF-1α, VP16-hHIF-1α) interacts with a wild-type HNF-4 bait (pBIND-hHNF-4-WT, GAL4-WT), but not with either a negative control bait (pBIND, GAL4) or a mutant HNF-4 [pBIND-hHNF-4-





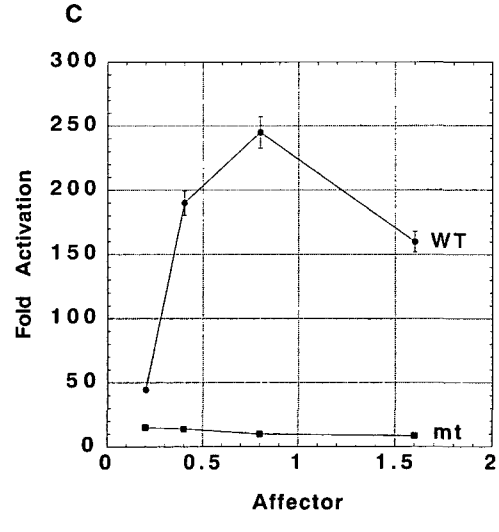


Fig. 1. **Structure and function of human HNF-4.** (A) Schematic representation of human HNF-4α2. The human HNF-4α2 cDNA encodes 465 amino acid residues. HNF-4α2 is composed of A/B, C, D, E, and F regions. The C region is the DNA-binding domain (amino acids 51–116). The F region is the hypoxia-responsive transactivation domain (amino acids 369–465). The numbers indicate amino acids. (B) Transactivation assaying of wild-type and mutant human HNF-4α2 in normoxia. The reporter plasmid of pEpoPLE (0.1 μg) and the affector plasmid of the pRC series (0.2, 0.4, 0.8, and 1.6 μg), pRC/CMV2, pRC-hHNF-4-WT, and pRC-mt-del(1–368) were transfected into Hep3B cells with pRSV-β-Gal (0.1 μg). Under normoxic conditions, luciferase activity was recorded and normalized with respect to β-galactosidase activity. Fold activation of pRC-hHNF-4-WT (WT, circles) and pRC-mt-del(1–368) (mt, squares) was calculated by dividing the corrected transcriptional activity of each sample by the average of the controls (pRC/CMV2). The results are expressed as the averages of triplicate determinations. Error bars indicate standard deviation. (C) Transactivation assaying of wild-type and mutant human HNF-4α2 in hypoxia. Transactivation assaying was performed as in (B). Under hypoxic conditions, luciferase activity was recorded and normalized with respect to β-galactosidase activity. The transcriptional activity without the affector plasmid in hypoxia is approximately fiftyfold higher than that in normoxia.

mt(460–463), GAL4-mt]. Similarly, Fig. 6B shows that ARNT (pACT-hARNT, VP16-hARNT) specifically binds to wild-type HNF-4 (GAL4-WT), but not to either a negative control (GAL4) or a mutant HNF-4 (GAL4-mt).

We have shown that the highly conserved TKQE motif (amino acids 460–463) of the hypoxia-responsive transactivation domain in human HNF-4 is essential for hypoxia-inducible Epo gene expression (Fig. 3B). In addition, we found *in vitro* and *in vivo* interactions of the TKQE motif in HNF-4 with HIF-1α and ARNT, in a sequence-specific manner (Figs. 5 and 6). This evidence indicates that human hypoxic signaling is transduced through a transcriptional apparatus formed by a complex of the HIF-1 heterodimer (HIF-1α and ARNT) with HNF-4. Direct protein–protein interactions are essential for the formation of a stable transcriptional complex, and thus for hypoxic signal transduction.

