Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells

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Here we describe the cloning and characterization of a PAS domain transcription factor termed endothelial PAS-1 (EPAS1). This protein shares 48% sequence identity with hypoxia inducible factor (HIF-1α) and lesser similarity with other members of the basic helix–loop–helix/PAS domain family of transcription factors. Like HIF-1α, EPAS1 binds to and activates transcription from a DNA element originally isolated from the erythropoietin gene and containing the sequence 5′-GCCCTACGTGCTGTCTCA-3′. Activation by both HIF-1α and EPAS1 is stimulated by hypoxic conditions. EPAS1 forms a heterodimeric complex with the aryl hydrocarbon nuclear transporter prior to transcriptional activation of target genes. EPAS1 expression is limited to the endothelium of mouse embryos and, in agreement with its cell type-specific expression pattern, is capable of specifically activating the transcription of the endothelial tyrosine kinase gene *Tie-2*. These observations raise the possibility that EPAS1 may represent an important regulator of vascularization, perhaps involving the regulation of endothelial cell gene expression in response to hypoxia.

[*Key Words:* PAS domain proteins; endothelial cell transcription; receptor tyrpsine kinase; Tie-2; hypoxia inducible factor; chromosome 2p16-21]

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Roughly a dozen proteins classified as basic helix–loop–helix (bHLH)/PAS domain transcription factors have been described in both vertebrates and invertebrates. Members of this class derive their name from the shared presence of a bHLH motif that specifies sequence-dependent recognition of DNA and a PAS domain composed of two imperfect repeats. PAS is an acronym derived from the first three proteins observed to contain this motif. These include the product of the *period* gene of *Drosophila melanogaster* (Jackson et al. 1986; Citri et al. 1987), the aryl hydrocarbon receptor nuclear transporter (ARNT) gene of mammals (Burbach et al. 1992), and the product of the fruit fly *single-minded* gene (Nambu et al. 1991).

The imperfect, direct repeats within the PAS domain are ~50 amino acids in length and contain a signature His–X–X–Asp sequence in each repeat. Three biochemical functions have been assigned to the PAS domain. First, it acts in concert with the HLH domain of bHLH/PAS proteins to form a dimerization surface (Reisz-Porszasz et al. 1994; Fukunaga et al. 1995; Lindebro et al. 1995). In the case of the *period* gene product, which lacks a bHLH domain, the PAS domain specifies heterodimerization with the product of the *timeless* locus (Gekakis et al. 1995; Myers et al. 1995). Interaction between the *period* and *timeless* gene products represents a crucial event in the control of circadian rhythm in fruit flies (Hunter-Ensor et al. 1996; Lee et al. 1996; Myers et al. 1996; Zeng et al. 1996). In contrast, the aryl hydrocarbon receptor (AHR) heterodimerizes with ARNT via PAS domain interactions (Fukunaga et al. 1995), producing a heterodimer that is competent for nuclear gene interaction. Second, the PAS domain mediates interaction with heat shock protein 90 (HSP-90). Several PAS domain proteins, including the *single-minded* gene product and the AHR, can be sequestered in the cytoplasm in an inactive state. Maintenance of the inactive state involves interactions between the PAS domain and HSP-90 (Perdew 1988; Henry and Gasiewicz 1993; Chen and Perdew 1994; McGuire et al. 1995). Finally, the PAS domain of the AHR facilitates high-affinity binding of certain xenobiotic compounds including dioxin (for review, see Hankinson 1995; Schmidt and Bradfield 1996).

PAS domain transcription factors perform diverse functions in a variety of cell types and organisms. The *period* gene product helps regulate circadian rhythm in fruit flies (Konopka and Benzer 1971), whereas the mammalian AHR provides response to xenobiotics by activating genes whose products facilitate detoxification (Schmidt and Bradfield 1996). A more recently described member of the PAS domain family, hypoxia inducible

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factor (HIF-1α), activates genes whose products regulate hematopoiesis in response to oxygen deprivation (Wang et al. 1995). In *Drosophila*, the *single-minded* gene product affects neurogenesis (Nambu et al. 1991) and the *trachealess* gene product controls the formation of tubular structures in the embryo (Isaac and Andrew 1996; Wilk et al. 1996).

The utilization of bHLH/PAS domain proteins in diverse species and physiological processes raises the possibility that this family of transcription factors might consist of many undiscovered members. Following this lead, we searched databases consisting of expressed cDNA sequences for the presence of new members of the bHLH/PAS domain family. Here we report the initial characterization of a new member of this protein family designated endothelial PAS domain protein 1 (EPAS1).

Results

cDNAs encompassing the coding region of the human EPAS1 were isolated by screening a HeLa cell cDNA library with a radiolabeled probe derived from an expressed sequence tag (#T70415) obtained from the GenBank database (see Materials and Methods). Multiple cDNA clones were isolated and subjected to DNA sequence analysis to derive the conceptually translated protein sequence of human EPAS1 shown in Figure 1A. The predicted M_r of the human EPAS1 was 96,528. A termination codon was located 24 nucleotides 5′ of the designated initiator methionine in the human sequence. cDNAs encoding the murine homolog were isolated from an adult mouse brain cDNA library using a probe obtained by RT–PCR with oligonucleotide primers de-

