Oxygen-regulated and Transactivating Domains in Endothelial PAS Protein 1: Comparison with Hypoxia-inducible Factor-1α*

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Endothelial PAS protein 1 (EPAS1) is a basic helix-loop-helix Per-AHR-ARNT-Sim transcription factor related to hypoxia-inducible factor-1α (HIF-1α). To analyze EPAS1 domains responsible for transactivation and oxygen-regulated function, we constructed chimeric fusions of EPAS1 with a GAL4 DNA binding domain, plus or minus the VP16 activation domain. Two transactivation domains were defined in EPAS1; a C-terminal domain (amino acids 828–870), and a larger internal domain (amino acids 517–682). These activation domains were interspersed by functionally repressive sequences, several of which independently conveyed oxygen-regulated activity. Two types of activity were defined. Sequences lying N-terminal to and overlapping the internal transactivation domain conferred regulated repression on the VP16 transactivator. Sequences lying C-terminal to this internal domain conveyed regulated repression and oxygen-regulated activity on the native EPAS1 C-terminal activation domain, but not the Gal/VP16 fusion. Fusions containing internal but not C-terminal regulatory domains manifested regulation of fusion protein levels. Comparison of EPAS1 with HIF-1α demonstrated a similar organization for both proteins, and for the C terminus defined a conserved RLL motif critical for induction. Overall, EPAS1 sequences were less inducible as their expression level was increased. Despite these quantitative differences, EPAS1 regulation appeared similar to HIF-1α, conforming to a model involving the modulation of both protein level and activity, through distinct internal and C-terminal domains.

Hypoxia-inducible factor-1 (HIF-1)1 is a transcriptional complex that plays a central role in oxygen-regulated gene expression (reviewed in Refs. 1–3). Affinity purification and molecular cloning of HIF-1 has revealed that the DNA binding complex consists of a heterodimer of proteins, HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT) (4). Both are members of the rapidly expanding PAS superfamily of basic helix-loop-helix proteins defined by the presence of two regions containing repeated sequences that share homology with the prototypical members from which the family’s name is derived, drosophila periodic, the aryl hydrocarbon receptor, the aryl hydrocarbon receptor nuclear translocator and drosophilin single minded (5). HIF-1 binds to hypoxia response elements containing the consensus BRCGTGV and activates the transcription of a wide variety of genes that encode products involved in hematopoiesis (erythropoietin), angiogenesis, and vasomotor control (vascular endothelial growth factor, nitric oxide synthase, and endothelins), energy metabolism (glycolytic enzymes and glucose transporters), catecholamine synthesis (tyrosine hydroxylase), and iron metabolism (transferrin) (for reviews see Refs. 1–3).

HIF-1 activation is mediated predominantly by post-translational processes affecting the a subunit (6–11). Understanding the interactions of these processes with the sensing and/or signal transduction processes is an important but potentially complex issue in which primary points of interaction need to be defined and distinguished from processes that are downstream consequences of such interactions. A key step in such analyses is the definition of functional domains within the molecules, in particular, regions that can independently confer the regulatory characteristic on a heterologous system. Several groups have now analyzed aspects of HIF-1α regulation and defined domains that can independently convey oxygen-regulated properties onto heterologous transcription factors such as the yeast GAL4 DNA binding domain (Gal) (7, 9, 11, 12).

Recent cloning experiments have identified several new members of the basic helix-loop-helix PAS family, the most similar to HIF-1α being a molecule first described as endothelial PAS protein 1 (EPAS1) (13), but also independently identified by other groups and termed member of PAS superfamily 2 (MOP2) (14), HIF-like factor (HLF) (15), and HIF-related factor (HRF) (16). The protein shares 48% sequence identity with HIF-1α, forms heterodimers with ARNT, and can activate transcription from a hypoxia response element (13). In hypoxic cells, EPAS1 protein levels are greatly up-regulated (17). Moreover, responses to chemical and pharmacological probes with known effects on HIF-1 activation are very similar (17), suggesting that one or more regulatory mechanisms are shared, and indicating that it should be informative to define regulatory and activation domains in EPAS1, and to compare their function with those in HIF-1α.

Here we show that fusion of EPAS1 sequences with Gal can confer hypoxia-inducible activity on a GAL-responsive reporter. Our analysis defines two transactivation domains for EPAS1 (a C-terminal transactivation domain and an internal transactivation domain), which are interspersed with sequences that possess repressive and regulatory properties, some of which can confer regulation of fusion protein levels. Overall these findings demonstrate a similar domain architec-
ture to HIF-1α. However, some Gal/EPAS1 fusions showed higher activity in normoxic cells and a lower amplitude of induction, particularly at higher levels of expression, indicating that there are quantitative differences in the activation characteristics of these molecules.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Hep3B cells were grown in minimal essential medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml).