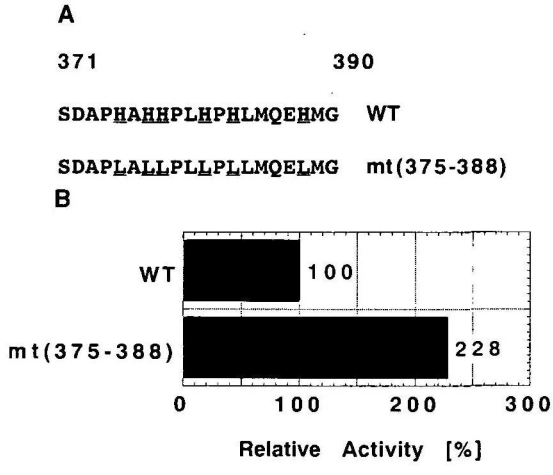


Fig. 2. **Function of histidine residues in human HNF-4.** (A) Amino acid 371–390 region of human HNF-4α2. The highly conserved histidine residues of the wild type (WT) were replaced by leucine residues. The resultant mutant was named mt(375–388). (B) Transactivation assaying of wild-type and mutant human HNF-4α2. The reporter plasmid of pEpoPLE (0.1 μg) and the affector plasmid of the pRC series (0.8 μg), pRC-hHNF-4-WT, and pRC-mt(375–388) were transfected into Hep3B cells with pRSV-β-Gal (0.1 μg). After exposure to hypoxic conditions, luciferase activity was recorded and normalized with respect to β-galactosidase activity. The results are expressed as the averages of triplicate determinations.

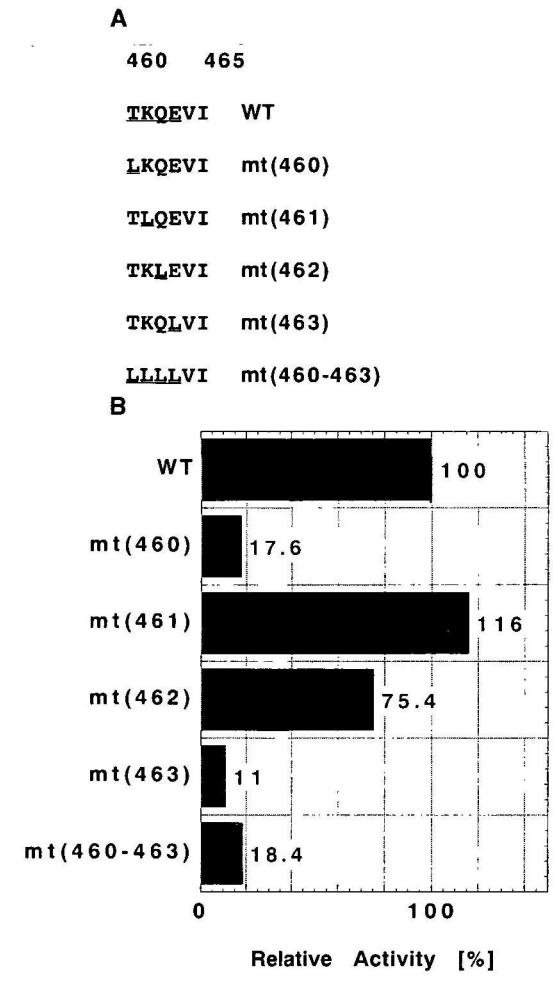


Fig. 3. **Function of the TKQE motif in human HNF-4.** The amino acid 460–465 region of human HNF-4α2. The highly conserved TKQE motif (460–463 amino acids) of the wild type (WT) was replaced by leucine residues. (B) Transactivation assaying of wild-type and mutant human HNF-4α2. The reporter plasmid of pEpoPLE (0.1 μg) and the affector plasmid of the pRC series (0.8 μg), pRC-hHNF-4-WT, pRC-mt(460), pRC-mt(461), pRC-mt(462), pRC-mt(463), and pRC-mt(460–463) were transfected into Hep3B cells with pRSV-β-Gal (0.1 μg). After exposure to hypoxic conditions, luciferase activity was recorded and normalized with respect to β-galactosidase activity. The results are expressed as the averages of triplicate determinations.

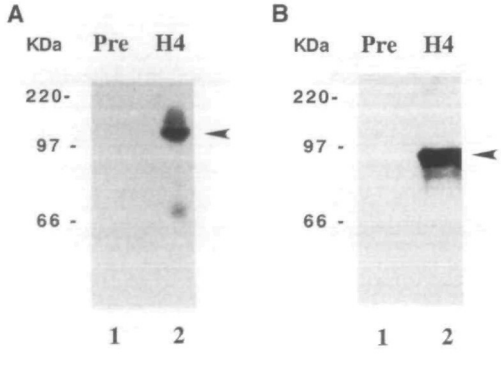


Fig. 4. **Immunoprecipitation and Western blotting.** Immunoprecipitation of a hypoxic Hep3B cell extract was performed using Protein A Sepharose 4 Fast Flow immobilized with rabbit preimmune serum (Pre) or anti-hHNF-4 antiserum (H4). After 12% SDS-PAGE, Western blotting was performed. The proteins were detected using monoclonal antibodies against hHIF-1α (A) and hARNT (B), as indicated by arrowheads.