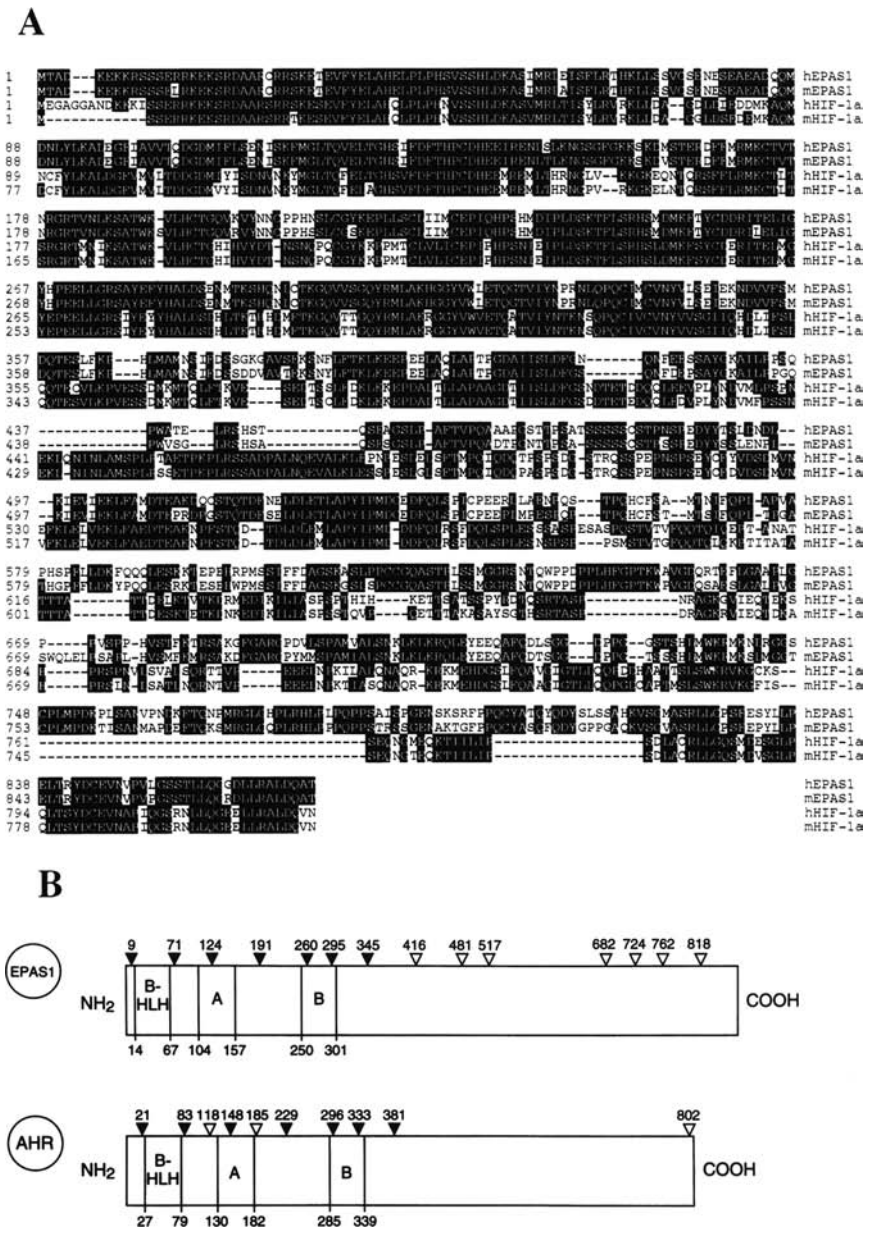

Figure 1. Sequence alignment and gene structure of EPAS1. (*A*) The predicted protein sequences of the human (h, GenBank accession no. U81984) and mouse (m, GenBank accession no. U81983) EPAS1 in single-letter amino acid code are shown aligned with those of the hypoxia inducible factor (HIF-1α). Identical residues are outlined in black. Amino acids are numbered on the *left*. (*B*) Schematic of the human EPAS1 protein containing bHLH and two imperfect repeats (A and B) composing the PAS domain is shown together with the positions at which introns (inverted triangles) interrupt the coding region of the gene. A second schematic represents the AHR gene product and the positions at which introns are found in the encoding gene (Schmidt et al. 1993). (▼) Intron locations that are conserved between the EPAS1 and AHR genes; (▽) nonconserved intron locations. Numbers refer to amino acids that are interrupted by introns (*above* schematic) or that compose the indicated domains (*below* schematic).

rived from the human EPAS1 cDNA sequence (see Materials and Methods). The predicted protein sequence of murine EPAS1 was aligned and compared with the human sequence in Figure 1A. The two proteins share 88% sequence identity. Database searches revealed that the human and murine EPAS1 proteins share extensive primary amino acid sequence identity with HIF-1α, a member of the bHLH/PAS domain family of transcription factors (Wang et al. 1995; Wenger et al. 1996). EPAS1 and HIF-1α share 48% primary amino acid sequence identity as revealed by the alignment shown in Figure 1A. Sequence conservation between the two proteins is highest in the bHLH (85%), PAS-A (68%), and PAS-B (73%) regions. A second region of sequence identity occurs at the extreme carboxyl termini of the EPAS1 and HIF-1α proteins (Fig. 1A). This conserved region in mHIF1α recently has been shown to contain a hypoxia response domain (Li et al. 1996). EPAS1 also shares sequence relatedness with other PAS domain proteins; however, the degree of similarity between EPAS1 and other family members is less striking than that between HIF-1α and EPAS1 (data not shown).

Genomic clones encoding the human EPAS1 transcript were isolated by screening bacteriophage libraries of human DNA. The intron–exon structure of the gene was established by comparison of DNA sequences obtained from the genomic DNA with that of the cDNA. The coding region of EPAS1 is specified by 15 exons. The exonic sequences mapped to six nonoverlapping bacteriophage lambda clones whose average insert size was 20 kb, indicating that the EPAS1 gene spans at least 120 kb of genomic DNA. The positions of the introns within the coding region are shown in Figure 1B. A comparison of the EPAS1 gene structure with that of the AHR (Schmidt et al. 1993) reveals that the positions of introns within the regions encoding the amino-terminal halves of the two proteins are highly conserved (Fig. 1B). In contrast, the portion of the EPAS1 gene specifying the carboxy-terminal half of the protein is interrupted by seven introns, whereas the AHR gene contains only a single intron in this region. Thus the 5′- ends of the two genes may have arisen from an ancient gene duplication event, whereas the 3′- regions have a more recent evolutionary origin.

Two methods were used to determine the chromosomal location of the human EPAS1 gene. Fluorescent in situ hybridization (FISH) analysis was performed using a biotinylated probe containing exons 8–14 of the EPAS1 gene. This analysis revealed a single hybridization signal over chromosome 2, bands p16–p21 (data not shown). As a second assay for gene localization, an oligonucleotide primer pair derived from exon 8 was used to amplify a segment of the EPAS1 gene from the genomic DNAs of a radiation hybrid panel. Computer-assisted analysis of the results indicated linkage of the EPAS1 gene to the D2S288 marker on chromosome 2p with a LOD score of 8.7 and a cR8000 value of 12.96. Thus, the data obtained from two independent mapping methods consistently positioned the EPAS1 gene on the short arm of chromosome 2 and indicate that the EPAS1 gene is nonsyntenic with the HIF-1α gene, which maps to chromosome 14q21-24 (Semenza et al. 1996).

The high degree of sequence similarity between the EPAS1 and HIF-1α proteins raised the possibility that they shared a common physiological function. To test this hypothesis, RNA blotting experiments were used to compare and contrast the distributions of EPAS1 and HIF-1α mRNAs in a variety of human tissues. As shown in Figure 2, an EPAS1 mRNA of ~5.8 kb was detected in all tissues examined with the single exception of peripheral blood leukocytes. Among the positive tissues, highly vascularized organs such as the heart, placenta, and lung showed the highest levels of EPAS1 mRNA (Fig. 2, top). A HIF-1α mRNA of ~4.4 kb was detected in all human tissues. In contrast to EPAS1 mRNA, however, peripheral blood leukocytes contained very high levels of HIF-1α mRNA. Likewise, we observed no enrichment of HIF-1α mRNA in highly vascularized tissues (Fig. 2, bottom).

