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6-Phosphofructo-2-kinase (*pfkfb3*) Gene Promoter Contains Hypoxia-inducible Factor-1 Binding Sites Necessary for Transactivation in Response to Hypoxia*

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The up-regulation of glycolysis to enhance the production of energy under reduced pO_2 is a hallmark of the hypoxic response. A key regulator of glycolytic flux is fructose-2,6-bisphosphate, and its steady state concentration is regulated by the action of different isozymes product of four genes (*pfkfb1–4*). *pfkfb3* has been found in proliferating cells and tumors, being induced by hypoxia. To understand the organization of *cis*-acting sequences that are responsible for the oxygen-regulated *pfkfb3* gene, we have studied its 5′-flanking region. Extensive analysis of the 5′ *pfkfb3* promoter sequence revealed the presence of putative consensus binding sites for various transcription factors that could play an important role in *pfkfb3* gene regulation. These DNA consensus sequences included estrogen receptor, hypoxia response element (HRE), early growth response, and specific protein 1 putative binding sites. Promoter deletion analysis as well as putative HREs sequences (wild type and mutated) fused to a c-*fos* minimal promoter unit constructs demonstrate that the sequence located from −1269 to −1297 relative to the start site is required for hypoxia-inducible factor 1 (HIF-1) induction. The effective binding of HIF-1 transcription factor to the HREs at −1279 and −1288 was corroborated by electrophoretic mobility shift assay and biotinylated oligonucleotide pull-down. In addition, HIF-1α null mouse embryo fibroblasts transfected with a full-length *pfkfb3* promoter-luciferase reporter construct further demonstrated that HIF-1 protein was critically involved for hypoxia transactivation of this gene. Altogether, these results demonstrate that *pfkfb3* is a hypoxia-inducible gene that is stimulated through HIF interaction with the consensus HRE site in its promoter region.

In eukaryotic cells exposure to a low oxygen environment induces a hypoxic response pathway through a hypoxia-inducible transcription factor (HIF)[1] (1). The active transcription factor is a heterodimeric protein complex composed of two subunits HIF-1α and HIF-1β. This dimer recognizes the hypoxia response element (HRE; 5′-ACGTG-3′) present in hypoxia-inducible promoters. The HIF-1β is a constitutively expressed protein, whereas the α subunit is rapidly degraded in normoxic conditions through the ubiquitin-proteasome system (2). The protein that initiates this degradation process is the tumor suppressor VHL (Von Hippel-Lindau), which is the recognition component of an E3 ubiquitin-protein ligase complex that targets HIF-1α for proteasomal degradation when HIF-1α prolines −564 and −402 are hydroxylated (3–5). This hydroxylation process is controlled by specific Fe^{2+}, oxoglutarate, and oxygen-dependent hydroxylase enzymes. Thus, stabilization of HIF-1α is induced by oxygen deficiency, allowing its nuclear translocation and dimerization with HIF-1β (6). Chelating or substituting Fe^{2+} with deferoxamine (7) and cobalt chloride (8), respectively, or inhibiting oxoglutarate with dimethyloxalylglycine reduces the hydroxylase activity and mimics the hypoxia effects.

There is now substantial evidence in support of the hypothesis that HIF-1 functions as a mediator of the adaptive response to hypoxia. Among all of the adaptations, transcriptional activation of genes associated to metabolism is of special interest. Many of these target genes promote cellular adaptation to reduced oxygen availability by increasing glucose uptake and glycolysis. Several genes encoding enzymes of the glycolytic pathway and glucose transport are activated by low pO_2: aldolase-A, phosphoglycerate kinase-1, pyruvate kinase M, lactate dehydrogenase A, phosphofructokinase L, and glucose transporter-1 (Glut-1) (1, 9–11).

High glycolytic flux is essential for tumor growth in hypoxic conditions, and many transformed cells display a high rate of glycolysis that is maintained even under aerobic conditions (Warburg effect) (12). Glycolytic flux is mainly controlled by 6-phosphofructo-1-kinase (13), with fructose-2,6-bisphosphate (Fru-2,6-P_2) being its most powerful allosteric activator (14, 15). These properties confer to this metabolite a key role in the control of the glycolytic pathway. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) is the bifunctional enzyme that catalyzes the synthesis and degradation of Fru-2,6-P_2 and hence critically regulates carbohydrate metabolism (14–16).

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[1] The abbreviations used are: HIF-1, hypoxia-inducible factor 1; Glut-1, glucose transporter-1; DFO, deferoxamine; HRE, hypoxia response element; E3, ubiquitin-protein isopeptide ligase; Fru-2,6-P_2, fructose-2,6-bisphosphate; PFK-2, 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase; mEF, mouse embryo fibroblast; DMEM, Dulbecco's modified Eagle's medium.

Four independent genes, *pfkfb1–4*, code for the different isoforms of the PFK-2 family. These isoforms show differences in their tissue distribution and kinetic properties in response to allosteric effector, hormonal, and growth factor signals (17). The *pfkfb3* gene product has the highest kinase/phosphatase activity ratio (18). This implies that in tissues where it is expressed, elevated Fru-2,6-P_2 levels are maintained, and consequently high glycolytic rates are sustained. Significantly, *pfkfb3,* is a ubiquitous gene constitutively expressed in proliferating tissues (19–24), in transformed cell lines (19, 25, 26), and in various tumors (27).