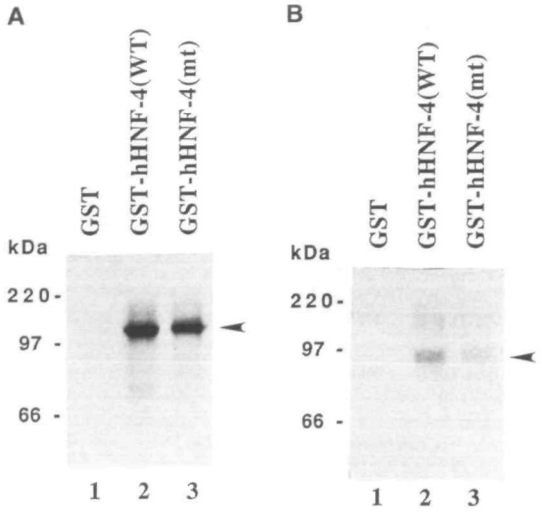


Fig. 5. **Affinity chromatography and SDS-PAGE.** The human HNF-4 cDNAs of the wild type and mt(460–463) mutant were cloned into the GST-fusion protein expression vector. The resultant GST-fusion proteins were named GST-hHNF-4(WT) and GST-hHNF-4(mt), respectively. ^{35}S-Methionine–labeled recombinant human HIF-1α and ARNT were independently bound to Glutathione-Sepharose 4B immobilized with GST, GST-hHNF-4(WT), and GST-hHNF-4(mt). Samples were analyzed by 12% SDS-PAGE. The gel was dried and then autoradiography was performed. Arrowheads indicate hHIF-1α (A) and hARNT (B) that were bound to the GST-fusion protein.

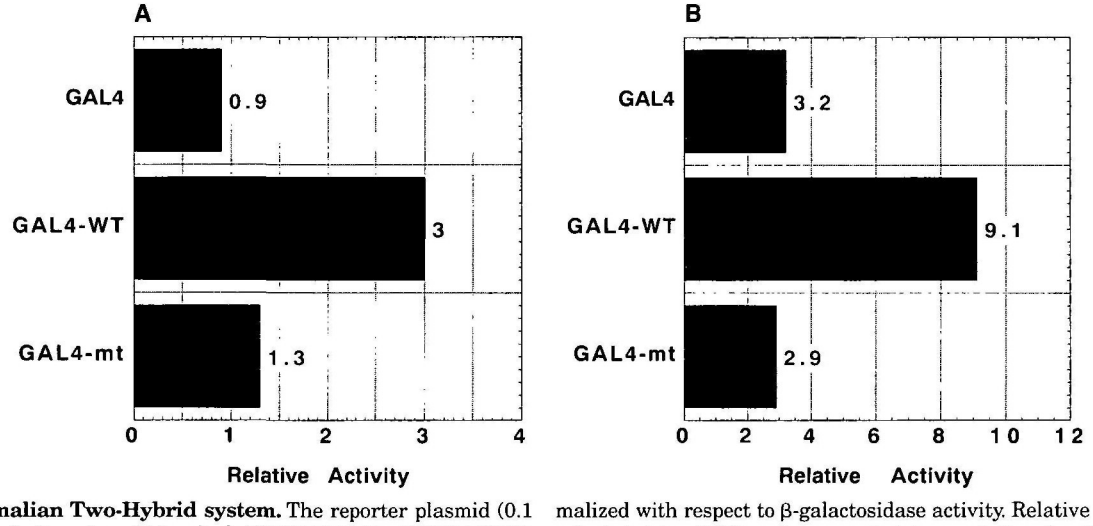


Fig. 6. **Mammalian Two-Hybrid system.** The reporter plasmid (0.1 μg) of pG5luc, bait vectors (0.1 μg) of pBIND (GAL4), pBIND-hHNF-4-WT (GAL4-WT), and pBIND-mt(460–463) (GAL4-mt), and prey vectors (0.1 μg) of pACT, pACT-hHIF-1α (A), and pACT-hARNT (B) were transfected into Hep3B cells with pRSV-β-Gal (0.1 μg). After exposure to hypoxic conditions, luciferase activity was recorded and normalized with respect to β-galactosidase activity. Relative activity was calculated by dividing the corrected transcriptional activity of each sample by the average of the controls (activity of pBIND plus pACT = 1.0, negative control). The results are expressed as the averages of triplicate determinations.

