Regulation of the prolyl hydroxylase domain protein 2 (*phd2*/*egln-1*) gene: identification of a functional hypoxia-responsive element

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The HIFs (hypoxia-inducible factors) are a family of heterodimeric transcription factors essential for the adaptation of cells to reduced oxygen supply. Three human PHDs (prolyl hydroxylase domain proteins, PHD1–PHD3) initiate oxygen-dependent degradation of HIF-α-subunits in normoxia. RNA interference directed against PHD2, but not PHD1 or PHD3, is sufficient to stabilize HIF-1α in normoxia. Therefore PHD2 is regarded as the main cellular oxygen sensor. PHD2 itself is up-regulated by hypoxia and may thus limit hypoxic signalling. By sequence analysis, we predicted a promoter approx. 3.5 kb 5′ of the translation start codon and a second promoter located in a CpG island immediately upstream of the coding sequence. A consensus HIF-1-binding site that is conserved in the murine *phd2* gene was detected in the CpG island. By electrophoretic mobility-shift assay, we demonstrated binding of HIF-1 to the putative HIF-1-binding site. In luciferase reporter vectors, the isolated upstream promoter was inactive in all cell lines tested unless 200 bp were deleted at the 3′-end. The downstream promoter was active and induced by hypoxia. In reporter vectors containing both promoter sequences, luciferase activity was equal to vectors containing only the downstream promoter. In cells transfected with a vector containing both promoters, a single luciferase transcript was detectable. This transcript had the same length as transcripts from a vector containing the downstream promoter only. We conclude that the *phd2* gene is transcribed exclusively from the downstream promoter that contains a functional hypoxia-responsive, *cis*-regulatory element. Our results establish that PHD2 is a direct HIF target gene.

Key words: egg-laying deficiency protein nine-like protein (EGLN), hydroxylation, hypoxia, hypoxia-inducible factor (HIF), oxygen, prolyl hydroxylase domain (PHD).

INTRODUCTION

HIF (hypoxia-inducible factor) is a transcription factor central to a large number of adaptive processes in a situation of reduced oxygen supply. Hypoxia is a feature of cardiovascular diseases associated with ischaemia and also plays an important role in tumour biology. Therefore HIF is currently regarded as an attractive target in the development of new therapeutic strategies (see [1–3] for reviews).

HIF is composed of an oxygen-sensitive α- and a constitutive β-subunit that is also known as arylhydrocarbon receptor nuclear translocator. Three related α-subunit paralogues have been characterized. For example HIF boosts red blood cell production by the induction of erythropoietin, and enhances transcription of angiogenic factors, e.g. vascular endothelial growth factor. Furthermore, HIF induces glucose transporters as well as virtually all glycolytic enzymes and thus favours anaerobic cell metabolism. More than 50 genes have been found to be HIF responsive (see [4] for a review). Inducibility by HIF is linked to the presence of a core HIF binding consensus sequence (ACGTG) in regulatory regions of HIF target genes. The most prominent example of a HIF binding HRE (hypoxia-responsive element) is the enhancer located 3′ to the *epo* gene that enabled the identification of HIF-1 [5,6].

The α-subunit of the active transcription factor HIF-1 is normally undetectable in the presence of oxygen whereas it becomes stable in hypoxia. The degradation process is triggered in normoxia by enzymatic hydroxylation of two conserved proline residues (Pro-402 and Pro-564 in human HIF-1α, [7–9]). Only hydroxylated HIF-α binds the pVHL (von Hippel–Lindau protein) that is part of an E3 ubiquitin ligase. After ubiquitination, HIF-α molecules are rapidly degraded by the proteasome [10,11]. A second control mechanism is the hydroxylation of Asn-803 in the C-terminal transactivation domain by the enzyme FIH-1 (factor inhibiting HIF-1) [12–14] that prevents the recruitment of transcriptional co-activator proteins.

The four human HIF-α hydroxylases that have been characterized so far (PHD1–PHD3 and FIH-1, where PHD stands for a prolyl hydroxylase domain protein) belong to a family of 2-oxoglutarate-dependent, non-haem iron-binding dioxygenases [15–17]. PHD1, PHD2 and PHD3 have also been termed HPH3, HPH2 and HPH1 [16], and EGLN2, EGLN1 and EGLN3 [18]. No substrates other than HIF-α have been reported to date. The PHDs are widely expressed in tissues [19,20]. PHD homologues, as well as HIF homologues, have been identified in all multicellular organisms investigated so far, termed EGL-9 (egg-laying deficiency protein 9) in *Caenorhabditis elegans* [15], CG1114 in *Drosophila melanogaster* [21] and SM-20 in rat [22]. In mouse, three PHDs with similarity to the human enzymes have been found by database analysis [18].

