Identification of a functional hypoxia-responsive element that regulates the expression of the *egl nine* homologue 3 (*egln3/phd3*) gene

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Low oxygen levels induce an adaptive response in cells through the activation of HIFs (hypoxia-inducible factors). These transcription factors are mainly regulated by a group of proline hydroxylases that, in the presence of oxygen, target HIF for degradation. The expression of two such enzymes, EGLN1 [EGL nine homologous protein 1, where EGL stands for egg laying defective (*Caenorhabditis elegans* gene)] and EGLN3, is induced by hypoxia through a negative feedback loop, and we have demonstrated recently that hypoxic induction of EGLN expression is HIF-dependent. In the present study, we have identified an HRE (hypoxia response element) in the region of the EGLN3 gene using a combination of bioinformatics and biological approaches. Initially, we isolated a number of HRE consensus sequences in a region of 40 kb around the human EGLN3 gene and studied their evolutionary conservation. Subsequently, we examined the functionality of the conserved HRE sequences in reporter and chromatin precipitation assays. One of the HREs, located within a conserved region of the first intron of the EGLN3 gene 12 kb downstream of the transcription initiation site, bound HIF *in vivo*. Furthermore, this sequence was able to drive reporter gene expression under conditions of hypoxia in an HRE-dependent manner. Indeed, we were able to demonstrate that HIF was necessary and sufficient to induce gene expression from this enhancer sequence.

Key words: EGL nine homologous protein 3 (EGLN3), HIF proline hydroxylase 3 (HPH3), hypoxia-inducible factor (HIF), hypoxia response element (HRE), proline hydroxylase domain 3 (PHD3), prolyl hydroxylase.

INTRODUCTION

Oxygen is indispensable for the correct functioning of a cell, and thus cells respond to hypoxia (1 % oxygen) by inducing evolutionarily conserved programmes of gene expression aimed at keeping the cell viable and restoring the oxygen supply. HIFs (hypoxia-inducible factors) are a group of basic helix–loop–helix Per-ARNT-Sim transcription factors that are central to this adaptive response [1]. HIFs are heterodimers of an oxygen-regulated α subunit (HIFα) and a constitutive β subunit (HIFβ/ARNT) that can also associate with other transcription factors not involved in oxygen homoeostasis [1,2]. In vertebrates, three different HIFα subunits (HIF1α, 2α and 3α) have been described [1]. Although these are thought to be similarly regulated by oxygen, their tissue distribution [3–5] and target genes [6,7] may differ. The importance of HIF factors, as well as the molecular machinery responsible for their oxygen-dependent regulation, is reflected in their high degree of conservation in multicellular animals from nematodes to mammals [1].

The oxygen concentration regulates the levels of HIFα protein, as well as their transactivation capacity [8]. Under normoxic conditions, HIFα subunits undergo hydroxylation of two specific proline residues [9–11], a modification that is mediated by the activity of a family of 2OG (2-oxoglutarate)-dependent hydroxylases termed PHDs (proline hydroxylase domains), HPHs (HIF proline hydroxylases) or EGLNs [EGL nine homologous proteins, where EGL stands for egg laying defective (*Caenorhabditis elegans* gene)] [12,13]. As a result, the hydroxyprolines are specifically recognized by the product of the VHL (von Hippel–Lindau) tumour suppressor gene, which forms part of an E3 ubiquitin ligase complex, targeting HIFα for degradation by the proteasome [9,10]. In addition to proline hydroxylation, HIFα subunits can also be hydroxylated at a conserved asparagine residue located in one of the two HIFα transactivation domains [C-TAD (C-terminal transactivation domain)] [14]. The enzyme responsible for asparagine hydroxylation, FIH (factor inhibiting HIF-1) [15], is also a 2OG-dependent hydroxylase, and this hydroxylation in the C-TAD of HIF prevents its interaction with the p300 transcriptional co-activator. Since molecular oxygen is a co-substrate in the reaction catalysed by EGLNs and FIH, these reactions are compromised in the absence of oxygen. Proline hydroxylation is particularly affected by hypoxia because of the elevated Michaelis–Menten constant, K_m for oxygen displayed by EGLNs [16]. Hence, under normoxic conditions, the efficient degradation of the α subunit promoted by the EGLNs impairs HIF transcription and its transcriptional activity is weakened due to FIH hydroxylation. On the other hand, a restricted oxygen availability leads to accumulation of HIFα due to the reduction in proline hydroxylation and an increase in its transcriptional activity through its interaction with p300. The stabilization of HIFα promotes its interaction with HIFβ subunits and p300, forming a complex that binds to specific sequences (HREs, hypoxia response elements) in target genes [17].

The hypoxic response includes the switch to anaerobic metabolism and the induction of angiogenesis and erythropoiesis among other events. Accordingly, functional HREs have been

Abbreviations used: ChIP, chromatin immunoprecipitation; CNS, conserved non-coding sequence; EGLN, EGL [egg laying defective (*Caenorhabditis elegans* gene)] nine homologous protein; HIF, hypoxia-inducible factor; FIH, factor inhibiting HIF-1; HRE, hypoxia response element; UTR, untranslated region; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau.

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identified in the regulatory region of the glucose transporter Glut-1 and in most of the genes encoding glycolytic enzymes such as aldolase A, phosphoglycerate kinase 1, enolase 1 and lactate dehydrogenase A [17]. Also regulatory regions of the genes encoding VEGF (vascular endothelial growth factor), a potent pro-angiogenic factor, and erythropoietin, a cytokine involved in the differentiation of red blood cells, contain functional HREs [17]. However, the list of oxygen-induced genes is not restricted to those involved in glycolysis or angiogenesis. The presence of functional HREs has also been demonstrated in many other genes involved in a wide range of biological activities such as those encoding P4Hα, BNIP3, Noxa, Nur77, inducible NOS, endothelial NOS, RORα4, c-Met, CXCR4, PAI1, DEC1/2, leptin, endothelin1, ceruloplasmin, transferrin and haem oxygenase 1. The HREs of all these genes contain the core sequence [A/G]CGT, which in most cases is ACGTG [17]. In addition, HIF transcription factors show preference for specific bases in the proximity of the core that has led to the description of the following consensus HRE sequence: [T/G/C][A/G]CGTG[CGA][GTC][GTC][CTG] [17].