Recombinant Plasmids Used in Mammalian Cells—The plasmids used are shown schematically in Fig. 1. The chimeric activator/reporter system used in transactivation assays was based on pGal (a plasmid based on pcDNA3, which contains an SV40 origin of replication and a cytomegalovirus promoter, truncated GAL4 gene encoding amino acids 1–147 followed by a polylinker bearing the rare restriction endonuclease sites, SacII, AscI, NotI), and the GAL-responsive luciferase reporter pUAS-tk-Luc (consisting of two copies of a 17-base pair Gal4 DNA binding site and the thymidine kinase promoter, pA3LUC) (19). To analyze the function of sequences from EPAS1 or HIF-1α, they were amplified by polymerase chain reaction using Pfu polymerase (Stratagene, La Jolla, CA) and forward oligonucleotides containing a SacII recognition sequence in the appropriate reading frame and reverse oligonucleotides containing an AscI recognition site and cloned into pGal, phIF-1α i was used as template for EPAS1 (13) and pBluescript/HIF-1α 3.2–377 (4) as template for HIF-1α sequences.

To analyze the regulatory function of EPAS1 or HIF-1α amino acids on the operation of a heterologous activation domain, sequences coding for the herpes simplex virus protein 16 amino acids 410–490 (VP16) were generated by polymerase chain reaction using Pfu polymerase with priming oligonucleotides incorporating in frame AscI and NotI restriction sites, and inserted 3′ to the EPAS1 sequence. The control plasmid pGalVP16 was produced by insertion of this polymerase chain reaction product directly into pGal, preserving the reading frame. All plasmids were subjected to in vitro transcription/translation reactions in the presence of [35S]-methionine and products analyzed by SDS-polyacrylamide gel electrophoresis to confirm the production of an appropriate fusion protein.

Plasmids bearing mutations were generated using a commercially available site-directed mutagenesis kit (QuickChange; Stratagene) and mutagenic oligonucleotides designed according to the manufacturer’s recommendations. Mutations in HIF-1α and EPAS1 were made in the context of pCOGTG/a775–826 (7) and pGal/EPAS819–870, respectively. These mutations were sequenced by the dyeexit method to confirm veracity.

A cytomegalovirus-promoted plasmid constitutively expressing β-galactosidase (pCMVβGal) was used as a transfection control.

**Transient Transfection for Functional Assays**—For transactivation assays, cells were transfected by electroporation using a 1 mF capacitor array charged at 375 V. For each transfection, approximately 105 cells were resuspended in 1 ml of RPMI 1640 containing a mixture of activator plasmid (ranging between 50 ng and 20 μg), reporter plasmid (50 μg) and the transfection control plasmid pCMVβGal (15 μg). After discharge of the capacitor, cells were left on ice for 10 min before being resuspended in the appropriate culture medium. Aliquots of this suspension were then used for parallel incubations. Conditions used for normoxic and hypoxic incubation were 5% CO2, balance air, and 1% O2, 5% CO2, balance N2, respectively. Chemicals were used at the following final concentrations: cobaltous chloride, 100 μM; desferrioxamine melydate, 100 μM; potassium cyanide, 1 mM; and sodium azide, 2 mM. Experimental incubations were for 16–18 h. All activator plasmids were tested in at least three independent transfection experiments.

**Luciferase and β-Galactosidase Assays on Mammalian Cell Extracts**—Luciferase activities in cell lysates were determined at room temperature using a commercially available luciferase assay system (Promega, Madison, WI), according to the manufacturer’s instructions, and a TD-20e luminometer (Turner Designs, Sunnyvale, CA). Relative β-galactosidase activity in lysates was measured using o-nitrophenyl-β-D-galactopyranoside (0.67 mg/ml) as substrate in a 0.1 M phosphate buffer (pH 7.0) containing 10 mM KCl, 1 mM MgSO4, and 30 mM β-mercaptoethanol incubated at 30 °C for 15–45 min. The Amaxo was determined after stopping the reaction by the addition of 0.3 mM sodium carbonate (final concentration).

**Analysis of Fusion Protein Levels in Whole Cell Extracts**—To achieve fusion protein levels sufficient for detection of certain activator plasmids, plasmids were amplified by co-transfection with a plasmid expressing the SV40 large T antigen, pCMV-TAg (20). Transfections were performed as above using doses of activator and amplifier plasmids ranging between 0.02 and 7 μg and 0.05 and 10 μg, respectively, to identify the range in which protein levels were detectable. After transfection cells were incubated for 48 h to allow plasmid amplification and expression. Cells were incubated in normoxia throughout or stimulated by addition of 100 μM desferrioxamine to the medium for the final 16 h.