Notably, PHD2 inhibition by RNAi (RNA interference), but not inhibition of PHD1 or PHD3, is sufficient to up-regulate HIF-1α in normoxia, indicating that the three enzymes are not simply redundant [23] and that PHD2 may be the main cellular oxygen sensor. PHD2 and PHD3 have been reported to be hypoxia-inducible [15,23,24]. Dysregulation of HIF-α in pVHL-deficient

Abbreviations used: EGLN, egg-laying deficiency protein nine-like protein; EMSA, electrophoretic mobility-shift assay; EST, expressed sequence tag; FIH, factor inhibiting HIF-1; HIF, hypoxia-inducible factor; HBS, HIF-1-binding site; HRE, hypoxia-responsive element; MEF, mouse embryonic fibroblasts; PHD, prolyl hydroxylase domain protein; pVHL, von Hippel–Lindau protein; RNAi, RNA interference; UTR, untranslated region; wt, wild-type.

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renal clear cell carcinoma cells is associated with the loss of hypoxic PHD2 and PHD3 induction [25], and the inhibition of HIF-α by RNAi also leads to a loss of induction of PHD2 and PHD3 in hypoxia [26]. These results suggest that HIF itself may mediate the induction and that HIF and PHDs form a feedback loop that limits hypoxic signalling and accelerates HIF degradation after reoxygenation. However, HIF-responsive elements in control of the *phd2* gene have not been identified yet.

In the present study, we demonstrate that a promoter element located in a CpG island approx. 0.5 kb upstream of the translation start site of the *phd2* gene is dominant in all cell lines tested. This promoter contains a HBS (HIF-1-binding site) that is the *cis*-acting regulatory element causing induction of the human *phd2* gene by hypoxia. Thus our results prove that PHD2 is a direct HIF target gene.

MATERIALS AND METHODS

Computational sequence analysis

Promoter region prediction and analysis of transcription factor binding sites in relevant parts of human and murine genes for PHD2 were performed with the GenomatixSuite software (Genomatix Software GmbH, Munich, Germany). Sequence alignments were performed with the tools of ClustalX program [27]. CpG islands within the genes of human and murine *phd2* were identified by means of the CpG-island-extraction algorithm as described previously [28]. The CpG island searcher is available at http://www.uscnorris.com/cpgislands/cpg.cgi. EST (expressed sequence tag) searching was performed using the Gene2EST BLAST server available at http://zurg.embl-heidelberg.de/

Cell culture

Human hepatoma cells HepG2, human ovarial carcinoma cells OVCAR3 and osteosarcoma cells U2OS were cultured in Dulbecco's modified Eagle's medium (Gibco, Karlsruhe, Germany) supplemented with 10 % (v/v) foetal calf serum (Gibco), 2 mM glutamine (Gibco), 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma, Deisenhofen, Germany). Normal MEF$^{+/+}$ (mouse embryonic fibroblasts) and the same cells bearing an inactivation of the *hif-1α* gene (MEF$^{-/-}$) were engineered by R. S. Johnson (Division of Biological Sciences, University of California at San Diego, La Jolla, CA, U.S.A.) and provided by R. H. Wenger (Institute of Physiology, University of Zürich, Zurich, Switzerland). MEFs were cultured in the same media as the human cells. Hypoxic incubations were performed either in an oxygen-regulated cell culture incubator (Heraeus, Hanau, Germany) or in an Invivo$_2$ 400 hypoxia workstation (Ruskinn Technologies, Leeds, U.K.).

Nuclear extract preparation and EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared from HepG2 cells incubated in a 3 % oxygen atmosphere for 4 h as described earlier [29]. Oligonucleotides for gel-shift assays were synthesized by MWG-Biotech (Ebersberg, Germany). Sequences were derived from the human *phd2* gene, containing the putative HBS (PHD2-HBS, nt 2747, GenBank® accession no. AF229245) or a mutated HBS (PHD2-HBSmut). Sequences for wt (wild-type) and mutant oligonucleotides were as depicted in Figure 1(A). As positive control, an oligonucleotide containing the HIF-1 responsive element (containing two HBSs) from the human transferrin gene (TfHBS) was used as described in [30]. The 5′-end labelling, annealing and binding reactions were performed as described previously [29]. Samples were resolved by electrophoresis on native 5 % polyacrylamide gels at room temperature (20 °C). Gels were dried and analysed by phosphoimaging (BAS 1000; Fuji, Düsseldorf, Germany). Specificity was tested by supershift experiments. For this purpose, 1 μl of undiluted monoclonal anti-HIF-1α antibody (BD Biosciences, Heidelberg, Germany) was added 60 min before the gel was run.