Importantly, EGLN1 and EGLN3 mRNAs, but not those for EGLN2 or FIH, are strongly induced by hypoxia in most cell types [18]. This effect is probably important for cellular adaptation to hypoxic conditions and is responsible for the increased oxygen-mediated HIFα degradation observed after long periods of hypoxia [19]. Indeed, the induction of EGLN3 mRNA expression by hypoxia is particularly significant when compared with other hypoxia-responsive genes [18,20,21]. Recently, it was shown that HIF-deficient cells [20] or cells in which HIFα mRNA was suppressed with small interfering RNA [22], did not up-regulate EGLN3 in response to hypoxia. Moreover, we found that VHL-deficient cells presented very high normoxic levels of EGLN3 that reverted upon restoration of VHL function [20,22]. Thus the hypoxic induction of EGLN3 appears to be mediated by HIF. The promoter of the rat EGLN3 gene (Sm-20) has recently been defined, yet no regulatory regions responsible for its hypoxic induction have been detected [23]. We describe here the identification of a functional HRE, located in the first intron of the EGLN3 gene, that is responsible for its hypoxic induction.

MATERIALS AND METHODS

Cell culture and reagents

Cells of the human HeLa cervix carcinoma cell line were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Basel, Switzerland), while the Chinese-hamster ovary-derived cell lines 4.5 and Ka13 [24] were grown in Ham's nutrient mixture, F-12 (Euroclone, Rehovot, Israel). In all cases, the culture medium was supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10 % (v/v) fetal bovine serum. Cells were grown at 37 °C in a humidified atmosphere containing 5 % CO_2. Experiments under hypoxic conditions (1 % O_2) were performed in an *In vivo* 400 Hypoxia Workstation (Ruskin Technology, Leeds, U.K.).

Plasmid constructs

To clone the EGLN3 promoter region, human genomic sequences from -3410 to $+170$ and -1465 to $+170$ relative to the transcription initiation site of the EGLN3 gene were amplified by PCR using primers 1+ 2 and 3 + 2 (Table 1) respectively. The PCR products were cloned into the pCR 2.1-TOPO TA plasmid (Invitrogen, Leek, The Netherlands) and then subcloned into the XhoI and NheI sites of the pGL3 basic luciferase plasmid (Promega, Mannheim, Germany) to generate pGL3-E3P3.5 and pGL3-E3P1 respectively.

Table 1 Primers used in the present study

The primers listed were used to amplify the regions indicated from human EGLN3 genomic fragment, except for primers 15 and 16, which were used to amplify the region indicated from human collagen proline-4 hydroxylase α, and primers 10 and 11, which were used to introduce mutations into the cloned enhancer A sequence.

No.	Target	Position	Dir.	Sequence
1	Promoter	-3410	F	CTCGAGCTAGCGCAGATGCACACTGGAAGACAATG
2	Promoter	$+147$	R	CTCGAGGTTTCTCGCAACTCTCGGGAGAAG
3	Promoter	-1465	F	GTTCTTGTCCTGGATGTCATTCCC
4	Enhancer A	$+12411$	F	TCCCTGGGCTGGACTGACCTTT
5	Enhancer A	$+12836$	R	CCTCCCCCAAGAAGCCACTGAAA
6	Enhancer A	$+12472$	F	TTCTCTGGTGACTGGGGTAGAGAT
7	Enhancer A	$+12728$	R	GAGCCCATGCAATTAGGCACAGTA
8	Enhancer D	$+739$	F	TCGGAGTCCGTTGTATGTCGACTT
9	Enhancer D	$+1099$	R	TGAAGGGCAGTATTAGGTGGCTTT
10	Mutant A	NA	F	TCACGCAGCGCTAGCAGCCCTGTCAC
11	Mutant A	NA	R	GTGACAGGGCTGCTAGCGCTGCGTGA
12	Promoter	-1685	F	CAAGGAGGGGTACCTCCTGCTCAA
13	Promoter	-1418	R	CCTCTCTGCCTCTGAGGGGAGAAA
14	Promoter	-2298	F	CAGCCTCCCGAGTAGCTGGGATTA
15	P4Hα prom	-329	F	ATCAAGGAGGCAAACTGAACAG
16	P4Hα prom	$+34$	R	ACTCGGAGCGGCTACTTCCTA

The sequences containing putative hypoxia-inducible regulatory sequences were amplified by PCR with the primers indicated in Table 1. The resulting amplified fragments were cloned into the pCR 2.1-TOPO TA plasmid (Invitrogen) and then subcloned into the BamHI and XhoI sites upstream of a minimal rat prolactin promoter (pProl-) to drive firefly luciferase expression [25]. In all cases, HeLa genomic DNA was used as a template for the PCRs. The mutant HRE form of enhancer A was generated by PCR with primers 10 and 11 (Table 1). The wild-type or mutant HRE enhancer A sequences were cloned into the XbaI site of pGL3-E3P3.5 to generate pGL3-E3P3.5A or pGL3-E3P3.5A* respectively.

Reporter assays

Cells were plated in six-well plates 24 h before transfection. Each plate was transfected with 9 μg of a DNA mixture containing 3 μg (HeLa cells) or 5 μg (4.5/Ka13 cells) of the indicated reporter plasmid and 0.1 μg of a plasmid encoding for *Renilla* firefly luciferase under the control of a SV40 promoter. Some 12–13 h after transfection, the cells were replated in 24-well plates and then transferred to hypoxic conditions (1 % oxygen) or left under normoxic conditions for an additional 24 h as indicated. Subsequently, the cells were harvested and the firefly luciferase activity was determined using a dual luciferase system (Promega) in which the firefly luciferase activity was normalized with respect to the *Renilla* luciferase activity. Each experimental condition was measured in duplicate.