These RNA blotting data indicate that, with few exceptions, most tissues express both EPAS1 and HIF-1α mRNAs. To determine whether this overlap extended to the cellular level, in situ mRNA hybridization was used to determine the cell type-specific expression patterns of the two gene products. Sections from day 11 and day 13 mouse embryos were examined first. Figure 3A shows a representative section from a day 11 embryo. EPAS1 transcripts were observed almost exclusively in endothelial cells of the intersegmental blood vessels separating the somites, the atrial and ventricular chambers of the heart, and the dorsal aorta. Extra-embryonic membranes, such as the yolk sac, which are highly vascularized, also expressed abundant levels of EPAS1 mRNA (Fig. 3B). In the developing brain of a day 13 embryo, endothelial cells of the highly vascularized choroid plexus contained abundant EPAS1 transcripts (Fig. 3C). The brain section shown in Figure 3C also revealed intense EPAS1 mRNA hybridization in the endothelial cells of a blood vessel lying along the edge of postmitotic neurons emanating from the lateral ventricle region. When a nearby section was hybridized with an antisense probe that was specific for the HIF-1α mRNA, only a diffuse signal somewhat over background was detected, indicating a low level of HIF-1α expression in many cell types (Fig. 3D). In contrast to the results with the EPAS1 probe, no concentration of HIF-1α mRNA was detected in the endothelial cells of the adjacent blood vessel (Fig 3; cf C and D). A differential expression pattern between EPAS1 and HIF-1α was also apparent in the region of the embryo containing the umbilicus. EPAS1 transcripts were detected in the endothelium of blood vessels within this structure (Fig. 3E), whereas HIF-1α mRNA was concentrated in the mesenchyme surrounding the vascular endothelium (Fig. 3F).

In tissues of adult mice, EPAS1 mRNA was also detected at high levels in endothelial cells yet was also present at lower levels in several additional cells types. For example, decidual cells of the placenta contained very high levels of EPAS1 mRNA, as did parenchymal tissue in the lung (data not shown). The distinction be-

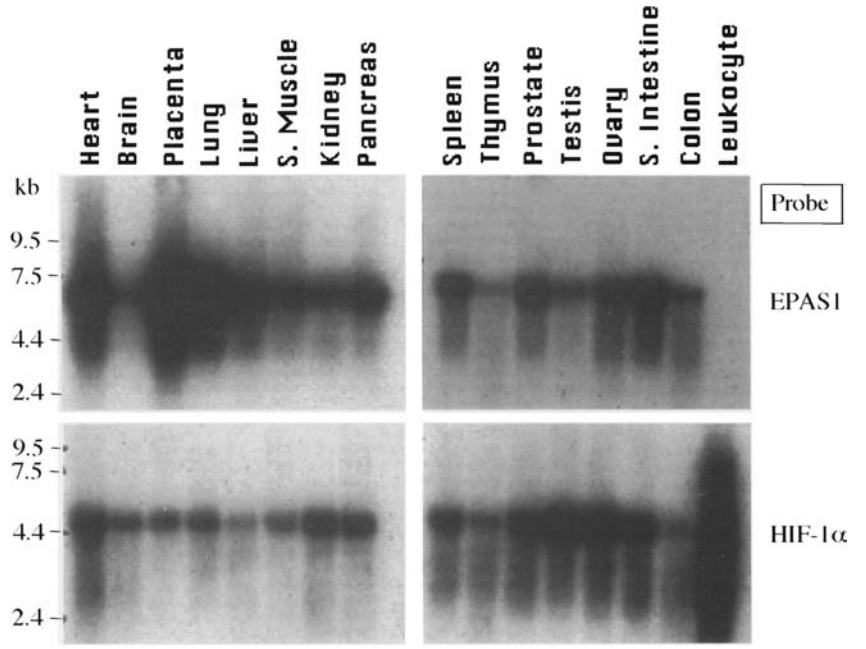

Figure 2. EPAS1 and HIF-1α mRNA in human tissues. Blots containing 2 μg of poly(A)-enriched RNA from the indicated tissues were hybridized with either an EPAS1 probe (*top* panels) or an HIF-1α probe (*bottom* panels). After hybridization and washing the filters were exposed to X-ray film for 17 hr (*top* panel) or 46 hr (*bottom* panel). The positions to which standards migrated and their sizes in kilobases are shown on the *right*.

tween EPAS1-expressing cell types and HIF-1α-expressing cells was also apparent in adult tissues. Figure 3G shows a section through the cortex of the kidney in which EPAS1 expression was detected in the mesangial cells. In contrast, HIF-1α expression was found in the cells of the collecting ducts (Fig. 3H). Taken together, these in situ mRNA hybridization results reveal very divergent patterns of EPAS1 and HIF-1α mRNA distribution.

The presence of bHLH and PAS domain motifs in EPAS1 raised the possibility that this protein might be capable of forming a complex with the ARNT (Hoffman et al. 1991) and that the resulting heterodimer might exhibit sequence-specific DNA binding. To test these predictions, EPAS1 and ARNT expression vectors were used to program a reticulocyte lysate. The EPAS1 expression vector was modified at its carboxyl terminus with a c-Myc epitope tag to facilitate immunological detection of the EPAS1 translation product. Radiolabeled methionine was included in the translation mix containing the ARNT mRNA, whereas unlabeled methionine was used in the EPAS1 reaction. After translation, the two reactions were mixed and subsequently incubated with a monoclonal antibody that recognizes the c-Myc epitope present on the EPAS1 protein. Under these conditions the c-Myc antibody was capable of immunoprecipitating the radiolabeled ARNT protein only when EPAS1-Myc protein was present in the reaction (Fig. 4A, cf. lanes 1 and 2).

The bHLH domains of HIF-1α and EPAS1 are nearly identical in primary amino acid sequence (Fig. 1A). Thus, to test for the ability of EPAS1 to form a functional heterodimer with ARNT, we used a HIF-1α response element derived from the 3′-flanking region of the erythropoietin gene (Semenza and Wang 1992) in gel mobility shift assays with in vitro-translated proteins. The data of Figure 4B show that a new complex was formed when both EPAS1 and ARNT were included in the DNA-binding reaction (lane 3) and that this complex was specifically recognized by an antipeptide antibody directed against the EPAS1 protein (lane 7). Competition experiments using a 100-fold excess of unlabeled competitor DNA containing the HIF-1α response element (lane 4), or a response element with three point mutations in this sequence (lane 5), indicated that EPAS1 exhibited sequence-specific binding properties. Taken together, the data of Figure 4 support the hypothesis that EPAS1 is capable of binding the HIF-1α response element in the presence of the ARNT protein.