The present study characterizes the 5′-flanking region of the *pfkfb3* gene and demonstrates its transcriptional regulation by HIF-1. Our data provide evidence that the consensus binding site located at −1279 and −1288 in the *pfkfb3* promoter is necessary for stimulation of this gene by hypoxia.

MATERIALS AND METHODS

Cell Culture—Human glioblastoma T98G and U-87 cell lines were obtained through the American Type Culture Collection (Manassas, VA). Mouse embryo fibroblasts (mEF wild type (+/+) or (+) and HIF-1α deficient (−/−) or (−) cell lines) were kindly provided by Dr. R. S. Johnson (University of California at San Diego, La Jolla, CA) (31). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Invitrogen), L-glutamine, and antibiotics and incubated in a humidified atmosphere of 10% CO_2 at 37 °C. Hypoxia simulation conditions were achieved by growing cells in DMEM supplemented with 200 μM deferoxamine (DFO), 200 μM $CoCl_2$, or 1 mM dimethyloxalylglycine (kindly provided by Peter Ratcliffe, Oxford, UK). For hypoxic treatment, cell culture plates were incubated in a modular incubator chamber (Billup-Rothenburg, Forma Scientific, Marietta, OH) and flushed with a gas mixture containing 2% O_2, 5% CO_2. Whole extracts were prepared from cultured cells as described below.

Quantitative Real Time PCR—Quantitative real time PCR was performed on RNA extracts from T98G cells. Total RNA was prepared from cultured cells under hypoxia (2% oxygen) (see above for details) for 0, 3, and 6 h. RNA was isolated according to the manufacturer's protocol (Ultraspec RNA Biotecx Laboratories). The concentration and purity of all RNA samples were determined using the $A_{260\ nm}/A_{280\ nm}$ ratio and formaldehyde gel electrophoresis. Five micrograms of total RNA was reverse transcribed using a Ready-To-Go First Strand Kit from Amersham Biosciences, using random primers. *pfkfb3* and *Glut-1* were specifically amplified by real time PCR using the probe/primer set (Hs0019079_ml) for human *pfkfb3* (NM_004566) and (Hs00197884_ml) for Glut-1 (NM_006516) (Applied Biosystems). The threshold cycle number (C_t) was obtained, and the relative quantity of the specific mRNA in each sample was calculated using the standard curve generated with the same primer/probe set on T98G total RNA. The relative expression of each gene was normalized to that of the TATA-binding protein gene (probe/primer: Hs99999910_ml), and gene expression in each sample was then compared with expression in basal conditions (T98G cells under hypoxia for 0 h).

HIF-1α RNA Interference Experiments—To knock down HIF-1α expression in U-87 cells, an expression vector (pCEP4; Invitrogen) containing small interference RNA oligonucleotides against HIF-1α mRNA was transfected into U-87 cells followed by selection in hygromycin (40 μg/ml)-containing medium. The vector was constructed by replacing the SV40 promoter with a cassette containing the U6 promoter and cDNA sequences corresponding to nucleotides 1543–1561 (NM-001530) to generate a looped small interference RNA against the human HIF-1α mRNA. Control cells were generated by using a similar vector containing scrambled cDNA sequences.

Plasmid Constructions—To expand up to 3566 the promoter region already available in our laboratory, we used a PCR-amplified fragment obtained from the cosmid clone CRI-JC2015 and subcloned into the previously published *PFKFB3*/−1198 pGL2-basic luciferase reporter construct (35). Primers utilized were named: *Fw-3661* 5′-GAACGTTTTAACCTGGCTATGGCTGGCACA-3′ (from −3661 to −3632) and *revoligo* 5′-CGTCTCCTTTCCCGGCCCTCGCAGTTT-3′ (from −994 to −1020). The fragment obtained was used as a substrate for a nested PCR reamplification using the *primer oligo BglII-Fw* 5′-GGAGTTAGAT*ct*CATTGGCTGGCAC-3′ (from −3572 to −3548) and the *primer revoligo* (lowercase letters indicate nucleotides changed to introduce restriction sites in the amplified fragment). PCR was performed at 95 °C for 2 min and then 35 cycles of 30 s at 95 °C, 30 s at 65 °C, 3 min at 72 °C, and 10 min at 72 °C. The reamplified fragment was cloned in a TOPO-TA vector (Invitrogen). A 2490-nt BglII-BglII fragment from this construct (corresponding to region from nucleotides −3566 to −1076 of the *pfkfb3* promoter) was subcloned in *PFKFB3*/−1198. The BglII-BglII common region of the 123-nt fragment was previously deleted from *PFKFB3*/−1198 construct. The new recombinant plasmid was named *PFKFB3*/−3566. *PFKFB3*/−1407 was generated by redigestion of the *PFKFB3*/−3566 construct using ApaI and subsequent religation. Positive clones were completely sequenced at both strands, using the dye terminator cycle sequencing kit (PerkinElmer Life Sciences), following the manufacturer's instructions. The reaction products were analyzed on a PerkinElmer ABI PRISM 377 automated DNA sequencer. *PFKFB3*/−1198, *PFKFB3*/−938, and *PFKFB3*/−148 constructs of the *pfkfb3* promoter cloned into the pGL2-basic vector (Promega) with the firefly luciferase gene as a reporter have been described previously (35). A pGL2-basic vector with the c-*fos* minimal promoter unit served as the basis for reporter constructs 29Wt-cfos and 29-Mut-cfos. The fragment of 29 nt from *pfkfb3* promoter (from −1297 to −1269) with the sequence: 5′-GCATGCGGG**ACGTG**ACGC**ACGTG**TGGCAG-3′ (containing the two putative HRE (marked in bold type)) was subcloned to obtain the 29Wt-cfos construct. The same 29 nucleotide fragment with a mutation in two base pairs in each of the HIF-1-binding sites (underlined) (5′-GCATGCGGGAATTGAGCGAATTGTGGCAG-3′) was used for the 29Mut-cfos construct. The identity of cloned products was confirmed by nucleotide sequence analysis. A green fluorescent protein plasmid that codes for the green fluorescent protein was used to monitor transfection efficiency