ChIP (chromatin immunoprecipitation)

For the ChIP assays, HeLa cells were grown on 10 cm plates until they reached 85 % confluence, at which point they were exposed to hypoxia (1 % oxygen) or left under normoxic conditions for a further 5 h. Subsequently, the cells were fixed with 1 % (v/v) formaldehyde (final concentration) for 12 min at 37 °C, which was stopped by the addition of 0.125 M glycine (final concentration). The cells were washed with cold PBS and then lysed by scraping in 1 ml of lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris/HCl, pH 8.1, and a protease inhibitor). Cell lysates were incubated on ice for 10 min and then sonicated to shear the DNA under conditions established to ensure that the DNA

fragments were between 200 and 1500 bp. After the removal of the insoluble material by centrifugation, 30 μl of each sample was removed and stored (input), while the rest was diluted in immunoprecipitation buffer (1 % Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris/HCl, pH 8.1). The lysates were precleared with preimmune serum and 200 μg of a Salmon Sperm DNA/Protein A agarose 50 % slurry (Upstate Biotechnology, Lake Placid, NY, U.S.A.) for 1 h at 4 °C. The samples were then immunoprecipitated twice, initially with whole rabbit serum for 6 h (IgG control) and then overnight at 4 °C with a polyclonal anti-HIF1 alpha antiserum (Abcam, ab2185). Immunocomplexes were recovered by the addition of 400 μg of Salmon Sperm DNA/Protein A agarose 50 % slurry to the samples that were then sequentially washed for 15 min in TSE I (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, and 150 mM NaCl), TSE II (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, and 500 mM NaCl) and buffer III (0.25 M LiCl, 1 % Nonidet P40, 1 % deoxycholate, 1 mM EDTA and 10 mM Tris/HCl, pH 8.1). Finally, the complexes were washed twice with TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA) and extracted twice with a buffer containing 1 % SDS and 0.1 M $NaHCO_3$. The eluates were pooled, and cross-linking was reversed by the addition of 200 mM NaCl (final concentration) and incubating overnight at 65 °C. The proteins were removed by the addition of proteinase K (30 μg/sample) for 2 h at 42 °C, and the DNA was extracted using Qiagen PCR extraction kit before eluting in 50 μl of water. Immunoprecipitated DNA was amplified by PCR using the primers indicated, and the PCR products were resolved by gel electrophoresis and visualized by ethidium bromide staining.

Computer identification of HREs

To generate a position-specific frequency matrix for HIF recognition, we aligned several sequences (100 bp) from known hypoxia-inducible genes: VEGF [26–28], aldolase A [29,30], phosphoglycerate kinase 1 [29,31], erythropoietin [32–35], haem oxygenase 1 [36], RORα4 [37], Glut-1 [38], P4Hα [39], BNIP3 [40] and c-Met [41]. In the case of VEGF, Epo, ROR4α, Glut-1, P4Hα and BNIP3, both human and rodent sequences were included in the alignment. We then calculated the residue frequencies at each position of the aligned sequences and compared them with the frequency expected by chance (χ^2 test). This analysis (Table 2) showed that, in addition to those positions corresponding to the [A/G]CGT core, several other positions presented a nucleotide composition significantly different from that expected by chance ($P < 0.05$). From this alignment, we constructed a 33 bp position-specific matrix with the frequencies observed at positions -8 to $+26$ ($+1$ corresponding to the first base of the [A/G]CGT core).

To identify potential regulatory sequences containing an HRE core, we first searched both DNA strands of the 40 kb human EGLN3 locus (-9675 to $+30326$ relative to transcription start) for the presence of the [A/G]CGT motif. This produced a list of 124 sequences for the human locus and 129 for the mouse locus. We then calculated a score for each one of the 33 bp genomic sequences containing an [A/G]CGT core using the position-specific matrix of frequencies (Table 2). The score was calculated as:

$$\left(\sum_{j=-8}^{j=+25} Freq_{ij}\right) - mi$$

where i is the nucleotide (adenosine, cytosine, guanosine or thymidine) at position j of the sequence analysed, and $Freq_{ij}$ is the frequency of nucleotide i at position j of the position-specific matrix of frequencies (read from Table 2, j corresponds to the position column). The positions are relative to the [A/G]CGT core, the first base of this core being (A or G) in position $+1$. Finally, mi is the minimum theoretical score value (5.27) for any sequence containing an [A/G]CGT core.

Thus, while the identification of a potential HRE is based solely on the presence of an [A/G]CGT core, the final score associated with each HRE-containing sequence is based on the comparison of the whole 33 bp sequence with the position-specific matrix (Table 2). For example, the score attributed to the sequence ACAGGGCTACGTGCGCTGCGTGAGGGTGGCAGC (enhancer A, see text for details) will be: $(0.00 + 0.13 + 0.06 + 0.5 + 0.31 + 0.25 + 0.19 + 0.44 + 0.81 + 1 + 1 + 1 + 0.94 + 0.5 + 0.56 + 0.25 + 0.5 + 0.06 + 0.38 + 0.75 + 0.06 + 0.25 + 0.56 + 0.44 + 0.5 + 0.31 + 0.06 + 0.25 + 0.44 + 0.5 + 0.19 + 0.13 + 0.69) - 5.25 = 8.758$.

Table 2 Position-specific frequency matrix

Position indicates the position relative to the first base of the HRE core. A, C, G, T, frequencies of these nucleotides at each position. P is the probability value obtained by comparison of the nucleotide frequency distribution at a determined position with that expected by chance (χ^2 test). Frequencies of nucleotides at the HRE core are shown in bold face.

Position	A	C	G	T	P
−8	0.000	0.630	0.250	0.130	0.003
−7	0.250	0.130	0.500	0.130	0.112
−6	0.060	0.500	0.310	0.130	0.058
−5	0.190	0.060	0.500	0.250	0.090
−4	0.130	0.560	0.310	0.000	0.009
−3	0.130	0.560	0.250	0.060	0.023
−2	0.190	0.190	0.560	0.060	0.029
−1	0.000	0.310	0.250	0.440	0.090
+1	**0.810**	**0.000**	**0.190**	**0.000**	**< 0.001**
+2	**0.000**	**1.000**	**0.000**	**0.000**	**< 0.001**
+3	**0.000**	**0.000**	**1.000**	**0.000**	**< 0.001**
+4	**0.000**	**0.000**	**0.000**	**1.000**	**< 0.001**
+5	**0.000**	**0.060**	**0.940**	**0.000**	**< 0.001**
+6	0.250	0.500	0.130	0.130	0.112
+7	0.000	0.310	0.560	0.130	0.009
+8	0.250	0.250	0.380	0.130	0.572
+9	0.130	0.130	0.250	0.500	0.112
+10	0.380	0.500	0.060	0.060	0.023
+11	0.250	0.380	0.190	0.190	0.682
+12	0.060	0.130	0.750	0.060	**< 0.001**
+13	0.310	0.130	0.500	0.060	0.058
+14	0.190	0.440	0.250	0.130	0.321
+15	0.560	0.250	0.190	0.000	0.015
+16	0.130	0.440	0.440	0.000	0.023
+17	0.130	0.310	0.500	0.060	0.058
+18	0.130	0.440	0.310	0.130	0.212
+19	0.310	0.310	0.310	0.060	0.392
+20	0.060	0.630	0.250	0.060	0.004
+21	0.190	0.190	0.440	0.190	0.392
+22	0.130	0.500	0.250	0.130	0.112
+23	0.190	0.310	0.250	0.250	0.919
+24	0.000	0.440	0.130	0.440	0.023
+25	0.190	0.690	0.000	0.130	0.001