At harvest, cells were cooled rapidly by rinsing with ice-cold phosphate-buffered saline, and removed by scraping with a rubber policeman. An ice-cold 7 mM urea, 10% glycerol, 1% SDS, 10 mM Tris, pH 6.8, buffer containing 5 mM dithiothreitol, 50 μM phenylmethylsulfonyl fluoride, and leupeptin, pepstatin, and aprotinin all at 0.1 μg/ml was added to the cell pellet, which was then disrupted using a hand-held homogenizer (Ultra-Turrax T8 with 5G dispersing tool; Janke & Kunkel GmbH, Staufen, Germany) for 20 s and then allowed to stand on ice for 5 min. Extract was either snap frozen on dry ice or stored or mixed with an

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**Fig. 1. Schematic representation of the chimeric activator and reporter plasmids used.** The restriction sites used in construction are indicated (see Experimental Procedures).
equal volume of 2× Laemmli sample buffer before SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto Immobilon-P mem-
brane (Millipore, Bedford, MA) by electrophoresis overnight at 20 V in
Towbin buffer containing 10% methanol and 0.005% SDS. Membranes
were blocked using phosphate-buffered saline supplemented with 5%
dry milk powder and 0.1% Tween 20 before indirect immunostaining.
Proteins were labeled with mouse monoclonal antibodies directed
against either the GAL4 DNA binding domain (RK5C1; Santa Cruz
Biotechnology) or EPAS1 amino acids 535–631 (190b) (17) followed by
peroxidase-conjugated swine anti-mouse immunoglobulin (DAKO). Per-
oxidase activity was detected by enhanced chemiluminescence (Super
Signal Ultra; Pierce).

RESULTS

EPAS1 Protein Sequences Confer Hypoxically Regulated
Transactivation When Fused to a GAL4 DNA Binding Domain
(Gal)—As a first step in the definition of regulatory domains and transactivation domains in EPAS1, a plasmid expressing a
fusion protein consisting of Gal linked to EPAS1 amino acids
19–870 was constructed (pGal/EPAS19–870), and activity was
tested by co-transfection with a Gal4-responsive reporter gene
(pUAS-tk-Luc) into Hep3B cells. For comparison, the activity of
a similar fusion between Gal and HIF-1α (pGal/HIF-1α 28–826) was
tested in a similar manner (Fig. 3A). Whereas chimeric genes containing deletions to amino acids
819 and 682 (pGal/EPAS19–819 and pGal/EPAS19–682)
showed inducible transactivation comparable with that of the
entire EPAS1 fusion (pGal/EPAS19–870), further C-terminal
deletions to amino acids 551, 495, and 416 removed most or all
activity. This indicated the existence of a powerful transactiv-
ating domain lying N-terminal to amino acid 682, and that sequences between amino acids 551 and 682 were necessary
for this function. The inducible activity of pGal/EPAS19–682
indicated the presence of at least one domain capable of convey-
ing inducible responses that lies N-terminal to amino acid 682,
but given the lack of transactivator function in the C-terminal
deletions to amino acids 551 and 495, and 416, these experiments
did not define the domains responsible for this regulation
further.

Seven N-terminal deletions of EPAS1 coding sequence were
tested in a similar manner (Fig. 3B). Striking differences in the
activity of these constructs were observed. Deletion of amino
acids 19 to 495 produced a large increase in activity, particularly
in normoxic cells, suggesting that this region contains

Fig. 2. Comparison of the transcriptional activity of Gal/EPAS1 and Gal/
HIF-1α fusion genes in Hep3B cells.
Activator plasmids encoded the indicated amino acids of EPAS1 or HIF-1α fused 3′
to the 147-amino-acid GAL4 DNA binding domain. Cells were co-transfected with an
activator plasmid, the GAL4-luciferase reporter plasmid (pUAS-tk-Luc) (50 ng),
and pCMVpGal (15 μg) (to provide a control to correct for variation in transfection
efficiency) and harvested after 16 h incubation in the presence of normoxia, hy-
opxia, 100 μM cobaltous ions, or 100 μM desferrioxamine. Different doses of each
activator plasmid between 0.05 and 20 μg were used as indicated. Bars indicate the
corrected luciferase activity (arbitrary units). Results obtained when 5 μg pGal
was used are shown for comparison.

Dose of Activator (μg)

| Dose (μg) | pGal | pGal/EPAS 19–870 | pGal/α28–826 |
|-----------|------|----------------|--------------|
| 0         | 0.05 | 0.05           | 0.05         |
| 0.25      | 0.25 | 0.25           | 0.25         |
| 1.0       | 1.0  | 1.0            | 1.0          |
| 5.0       | 5.0  | 5.0            | 5.0          |

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sequences capable of exerting a functionally suppressive effect (compare pGal/EPAS19–870 and pGal/EPAS495–870). Whereas further deletion to amino acid 517 caused only a small effect on activity, deletion to amino acid 551 (pGal/EPAS517–870) produced a complete loss of this transactivating function, suggesting that the N-terminal boundary of this transactivating region resides between 517 and 551. With further N-terminal deletions an increase in activity was observed indicating the existence of a second transactivation domain capable of operation in isolation, at the C terminus of EPAS1. Comparison of the activities of pGal/EPAS551–870, pGal/EPAS682–870, pGal/EPAS724–870, pGal/EPAS819–870, and pGal/EPAS828–870 indicated that this transactivating function was contained within amino acids 828–870, whereas amino acids 682–827 were functionally repressive. An inducible response was observed with both plasmids pGal/EPAS724–870 and pGal/EPAS819–870 but not plasmid pGal/EPAS828–870 located a minimal sequence, which could confer inducible behavior on this transactivator between amino acids 819 and 828. In view of this evidence for regulatory C-terminal sequences, the low amplitude of induction observed for highly active plasmids pGal/EPAS495–870 and pGal/EPAS517–870 appeared surprising. We therefore retested pGal/EPAS495–870 using low doses as described above, and noted that as overall activity was reduced there was a substantial increase in the amplitude of the inducible response (Table I).