Transfections and Luciferase Assays—Transfections were performed using polyethylenimine or Lipofectamine 2000 (Invitrogen) and DMEM following the supplier's protocols. The different promoter-reporter fusion plasmids (1 μg) and 60 ng of the pSV40-β-galactosidase control vector (Promega, Madison, WI) were co-transfected into cells. Four hours later, the cells were washed twice with phosphate-buffered saline and maintained in DMEM (basal condition). For hypoxia stimulation assays, transfected cells were maintained in DMEM with 200 μM DFO (Sigma) or 200 μM cobalt chloride (Aldrich) or hypoxia (2% O_2) for 16 h. At the times indicated, the cells were washed twice with phosphate-buffered saline and lysed in lysis buffer. Luciferase activity was measured in supernatant extracts. Co-transfection with pSV-β-galactosidase plasmid DNA was carried out to normalize transfection efficiencies in different transfectants. The transfections were performed at least in triplicate, and the individual values were averaged to give the result of one experiment. The protein content of each sample was determined using a BCA protein kit (Pierce). Luciferase activity was measured in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). β-Galactosidase activity was determined in 3 μl of cell extract using the luminescent β-galactosidase Clontech detection kit II (Clontech, Palo Alto, CA). The data are presented as the means ± S.E. At least three separate experiments with each plasmid DNA preparation were performed.

Analysis of exogenous HIF-1α and HIF-1β protein effects on *pfkfb3* promoter constructs were performed using co-transfection experiments. For these co-transfection experiments, *PFKFB3*/−3566 and pcDNA3-HA-HIFα(401Δ603) and/or pARNT constructs were transfected into T98G using various amounts of each plasmid such that all cells received a total of 1 μg plasmid DNA. HA-HIFα(401Δ603) and pARNT pcDNA3 protein expression vectors contain an oxygen-dependent degradation domain deleted HIF-1α and HIF-1β c-DNA human sequences, respectively, downstream of a cytomegalovirus promoter. Both constructs were kindly provided by F. Bunn and L. E. Huang (Harvard Medical School, Boston, MA).

Western Blot Analysis—Total cellular protein was obtained from T98G cells using 500 μl of lysis buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol with 2% SDS), scraped, and heated for 5′ at 95 °C. 30 μg of total cellular protein were separated on 8% SDS-polyacrylamide gel under reducing conditions and then transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was probed with a specific polyclonal antibody against the C terminus of uPFK-2 (26) at 1:50 dilution and visualized with horseradish peroxidase-conjugated sheep anti-rabbit antibody. α-Tubulin and HIF-1α were detected using monoclonal anti-α-tubulin (Calbiochem) and anti-HIF1-α (Transduction Laboratories) antibodies (1:250 dilution), respectively. Bound antibody was visualized with horseradish peroxidase-conjugated donkey anti-mouse. The antigen-antibody complexes were developed by enhanced chemiluminescence using ECL (Amersham Biosciences).

Isolation of Whole Cell Extracts for Gel Retardation Assay—Whole cell extracts were isolated from either untransfected (basal condition) or 48 h after transfection with HIF-1α(401Δ603) and HIF-1β protein ex-

pression vectors. Briefly, T98G cells were washed twice with chilled phosphate-buffered saline and harvested by scraping using 500 μl of cold phosphate-buffered saline and then pelleted by centrifugation at 150 $\times$ *g* for 5′. The cell pellet was resuspended with 50 μl of an extraction solution containing 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 25% glycerol. The cells were broken by passing the cell suspension through a 25-gauge needle at 4 °C. The homogenate was centrifuged at 12,000 $\times$ *g* for 15′ at 4 °C. The clear supernatant was used as whole cell extract for gel retardation assay. The cell extracts were stored at −80 °C. Protein concentrations were measured spectroscopically using Bio-Rad protein reagents.