Quantitative PCR

Mouse embryonic fibroblasts were incubated in an atmosphere of 1 % oxygen for 4 h. Total RNA was isolated following a standard method [31]. RNA (1 μg) was reverse transcribed with oligo-(dT) and Transcriptor Reverse Transcriptase (Roche, Penzberg, Germany). The coding sequence of mouse PHD2 was assembled from ESTs published previously [18]. Primer sequences were: (forward) 5′-GACCGGCGTAACCCTCATG-3′ and (reverse) 5′-TTGCTGACTGAATTGGGCTTG-3′. Mouse PHD2 mRNA expression was quantified using the qPCR Mastermix for SYBR Green I (Eurogentech, Seraing, Belgium) and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Murine ribosomal *L28* was used as a housekeeping gene. PCRs were set up in a final volume of 25 μl and contained 2 μl cDNA, 1× reaction buffer with SYBR Green I and 20 pmol of each forward and reverse primer. PCR conditions were set to 10 min at 95 °C followed by 40 PCR cycles (15 s at 95 °C and 1 min at 60 °C). Amplification of one specific PCR product was confirmed by melting-point analysis. Data were analysed using the delta-delta Ct method (where Ct stands for cycle threshold).

Plasmid construction

Fragments of the *phd2* gene used in this study were amplified by PCR using DNA isolated from bacterial artificial chromosome clone RP11-295G20 (GenBank® accession no. AL445524; Wellcome Trust Sanger Institute, Hinxton, U.K.) provided by RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany). Luciferase reporter gene constructs were obtained by cloning the region of interest into the pGL3-family of vectors (Promega/Boehringer Ingelheim, Heidelberg, Germany). Mutations of the HBS plasmids were produced by site-directed mutagenesis utilizing the oligonucleotide primers PHD2-HBSmut (sense) and PHD2-HBSmut (antisense) that had also been used for EMSA. Since the HBS is located in a CpG-rich region, mutagenesis PCR was performed with Thermal Ace® polymerase (Invitrogen, Karlsruhe, Germany) for amplification of GC-rich templates according to the manufacturer's instructions. The numbering of all plasmids is according to the mRNA registration by Dupuy et al. (GenBank® accession no. AF229245) [33]. All plasmids containing PCR-inserts or mutations respectively were sequence-verified (Seqlab, Goettingen, Germany).

In detail, the following plasmids were constructed: for the subset of 5′-promoter reporter vectors, a region of −1535 to +1 flanked XhoI and HindIII was cloned into pGL3-basic. This sequence contains an internal HindIII site. Thus incomplete digestion with HindIII resulted in pGL3b(−1535/1)P2P and pGl3b-(−1535/−191)P2P. A SacI–XhoI PCR-fragment spanning nt −985 to +137 was ligated into pGL3-basic and was named pGL3b(−985/137)P2P. For pGl3b(1454/3172)P2P-wt, the designated PCR product flanked XhoI and HindIII was amplified and ligated into pGL3-basic. The corresponding plasmid pGl3b-(1454/3172)P2P-mt containing the mutation of the HBS was generated as described above.

Reporter constructs possessing the two putative regulatory regions in the endogenous context [pGL3b(−1535/3172)P2P-wt and pGL3b(−1535/3172)P2P-mt respectively] were derived by a two-step cloning strategy. A XhoI–BamHI flanked PCR-product