The combined results of the N-terminal and C-terminal deletions of EPAS1 sequence suggested the presence of two domains in EPAS possessing both regulatory and transactivating potential; a powerful internal domain contained within exon 11 (amino acids 517–682) and a weaker domain contained within the C-terminal exon (amino acids 819–870). These experiments also demonstrated the presence of two regions capable of exerting functionally suppressive effects on transactivation, sequences N-terminal to amino acid 495 and sequences lying between amino acids 682 and 819.

To test whether the internal transactivation domain could function independently and to define the functional sequences in more detail, a further series of plasmids was constructed and tested in Hep3B cells. pGal/EPAS517–682 showed powerful inducible transactivation, confirming that this internal transactivation domain could function independently and indicating that it also contained regulatory sequences. Deletions from the

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Transcriptional activity of different Gal/EPAS1 fusion genes. A, C-terminal deletions and B, N-terminal deletions. Activator plasmids encoding the indicated amino acids of EPAS1 fused 3′ to the 147-amino acid GAL4 DNA binding domain or the GAL4 DNA binding domain alone. Hep3B cells were co-transfected with an activator plasmid (1 μg), the GALA-luciferase reporter plasmid (pUAS-tk-Luc) (50 μg) and pCMVβGal (15 μg) (transfection control) and harvested after 16 h incubation in the presence of normoxia, hypoxia, 100 μM cobaltous ions, or 100 μM desferrioxamine. Bars show corrected luciferase activity (arbitrary units).

**Table I**

| Plasmid | Dose (μg) | Normoxic Activity | Fold induction by |
|---------|-----------|------------------|------------------|
|         |           |                  | Hypoxia          | Cobalt           | Desferrioxamine |
| pGal/EPAS1 | 0.05     | 16.0             | 4.4              | 4.7              | 11.3           |
| pGal/EPAS1 | 0.25     | 53.5             | 4.9              | 5.0              | 15.2           |
| pGal/EPAS1 | 1.0      | 231              | 2.7              | 2.2              | 6.7            |
| pGal/EPAS1 | 5.0      | 534              | 1.6              | 0.7              | 2.8            |
| pGal/EPAS1 | 20       | 497              | 1.2              | 0.4              | 1.6            |

Dependence of the amplitude of induction of a Gal fusion protein containing EPAS495–870 on the dose of activator plasmid used

A co-transfection was performed as described in Fig. 3 using the indicated doses of the activator plasmid expressing amino acids 495–870 of EPAS1 fused to the GAL4 DNA binding domain. The corrected, relative normoxic activity is shown in column 2. Fold induction was calculated as a ratio of the corrected luciferase counts produced by cells stimulated with hypoxia, 100 μM cobaltous ions or 100 μM desferrioxamine to those obtained when the cells were cultured without stimulation.
C terminus and N terminus of this region both produced a decrease in activity, indicating that this entire domain was necessary for maximal activity (Fig. 4). Interestingly, the N-terminal deletion (pGal/EPAS9–682) showed enhanced transactivation in normoxic cells and complete loss of the inducible response, defining a short sequence (amino acids 517–534) as critical for the regulatory property.

Domains from EPAS1 Can Confer Hypoxic Regulation on the Heterologous VP16 Transactivator—Though experiments described above indicated that the N-terminal 495 amino acids of EPAS1 sequence had functionally suppressive effects on the internal and C-terminal transactivation domains and might contribute to regulation, such a function could not be demonstrated independently because deletion of the native transactivation domains of EPAS1 removed all activity. To enable further analysis of this possibility, a second set of fusions was created in which the powerful C-terminal 80 amino acid transactivator from herpes simplex virus protein 16 (VP16) was linked to the C terminus of the Gal/EPAS1 fusion proteins.

First, the N-terminal sequence of EPAS1 (amino acids 9 to 517) was tested by creation of pGal/EPAS9–517/VP16. When compared with the activity of a plasmid encoding the Gal/VP16 fusion alone, inclusion of EPAS1 amino acids 9 to 517 had a profoundly suppressive effect on transactivation in normoxic conditions. On stimulation by hypoxia, cobaltous ions, or desferrioxamine the extent of repression was reduced (Fig. 5A), indicating that this sequence could convey regulatory effects in isolation and that these effects could be conveyed on a heterologous transactivator.