Gel Retardation Assay (Electrophoretic Mobility Shift Assay)—Double-stranded oligonucleotides were prepared by mixing equal amounts of complementary single-stranded DNAs in 50 mM NaCl, heating to 70 °C for 15 min, and cooling at room temperature. Oligonucleotides utilized contained the sequence from −1297 to −1269 of the *pfkfb3* gene promoter, which include two consensus HRE boxes (in bold type, sequence of sense strand): 5′-GCATGCGGG**ACGTG**ACGC**ACGTG**TGGCAG-3′ and a mutated consensus (nucleotides underlined) 5′-GCATGCGGGAATTGAGCGAATTGTGGCAG-3′.

The sequence of the control oligonucleotide EPO-1 is: 5′-GCCCTACGTGCTGTCTCA-3′. The annealed oligonucleotides were labeled with ^{32}P in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions were carried out in a reaction mixture containing 5 μg of whole protein extract, incubated in the presence of binding buffer (100 mM Tris, pH 7.9, 250 mM NaCl, 50% glycerol, and 5 mM dithiothreitol) with 1 μg of poly(dI-dC)-poly(dI-dC) for 10 min at room temperature. ^{32}P-Labeled DNA probes (50,000 cpm) where added for 30 min at room temperature. In reactions including antibodies, rabbit polyclonal antibody against HIF-1β protein (Abcam, Novus-Biologicals) was added and incubated for 30 min at room temperature with the reaction mixture. The samples were immediately separated using low ionic strength 4% polyacrylamide to analyze for DNA-protein complex. A bromphenol blue-xylene cyanol dye solution was added to empty spaces flanking the sample wells to provide an indication of the electrophoresis process. The gels were electrophoresed at 20 mA.

Biotinylated Oligonucleotide Precipitation Assay—Six hours after 2% O_2 hypoxia, normoxia, or $CoCl_2$ treatment, T98G cells were lysed with HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, and 0.25% of Nonidet P-40) containing protease and phosphatase inhibitors. Supernatants were collected after 10 min of centrifugation. The cell extracts were incubated with 1 μg of biotinylated double-stranded oligonucleotides (containing the sequence from −1297 to −1269 of *pfkfb3* gene promoter) and 10 μg of poly-(dI-dC)-poly(dI-dC) for 16 h. DNA-bound proteins were collected with streptavidin-Sepharose beads (Amersham Biosciences) for 1 h, washed with HKMG buffer, and separated on a SDS-polyacrylamide gel. Human anti-HIF-1α antibody was used to identify the DNA-bound proteins by Western blot.

Computerized Search for Nuclear Factor-binding Sites in the pfkfb3 Promoter—Potential nuclear factor-binding sites were found using the TRANSFAC library of binding sites and MatInspector software. Notably, the following TRANSFAC consensus sequences were identified among others: cEBP, (C/T)AA(C/T); early growth response, GCGCGGGCG; estrogen receptor, GGTACAnnnTGACC; HRE, ACGTG; NFKB, GGGACTCTC; specific protein 1, GG(G/C)(C/G)GG.

Data Analysis and Statistics—Fold of induction values obtained from different promoter fragments constructs were compared using an analysis of variance previous logarithmic transformation of data values, after testing their normal distribution. Comparison among the different constructs was performed applying a Scheffe's multiple comparison test. $\alpha = 0.05$ was selected as statistically significant level. A two-sample *t* test was used to compare fold induction obtained from 29-Wt-cfos and 29Mut-cfos constructs.