The ability of EPAS1 to *trans*-activate a reporter gene containing the HIF-1α response element was tested by transient transfection. Expression vectors in which either EPAS1, HIF-1α, or ARNT were placed under the control of a cytomegalovirus promoter were constructed (Fig. 5A). Two luciferase reporter constructs were prepared. One contained nucleotides −105 through +58 of the herpes simplex virus thymidine kinase promoter (McKnight et al. 1981) linked to three copies of the HIF-1α response element from the erythropoietin gene (pRE-tk-LUC; Fig. 5A). The other contained a TATA sequence from the adenovirus major late gene promoter (Lillie and Green 1989) linked to the same three HIF-1α response elements (pE1B-LUC; Fig. 5A). Combinations of these plasmids were then transfected into cultured human embryonic kidney 293 cells and the expression of luciferase enzyme activity was monitored in cell lysates 16–20 hr post-transfection. The data of Figure 5B show that EPAS1 induced a 12-fold increase in luciferase enzyme activity when transfected in the absence of the ARNT vector. Cotransfection of the ARNT expression vector

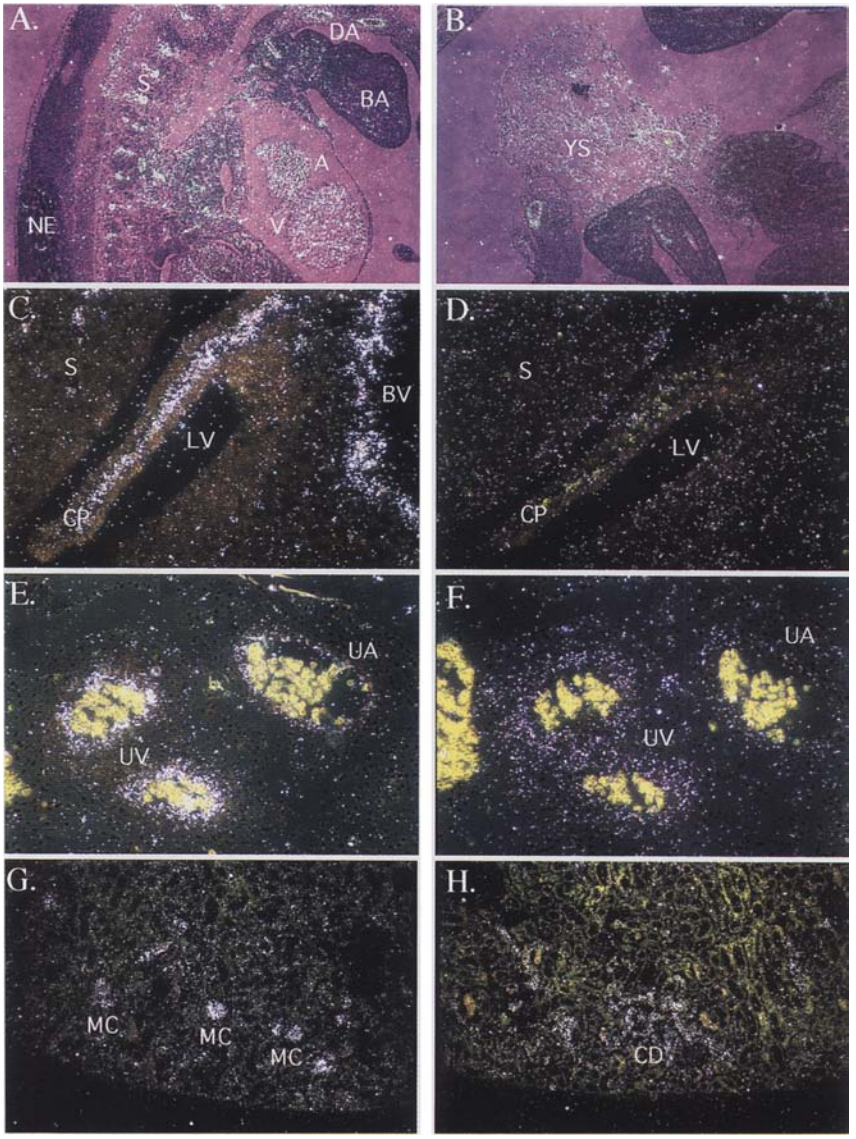

Figure 3. In situ mRNA hybridization analyses with EPAS1 and HIF-1α antisense probes in embryonic and adult tissues. (*A*) Sagittal section through a day 11 embryo showing cells expressing EPAS1, including blood vessels interdigitated with the somites (S), the dorsal artery (DA), and the endothelium of the atrial (A) and ventricle (V) chambers of the heart. (BA) Mandibular component of the first branchial arch; (NE) neuroepithelium of the neural tube. Bar, 280 μm. (*B*) Sagittal section through day 11 embryo showing EPAS1 mRNA localized to the extra-embryonic yolk sac (YS). Bar, 280 μm. (*C*) Sagittal section through day 13 brain showing EPAS1 transcripts in the choroid plexus (CP) within the lateral ventricle (LV) and high-level expression in a blood vessel (BV). Also shown is the location of the striatum (S). Bar, 70 μm. (*D*) Sagittal section close to that shown in *C* probed with HIF-1α antisense RNA. A weak signal slightly over background was evident throughout tissue. Bar, 70 μm. (*E*) Sagittal section through day 13 embryo showing umbilical cord and expression of EPAS1 mRNA in endothelial cells of the umbilical artery (UA) and veins (UV). Bar, 140 μm. (*F*) Section adjacent to *E* showing expression of HIF-1α mRNA in mesenchyme surrounding the blood vessels of the umbilical cord. Bar, 140 μm. (*G*) Sagittal section through cortex of adult kidney showing EPAS1 transcripts in mesangial cells (MC). Bar, 140 μm. (*H*) Sagittal section adjacent to *G* showing HIF-1α mRNA in the collecting duct cells (CD). Bar, 140 μm.

with low levels of EPAS1 expression vector did not increase the EPAS1-mediated induction of luciferase activity (data not shown), suggesting that this cell line might contain adequate amounts of endogenous ARNT to support heterodimer formation with EPAS1. A sevenfold stimulation of luciferase activity was also obtained when larger amounts of the HIF-1α expression plasmid were introduced into 293 cells (Fig. 5B). The introduction of three point mutations into the core sequence of the hypoxia response element eliminated both EPAS1 -dependent and HIF-1α-dependent activation of the reporter gene (data not shown).

The potential of HIF-1α to induce expression of target genes is increased by both hypoxia and pharmacological compounds that mimic hypoxia in cells, such as desferrioxamine (DFX) and cobalt chloride (Wang et al. 1995). To determine whether EPAS1 activity might also be stimulated by these agents, 293 cells were incubated under hypoxic conditions or treated with desferrioxamine (DFX) or $CoCl_2$ after transfection with the plasmids shown in Figure 5A. Treatment of cells under conditions that mimic hypoxia increased expression from the luciferase construct in the absence of exogenous EPAS1 or HIF-1α (Fig. 5B). This *trans*-activation presumably arises from endogenous HIF-1α or EPAS1 proteins whose mRNAs are present in 293 cells (data not shown). As noted above, introduction of the EPAS1 expression vector led to 5–10 times higher levels of luciferase activity over those seen in mock-transfected cells. An extra two- to fourfold stimulation of luciferase expression was observed upon pretreatment with $CoCl_2$, DFX, or hypoxia relative to that measured in EPAS1-transfected but untreated cells (Fig. 5B). Of the three conditions, treatment with $CoCl_2$ led to a slightly larger increase in EPAS1 activity, resulting in a fourfold higher level of luciferase activity over that detected in untreated cells. As has