DISCUSSION

Hypoxic Signal Transduction—Mammalian oxygen sensors have not been identified. However, bacterial oxygen sensors have been cloned and characterized. An oxygen sensor of *Rhizobium meliloti* is a heme protein (FixL) with kinase activity (*29–33*). Another oxygen sensor is an oxygen-sensing transcription factor (FNR) of *Escherichia coli*, which is an iron-sulfur protein (*34, 35*). Dissociation of oxygen from iron is thought to trigger a conformational change in both FixL and FNR.

In contrast to the above, there is no direct evidence of human oxygen sensors. Epo gene expression in response to hypoxia is one of the best models for studying the human oxygen-sensing mechanism. Hypoxia-activated human Epo promoter (*12, 13*) and enhancer (*16, 22*) have already been identified. Among the binding factors, HIF-1 (*23*) and HNF-4 (*16*) are known to be involved in the hypoxic response. In addition, we had found that HIF-1 and HNF-4 play a synergistic role in Epo gene expression, and that HNF-4 interacts with HIF-1α during hypoxia (*25*). These results suggest that a putative oxygen sensor may transduce an unknown hypoxic signal to HIF-1α and HNF-4. As a result, a conformational change of HIF-1α and HNF-4 may be induced, which brings about their tight interactions.

Another possible mechanism is hypoxia-inducible stabilization of HIF-1α (*36, 37*). However, it is unknown why proteolysis is blocked in response to hypoxia. The protein stability of HIF-1α may be modulated through interactions with von Hippel-Lindau (VHL) tumor suppressor gene product (*38*), p53 (*39*), and HNF-4 (*25*) in hypoxia.

Interactions of HNF-4 with HIF-1α and ARNT—The deletion mutant (amino acids 1–355) of HNF-4 functioned as a dominant-negative mutant for hypoxia-inducible Epo gene expression (*16*). In this study, we have shown that the

F region (amino acids 369–465) is a hypoxia-responsive transactivation domain. However, it has been reported that two regions, amino acids 1–24 and amino acids 128–366, are transactivation domains for constitutive apoB and apoCIII genes (*40*). The F region functions as a hypoxia-responsive transactivation domain for the Epo gene, as shown in this study, and as a negative regulatory domain for the apoB and apoCIII genes (*40*). These results strongly suggest that transcription factor HNF-4 has dual functionality, using different domains. For hypoxia-inducible Epo gene expression, the F region functions as a hypoxia-responsive transactivation domain through interactions with HIF-1α and ARNT, as shown in this study.

HIF-1 is a heterodimer composed of HIF-1α (*23*) and ARNT (*24*). In this study, we have shown that HNF-4 interacts with HIF-1α and ARNT independently by means of a mammalian two-hybrid system and pull-down binding assays. It had also been found that HNF-4 interacts with HIF-1α in an oxygen-dependent manner (*25*). In addition, Epo gene expression in response to hypoxia is regulated through the synergistic role of HIF-1 and HNF-4 on the Epo enhancer (*25*). These results strongly suggest that transduction of hypoxic signaling, from a putative oxygen sensor, influences the interactions between HIF-1 and HNF-4. As a result, it is likely that a stable transcriptional machinery is formed in response to hypoxia and that high-level Epo gene expression is induced.

The *in vitro* and *in vivo* assays in this study indicated that the essential TKQE motif (amino acids 460–463) of the hypoxia-responsive transactivation domain in HNF-4 plays a role in specific interactions with HIF-1α and ARNT. At present, it is unclear how the TKQE motif of HNF-4 interacts with HIF-1α and ARNT. In addition, we have not determined what kind of signaling may be involved in the protein–protein interactions, and activation of HIF-1α, ARNT, and HNF-4, *e.g.* kination, electron transfer, *etc.* Further analyses of the signal transduction and structural biology of these proteins and their associated cofactors may reveal the molecular basis of the interactions and activation mechanisms.

We wish to thank Drs. G.L. Semenza, O. Hankinson, and G.U. Ryffel for providing the human HIF-1α, ARNT, and HNF-4α2 cDNAs, respectively. We also thank Dr. S. Ishii for providing the *Escherichia coli* strain BL21(DE3) harboring chaperone GroESL.