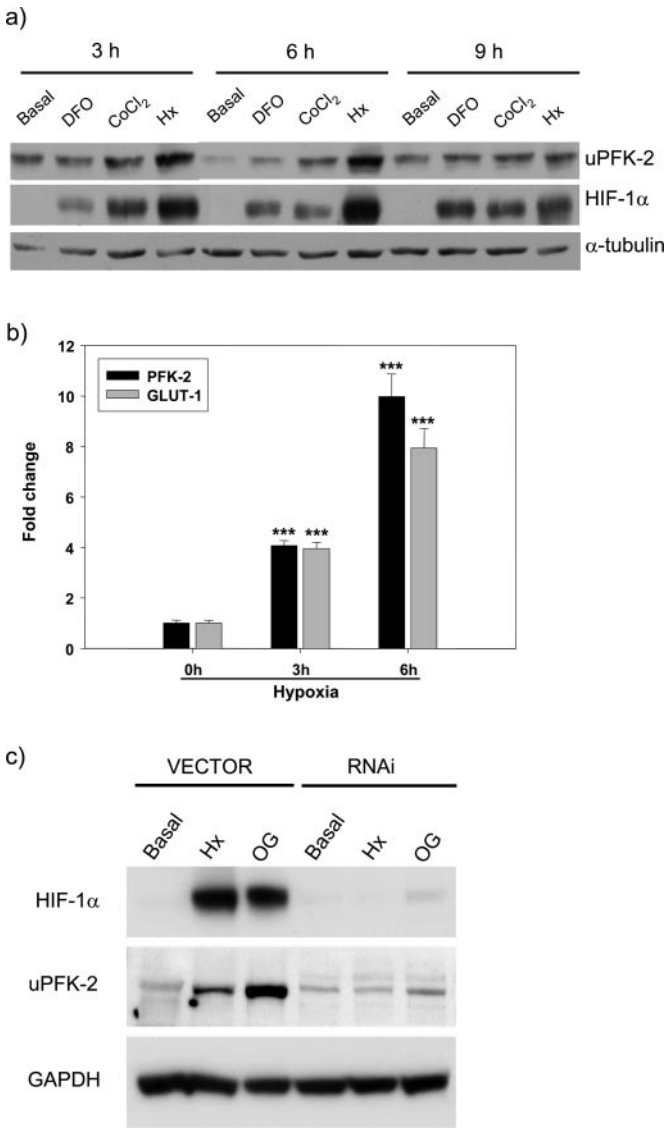
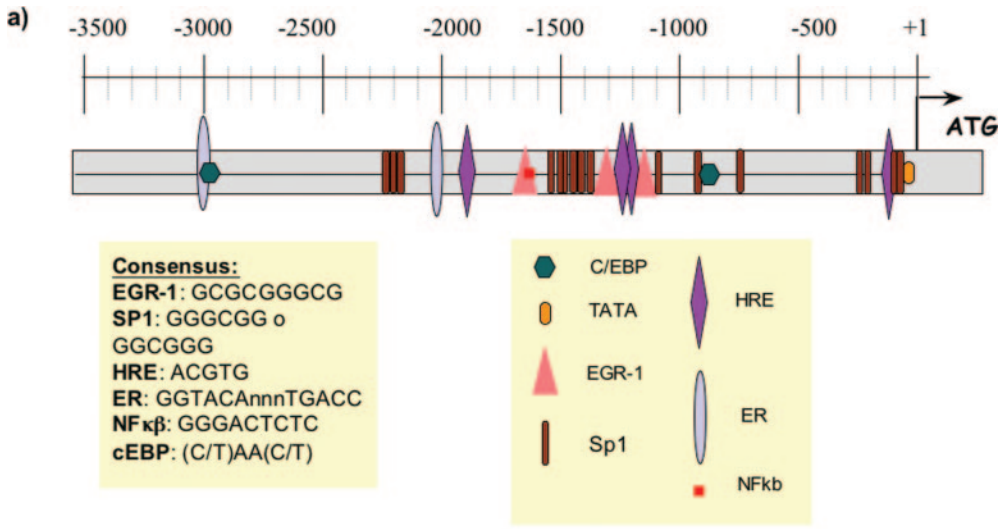


FIG. 1. **Expression of HIF-1 and *pfkfb3* in glioblastoma cell lines.** *a*, Western blot against uPFK-2 and HIF-1α from T98G whole cell extracts maintained in normoxia (basal) or incubated for 3, 6, and 9 h in the presence of 200 μM DFO, 200 μM cobalt chloride ($CoCl_2$), and hypoxia (2% O_2; *Hx*). Antibody against α-tubulin was used for loading normalization. *b*, real time PCR analysis of RNA isolated from T98G cells after 0, 3, or 6 h of hypoxia (2% O_2). The *black bars* represent hypoxic samples of *pfkfb3* RNA, and the *gray bars* represent hypoxic samples of Glut-1 RNA. The data represent the fold change *versus* 0 h hypoxia (normoxia), and the values are normalized to TATA box-binding protein (means ± S.E., $n = 3$ for each condition). Statistically significant effects ($p < 0.001$, ***) of hypoxia were observed compared with normoxia conditions. *c*, expression of *pfkfb3* in HIF-1α RNAi suppressed cells. Western blot against uPFK-2 and HIF-1α of cell extracts from U-87 cells transfected with either a control vector or a HIF-1α RNAi vector exposed for 16 h to 2% O_2 (hypoxia), 1 mM dimethyloxalylglycine (*OG*), or maintained in normoxia (*Basal*). Antibody against glyceraldehyde-3-phosphate dehydrogenase was used as loading normalization.

RESULTS

Effect of Deferoxamine, Cobalt Chloride, Dimethyloxalylglycine, and Hypoxia on uPFK-2 Expression in Glioblastoma Cells—To examine the effects of hypoxia on *pfkfb3* gene expression, we used hypoxia (2% O_2) and the hypoxia-mimics: cobalt chloride ($CoCl_2$), a transition metal, DFO, an iron chelator, and dimethyloxalylglycine, a cell-permeable competitive inhibitor of oxoglutarate. These reagents inhibit HIF-1 prolyl hydroxylases activity (7). Human glioblastoma T98G and U-87 cell lines were chosen because of positive gene expression for *pfkfb3* (22). Western blot results from T98G (Fig. 1*a*) show the accumulation of HIF-1α subunit after 3, 6, and 9 h of treatment with deferoxamine, cobalt chloride, or hypoxia. Similarly, uPFK-2 isozyme expression increased significantly in the same conditions. To assess mRNA levels in hypoxia conditions, quantitative real time PCR was used. As shown in Fig. 1*b* hypoxia treatment produced significant increases on *pfkfb3* mRNA levels, around 10-fold at 6 h. Similar results were achieved using *Glut-1*, another HIF target, as a positive control of hypoxia response. These results indicate that hypoxia and hypoxia mimics produce an increase of



b)