Other portions of the EPAS1 molecule were assayed for regulatory activity in a similar way by inserting three portions of EPAS1 sequence (corresponding to exons 2–6, 7–11, and 12–15, and covering all except the N-terminal 8 amino acids) between the Gal and VP16 domains (pGal/EPAS2–295/VP16, pGal/EPAS295–682/VP16, and pGal/EPAS682–870/VP16). Results are shown in Fig. 5B. In comparison with pGal/VP16, both pGal/EPAS9–295/VP16 and pGal/EPAS682–870/VP16 had rather similar activity, which was not inducible. In contrast, pGal/EPAS295–682/VP16 had much reduced activity in normoxic cells and showed induction by all three stimuli. These results suggested that repressive and regulatory properties of the internal domain of EPAS1 could be conferred on a heterologous transactivator. In contrast, comparison of the activity of pGal/EPAS682–870/VP16 with the simple Gal/EPAS fusions (Fig. 3B) indicated that sequences from the C-terminal domain of EPAS1, which exerted powerfully repressive and regulatory effects on the native C-terminal transactivation domain, had little or no such effects on the VP16 transactivator.

In an attempt to pin-point shorter sequences with regulatory potential, sequences corresponding to exons 7–11 were individually inserted in-frame into the Gal/VP16 fusion and tested in an analogous manner. To maximize the chance of observing subsequences with the ability to convey regulation in isolation, plasmids were tested at several doses. Results are shown for cells transfected with 100 ng in Fig. 5C; identical results were obtained with 10 ng. Though sequences corresponding to exon 7 were suppressive in this assay, no individual exon conveyed regulation at any dose with the exception of exon 11 (amino acids 517–682).

Finally, the effect of removing individual exons from the internal domain (exons 7–11) was tested (Fig. 6). Removal of exon 11 (creating pGal/EPAS9–517/VP16) led to much higher activity in normoxic cells and greatly reduced inducibility. Removal of successive exons from the N terminus led to a more progressive increase in activity in normoxic cells and a progressive reduction in the extent of induction. The results therefore suggest that multiple sequences within exons 7–11 contribute to regulatory properties of this domain. Sequences lying both N-terminal and C-terminal to amino acid 517 were able to confer the inducible property in isolation, though the effect was only modest for the N-terminal portion.

These data indicate an organization for EPAS1 in which two transactivation domains are surrounded by sequences that have repressive effects and that confer regulation. Previous analyses of the C-terminal domain (amino acids 530–826) of HIF-1α have defined a similar organization (7, 9). In our analysis of HIF-1α we also showed that amino acids 28–530 confer a suppressive effect on the endogenous transactivating capacity (t1) of the human glucocorticoid receptor DNA binding domain (amino acids 1–500) (7). The power of this suppressive effect almost totally obliterated the activity of this relatively weak transactivator making it difficult to ascribe any regulatory function to this region of HIF-1α. We therefore sought to extend the current comparison of EPAS1 with HIF-1α by analyzing further the functional effects of HIF-1α sequences on the Gal/VP16 fusion.

Comparison with Regulatory Domains in HIF-1α—To permit a direct comparison with the current analysis of EPAS1, we
made similar Gal/VP16 fusions bearing HIF-1α amino acids and tested their function in Hep3B cells. During the course of this work, the intron-exon structure of HIF-1α was published (25). Constructs were first designed to follow from our previous analysis of HIF-1α. Subsequently, as with EPAS1, the HIF-1α sequences tested were based on the known intron-exon structure. Fig. 7A shows the effect of N-terminal HIF-1α sequences (amino acids 13–553). These sequences had a profoundly suppressive effect in the system and conferred high levels of regulation. Though this effect was similar to that observed for EPAS1, the HIF-1α sequence was associated with a higher degree of suppression and a higher amplitude of regulation. Fig. 7B shows a similar analysis for other regions of HIF-1α. As reported previously, we found that pGal/EPAS1–517/VP16 showed regulated activity. Such regulated activity was again observed with sequences lying N-terminal to this region (pGal/a530–552/VP16) but not C-terminal to this region (pGal/a552–813/VP16). Interestingly, HIF-1α C-terminal sequences, which confer repression and high level regulation on the native transactivation domains (but excluding a subdomain, amino acids 530–572, necessary for regulation of protein level of a Gal/ HIF-1α fusion) (7), had little or no effect on the Gal/VP16 fusion (pGal/a572–774/VP16, Fig. 7B).