REFERENCES

1. Miyake, T., Kung, C.K.-H., and Goldwasser, E. (1977) Purification of human erythropoietin. *J. Biol. Chem.* **252**, 5558–5564
2. Schuster, S.J., Badiavas, E.V., Costa-Giomi, P., Weinmann, R., Erslev, A.J., and Caro, J. (1989) Stimulation of erythropoietin gene transcription during hypoxia and cobalt exposure. *Blood* **73**, 13–16
3. Bondurant, M.C. and Koury, M.J. (1986) Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. *Mol. Cell. Biol.* **6**, 2731–2733
4. Beru, N., McDonald, J., Lacombe, C., and Goldwasser, E. (1986) Expression of the erythropoietin gene. *Mol. Cell. Biol.* **6**, 2571–2575
5. Goldberg, M.A., Glass, G.A., Cunningham, J.M., and Bunn, H.F. (1987) The regulated expression of erythropoietin by two human hepatoma cell lines. *Proc. Natl. Acad. Sci. USA* **84**, 7972–7976
6. Kambe, T., Tada, J., Chikuma, M., Masuda, S., Nagao, M., Tsuchiya, T., Ratcliffe, P.J., and Sasaki, R. (1998) Embryonal carcinoma P19 cells produce erythropoietin constitutively but express lactate dehydrogenase in an oxygen-dependent manner. *Blood* **91**, 1185–1195
7. Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S.D., Kaufman, R.J., Mufson, A., Seehra, J., Jones, S.S., Hewick, R., Fritsch, E.F., Kawakita, M., Shimizu, T., and Miyake, T. (1985) Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* **313**, 806–810
8. Lin, F.-K., Suggs, S., Lin, C.-H., Browne, J.K., Smalling, R., Egrie, J.C., Chen, K.K., Fox, G.M., Martin, F., Stabinsky, Z., Badrawi, S.M., Lai, P.-H., and Goldwasser, E. (1985) Cloning and expression of the human erythropoietin gene. *Proc. Natl. Acad. Sci. USA* **82**, 7580–7584
9. Goto, M., Akai, K., Murakami, A., Hashimoto, C., Tsuda, E., Ueda, M., Kawanishi, G., Takahashi, N., Ishimoto, A., Chiba, H., and Sasaki, R. (1988) Production of recombinant human erythropoietin in mammalian cells: host-cell dependency of the biological activity of the cloned glycoprotein. *Bio/Technology* **6**, 67–71
10. McDonald, J.D., Lin, F.-K., and Goldwasser, E. (1986) Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene. *Mol. Cell. Biol.* **6**, 842–848
11. Shoemaker, C.B. and Mitsock, L.D. (1986) Murine erythropoietin gene: cloning, expression, and human gene homology. *Mol. Cell. Biol.* **6**, 849–858
12. Tsuchiya, T., Ochiai, H., Imajoh-Ohmi, S., Ueda, M., Suda, T., Nakamura, M., and Kanegasaki, S. (1992) *In vitro* reconstitution of an erythropoietin gene transcription system using its 5′-flanking sequence and a nuclear extract from anemic kidney. *Biochem. Biophys. Res. Commun.* **182**, 137–143
13. Tsuchiya, T., Okada, M., Ueda, M., and Yasukochi, Y. (1997) Activation of the erythropoietin promoter by a point mutation from GATA to TATA in the −30 region. *J. Biochem.* **121**, 193–196
14. Tsuchiya, T., Ueda, M., Ochiai, H., Imajoh-Ohmi, S., and Kanegasaki, S. (1993) Erythropoietin 5′-flanking sequence-binding protein induced during hypoxia and cobalt exposure. *J. Biochem.* **113**, 395–400
15. Beru, N., Smith, D., and Goldwasser, E. (1990) Evidence suggesting negative regulation of the erythropoietin gene by ribonucleoprotein. *J. Biol. Chem.* **265**, 14100–14104
16. Galson, D.L., Tsuchiya, T., Tendler, D.S., Huang, L.E., Ren, Y., Ogura, T., and Bunn, H.F. (1995) The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. *Mol. Cell. Biol.* **15**, 2135–2144
17. Sladek, F.M., Zhong, W., Lai, E., and Darnell, Jr., J.E. (1990) Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **4**, 2353–2365
18. Hata, S., Tsukamoto, T., and Osumi, T. (1992) A novel isoform of rat hepatocyte nuclear factor 4 (HNF-4). *Biochim. Biophys. Acta* **1131**, 211–213
19. Chartier, F.L., Bossu, J.-P., Laudet, V., Fruchart, J.-C., and Laine, B. (1994) Cloning and sequencing of cDNAs encoding the human hepatocyte nuclear factor 4 indicate the presence of two isoforms in human liver. *Gene* **147**, 269–272
20. Drewes, T., Senkel, S., Holewa, B., and Ryffel, G.U. (1996) Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. *Mol. Cell. Biol.* **16**, 925–931
21. Hertz, R., Magenheim, J., Berman, I., and Bar-Tana, J. (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4α. *Nature* **392**, 512–516
22. Maden, A. and Curtin, P.T. (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
23. Wang, G.L., Jiang, B.-H., Rue, E.A., and Semenza, G.L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS het-