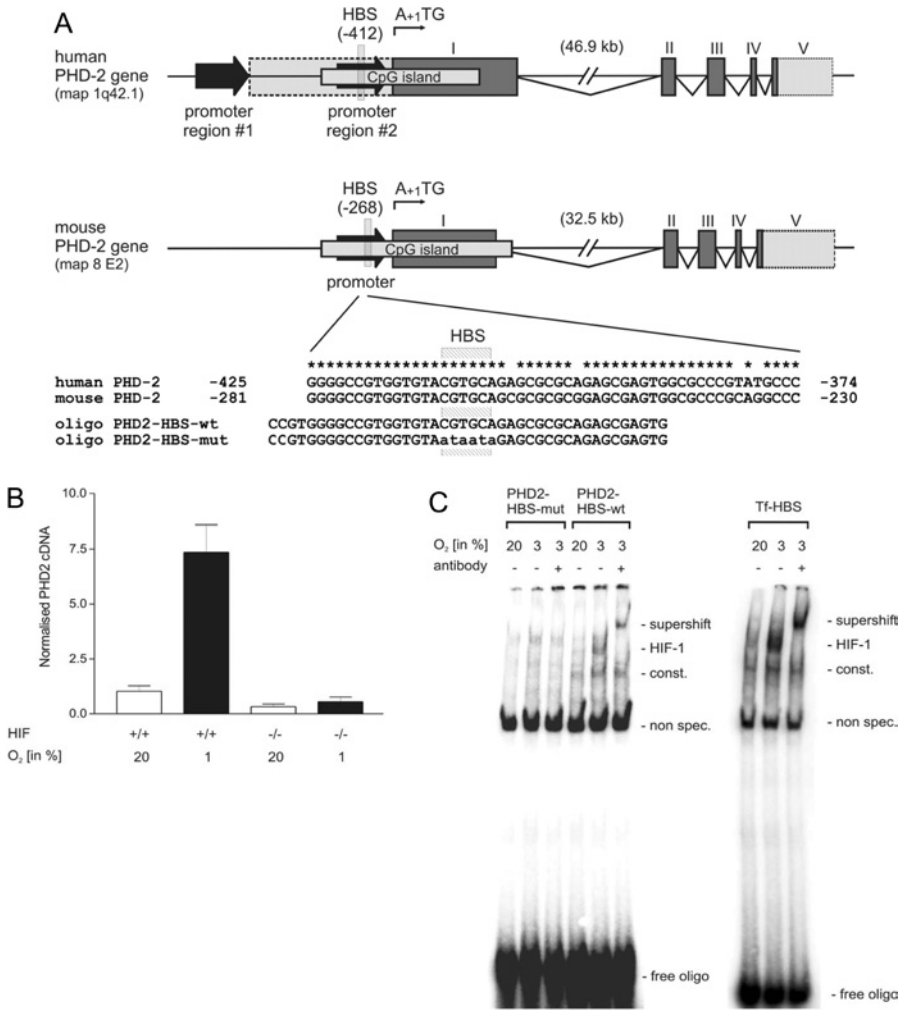

Figure 1 HIF-dependence of PHD2 expression

(**A**) The *phd2/egln-1* gene in human and murine genome. Although in mouse the analysis of nucleotide sequences recognizes a single promoter located in a CpG island closely upstream of the translational start site, analysis of the human gene leads to prediction of one promoter 3.5 kb upstream of the translational start site and an alternative promoter in a CpG island approx. 0.5 kb 5′ of the translational start site. The HBS is conserved between species. (**B**) PHD2 expression in mouse embryonic fibroblasts containing wt ($HIF^{+/+}$) or inactivated ($HIF^{-/-}$) *hif-1α* gene. The cells were incubated in normoxia (20 % O_2) or hypoxia (1 % O_2). Total mRNA was subjected to reverse transcription and quantitative PCR. Relative amount of PHD2 cDNA was calculated by the delta-delta Ct method. Individual PHD2-Ct values were normalized to corresponding L28 Ct-values. Normalized PHD2 values were set in relation to the mean of the $HIF^{+/+}$ normoxia group. (**C**) EMSAs with nuclear extracts from HepG2 cells. Cells were incubated at 20 or 3 % oxygen for 4 h. Oligonucleotides used were: PHD2-HBSmut (mutated HBS, negative control), PHD2-HBS-wt (putative HBS) and Tf-HBS (positive control). Specificity of the HIF-1 signal was demonstrated by supershift analysis with an anti-HIF-1α antibody. HIF-1, specific signal; const., constitutive DNA-binding; non spec., non-specific DNA-binding. All samples were run on the same gel. The Tf-HBS part of the image was electronically brightened.

corresponding to (−1535/1454) was ligated into pGL3-basic utilizing an internal BamHI site at 1454 of the *phd2* gene. Subsequently, a cassette containing (1454/3172) and the *luc* gene was released with BamHI from pGL3b(1454/3172)P2P-mt and cloned into the intermediate to gain the full-length vector. The corresponding wt was obtained by replacing an endogenous SfiI fragment including the wt HRE derived from SfiI-digested pGl3b(1454/3172)P2P-wt.

Reporter gene plasmids carrying the HRE-regulatory region in *trans*-position, i.e. downstream of an SV40 promoted luciferase gene, were derived by a recombinant cloning strategy. An internal BamHI site in position 1454 was used to release a BamHI–XhoI fragment of pGl3b(1069/3172)P2P-wt and of pGl3b(1069/3172)-P2P-mt and ligated into pGL3-prom opened with BamHI–SalI. The resulting plasmids were termed pGl3-prom-3′E(1454/3172)-P2P-wt and pGl3-prom-3′E(1454/3172)P2P-mt respectively. Purity and concentration of all plasmids transfected into cells was estimated by agarose gel electrophoresis.

Transfection and luciferase assays

Transient transfections were performed on 100 mm dishes using Fugene 6 (Roche Applied Science, Mannheim, Germany) transfection reagent by following the manufacturer's instructions. To normalize for transfection efficiency, 3 μg of each reporter construct was co-transfected with 1.5 μg of a cytomegalovirus-promoter-driven β-GAL expression plasmid (a gift from P. J. Ratcliffe, The Henry Wellcome Building of Genomic Medicine, University of Oxford, Oxford, U.K.). Transfected cultures were grown overnight, trypsinized the following day and split into two equal parts and placed on two 6-well plates, i.e. from a single transfected plate 12 separate cell culture wells were produced which allowed the incubation of cells from the same transfection either in normoxia or in hypoxia. Cells were allowed to become adherent and subsequently incubated for 16 h either under normoxic or hypoxic (1 % O_2) conditions. Cell lysates were prepared with passive lysis buffer (Promega) and aliquots were assayed

for luciferase expression with a Microlumat LB96P luminometer (EG-G Berthold, Bad Wildbad, Germany). Additionally, β-GAL expression for each sample was measured. Data are expressed in relative light units (luciferase counts/β-GAL expression). Bars indicate means $\pm$ S.D. for six separate cell culture wells. All experiments were performed repeatedly so that all results were confirmed in at least one completely independent experiment.