Sequence comparison indicated a high degree of conservation in the intron-exon boundaries of the two genes. However, the sequence of several portions of the internal region is poorly conserved between HIF-1α and EPAS1 (Fig. 10), and in particular, HIF-1α contains a number of nonconserved sequences inserted into the N-terminal portion of exon 10. In an attempt to define sequences that might contribute to the enhanced inducibility observed for the HIF-1α fusions, we extended the comparison by testing individually subsequences covering HIF-1α amino acids 294 to 698 (corresponding to exons 8–12). As with EPAS1 exon 11, the corresponding HIF-1α exon 12 conferred regulation in isolation (Fig. 7C). However, none of the other individual subsequences (including that containing the nonconserved portion of exon 10) conferred high level regulation in isolation, though HIF-1α exon 8 was (like EPAS1 exon 7) profoundly suppressive, and HIF-1α exon 9 showed modest inducible activity. Nevertheless when considered together, HIF-1α sequences 345–553, corresponding to exons 8–11, conferred substantially greater inducibility than the corresponding portion of EPAS1 (compare Fig. 7D with Fig. 6).

The main contrast between EPAS1 and HIF-1α was therefore in the overall level of inducibility. Nevertheless, there were many similarities in the organization of the two molecules that involved transactivation domains interspersed by sequences that have repressive effects and that confer regulation. In each case, these repressive sequences appeared to be of two types; internal sequences whose action was clearly evident on the heterologous Gal/VP16 fusions, and sequences lying C-terminal to the internal transactivation domain whose repressive action could not be transferred in that way. Our previous analysis of the C-terminal domain (amino acids 530–826) of
HIF-1α had defined two types of regulatory mechanism. Sequences lying C-terminal to the internal transactivation domain had an action that was not dependent on changes in protein level, whereas those overlapping the internal transactivation domain could also confer regulation of fusion protein level. We therefore tested the ability of different regulatory sequences from EPAS1 to confer regulation on Gal fusion protein levels.

Regulated Fusion Protein Levels Contribute to the Inducible Activity Conveyed by Internal EPAS1 Domains—Whole cell extracts were prepared from Hep3B cells transfected with plasmids that express chimeric proteins containing domains from EPAS1 fused to Gal and subjected to Western blot analysis. Although a clear signal was obtained from the Gal protein in cells transfected with pGal, products of cells transfected with the majority of fusion proteins were below the limit of detection, precluding an assessment of whether levels were regulated in response to exposure to hypoxia, cobalt, or desferrioxamine. To increase expressed fusion protein levels into a range that could be detected by Western analysis, Hep3B cells were co-transfected with the activator plasmid and an amplifying plasmid, pCMV-TAg, after which the cells were split for parallel incubations in normoxia and stimulating conditions. The expression of plasmids containing either the N-terminal portion (amino acids 9 to 517) or the C-terminal portion (amino acids 517 to 870) of EPAS1 fused to Gal were first tested (Fig. 8). Regulation of Gal fusion protein level was observed with pGal/EPAS517–870, but not pGal/EPAS9–517. To analyze this further, a series of Gal/EPAS1 fusions with different EPAS1 N-terminal deletions was examined. Three plasmids expressing fusion proteins containing successive N-terminal deletions to EPAS1 amino acids 345, 495, and 517 all manifested regulated fusion protein level, whereas two plasmids expressing further deletions to amino acids 682 and 819 expressed unregulated levels of fusion protein (Fig. 8). We also conducted titrations in which plasmids were expressed at varying levels. The same results were obtained even when plasmid concentrations used in transfections were adjusted such that expression levels were at the limit of detection by Western analysis (data not shown). Using higher plasmid doses, a dependence of inducible expression on overall level of expression was again observed. An example of such a titration for the plasmid pGal/EPAS495–870 is shown in the bottom panel of Fig. 8. The fusion protein level is seen to be inducible by desferrioxamine only at the lower levels of expression.

Definition of Amino Acids Critical to the Regulated Function of the C Terminus of EPAS1 and HIF-1α—Our functional analysis of EPAS1 and the comparison with HIF-1α, indicates that in certain regions sequence comparison should give clues as to functionally critical residues. Nevertheless, the large size of some of the regulatory domains together with a level of redundancy among subsequences makes this a relatively difficult task for the internal regulatory domains. However, at the C terminus of EPAS1 our experiments showed that addition of nine amino acids was sufficient to confer regulation on an otherwise constitutive transactivation domain (Fig. 3B). Comparison of published data suggested that this might also be true of HIF-1α (7, 9). To test this directly, we tested the activity of pGal/ε775–826 and pGal/ε786–826 (Fig. 9A). Results indicated that, as with EPAS1, addition of this short sequence to the N terminus of the transactivation domain conveyed regulation on an otherwise constitutively active region. The effects of scanning mutations through this region and the effect of mutating a single conserved cysteine lying close to this region (HIF-1α, Cys-800) are illustrated in Fig. 9C. Mutation of the cysteine residue, Cys-800, to alanine produced a modest reduc-
tion in activity but did not ablate inducibility. Relatively modest effects were also observed with mutations affecting conserved serines (HIF-1α, PSD775–777; GQS784–786), and a nonconserved cysteine (HIF-1α, LAC778–780). In contrast, a much greater effect was observed with mutation of the conserved sequence arginine-leucine-leucine. Mutation of this sequence in the context of either the HIF-1α C terminus or the EPAS1 C terminus (HIF-1α, RLL781–783; EPAS1, RLL825–827) greatly increased normoxic activity and essentially recreated constitutive transactivation.