erodimer regulated by cellular O_2 tension. *Proc. Natl. Acad. Sci. USA* **92**, 5510–5514
24. Hoffman, E.C., Reyes, H., Chu, F.-F., Sander, F., Conley, L.H., Brooks, B.A., and Hankinson, O. (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252**, 954–958
25. Zhang, W., Tsuchiya, T., and Yasukochi, Y. (1999) Transitional change in interaction between HIF-1 and HNF-4 in response to hypoxia. *J. Hum. Genet.* **44**, 293–299
26. deWett, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725–737
27. Edlund, T., Walker, M.D., Barr, P.J., and Rutter, W.J. (1985) Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5′ flanking elements. *Science* **230**, 912–916
28. Yasukawa, T., Kanei-Ishii, C., Maekawa, T., Fujimoto, J., Yamamoto, T., and Ishii, S. (1995) Increase of solubility of foreign proteins in *Escherichia coli* by coexpression of the bacterial thioredoxin. *J. Biol. Chem.* **270**, 25328–25331
29. Gilles-Gonzalez, M.A., Ditta, G.S., and Helinski, D.R. (1991) A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. *Nature* **350**, 170–172
30. Tamura, K., Nakamura, H., Tanaka, Y., Oue, S., Tsukamoto, K., Nomura, M., Tsuchiya, T., Adachi, S., Takahashi, S., Iizuka, T., and Shiro, Y. (1996) Nature of endogenous ligand binding to heme iron in oxygen sensor FixL. *J. Am. Chem. Soc.* **118**, 9434–9435
31. Nakamura, H., Saito, K., Ito, E., Tamura, K., Tsuchiya, T., Nishigaki, K., Shiro, Y., and Iizuka, T. (1998) Identification of the hydrophobic amino acid residues required for heme assembly in the rhizobial oxygen sensor protein FixL. *Biochem. Biophys. Res. Commun.* **247**, 427–431
32. Miyatake, H., Kanai, M., Adachi, S., Nakamura, H., Tamura, K., Tanida, H., Tsuchiya, T., Iizuka, T., and Shiro, Y. (1999) Dynamic light-scattering and preliminary crystallographic studies of the sensor domain of the haem-based oxygen sensor FixL from *Rhizobium meliloti*. *Acta Crystallogr.* **D55**, 1215–1218
33. Miyatake, H., Mukai, M., Park, S.-Y., Adachi, S., Tamura, K., Nakamura, H., Nakamura, K., Tsuchiya, T., Iizuka, T., and Shiro, Y. (2000) Sensory mechanism of oxygen sensor FixL from *Rhizobium meliloti*: crystallographic, mutagenesis and resonance Raman spectroscopic studies. *J. Mol. Biol.* **301**, 415–431
34. Khoroshilova, N., Beinert, H., and Kiley, P.J. (1995) Association of a polynuclear iron-sulfur center with a mutant FNR protein enhances DNA binding. *Proc. Natl. Acad. Sci. USA* **92**, 2499–2503
35. Melville, S.B. and Gunsalus, R.P. (1996) Isolation of an oxygen-sensitive FNR protein of *Escherichia coli*: interaction at activator and repressor sites of FNR-controlled genes. *Proc. Natl. Acad. Sci. USA* **93**, 1226–1231
36. Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., and Ratcliffe, P.J. (1997) Activation of hypoxia-inducible factor-1: definition of regulatory domains within the α subunit. *J. Biol. Chem.* **272**, 11205–11214
37. Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **95**, 7987–7992
38. Maxwell, P.H., Wiesener, M.S., Chang, G.-W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399**, 271–275
39. An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., and Neckers, L.M. (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1α. *Nature* **392**, 405–408
40. Hadzopoulou-Cladaras, M., Kistanova, E., Evagelopoulou, C., Zeng, S., Cladaras, C., and Ladias, J.A.A. (1997) Functional domains of the nuclear receptor hepatocyte nuclear factor 4. *J. Biol. Chem.* **272**, 539–550