Statistical analysis of data

Experimental data were analysed with the Prism™ GraphPad (version 4.01) software. Data from reporter assays were analysed by the ANOVA test followed by the Tukey test. The P values obtained in these analyses are indicated in the text and Figures. Other statistical tests were applied to specific datasets as indicated in the text (t test and χ^2 test).

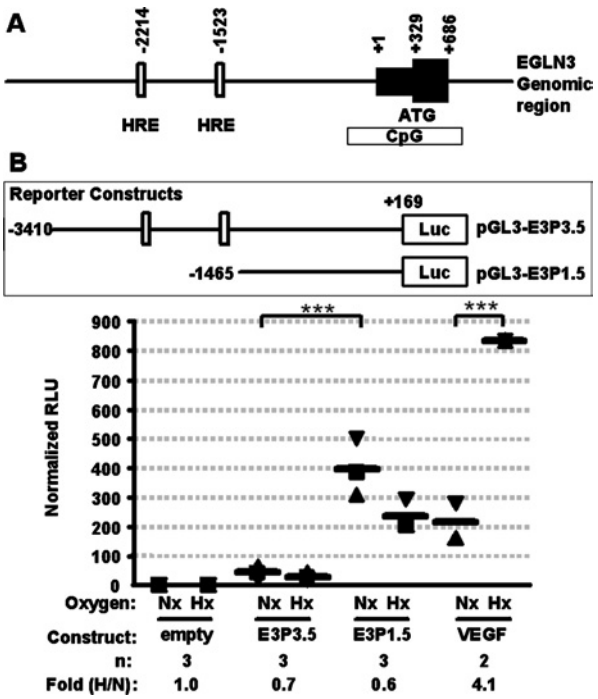

Figure 1 The EGLN3 promoter region is not induced by hypoxia

(**A**) Diagram of the human EGLN3 promoter region, in which the numbers indicate the nucleotide positions relative to the transcription initiation site. Black boxes represent the first EGLN3 exon comprising residues +1 to +686. The small black box corresponds to the 5′-UTR (from +1 to +329) and the large one to the coding sequence (from +329 to +686). CpG, location of a CpG-rich region according to the UCSD Genome Browser (http://genome.ucsc.edu). Open boxes indicate the localization of putative HRE core sequences. The regions indicated, −3410 to +169 and −1465 to +169, in the EGLN3 genomic fragment were cloned into pGL3 basic vectors upstream of the firefly luciferase gene to generate pGL3-E3P3.5 and pGL3-E3P1.5 respectively. (**B**) HeLa cells were transfected with the fragments indicated from EGLN3 or from the VEGF-A promoter cloned into the pGL3 basic reporter plasmid. Upper panel: diagram of the pGL3-E3P3.5 and pGL3-E3P1.5 constructs. Lower panel: after transfection, the cells were cultured under normoxic conditions (Nx) or in an atmosphere of 1 % oxygen (Hx) for 24 h before analysing the luciferase activity. The mean results from '*n*' independent experiments, each performed in duplicate (solid bar) are shown. Each type of symbol represents data from an individual experiment. In order to compare between different experiments, the ratio of firefly and *Renilla* luciferase activities for each sample was normalized to the ratio obtained for the control (empty vector under normoxia). ***, statistically significant differences ($P < 0.001$) between the two indicated samples. The fold induction of hypoxia versus normoxia for each sample is shown.

RESULTS

EGLN3 gene promoter activity is not induced by hypoxia

In order to study the regulation of EGLN3 transcription by hypoxia, we cloned the putative promoter region of the human EGLN3 gene. To this end, we PCR-amplified the region between nt −3410 and +170 (E3P3.5) relative to the EGLN3 transcription initiation site and cloned this upstream to a firefly luciferase reporter gene (Figure 1A). This genomic region contains two putative HRE that match the [A/G]CGT consensus. We then also generated a reporter construct containing the genomic region −1465 to +170 (E3P1.5) that lacks these two putative HREs. Both genomic regions E3P3.5 and E3P1.5 acted as promoters capable of driving luciferase expression above control levels (Figure 1B). Interestingly, stronger promoter activity was observed with the shortest fragment (E3P1.5), suggesting the presence of a negative regulatory sequence in the longer fragment (E3P3.5). However, in contrast with promoters induced by hypoxia (e.g. the VEGF-A promoter), hypoxia did not induce promoter activity of either E3P1.5 or E3P3.5 (Figure 1B). Thus it appears that the regulatory sequences responsible for the induction of EGLN3 expression under hypoxic conditions are not located in this promoter region.

Identification of putative HREs in the EGLN3 genomic region

In view of the results presented above, we reasoned that an enhancer region might be responsible for the induction of EGLN3 by hypoxia. Since enhancers can be located upstream or downstream of and even far from the transcriptional initiation site, we employed a strategy based on bioinformatics − to search for putative HREs in a large genomic region of the human EGLN3 locus (*Homo sapiens* chromosome 14 genomic contig NT_026437.11, gi 29736559:14310000–14350000). This region extends from 9 kb upstream of the transcription initiation site to 3.5 kb downstream of the last exon of the gene. Since the probability of encountering a short motif such as the canonical HRE [A/G]CGT over 40 kb is very large, we first generated an extended HRE consensus motif to screen this genomic fragment. For this, we selected a set of well-characterized hypoxia-induced regulatory sequences from the alignment of the promoters of several hypoxia-induced genes (see the Materials and methods section). Analysis of the sequences revealed that, in addition to the [A/G]CGT core, other positions extending over 33 bp showed a significant degree of conservation ($P < 0.05$, χ^2 test). From this alignment, we generated a 33 bp position-specific probability matrix based on the frequencies observed for the nucleotides at each position of the aligned sequences around and including the HRE core motif (Table 2).