Transient transfections for subsequent luciferase Northern blotting were performed following a similar transfection procedure. Cells growing in 100 mm dishes were transfected with 3 μg of the indicated reporter constructs. Plates were grown overnight and divided equally on two 150 mm culture dishes. Corresponding cultures were exposed to either normoxia or 1 % oxygen for 16 additional hours, cells were then lysed and total RNA was isolated as described above.

Northern blotting

Human osteosarcoma U2OS cells and human ovarial carcinoma cells OVCAR3 were incubated in a normoxic (20 % O_2) or hypoxic (1 % O_2) atmosphere for 4 h. Total RNA was isolated as described above. mRNA was purified using 0.5 mg of total RNA (Oligotex mRNA Midi; Qiagen, Hilden, Germany) following the manufacturer's instructions. mRNA (4 or 6 μg) of each sample was subjected to electrophoresis in denaturing 1 % agarose gels containing 0.7 M formaldehyde. RNA transfer, prehybridizations and hybridizations were performed exactly as described previously [29]. A hybridization probe was generated by restriction digest of pcDNA3-PHD2 with NotI and EcoNI and thus contained nt 3543–3800 (GenBank® accession no. AF229245). Ribosomal protein L28 mRNA was used to confirm equal loading and transfer as described previously [32]. The L28 hybridization probe was amplified from HepG2 cDNA by PCR. For detection of luciferase mRNA, 20 μg of total RNA was resolved on denaturing agarose gels. The hybridization probe for luciferase mRNA was excised from pGL3-basic using EcoNI and thus encompassed nt 646–1046 (GenBank® accession no. U47295). In this case equal loading was verified by ethidium bromide staining of 28 and 18 S rRNA. All probes were labelled using a commercial DNA labelling kit (MBI Fermentas, St. Leon Rot, Germany). Filters were analysed by phosphoimaging.

RESULTS

The prime objective of the present study was to identify the HRE(s) conferring hypoxic induction of the *phd2*/*egln-1* gene. Interestingly, two GenBank® entries have been deposited (GenBank® accession nos. AF229245 and AJ310543) that do not differ regarding the coding sequence of PHD2. However, one of these sequences contains an unusually large 5′-UTR (5′-untranslated region) [33], which has been confirmed experimentally by anchored PCR using an embryonic cDNA as template. The other has been assembled from ESTs and has a short 5′-UTR but an extended 3′-UTR [18].

To define regulatory sequences, we analysed genomic DNA from approx. 8 kb upstream relative to the translation start site to a region approx. 5 kb downstream of the human PHD2 coding sequence. Two putative promoter regions were predicted, one of which contains a cellular TATA box and is located 3.5 kb upstream of the translation start site, whereas the second promoter region is contained in a CpG island 0.5 kb 5′ of the translation start codon. Transcription factor binding analysis identified one HIF-binding motive (ACGTG) located in the downstream promoter 412 bp upstream of the start codon (Figure 1A).

Importantly, the potential HBS within the CpG island is conserved in mouse *phd2* sequence. In line with this computational analysis, we show by comparison of mouse embryonic fibroblasts carrying a null mutation for the HIF-1α gene with wt cells [34] that the hypoxic induction of mouse *phd2* expression is exclusively dependent on the presence of HIF-1α (Figure 1B).

To demonstrate the functional importance of the putative HBS, it was a prerequisite to prove binding of HIF-1α to the sequence under investigation. In EMSAs, oligonucleotides derived from the human *phd2* sequence containing the wt HBS bound a protein complex from hypoxic HepG2 cells which was supershifted on the addition of a HIF-1α antibody (Figure 1C). Binding of HIF-1 was abolished by mutation of the HBS.

We went on to dissect the function of the two putative promoters, to analyse their interplay, and their oxygen sensitivity by means of luciferase reporter gene assays. The promoter regions were cloned separately or in combination, 5′ to a promoterless luciferase gene. The expression of the luciferase gene was analysed after normoxic and hypoxic incubation. In all cell lines tested, the upstream promoter was inactive. In contrast, the downstream promoter was moderately active in normoxia but strikingly induced by hypoxia (Figure 2A). In all cell lines, hypoxic induction was dependent on the presence of the HBS located in the CpG island. We also used vectors that contained both putative promoters and demonstrated that the activity of this construct was essentially indistinguishable from constructs containing only the downstream promoter region (Figure 2B). When we deleted the terminal 200 bp of the upstream promoter it became active but remained unresponsive to hypoxia (Figure 2C). This result may indicate that the promoter region between -200 and $+1$ contains a repressor element that blocks promoter activity in all cell lines that we have analysed. To further delineate the properties of the downstream promoter region, we produced a vector that contained the luciferase gene driven by a heterologous SV40 promoter followed by the regulatory sequence in a 3′ position. In this experiment, the downstream sequence conferred hypoxic inducibility on the SV40 promoter, i.e. the region under investigation can, at least in this artificial reporter gene context, have enhancer function (Figure 2D).