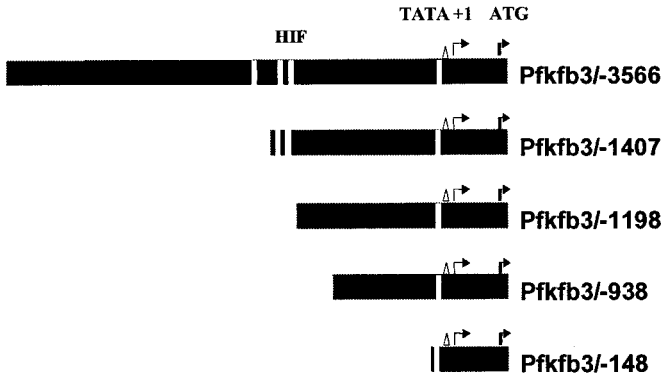
Position, bp	HRE sequence
-107	CCCC**ACGTG**GAAG
-1279/-1288	GG**ACGTG**AGCG**ACGTG**
-1902	CTGT**ACGTG**TGCA

FIG. 2. **Analysis of the 5′ *pfkfb3* promoter region.** *a*, putative regulatory elements in the *pfkfb3* gene promoter. *b*, compilation of HIF-1-binding sites.

uPFK-2 levels in this cell line, as was described for Hep-3B cells (11). Moreover, to evaluate the direct effect of endogenous *pfkfb3* gene as an HIF target, expression of *pfkfb3* was assayed in HIF-1α RNAi suppressed cells. Fig. 1*c* shows Western blot analysis from HIF-1α RNAi suppressed U-87 cells exposed to hypoxia or dimethyloxalylglycine. When using these cells, a complete correlation between the inhibition of HIF-1α expression and a decrease on uPFK-2 levels was observed, whereas levels of uPFK-2 increased in the control-vector transfected cells. Taken together, these results demonstrate the importance of HIF-1α subunit in the transcription regulation of endogenous *pfkfb3* gene in glioblastoma cells.

Identification of Sequences Required for Hypoxia-inducible Transcription from the pfkfb3 Promoter—We next focused our attention on detailed *pfkfb3* promoter analysis, being of particular interest the search for putative HIF-binding sequences. For this purpose, we obtained various fragments from the promoter region and constructed luciferase expression vectors containing up to 3566 bp of the 5′-flanking region of the human *pfkfb3* gene. The fragments were obtained using PCR amplification of the BAC cosmid CRJ2015 (containing the whole *pfkfb3* promoter region). As shown in Fig. 2*a*, an extensive analysis of 5′ *pfkfb3* promoter sequence, using the computer data base TRANSFAC, version 3.2, revealed the presence of several putative consensus binding sites for various transcription factors likely relevant in *pfkfb3* gene regulation. These DNA consensus sequences included estrogen receptor, HRE, early growth response, and specific protein 1-binding sites. Among all the putative response sequences found in the *pfkfb3* promoter region, estrogen receptor and HRE are of special interest because of the implication of these factors in the transcriptional regulation of the gene. Fig. 2*a* also indicates the presence of DNA-binding sites containing the core consensus sequence for other transcription factors that have been implicated in the induction of numerous genes in response to hypoxia. Most notable are sequences for NF$\kappa\beta$, specific protein 1, and cEBP. In Fig. 2*b* the positions of hypoxia response element sequences located in the *pfkfb3* promoter are shown. A total of four HREs that are 100% homologous to the consensus HIF binding site (-ACGTG-) are present. The previously reported analysis of published *pfkfb3* promoter included a −1198-bp region that contained a putative HRE at position −107. Spanning the region of study up to 3566 bp revealed interesting new potential hypoxia binding sites at positions −1279, −1288, and −1902 that appeared to be good candidates to contribute to the *pfkfb3* stimulation by hypoxia.

Response of Nested Deletions of the 5′-Flanking Region of pfkfb3 Gene Promoter to Deferoxamine and Cobalt Chloride Treatments—To delimit the promoter region mediating activation by $CoCl_2$ or DFO, different fragments of the *pfkfb3* promoter were generated and cloned upstream of a luciferase reporter vector. T98G cells were transiently transfected with these reporter constructs and 60 ng of β-galactosidase expression vector to normalize transfection efficiencies. For each construct, the fold increase in luciferase activity elicited by either $CoCl_2$ or DFO treatment was determined over basal luciferase activity from each promoter construct. As shown in Fig. 3, luciferase activities from cells transfected with constructs larger than −1407 nucleotides (*PFKFB3*/−3566 and *PFKFB3*/−1407) and treated with DFO and $CoCl_2$ are comparable and significantly higher than those transfected with constructs shorter than −1407 nt (*PFKFB3*/−1198, *PFKFB3*/−938, and *PFKFB3*/−148). Thus, 25 and 15-fold inductions were observed for *PFKFB3*/−3566 and *PFKFB3*/−1407 constructs, respectively, after DFO treatment. Similarly, 59- and 37-fold inductions were obtained for the same constructs, respectively, after $CoCl_2$. No statistically significant differences were found among the two longest constructs. Results using *PFKFB3*/−1198 construct give 3- and 3.5-fold in DFO and $CoCl_2$ treatments, respectively. Finally, DFO treatment of the smallest constructs, *PFKFB3*/−938 and *PFKFB3*/−148, produced 50 and 30%, respectively, of the luciferase activity observed in its basal conditions. Similar results were obtained following treatment with $CoCl_2$: 90% using *PFKFB3*/−938 and 1.6-fold induction in *PFKFB3*/−148. Statistical analysis revealed that *PFKFB3*/−1198, *PFKFB3*/−938, and *PFKFB3*/−148 were not