By comparing the nucleotide composition of putative HREs with this position-specific frequency matrix, a score can be assigned to any sequence that will reflect its similarity to known functional hypoxia-responsive sequences. The scoring method used assigned a value of zero to sequences lacking the highly conserved [A/G]CGT core and increasing values to sequences that were more similar to those observed in the known hypoxia-responsive regulatory regions. In this way, the scores obtained for several 33 bp-long sequences containing functional HREs from known hypoxia-induced genes (9.871 ± 1.222, $n = 17$) were significantly higher ($P < 0.0001$, t test) than those obtained for a group of random sequences containing the [A/G]CGT core (5.809 ± 0.772, $n = 17$; Figure 2A).

To identify potential functional HREs, we first identified 33 bp stretches that contained an [A/G]CGT core in the EGLN3 genomic fragment. This analysis resulted in a list of 124 putative hypoxia-responsive sequences. We then calculated the score for each sequence by comparison with the position-specific frequency matrix (see the Materials and methods section for details) and sorted each putative responsive sequence according to its value. Since the hypoxic response of EGLN3 is conserved in rodent cells, we reasoned that the hypoxia-responsive sequence in the human EGLN3 locus should be conserved in the equivalent mouse locus. Thus we also searched for sequences containing HREs along 32 kb of the mouse EGLN3 genomic region (*Mus musculus* chromosome 12 genomic contig NT_039551.3, gi 5176690: 13190000–13158000) in the same manner as for the human sequence. We identified 129 putative murine HREs from this genomic fragment. In order to determine whether any of the putative HREs was conserved between human and mouse, we compared them using the BLAST 2 SEQUENCES program [42]. Instead of comparing each one of the 124 putative human HREs with all the 129 putative mouse HREs, we concatenated all human HREs in a single artificial sequence and compared it with the concatenated mouse HREs in a single step (Figure 2B). Only five sequences were conserved between the two species (Figures 2B

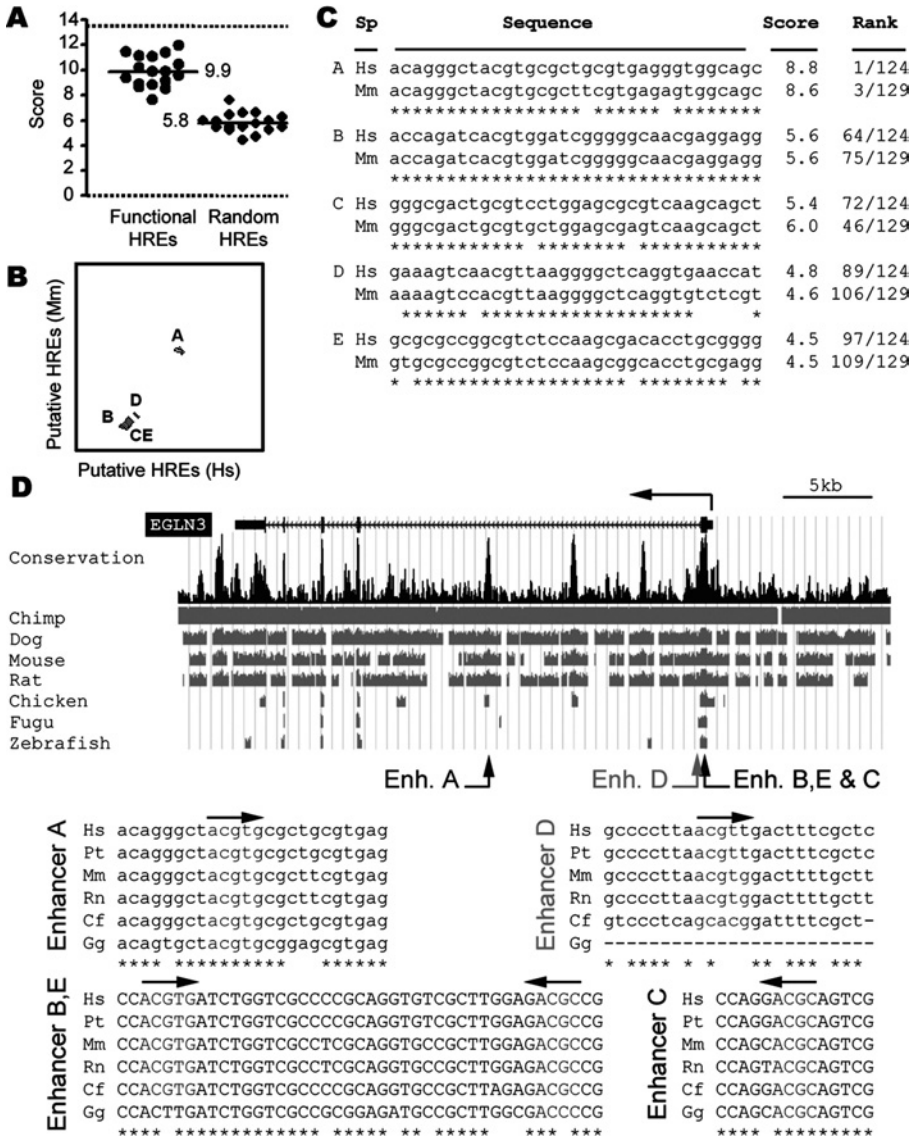

Figure 2 Computer identification of HRE in the EGLN3 locus

(**A**) The data represent the scores obtained by comparing the sequences from VEGF-A, BNIP3, P4Hα, ROR4α, c-Met, PGK1, Glut-1, promoters (functional HREs; ●) or from random sequences containing a core [A/G]CGT motif (random HREs; ▲), to the position-specific frequency matrix. The horizontal line represents the mean value for each group of data and the number represents its value. The difference between their means was statistically significant ($P < 0.001$, t test). (**B**) BLAST comparison of the concatenated 124 putative HRE sequences found in the human 40 kb EGLN3 genomic region versus the concatenated 129 sequences found for the mouse locus. (**C**) Alignment of the five HRE sequences identified in (**B**) that are conserved between human (Hs) and mouse (Mm). The score for each sequence, according to the position-specific frequency matrix, as well as their rank, is shown. (**D**) Genomic region of the EGLN3 locus (adapted from UCSD Genome Browser, http://genome.ucsc.edu). Boxes are open reading frames. The size of the box indicates whether it is UTR or CDS, as indicated in Figure 1. The arrow indicates the direction of transcription. The EGLN3 gene structure, including transcription start site, is based on RefSeq NM_022073. Conservation of sequences among different species is indicated by the black histogram, and the individual homology between different species and human is indicated by grey histograms. The localization of enhancers A–D in the EGLN3 genomic region is indicated by arrows. The sequence conservation of the five putative enhancers between different species is shown; the arrows above the HREs indicate their direction.

and 2C), and these were named enhancers A–E in decreasing order of the scores obtained for each (Figure 2C).