We have noted that independent groups have found two bands by Northern blotting for PHD2 in human [33] and in murine [20] tissues. These transcripts had a length of approx. 4 and 2 kb respectively. Another report shows that three bands are detectable [23], which is consistent with our own PHD2 Northern blot results (Figure 3A). Interestingly, our experiments showed that all of these bands were induced by hypoxia.

To determine whether transcripts are produced from the upstream promoter when it is not in isolation, we transfected cells with luciferase vectors that contained both promoter regions or the isolated downstream promoter and performed luciferase Northern blotting. We detected a single transcript that had the same length in all samples, indicating that all transcripts originated from the downstream promoter (Figure 3B).

The activity of a single hypoxia-responsive promoter was in harmony with the observation that all transcripts were hypoxia-inducible, but it did not help to interpret the presence of three detectable PHD2 transcripts by Northern blotting. Revisiting the GenBank® entries AF229245 and AJ310543, we found that three poly(A)$^+$ (polyadenylated) signals have been reported which are located 60, 500 and 2630 bp downstream of the translation stop signal. The use of the downstream promoter together with these alternative poly(A)$^+$ signals is predicted to result in three transcripts, which are approx. 1.9, 2.3 and 4.4 kb in length, which is well in line with our Northern blotting results. To test this hypothesis, we used U2OS mRNA samples for reverse transcription