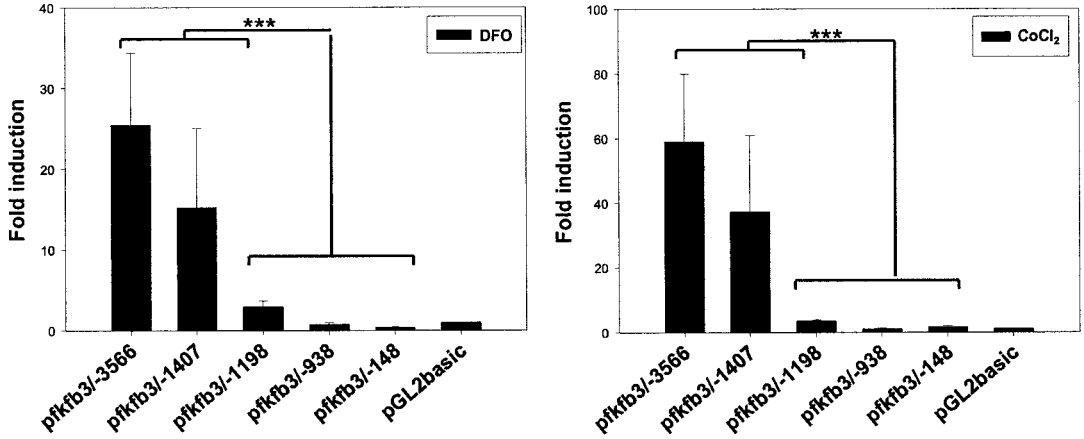


FIG. 3. ***pfkfb3* promoter sequences mediate transcriptional responses to hypoxia.** The diagram shows the structures of the pGL2-basic luciferase expression vectors containing various lengths of 5′-flanking regions of the human *pfkfb3* gene promoter. The reporter constructs and 60 ng of β-galactosidase expression vector were transiently transfected into T98G cells. The cells were incubated for 16 h with DFO and $CoCl_2$ or maintained with DMEM. Transfections were performed at least in triplicate, and the individual values were averaged to give the result of one experiment. In each experiment, the individual data were calculated as the means of at least triplicates and expressed as the ratio of luciferase to β-galactosidase activity measured in the same cell lysate. The results are the means ± S.E. for at least three independent experiments. The results are expressed as fold of induction compared with corresponding basal (normoxia) condition. *PFKFB3*/−3566 and *PFKFB3*/−1407 have a significant effect of $p < 0.001$, compared with *PFKFB3*/−1198; *PFKFB3*/−938 and *PFKFB3*/−148 constructs. Nonstatistically significant effects were observed between *PFKFB3*/−3566 and *PFKFB3*/−1407 or among *PFKFB3*/−1198, *PFKFB3*/−938 and *PFKFB3*/−148.

significantly different. Thus, when constructs containing the putative HRE located at −1279 and −1288 were compared with the smallest constructs, statistically significant differences were obtained ($p < 0.001$). Fold induction results obtained with *PFKFB3*/−1198, *PFKFB3*/−938, and *PFKFB3*/−148 were negligible, pointing out that the first 5′ −1198 nt of the *pfkfb3* promoter are not essential in the hypoxic response. In addition given that *PFKFB3*/−3566 and *PFKFB3*/−1407 responses are not significantly different, the putative HRE located at −1902 may be not relevant for physiological hypoxic response in the *pfkfb3* gene.

Effect of Overexpression of Exogenous HIF Protein on the pfkfb3 Gene Promoter—To test whether exogenous HIFα overexpression could cause the same stimulatory effects on the *pfkfb3* promoter as those observed with DFO or $CoCl_2$ treatments, HIF subunits expression vectors were assayed. *PFKFB3*/−3566 promoter construct was co-transfected with pcDNA3-HA-HIFα(401Δ603) and/or pcDNA3-HIF1β and 60 ng of β-galactosidase expression vector. The pcDNA3-HA-HIFα-(401Δ603) construct has a deletion of the entire oxygen-dependent degradation domain of the α-subunit. This deletion allows complete stabilization of HIF-1α under normoxic conditions. Consequently an accumulation of the overexpressed α-subunit is achieved. Co-transfection of *PFKFB3*/−3566 with the deleted HIF-1α showed a 31-fold in luciferase activity in contrast to the 12-fold of *PFKFB3*/−3566 observed upon treatment with $CoCl_2$ (Fig. 4). On the other hand, no significant additive stimulation was observed when HIF-1α(401Δ603) and HIF-1β were co-transfected with *PFKFB3*/−3566, indicating that the levels of endogenous HIF-1β are sufficient for full stimulation when cells are expressing constitutively active HIF-1α.