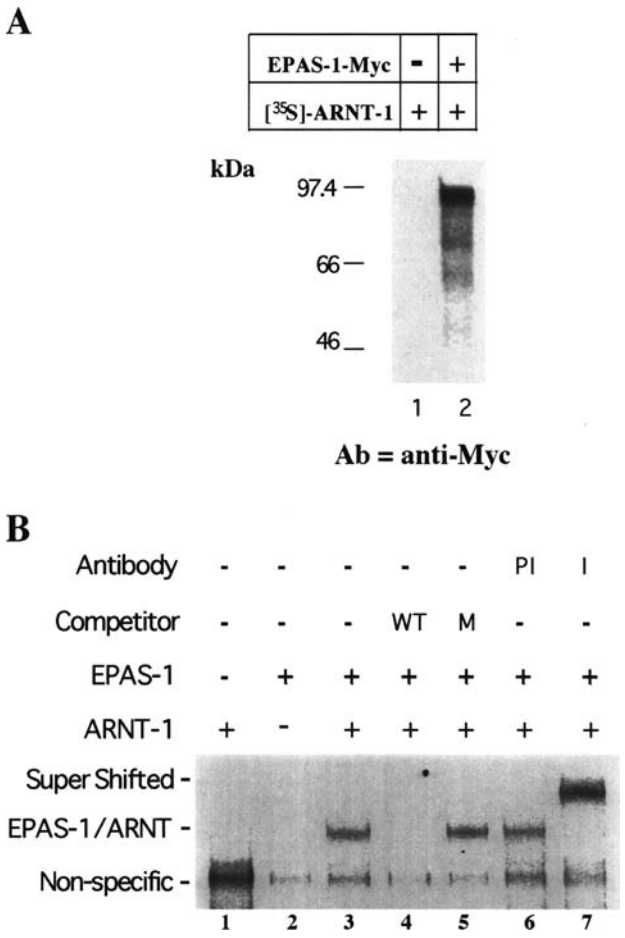

Figure 4. EPAS1 heterodimerizes with ARNT and binds to a hypoxia response element sequence. (*A*) Coimmunoprecipitation of EPAS1 and ARNT. EPAS1 protein containing a c-Myc epitope tag at the carboxyl terminus was produced by in vitro translation and mixed with similarly produced but radiolabeled ARNT. The mixture was subjected to immunoprecipitation with a monoclonal antibody that recognizes the c-Myc epitope and bound proteins were analyzed by gel electrophoresis and autoradiography. ^{35}S-Labeled ARNT is immunoprecipitated only in the presence of EPAS1–Myc (lane *2*) and not when this protein is replaced with unprogrammed reticulocyte lysate in the mixture (lane *1*). (*B*) Gel retardation assay showing ARNT-dependent and sequence-specific DNA binding by EPAS1. EPAS1 (M_r=118,000) and ARNT (M_r=~90,000) were produced by in vitro translation and incubated with a ^{32}P-labeled probe containing the sequence 5′-GCCCTACGTGCTGTCTCA-3′. Incubation of ARNT (lane *1*) or EPAS1-1 (lane *2*) with probe produces only a background band. The same nonspecific band was observed when unprogrammed reticulocyte lysate was incubated with the probe (data not shown). A mixture of the two proteins produces a complex (lane *3*) that is abolished by a 100-fold mass excess of unlabeled probe (lane *4*) but not by a similar excess of a mutant (5′-GCCCTAAAAGCTGTCTCA-3′) probe (lane *5*). The migration of the new complex is slowed when an antipeptide antibody (I) directed against EPAS1 is included in the reaction mixture (lane *7*) but not by the corresponding preimmune (PI) serum (lane *6*).

been observed in previous studies (Forsythe et al. 1996; Jiang et al. 1996), hypoxic conditions also stimulated the ability of HIF-1α to *trans*-activate the target gene containing the hypoxia response element (Fig. 5B).

The EPAS1 expression vector was also tested for its ability to activate a reporter gene (pRE-Elb-LUC, Fig. 5A) following transfection into murine hepatoma cells (Hepa1c1c7) that express ARNT, as well as in a mutant line derived from these parental cells that does not express ARNT (c4 variant; Legraverend et al. 1982). Expression of EPAS1 in the Hepa1c1c7 cells led to a ninefold increase in luciferase activity (Fig. 5C). Transfection of EPAS1 alone into c4 cells increased luciferase enzyme activity only slightly (1.8-fold), whereas cotransfection of EPAS1 and ARNT led to a 12-fold stimulation of activity (Fig. 5C). These findings are consistent with the interpretation that EPAS1 forms an active heterodimeric transcription factor with ARNT, and they confirm the results showing heterodimerization of these two proteins obtained in coimmunoprecipitation and gel mobility shift assays (Fig. 4).

The experiments demonstrating the functional activity of EPAS1 utilized a hypoxia response element derived from the erythropoietin gene, which is a known target gene for HIF-1α (Semenza and Wang 1992). Despite the activity of EPAS1 in these assays, as well as the high degree of sequence similarity between HIF-1α and EPAS1, the in situ mRNA hybridization results indicate that the two proteins are expressed in different cell types and thus might activate different target genes. The high level of expression of EPAS1 in endothelial cells raises the possibility that the EPAS1 protein might activate genes whose expression is limited to endothelial cells. To test this hypothesis, we transfected 293 cells with a c-Myc-tagged EPAS1 expression vector and a marker gene composed of the promoter and intron 1 enhancer of the *Tie-2* gene linked to β-galactosidase. *Tie-2* encodes a tyrosine kinase receptor that is specifically expressed in cells of endothelial lineage (Dumont et al. 1992; Maison-Pierre et al. 1993; Sato et al. 1993; Schnurch and Risau 1993). The data of Figure 6A show that EPAS1 potently stimulated expression of the *Tie-2*-driven reporter gene, and that the degree of stimulation correlated with the level of immunodetectable EPAS1 in the transfected cells (Fig. 6B). Surprisingly, little or no transcriptional activation of the *Tie-2* reporter gene by HIF-1α was detected (Fig. 6A), even though equivalent amounts of HIF-1α and EPAS1 proteins were expressed in the 293 cells (Fig. 6B).