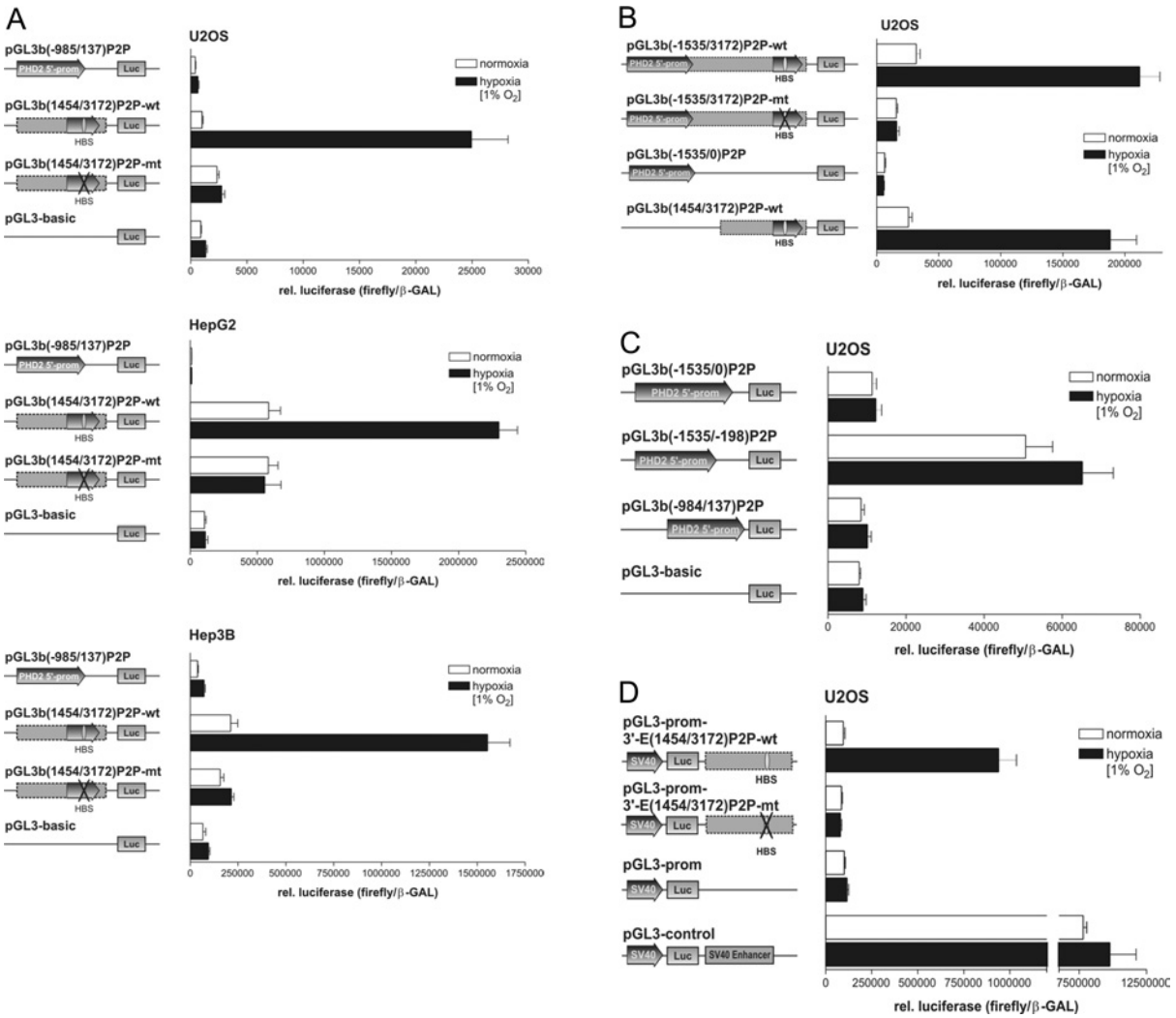

Figure 2 Luciferase reporter gene assays

The regulatory DNA regions of the human *phd2* gene were cloned into luciferase reporter vectors, which were transiently transfected into cells. 24 h after transfection, cells were incubated overnight under normoxia or hypoxia. β-galactosidase served as a transfection control. Bars represent means $\pm$ S.D. for six separate cell culture wells. (**A**) The function of the promoter located approx. 3.5 kb upstream of the translational start site (PHD2 5′-prom) when compared with the downstream promoter region which contains either the wt HBS (wt) or a mutation of the HBS (mt) in U2OS, HepG2 and Hep3B cells. For example, a pGL3-basic vector that contains position -985 to position $+137$ of the putative regulatory region of PHD2 (numbering according to GenBank® accession no. AF229246) is termed pGL3b(-985/137)P2P. (**B**) Luciferase expression was analysed in U2OS cells transfected with reporter vectors containing both regulatory regions or the isolated downstream promoter element. (**C**) The isolated upstream promoter was compared with a truncated version of the same regulatory region. (**D**) The downstream promoter region in a position downstream of the luciferase gene conferred hypoxic induction on an SV40 promoter, demonstrating enhancer activity of the region under investigation.

with oligo(dT) or specific oligonucleotides binding upstream of the 3′ promoter. Although we could readily amplify PCR products from the extended 3′-UTR, we were not able to generate PCR products from the region between the two promoters (results not shown). These results suggest that indeed only the downstream promoter is active in cells and that the different transcripts arise from the use of distinct poly(A)$^+$ signals.

DISCUSSION

The human HIF-α prolyl-4-hydroxylase PHD2 is of high biomedical relevance since it is the enzyme that down-regulates the oxygen-sensitive transcription factor subunit HIF-α [23] in normoxia. Besides the reports mentioned above further studies have demonstrated the induction of PHD2 and of another HIF-prolyl-4-hydroxylase (PHD3) by hypoxia in various cell lines and by use of different experimental techniques [35–37]. It has also been shown that dysregulation of HIF-1α is associated with the loss of hypoxic induction of PHD2. Inactivation of pVHL, a protein necessary for oxygen-dependent degradation of HIF-1α, leads to high normoxic levels of HIF-1α and PHD2 [25]. RNAi directed against HIF-1α has also been reported to eliminate hypoxic PHD2 induction [26]. These results suggest that PHD2 may be a target gene of HIF-1. The evidence is, however, circumstantial. The classification of PHD2 as a HIF target gene requires the experimental identification of a *cis*-regulatory HRE in the *phd2*/*egln-1* gene, which was the aim of the present study. Importantly, presence of a consensus HIF-binding motif in a regulatory DNA domain is not sufficient to demonstrate hypoxic induction by HIF, as it has been shown that erythroid 5-aminolevulinate synthase is hypoxia-inducible but HIF-independent although a putative HBS is found in the promoter of this gene [38].

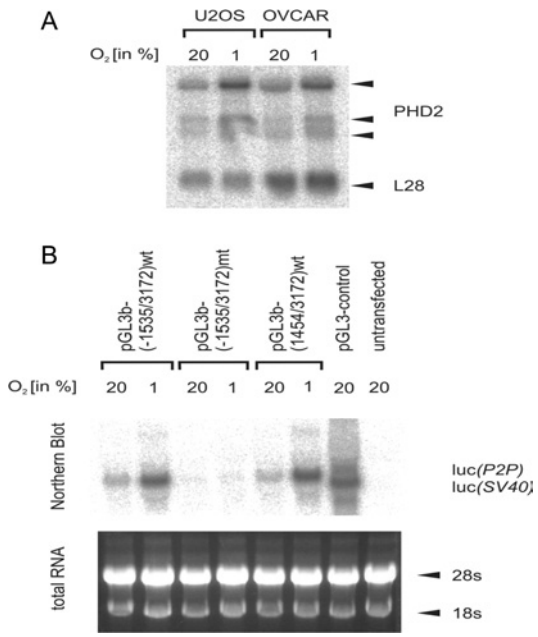

Figure 3 Analysis of PHD2 promoter function by Northern blotting

(**A**) PHD2 Northern blot. Human osteosarcoma (U2OS) and ovarian carcinoma (OVCAR3) cells were subjected to normoxic (20 % O_2) or hypoxic (1 % O_2) incubation for 4 h. mRNA was isolated as described in the Materials and methods section. mRNAs (4 μg U2OS or 6 μg OVCAR3) were run in each lane. Hybridization probes corresponded to PHD2 nt 3543–3800, GenBank® accession no. AF229245. The PHD2 transcripts are approx. 4.1, 2.3 and 1.8 kb. The constitutively expressed ribosomal protein L28 was used as a loading and transfer control. (**B**) Luciferase Northern blot U2OS cells were transfected as described in the Materials and methods section. The cultures were subjected to normoxic or hypoxic incubation overnight. Total RNA was isolated and 20 μg was resolved in each lane.