Of these five putative hypoxia-responsive sequences conserved between human and mouse, only enhancer A reached a score similar to that found for other functional HREs (Figures 2C and 2A). In addition, enhancer A had the highest score of all the putative HREs found in the EGLN3 genomic region (Figure 2C). The other four sequences (enhancers B–E) had a score that was significantly below that of the functional HREs (Figures 2A and 2C; $P < 0.01$). We examined the genomic location of these sequences, as well as their conservation across a broad range of species (Figure 2D). Three of the five putative HREs (enhancers B, C and E) were located in the coding sequence of the first exon of human EGLN3 (at positions +563, +453 and +528 respectively), while the other two conserved regions were situated in the first intron of human EGLN3 (enhancer D at +870 bp and enhancer A at +12588 bp), downstream of the transcription initiation site (Figure 2D). Significantly, enhancers A and D were located in a long 400 bp conserved region and such regions, called CNS (conserved non-coding sequences), often correspond to dispersed transcriptional regulatory elements [43].

Closer analysis of the evolutionary conservation of the putative HREs (Figure 2D) revealed that the sequence located at + 12 kb (enhancer A) was highly conserved throughout evolution from birds to humans. In contrast, despite being located in a CNS, the core of the HRE located at + 870 (enhancer D) was not conserved in dog or chicken (Figure 2D). Since the other three sequences (enhancers B, C and E) were located in the coding sequence of EGLN3, their conservation might reflect the need to maintain the protein structure of EGLN3 rather than a conserved regulatory function. Even so, the core HRE of enhancers B and E was not conserved in chicken (Figure 2D).

In summary, through this bioinformatics approach, we identified an evolutionarily conserved region (enhancer A) containing a putative hypoxia-inducible sequence in the first intron of the EGLN3 gene.

Enhancer A is up-regulated by hypoxia in an HRE-dependent manner

In order to probe the functionality of enhancer A, we cloned the sequence + 12411 to + 12836 (containing the CNS) from the human EGLN3 gene upstream of a minimal promoter to drive luciferase expression in a reporter construct (Figure 3A). The expression of luciferase from this construct was strongly induced under hypoxic conditions, increasing by a mean of 11.9-fold (Figure 3B, $P < 0.001$ when comparing normoxic versus hypoxic values). In contrast, the other putative HRE-containing sequences derived from the EGLN genomic region (Figure 3A), such as enhancer D (+ 738 to + 1099; Enh.D), those located in the promoter regions (− 1685 to − 1418 and − 2298 to − 1418; Prom.1 and Prom.2 respectively), and those from the coding sequence (enhancers B, C and E) were unable to induce luciferase expression in response to hypoxia (Figure 3B and results not shown). To confirm that the induction in response to hypoxia observed with the enhancer A construct was mediated by the putative HRE in this sequence, we generated a mutant construct in which the central ACGT core was mutated to TAGC (Enh. A*). This mutant construct was no longer able to induce luciferase expression in hypoxic conditions (Figure 3B). Thus these results indicate that enhancer A contains a functional HRE.

We therefore examined whether enhancer A was able to drive hypoxic gene expression in the context of its natural relative location. Hence, we cloned the enhancer A sequence into the pGL3-E3P3.5 construct (Figure 1A), downstream of the luciferase gene (Figure 3C). Reporter assays with this construct revealed that enhancer A was able to increase luciferase expression in response to hypoxia (a mean of 2.4-fold, $P < 0.001$) regardless of its position relative to the promoter (Figure 3C). Furthermore, in these experiments, enhancer A increased transcription from the natural EGLN3 promoter.

HIF1α binds to the enhancer A sequence *in vivo*

For the enhancer A sequence identified to be a true HRE, it must be recognized by HIF *in vivo*. To test this possibility, we performed ChIP experiments on HeLa cells grown under normoxic or hypoxic (1 % oxygen) conditions (Figure 4). After treatment, chromatin was immunoprecipitated with anti-HIF1α antibodies and the bound DNA was identified by PCR amplification with primers specific for enhancer A (primers 6 and 7, Table 1), enhancer D (primers 8 and 9, Table 1) or the hypoxia-induced region of the P4Hα (primers 15 and 16, Table 1) as a control. Under hypoxic conditions, endogenous HIF1α bound to the HREs in enhancer A and the P4Hα promoter, but not to that in enhancer D (Figure 4). Moreover, both in the case of the control P4Hα promoter and that of enhancer A, HIF1α binding was strongly diminished under normoxic conditions (Figure 4). These results suggested that enhancer A is indeed a functional regulatory sequence that responds to hypoxia *in vivo*.