Discussion

This report describes the isolation and initial characterization of a PAS domain transcription factor termed EPAS1. This protein shares 48% sequence identity with HIF-1α and, like HIF-1α, binds to and activates transcription from a DNA element containing the sequence 5′-GCCCTACGTGCTGTCTCA-3′. Transcriptional activation by both proteins, at least as judged by the transient transfection assays we have employed, is stimu-

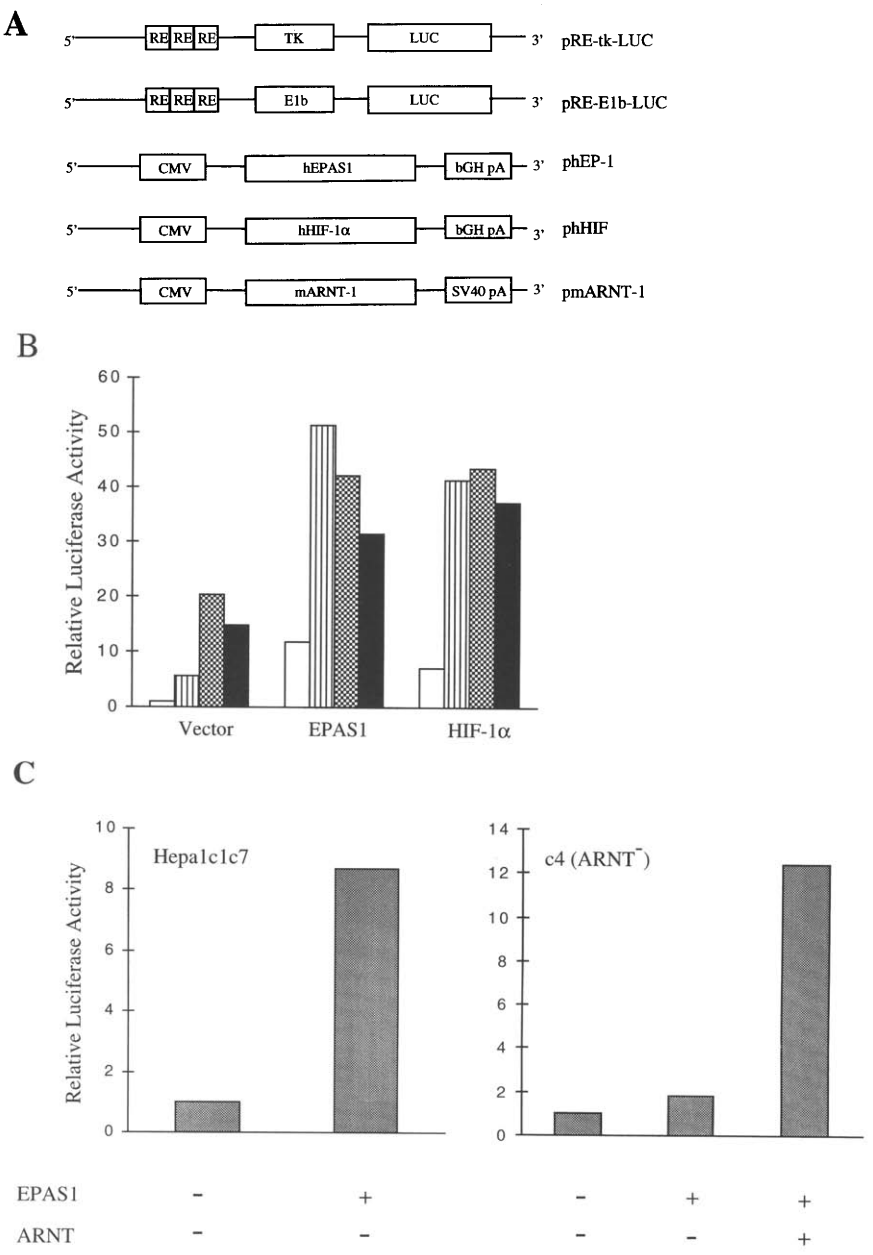

Figure 5. *Trans*-activation of reporter genes by EPAS1. (*A*) Expression vectors and reporter gene constructs. (CMV) Immediate early region promoter from human cytomegalovirus; (EPAS1) cDNA encoding EPAS1 protein with or without a c-Myc epitope (EQKLISEEDL); (bGH) transcription termination and polyadenylation sequences from bovine growth hormone gene; (HIF-1α) cDNA encoding HIF-1α protein with or without a c-Myc epitope; (RE) 50-bp hypoxia response element from the human erythropoietin gene; (TK) herpes simplex virus thymidine kinase gene promoter; (LUC) gene encoding firefly luciferase. (*B*) Activation of reporter gene (pRE-tk-LUC, 100 ng per dish) by EPAS1 (5 ng per dish) and HIF-1α (50 ng per dish) expressed in untreated 293 cells (open bars), $CoCl_2$-treated cells (hatched bars), DFX-treated cells (stippled bars), or cells grown in low oxygen (hypoxia; solid bars). The data are from one of six experiments. (*C*) ARNT dependence of reporter gene activation by EPAS1 and HIF-1α. Lines of mouse hepatoma cells that express ARNT (Hepa1c1c7) or do not express this protein (c4 variant) were transfected with the indicated expression vectors and a reporter gene (pRE-Elb-LUC) and the amount of luciferase enzyme activity determined by luminometry.

lated by hypoxic conditions. EPAS1 heterodimerizes with ARNT and requires ARNT for transcriptional activation of target genes. EPAS1 is preferentially expressed in endothelial cells and specifically activates transcription of the *Tie-2* gene, which has been shown to be exclusively expressed in endothelial cells.

More than a dozen proteins have been identified in invertebrates and vertebrates that contain contiguous bHLH and PAS domains. This family of transcription factors can be subdivided into AHR-like proteins and ARNT-like proteins. In general, gene activation occurs when a member of the AHR-like class heterodimerizes with a member of the ARNT-like class (Schmidt and Bradfield 1996), although homodimerization of ARNT has been shown, under certain experimental conditions, to lead to activation through an E-box DNA sequence motif (Antonsson et al. 1995; Sogawa et al. 1995). EPAS1 belongs to the AHR-like class of proteins and is hereby shown to form a functional heterodimer with the ARNT protein (Figs. 4 and 5C). We do not yet know whether EPAS1 will form a complex with the recently described ARNT-2 protein (Hirose et al. 1996), or with other as yet unidentified members of this class of proteins.

Among AHR-related proteins, EPAS1 shares highest sequence similarity with HIF-1α (Fig. 1A), a bHLH/PAS domain transcription factor that was isolated based on its ability to activate gene expression in response to hypoxia (Wang and Semenza 1995; Wang et al. 1995). The highest degree of sequence similarity between EPAS1 and HIF-1α is centered on the bHLH domains of the two