The *phd2* gene has been localized as the 12th open reading frame on chromosome 1 (C1ORF12) and is reported to contain five exons [33]. According to the report, exon 1 includes 4047 bp with the translational start site in position 3157, thus the initial 3156 bp are an unusually large 5′-UTR. Importantly, this 5′-UTR has been verified experimentally by PCR walking and anchored PCR using a random embryonic total cDNA library. Consequently, the study implies that an active promoter is situated upstream to the 5′-UTR. However, searching EST databases with the 5168 bp PHD2 mRNA sequence (GenBank® accession no. AF229245) results in retrieval of more than 300 ESTs none of which contains a sequence upstream to the CpG island overlapping the translation start site.

Presence of a CpG island spanning the terminal region of the 5′-UTR and the beginning of the coding sequence is a hallmark of mammalian promoters [39,40]. Consequently, promoter prediction programs recognize this region as a second promoter. Interestingly, this prediction is in line with a second GenBank® PHD2 mRNA sequence assembled from ESTs (GenBank® accession no. AJ310543), which gives a 5′-UTR of approx. 100 bp.

Embedded in this CpG island, we have identified a putative HBS, by sequence analysis, situated −412 bp relative to the translational start site. Interestingly, the HBS as well as the surrounding sequence is conserved in the murine *phd2* gene (Figure 1A), which suggests functional relevance. Indeed we have shown that hypoxic induction of murine *phd2* is dependent on HIF-1.

Our sequence analysis suggested that two promoters are located upstream of the PHD2 coding sequence. The upstream promoter element which is located approx. 3.5 kb 5′ of the transcription start site is inactive in our experimental setting. Interestingly, truncation of this promoter region had an activating effect. It seems possible that a repressor element inhibited transcription initiation by this promoter region. Our results do not preclude the possibility that this promoter is active in a different cellular background or at a different stage of development. The dominant promoter, however, is situated in a CpG island immediately 5′ of the translation start site and contains a functional HRE. The different transcripts detected by Northern blotting in previous reports [20,23,33] and in our experiments are explained by the use of three separate poly(A)$^+$ signals. The possibilities for the production of distinct PHD2 transcripts are summarized in Figure 4.

Two further reports add complexity to the situation: alternative splicing has been described in [41] for exons 3 and 4. We have not reproduced these data, but as these exons span 207 bp only, alternative splicing cannot account for the different transcripts we have noted. Secondly, the existence of a gene termed *scand2* that has probably evolved by retroposition of the *phd2* gene has been demonstrated [33]. *Scand2* is located on chromosome 15q25, and several transcript variants have been reported. The functions of this gene as well as its regulation are unclear so far. However, since large blocks of nucleotide sequence are highly conserved, it seems mandatory to align the *phd2* and *scand2* sequences and to choose hybridization probes and oligonucleotide primers from unique regions to avoid confounding results.

In essence, we have identified the dominant promoter of the *phd2* gene 0.5 kb 5′ of the translational start site. The promoter

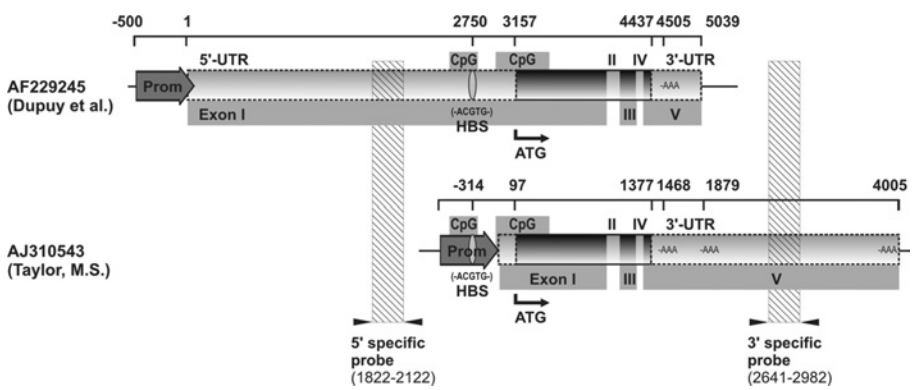

Figure 4 Illustration of the two GenBank® entries potentially leading to distinct PHD2 mRNA species

A transcript containing the extended 5′-UTR and the extended 3′-UTR would encompass 7.5 kb and has not been detected yet. The '5′-specific probe' and '3′-specific probe' designate regions of the *phd2* gene used to detect transcripts containing the extended 5′-UTR and the extended 3′-UTR respectively.

is located in a CpG island, contains a functional HBS, and thus confers hypoxic inducibility. By identification of this *cis*-acting regulatory element, PHD2 is classified as a direct HIF target gene. As PHD2 seems essential in the regulation of HIF-1, our findings may add to the understanding of mammalian oxygen sensing.

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