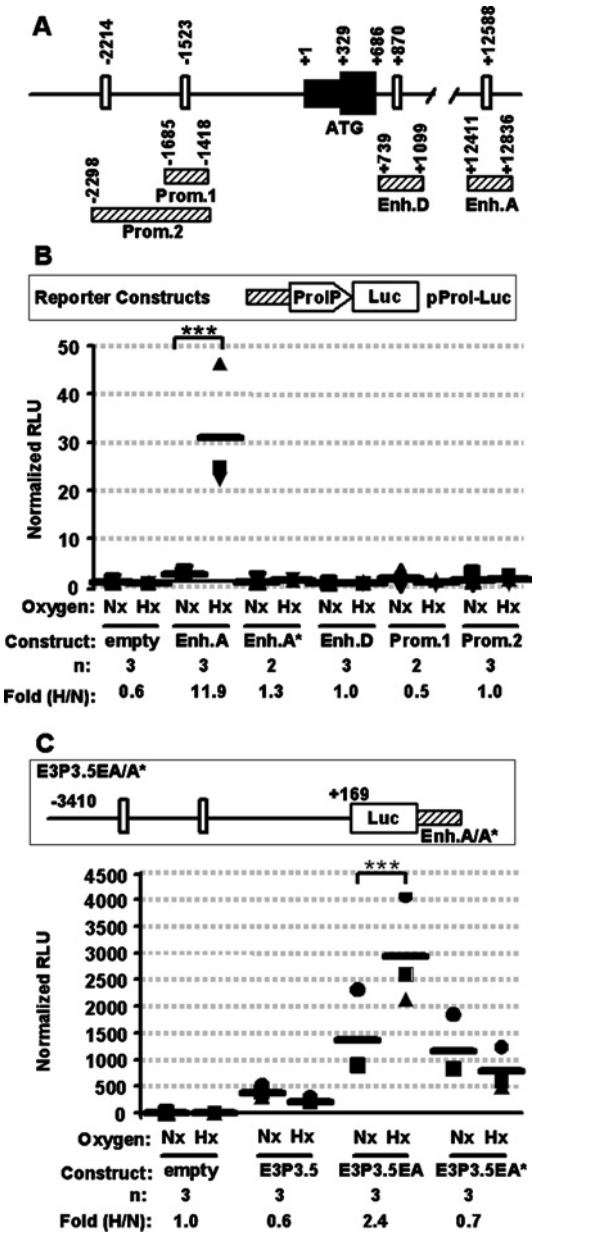

Figure 3 Enhancer A is induced by hypoxia

(**A**) Diagram of the *H. sapiens* EGLN3 genomic region in which the numbers indicate nucleotide positions relative to the transcription initiation site, as shown in Figure 1. The indicated regions (hatched boxes) containing putative hypoxia-responsive sequences were cloned into the pProI plasmid upstream of the rat minimal prolactin promoter. (**B**) HeLa cells were transfected with the fragments from the EGLN3 genomic region indicated, cloned upstream of a minimal promoter (open pointed box). A diagram of the constructs is depicted in the Figure, in which the hatched box represents any of the elements from Figure 3(A). After transfection, the cells were cultured under normoxic (Nx) conditions or in an atmosphere of 1 % oxygen (Hx) for 24 h before analysing the luciferase activity. Enh. A* is a reporter construct derived from enhancer A (Enh. A) in which the core ACGT was mutated to TAGC. The data are presented as indicated in Figure 1. (**C**) The enhancer A sequence, or its mutated form, was cloned into pGL3-E3P3.5 (see Figure 1A) downstream of the firefly luciferase gene as depicted in the Figure (hatched box). HeLa cells were transfected with the indicated constructs and treated as in (**B**). The data are presented as indicated in Figure 1.

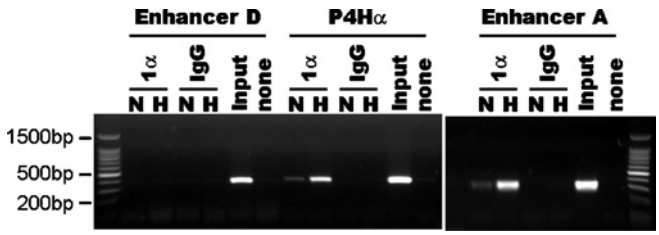

Figure 4 HIF1α binds to enhancer A *in vivo*

HeLa cells were cultured under normoxic (N) or hypoxia (H) conditions for 12 h and then fixed to cross-link proteins to DNA. Cells were lysed and their DNA fragmented by sonication. Cell lysates were then immunoprecipitated with control IgGs (IgG) or a polyclonal antiserum raised against HIF1α (1α). Co-immunoprecipitated DNA was then amplified by PCR with primers specific for enhancer A (primers 6 and 7, Table 1), enhancer D (primers 8 and 9, Table 1) or collagen proline-4 hydroxylase α promoter (P4Hα; primers 15 and 16, Table 1). Input, sample of fragmented genomic DNA before immunoprecipitation. None, PCR without template. The experiment was repeated three times yielding similar results.

HIF is necessary and sufficient to induce enhancer A

It is known that the induction of EGLN3 by hypoxia is mediated by HIF [20,22]. The results we presented above suggest that enhancer A located at + 12 kb inside the first intron of the EGLN3 gene was the regulatory region responsible for the induction of EGLN3 in response to hypoxia. Therefore we studied whether HIFα was necessary to induce enhancer A-mediated transcription by analysing the response to hypoxia of constructs driven by enhancer A in Ka13 Chinese-hamster ovary cells. These cells are deficient in HIF activity due to their incapacity to express the HIF1α and HIF2α genes [24]. For comparison, these constructs were also transfected into a Ka13-derived cell line, 4.5 cells, in which the HIF deficit is complemented by the expression of HIF1α. [24]. Of these lines, the activity of enhancer A was only induced by hypoxia in the 4.5 cells that expressed functional HIF (Figure 5A). We then determined whether HIF was sufficient to induce enhancer A by transfecting HeLa cells with the enhancer A reporter constructs in combination with a plasmid encoding an oxygen-independent HIF1α construct. This mutant HIF is resistant to oxygen-mediated degradation due to the mutation of Pro^{402} and Pro^{564} to Ala. [20]. HIF1α expression was sufficient to induce wild-type enhancer A activity under conditions of normoxia, but not the activity of the HRE-mutant enhancer A construct (Figure 5B). Similar results were obtained with constructs where enhancer A was situated downstream of the firefly luciferase reporter gene (results not shown). Thus HIF1α did appear to be necessary and sufficient to induce EGLN3 enhancer activity.

DISCUSSION

The adaptive response to hypoxia mediated by HIF involves the induction of a large number of genes, including those required for anaerobic glycolysis and angiogenesis. HIF also mediates the induction of EGLN1 and EGLN3, both involved in its regulation, which is indicative of the existence of a negative feedback loop to maintain strict control over HIF activity.