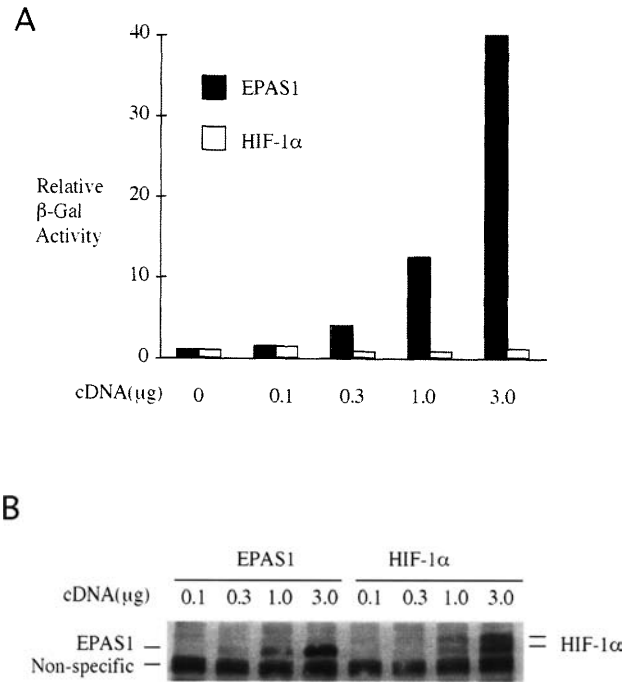

Figure 6. Activation of *Tie-2* reporter gene by EPAS1. (*A*) The indicated amounts of expression vectors containing EPAS1 cDNA (solid bars) or HIF-1α cDNAs (open bars) with c-Myc epitope tags were transfected into 293 cells together with a constant amount (1 μg) of a reporter gene construct containing 10.3 kb of regulatory DNA derived from the murine *Tie-2* gene linked to the *E. coli* β-galactosidase gene. Levels of bacterial β-galactosidase gene activity were determined by luminometry after heat inactivation (50 min, 48°C) of endogenous β-galactosidases. (*B*) Immunoblotting of transfected cell lysates with anti-Myc antibody. Aliquots (100 μg) of the whole cell lysates used to measure β-galactosidase activity in *A* were separated by electrophoresis on an SDS–polyacrylamide (8%) gel, transferred to a nylon membrane, and blotted with an anti-Myc antibody. EPAS1–cMyc migrates with an apparent M_r of 118,000, a value in good agreement with that determined by in vitro translation of synthetic EPAS1 mRNA. HIF-1α migrates as a doublet with apparent M_rs of 118,000 and 120,000 (Wang et al. 1995). The data of *A* and *B* represent one of three experiments.

proteins, presumably accounting for the ability of EPAS1 to bind to the HIF-1α response element (Fig. 4B) and to activate reporter genes containing multiple hypoxia response elements (Fig. 5). HIF-1α has been shown to function as a more potent activator when expressed in cells grown under hypoxic conditions (Forsythe et al. 1996; Jiang et al. 1996). The mechanism of this enhancement has not yet been determined. It is clear, however, that abundant HIF-1α mRNA can be found in many cell lines that contain little if any HIF-1α protein (Wood et al. 1996; H. Tian, unpublished observations). Thus, hypoxia may somehow stabilize HIF-1α protein or stimulate the translation of HIF-1α mRNA. The activity of EPAS1 is also enhanced in cells grown under hypoxic conditions (Fig. 5B), suggesting that it may be subject to the same regulatory influences as HIF-1α.

EPAS1 is predominantly expressed in highly vascularized tissues of adult humans (Fig. 2), and in endothelial cells of the embryonic and adult mouse (Fig. 3). Moreover, EPAS1 functions as a potent activator of the *Tie-2* gene (Fig. 6), which is known to be selectively expressed in endothelial cells. *Tie-2* encodes a tyrosine kinase receptor that is thought to be activated by as yet unidentified vascular growth factors (Dumont et al. 1992; Maison-Pierre et al. 1993; Sato et al. 1993). The *Tie-2* regulatory DNA sequences that were tested in the present study have been shown to facilitate endothelial cell specific gene activation (T.N. Sato, unpubl.). Taken together, these observations raise the possibility that EPAS1 may play an important role in the ontogenic specification of the endothelium. At least five endothelial cell-specific tyrosine kinase genes, including *Flk-1*, *Tie-1*, *Tie-2*, *Flt-1*, and *Flt-4*, have been isolated and shown to be important for the formation of cells of the endothelial lineage (Dumont et al. 1994). Of these five genes, in situ mRNA hybridization experiments have revealed a temporal hierarchy in which *Flk-1* expression precedes that of the other kinases (Dumont et al. 1995). Consistent with this hierarchy, disruption of the *Flk-1* gene in mice leads to abnormalities in both hematopoiesis and vasculogenesis (Shalaby et al. 1995). Disruption of the *Tie* genes leads to defects in endothelial cell differentiation and angiogenesis (Sato et al. 1995), and mutation of the *Flt-1* gene affects assembly of the vascular endothelium (Fong et al. 1995). The activation of *Tie-2* by EPAS1 suggests that the EPAS1 gene may lie upstream of the *Tie-2* gene in the regulatory pathway leading to the formation of the embryonic vasculature. Similar *trans*-activation experiments using the promoter of the *Flk-1* gene may reveal whether EPAS1 is distal to all members of this tyrosine kinase family, thus acting as a master regulator of endothelial cell differentiation. Expression is particularly striking in the embryonic choroid plexus, suggesting that EPAS1 may also play a crucial role in the formation of the endothelium giving rise to the blood brain barrier.

What is the role of EPAS1 in adult mammals? We have observed EPAS1 mRNA in the vasculature of adult mice and at lower levels in several different cell types (Fig. 3). We hypothesize that EPAS1 may facilitate response to oxygenation in these tissues or to other hemodynamic stimuli. This interpretation is based primarily on the ability of hypoxic conditions to stimulate the activity of EPAS1 in 293 cells (Fig. 5B) and on the precedence established for the closely related HIF-1α transcription factor (Forsythe et al. 1996; Jiang et al. 1996). In the case of HIF-1α, its most clearly defined target is the gene encoding erythropoietin. Under hypoxic conditions, HIF-1α is believed to induce erythropoietin gene expression in the kidney, leading to synthesis and secretion of erythropoietin and enhanced erythroid cell differentiation in the bone marrow (Semenza 1994). Although we have provided evidence that the gene encoding a vascular growth factor receptor (*Tie-2*) might be a target for EPAS1 activation (Fig. 6), the identification of true in vivo target genes of EPAS1 will require additional work.

Numerous questions remain unresolved regarding the

functional roles of HIF-1α and EPAS1. Both proteins may themselves be regulated by metabolites that originate as a function of intracellular redox state, or by post-transcriptional mechanisms influenced by oxygenation. EPAS1 and HIF-1α may, in some cases, regulate similar genes in different cell types, and in other cases distinct sets of target genes. It has been reported, for example, that growth of cells under hypoxic conditions results in the activation of the vascular endothelial cell growth factor and its receptor (see references in Namiki et al. 1995 and Brogi et al. 1996). The observations that EPAS1 is preferentially expressed in the endothelium (Fig. 3), is stimulated by hypoxic conditions (Fig. 5), and specifically activates a *Tie-2*-driven reporter gene (Fig. 6) indicate that this genetic circuit may be regulated by EPAS1 in the vasculature. The mechanism underlying the ability of EPAS1, but not HIF-1α, to stimulate expression of *Tie-2* remains to be determined but may be attributable to an EPAS1-specific response element in the *Tie-2* gene or to the discriminatory interaction of EPAS1 with cofactors present in human embryonic kidney 293 cells. Because both HIF-1α and EPAS1 are able to activate transcription from a HIF-1α-response element in these same cells, the discriminatory activity of EPAS1 on *Tie-2*-directed gene expression is likely dictated by *Tie-2* regulatory DNA sequences. These data reveal a significant difference between EPAS1 and HIF-1α, and are consistent with the prediction that EPAS1 may function in the vasculature, whereas HIF-1α may function in extravascular tissues.