DISCUSSION

In these experiments, we have analyzed the activation and regulatory domains of EPAS1, and compared their function with those of HIF-1α.

Analysis of transactivation defined two domains in EPAS1, one lying at the C terminus within exon 15 and the other in an internal region corresponding broadly to exon 11. A similar arrangement exists in HIF-1α (7, 9). For the C-terminal domain, conservation between the genes was striking with activity residing in a very similar sequence of around 40 amino acids. In contrast, the internal activation domain of EPAS1 was not so similar to that identified in HIF-1α. A sequence of 165 amino acids was necessary for maximal activity of the internal activation domain of EPAS1, whereas for HIF-1α the highest level of activity was observed with a short 30–40-amino acid region corresponding to the N-terminal portion of the EPAS1 domain. Thus for EPAS1, a proline rich domain in the C-terminal portion of exon 11 (which is not present in the corresponding exon 12 of HIF-1α) contributes to transactivation.

Analysis of regulation defined at least three EPAS1 sequences, which could independently convey oxygen-regulated activity. These were located within and N-terminal to the internal activation domain and between the activation domains. Two types of activity were distinguished. Sequences overlapping and extending N-terminal to the internal transactivation domain conveyed regulation on the Gal/VP16 fusion, indicating that their operation was not dependent on a specific interaction with EPAS1 transactivation domains. In contrast, sequences lying adjacent to and conveying regulation on the C-terminal EPAS1 transactivation domain had no action on the heterologous Gal/VP16 system, indicating a different mode of operation.

Comparison with regulatory domains of HIF-1α showed striking similarities. First, HIF-1α regulatory domains also manifest a dual pattern of activity where the internal regulatory sequences conveyed regulation on the Gal/VP16 fusion but those at the C terminus did not. Second, the position of C-
FIG. 8. Expression of Gal/EPAS1 fusion proteins in transfected Hep3B cells. Cells were transfected with activator plasmid and pCMV-TAg and incubated for 48 h in normoxia with or without 100 µM desferrioxamine applied for the last 16 h (N and D, respectively). Results from untransfected cells (U) are also shown. Whole cell extracts (50 µg of protein) were analyzed by SDS-polyacrylamide gel electrophoresis and expression fusion proteins were detected by enhanced chemiluminescence after indirect immunostaining with the specified mouse monoclonal antibody followed by peroxidase-conjugated goat anti-mouse immunoglobulin. The four panels at the top show results obtained after transfection with the indicated constructs, using monoclonal antibody RK5C1 directed against the GAL4 DNA binding domain. Results for pGal/EPAS517–870 and pGal/EPAS345–870 identified with monoclonal antibody 190b against human EPAS1 are shown in the two middle panels. The effects of overall expression on the amplitude of regulation are illustrated in the bottom panel. Transfections were performed with increasing doses of the pGal/EPAS495–870 activator plasmid and a fixed dose of pCMV-TAg. In each panel, the position of the relevant fusion protein is indicated by a closed arrow; in the middle panels, open arrows indicate the position of the endogenous EPAS1 protein.

Terminal and internal regulatory domains was similar, with internal regulatory sequences overlapping and extending N-terminal to the internal transactivation domain. In a previous analysis of HIF-1α, we focused our analysis on the function of residues C-terminal to amino acid 530 and defined regulatory sequences that overlapped the internal transactivation domain (7). In those experiments we found that sequences lying N-terminal to amino acid 530 greatly reduced activity when fused to a truncated glucocorticoid receptor, but levels of activity were so low that regulatory effects could not be assayed. Using the Gal/VP16 system, the regulatory effects of these sequences were clearly demonstrated (Fig. 7). These data are consistent with a recently published analysis of HIF-1α in which an extensive domain responsible for oxygen-regulated proteolytic degradation was defined between amino acids 401 and 603 (11). Interestingly, in the context of the native molecule, portions of this domain were shown to act independently to confer partial instability (11). The present analysis of the independent operation of subsequences is consistent with that observation, and establishes a third point of similarity between HIF-1α and EPAS1. In each case, independently operating subsequences within the internal regulatory domain could be defined, which were capable of conveying regulatory activity on the heterologous Gal/VP16 fusion.