Here, we describe the identification of an evolutionarily conserved enhancer sequence responsible for the hypoxia-mediated induction of EGLN3. Interestingly, this enhancer was located in the first intron of the EGLN3 gene, more than 12 kb downstream the transcription initiation site. It is well known that enhancers are capable of influencing transcription at such a distance from the promoter, regardless of their relative position with respect to the transcription initiation site (upstream or downstream). However, this is unusual among hypoxia-regulated genes in which most of the HREs described lie in the promoter region of the gene regulated, usually within 1 kb of the transcription initiation site. An important exception is the hypoxia-inducible sequence of the erythropoietin gene that is located in its 3′-UTR (3′-untranslated region) at 3 kb downstream of the transcription initiation site [32]. Many models for 'action at a distance' have been proposed [44]. In most of the cases, it is supposed that the DNA between the enhancer and the promoter loops out to allow the activator proteins bound to the enhancer to come into contact with proteins (including the general transcription machinery) bound to the promoter. In fact, this model has been described for the Epo gene [45]. The binding of Sp1 and Smad3 transcription factors to Epo promoter is required for efficient hypoxic induction through the HRE located at the 3′-UTR gene region. This co-operation is due to physical interaction between these transcription factors and HIF1α. The physical contact between the upstream promoter and the 3′ downstream enhancer is mediated by Sp1 and Smad3 factors, and would occur upon bending of the DNA intervening sequences [45]. Hence, a similar mechanism might explain the

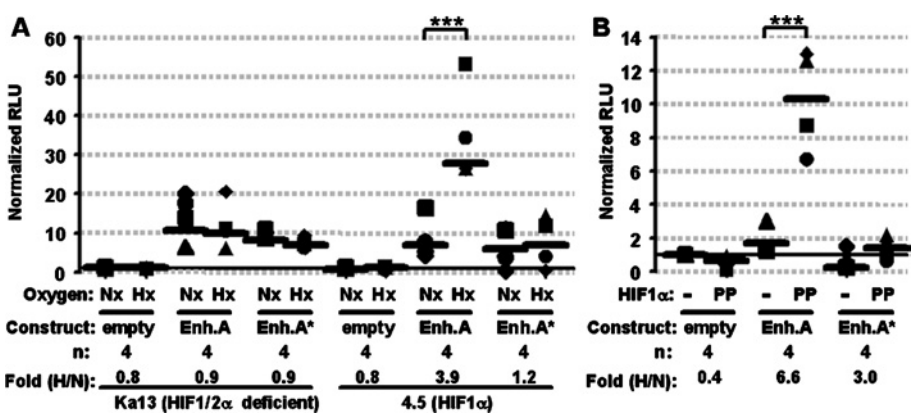

Figure 5 HIFα is necessary and sufficient to induce enhancer A activity

(**A**) HIF1α-deficient Ka13 cells or HIF1α-competent 4.5 cells were transfected with the constructs indicated (see Figure 3A for details). After transfection, the cells were cultured under normoxic conditions (Nx) or in an atmosphere of 1 % oxygen (Hx) for 24 h before analysing the luciferase activity. Results are represented as indicated in Figure 1. (**B**) HeLa cells were transfected with the indicated constructs derived from enhancer A (see Figure 3A for details) together with a plasmid encoding a stable HIF construct (HIF PP). After transfection, the cells were cultured under normoxic conditions for 48 h before analysing the luciferase activity. Results are represented as indicated in Figure 1.

regulation of the EGLN3 gene by HIF acting through the enhancer described herein.

Although we cannot rule out the possibility that other sequences exist which control the induction of EGLN3 by hypoxia, the one described here is likely to be functionally relevant because endogenous HIF1α binds to it *in vivo* in an oxygen-dependent manner (Figure 4). In agreement with our data (Figure 1), a region upstream of the transcription initiation site of the Sm20 gene, the rat orthologue of the EGLN3, was shown to have promoter activity dependent on Sp1/Sp3 sites [23]. Moreover, a negative regulatory region was observed between -3800 and -1100 of the rat promoter that seems to be conserved in the human promoter (Figure 1). However, while Sm20 (EGLN3) is known to be induced by hypoxia in rat cells [21], how this promoter is regulated by hypoxia and whether functional hypoxia-inducible sequences exist is still unclear [23]. Thus our results are the first to explain the mechanism behind EGLN3 induction at low oxygen tensions and constitutive HIF activity.

The atypical location of the HRE within the EGLN3 locus, and its distance from the transcription initiation site, made its identification difficult by conventional biological approaches. The successful identification of a hypoxia-regulated sequence by adopting a bioinformatics approach corroborates the power of these strategies in the search for regulatory regions in genomic sequences. We first generated a quantitative consensus sequence (position-specific frequency matrix) through the alignment of several hypoxia-regulated sequences. This consensus allowed us, not only to identify putative HREs, but also to sort them according to their similarity to known functional HREs. We then investigated the evolutionary conservation of these putative HREs since the evolutionary conservation of regulatory sequences is of paramount importance to identify biologically relevant regulatory sequences [43]. Here, the combination of both approaches resulted in the identification of a functional HRE. Significantly, the position-specific frequency matrix described in this work might aid in the future identification of hypoxia-regulated regions in other genes.

One further conclusion from our work is that the core HRE ([A/G]CGT) is necessary, but not sufficient, to confer oxygen sensitivity. This is supported by the fact that several sequences containing this core were not able to drive luciferase expression in response to hypoxia (Figure 3) or to bind HIF1α *in vivo* (Figure 4 and results not shown). These results are in agreement with previous studies of other hypoxia-inducible promoters [30]. Therefore sequence requirements other than the core HRE are probably required for the efficient recruitment of HIF1α. This might explain why the alignment of functional HREs showed that, in addition to the [A/G]CGTG core, the frequency of nucleotides at other specific positions was significantly greater than that expected by chance. Conserved sequences other than the core HRE in hypoxia-inducible sequences have been identified previously [37,46], although these often differed and do not match with that described here. Nevertheless, it is probable that, in addition to the HIF-binding site, these conserved sequences play a major role in HIF-mediated transcription. In agreement with this, mutation of these abolishes the hypoxic response of the isolated regulatory sequences [37,46]. Finally, the conservation of residues might also indicate that factors other than HIF are required for hypoxic induction of these sequences. In fact, for some genes, it has been shown that the binding of transcription factors such as AP-1 [47] or Sp1 [37] is required for the HIF-mediated response to hypoxia.

In summary, we have identified, by a combination of bioinformatics and biological approaches, a CNS in the first intron of the EGLN3 gene that binds HIF1α *in vivo* and drives transcription *in vitro* in response to hypoxia. Thus we conclude that this enhancer region, located 12 kb downstream of the transcription initiation site, is responsible for the HIF-mediated hypoxic induction of EGLN3.

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