Materials and methods

cDNA and genomic cloning, chromosomal mapping

In the course of screening for genes that are differentially expressed in prostate adenocarcinoma versus normal tissue, a cDNA encoding a bHLH/PAS domain protein was isolated. Database searches generated several expressed sequence tags that showed sequence similarity to this family of transcription factors. EPAS1 cDNAs correspond to the human expressed sequence tag #T70415 in the GenBank collection and were isolated by a combination of RT–PCR and screening of a HeLa cell cDNA library (Yokoyama et al. 1993) with standard methods. Similar approaches were used to isolate the murine homolog from a commercially available mouse adult brain cDNA library (#837314, Stratagene Corp., La Jolla, CA). A human HIF-1α cDNA was generated by ligation of an amplified cDNA fragment to expressed sequence tag hbc025 (Takeda et al. 1993). Bacteriophage λ clones harboring genomic DNA inserts corresponding to the human EPAS1 gene were isolated by screening a commercially available fibroblast genomic library (λ FIXII vector, #946204, Stratagene Corp.)

Fluorescence in situ hybridization to identify the chromosomal localization of the human EPAS1 gene was carried out as described previously (Craig and Bickmore 1994). This analysis indicated hybridization to the short arm of chromosome 2, bands p16–21. To confirm the assignment, a 269-bp segment of exon 8 from the EPAS1 gene was amplified from the 83 genomic DNAs of a radiation hybrid panel (Stanford G3 panel, Research Genetics, Huntsville, AL) using oligonucleotide primers (5′-TGGTGGCACACCCCTGCCCC-3′ and 5′-TGAGTCTGGGAAGCTTGGGC-3′) and a thermocycler program consisting of 35 cycles of 94°C for 1 min, 68°C for 1 min. Analysis of the results via an e-mail server at Stanford University indicated linkage to the D2S288 marker (logarithm of the odds score of 8.7, cR_8000 value of 12.96), which is located ~82 cM from the telomere of the short arm of chromosome 2 (MIT Center for Genome Research, Cambridge, MA).

RNA blotting and in situ hybridization

Human multiple tissue RNA blots (Clontech Laboratories, Palo Alto, CA) were probed with EPAS1 and HIF-1α cDNA probes using Rapid-Hyb from Amersham Corp. (Arlington Heights, IL). For in situ mRNA hybridization, mouse tissues were fixed in 4% paraformaldehyde, sectioned at 5 μm thickness, and subjected to in situ mRNA hybridization as described (Berman et al. 1995). A ^{33}P-labeled antisense RNA probe recognizing the EPAS1 mRNA was derived by in vitro transcription of an ~300-bp DNA fragment encoding amino acids 225–327 of the sequence shown in Figure 1A. A segment of the murine HIF-1α cDNA encoding amino acids 41–125 was isolated by RT–PCR using mRNA template isolated from embryonic day 10 mouse embryo.

Coimmunoprecipitation experiments

Human EPAS1 and mouse ARNT proteins were generated in vitro using a transcription–translation kit (TNT System, Promega Corp., Madison, WI). cDNAs (see Fig. 5A) encoding full-length proteins were subcloned into the pcDNA3 vector (Invitrogen Corp., San Diego, CA) prior to coupled transcription/translation. For immunoprecipitation, ~5 μl of each reaction was transferred to a separate tube, mixed well, and subsequently diluted by the addition of 500 μl of ice-cold buffer (20 mM HEPES–KOH at pH 7.4, 100 mM KCl, 10% (vol./vol.) glycerol, 0.4% (vol./vol.) Nonidet P-40, 5 mM EGTA, 5 mM EDTA, 100 μg/ml of bovine serum albumin, 1 mM dithiothreitol) (Huang et al. 1993). The diluted mixture was incubated with 1 μl (0.1 μg) of anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hr at 4°C. A 10-μl aliquot of beads (~4×10^6 in number; Dynal Corp., Lake Success, NY) coated with rat antimouse IgG1 antibody was then added followed by a further incubation for 1 hr at 4°C. Beads were washed three times with 1.5 ml of the above buffer, and bound proteins were analyzed subsequently by electrophoresis through 8% polyacrylamide gels containing SDS.

Gel retention assays

EPAS1 and ARNT cDNAs were translated in vitro as described above. Gel retention assays were performed as described previously (Semenza and Wang 1992) using a double-stranded oligonucleotide probe radiolabeled with the Klenow fragment of *Escherichia coli* DNA polymerase I and containing an HIF-1α binding site (5′-GCCCTACGTGCTGTCTCA-3′) from the erythropoietin gene (Semenza and Wang 1992). For supershift assays, a polyclonal antibody was raised against residues 1–10 of the human EPAS1 protein (Fig. 1A) by standard methods, and 1 μl of serum was added to the gel retention reaction mixture prior to the 30-min incubation at 4°C. A preimmune serum served as a negative antibody control.

Transient transfection assays

The pTK-RE3-luc reporter plasmid was constructed by inserting three copies of a 50-nucleotide hypoxia-inducible enhancer from the erythropoietin gene (Semenza and Wang 1992) into

pGL3-TK (a kind gift from Dod Michael, University of Texas Southwestern Medical Center, Dallas). The *Tie-2*–β-galactosidase reporter gene pT2HLacZpA1I.7, containing 12.3 kb of promoter (2 kb) and intron 1-derived enhancer (10.3 kb) DNA from the murine *Tie-2* gene, was a kind gift of Thomas Sato (Beth Israel Hospital, Boston, MA). Human embryonic kidney 293 cells (ATCC CRL#1573) were cultured in Dulbecco's modified Eagle medium (DMEM, low glucose; GIBCO-BRL) supplemented with 10% fetal calf serum. The murine hepatoma cell line Hepa1c1c7 and the c4 ARNT-deficient mutant derived from this line were maintained as described previously (Legraverend et al. 1982). Approximately 24 hr before transfection, cells were inoculated in 12-well plates at a density of 120,000 cells per well. Plasmid DNA (1–10 μg) was transfected into cells using a kit (MBS, Stratagene Corp.). Cells were allowed to recover for 3 hr at 35°C in a 3% CO_2 atmosphere. Where indicated, 125 μM $CoCl_2$ (#C3169, Sigma Chem. Corp., St. Louis, MO) or 130 μM DFX (#D9533, Sigma) were added to cells at this time and the incubation continued for an additional 16 hr in atmospheres containing 20% or 1% O_2. Luciferase and β-galactosidase enzyme activities were determined according to the manufacturer's instructions (Tropix, Bedford, MA). Reporter gene expression was normalized by cotransfection of a β-galactosidase expression vector (pCMV–β-gal) and/or to expression obtained from the pGL3-Control plasmid (Promega Corp.). Levels of expressed c-Myc epitope-tagged EPAS1 or HIF-1α were assessed by immunoblotting with the anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA) using a protocol supplied by the manufacturer.

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