The comparative analysis also highlighted differences between the molecules. For instance a striking difference was observed in the dose-dependent activity of Gal fusions to EPAS1 (amino acids 19–826) or HIF-1α (amino acids 28–826) (Fig. 2). The extent of induction varied markedly with the dose of transfected Gal/EPAS1 plasmid, such that at high plasmid doses the fusion protein displayed essentially constitutive activity, whereas at low doses it showed clear inducible activity. In contrast, the HIF-1α fusion showed higher levels of inducibility than EPAS1 which were maintained at all doses tested. Such behavior suggests that interaction of EPAS1 with the sensing/signal transduction system involves some readily saturable process. This could explain why inconsistent degrees of induction by hypoxia are seen when forced overexpression of EPAS1 drives high levels of hypoxia response element-dependent reporter gene expression (13, 14, 17). It might also explain patterns of gene expression seen in vivo. For instance, several groups have reported in situ mRNA studies in which high levels of EPAS1 expression appear to be associated with high levels of mRNA for the EPAS1 target gene vascular endothelial growth factor, even in situations where the cells are not obviously hypoxic (15, 26).

Analysis of individual sequences also showed lower inducibility in both the C-terminal domain and the internal regulatory domains of EPAS1. This was most striking for the N-terminal portion of the internal domain. We considered whether the poor conservation in EPAS1 of HIF-1α exon 10, which encodes a substantial portion of the oxygen degradation domain (11), might account for their different behavior. However, our analysis of individual exons did not define a particular exon as responsible for this difference.

The exon analysis did reveal one unexpected but striking finding. EPAS1 exon 7 was very strongly repressive in these assays although it did not confer regulation. Similar results were obtained with HIF-1α exon 8, which lies outside the degradation domain. We have not yet defined the mechanism of action of these exons, though in isolation they do not affect fusion protein level (data not shown). Interestingly these regions align with, and show similarity to, a region of AHR that is also strongly repressive, and corresponds to the ligand and HSP90 binding domain (27, 28).

Overall, when the functional analysis was compared with the
extent of sequence similarity, a good correlation was observed (Fig. 10). Outside the basic helix-loop-helix PAS domains (which are known to possess similar dimerization and DNA binding properties) (13, 15), the highest levels of sequence similarity are seen in the N-terminal portion of EPAS1 exon 11 (HIF-1α exon 12), and in exon 15 (both molecules), which are the regions that show the clearest functional resemblance in these studies. The less well conserved region corresponding to HIF-1α exons 9–11 and EPAS1 exons 8–10 showed a greater difference in function.

The detailed deletional analysis of the two molecules highlighted short conserved sequences lying immediately adjacent to the activation domains that contained minimal elements necessary for regulated activity. In a previous analysis of HIF-1α (7), we mutated all phosphoacceptor amino acids in an internal regulatory region close to the internal activation domain and found no functional effects. In the current analysis, we focused our attention on the sequences lying adjacent to the C-terminal activation domain. The addition of a further nine amino acids in EPAS1 or eleven amino acids in HIF-1α was found to confer regulation on the otherwise constitutive domains (compare EPAS1 sequences 819–870 and 828–870, and HIF-1α sequences 775–826 and 786–826). In the context of these minimal sequences, we found that neither of two conserved serine residues was necessary for the inducible property. Because protein oxidation, possibly involving sulfhydryl chemistry, is an attractive candidate for HIF-1α regulation (6, 8, 29–31), we mutated each of two cysteine residues in or adjacent to the region and again found little effect on the inducible characteristics of this domain. In contrast however a conserved RLL sequence was critical for induction. At present we are unable to determine the molecular basis for this finding, though it is of interest that the C-terminal activation domain contains several dileucine repeats, that leucine-rich regions are known to be important in the interaction of co-activators such as p300 with transcription factors (32), and that p300 can interact with this domain of HIF-1α and EPAS1.2

Existing data on HIF-1α support a model in which oxygen-dependent regulation of HIF-1α abundance, mediated by the proteasome acting on an internal degradation domain, serves to amplify a further oxygen-regulated activity at the C termi-
The overall similarity between EPAS1 and HIF-1α regulatory sequences, in which internal regulatory sequences conveyed inducible characteristics on Gal/VP16, but C-terminal regulatory sequences did not, led us to consider a similar model for EPAS1 regulation. We therefore tested for oxygen-dependent regulation of Gal/EPAS fusion protein abundance. We found that Gal/EPAS fusions containing N-terminal EPAS deletions to amino acids 345, 495, and 517 showed a regulated level of protein product. This was not seen with further deletions to amino acids 682 or 819 suggesting that sequences lying N-terminal to amino acid 682 were necessary for this effect. The extent of regulation was lower than has been described for HIF-1α fusions (11) and, as described for functional assays, appeared to saturate as levels of expression were increased. We did not observe such regulation with a Gal fusion to amino acids 9–517. The inability to detect fusion proteins at low levels of expression, coupled with the demonstration of reduced regulation at high levels of expression indicates that regulatory effects occurring through changes in protein level might easily be missed and illustrates a common problem in transfection studies of inducible systems. Overall, however our results indicate that internal sequences in EPAS1 can support regulation through changes in protein level. Taken together with the different activities of internal and C-terminal regulatory sequences on the Gal/VP16 fusion, they are consistent with the dual activation model proposed above, and suggest quantitative rather than qualitative differences in the mechanisms of activation for EPAS1 and HIF-1α.

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