Searching the Functional HRE in the Human pfkfb3 Promoter—The region from −1269 to −1297 seemed to be a good candidate to contain the major HIF responsive element because of the proximity of the two HRE sequences. To test the hypoxia-responsiveness of this region, we used a 29-nt fragment of the *pfkfb3* in front of a c-*fos* minimal promoter unit in a luciferase reporter vector. This fragment, encompassing −1269/−1297, relative to the transcription start site, was used to create reporter constructs 29Wt-cfos and 29Mut-cfos. Constructs 29Wt-

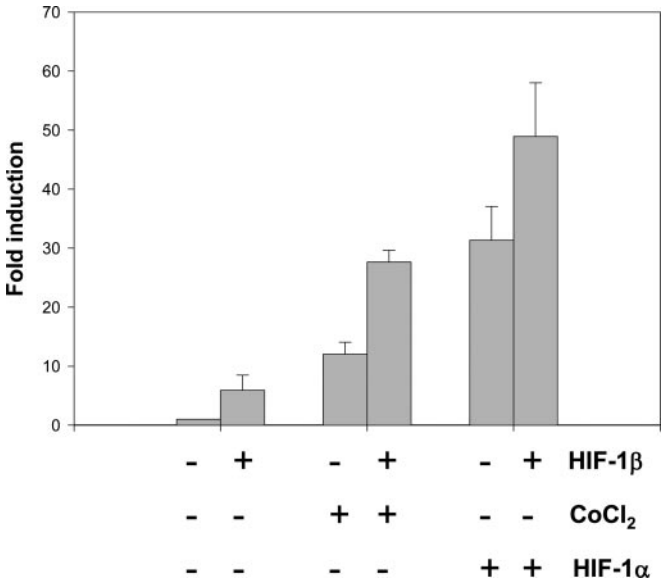


FIG. 4. **Effect of exogenous HIF-1α overexpression on *PFKFB3*/−3566 promoter fragment.** T98G cells were transiently transfected with *PFKFB3*/−3566 construct. When indicated cells were co-transfected with 330 ng of pCDNA3-pARNT (*HIF-1β*). Some cells were co-transfected with 330 ng of p(HA)HIF-1α(401Δ603) (*HIF-1α*). The total amount of transfected DNA was kept at 1 μg by addition of empty vector. Four hours after transfection the medium was changed, and the cells were cultured in serum free medium in the absence or presence of 200 μM of $CoCl_2$ for 16 h. Transfections were performed at least in triplicate, and the individual values were averaged to give the result of one experiment. In each experiment, the individual data were calculated as the means of triplicates and expressed as the ratio of luciferase to β-galactosidase activity measured in the same cell lysate. The results are the means ± S.E. for at least three independent experiments. The results are expressed as fold of induction compared with *PFKFB3*/−3566 basal (normoxia) condition.

cfos and 29Mut-cfos are identical except that the latter contains a mutation in two base pairs in each of the HIF-1-binding sites.

To study the enhancer activity, we measured relative luciferase units of T98G cells transfected with 29Wt-cfos or 29Mut-cfos constructs and 60 ng of β-galactosidase expression vector to normalize transfection efficiencies, in the presence or absence of DFO or $CoCl_2$. As shown in Fig. 5*a*, although wild type and mutated constructs display similar basal reporter activities, a 294- or 330-fold increase was detected in the 29Wt-cfos construct following DFO or $CoCl_2$ treatment. Therefore, as expected, the wild type construct was induced by hypoxia but not the mutated construct. To corroborate the importance of HIF-1-binding sites located at −1279 and −1288, we designed another approach using either a wild type mEF cell line or one with a deletion of the HIF-1α gene (31). Transient transfection of 29Wt-cfos construct showed a 16-fold induction following treatment with $CoCl_2$ in the mEF/HIF (+) cell line whereas no induction was observed in mEF/HIF (−) cells. As expected, transient transfection of 29Mut-cfos construct in both models did not show any increase in luciferase activity over the basal response (Fig. 5*b*). Altogether these results demonstrate the direct implication of HIF to these HREs for *pfkfb3* hypoxia response.

HIF-1 Binds to the HRE Sequences Located at −1279 and −1288 in the pfkfb3 Promoter—To unequivocally demonstrate the binding of HIF-1 proteins to these consecutive HRE sequences, two different approaches were undertaken. First, a probe consisting of 29 nucleotides from the *pfkfb3* promoter that contains the HREs was used in an electrophoretic mobility shift assay together with whole cell extracts overexpressing HIF-1α (401Δ603) and HIF-1β. As shown in Fig. 6*a*, a slow migrating band doublet appeared only in the presence of HIF-1α (401Δ603) and HIF-1β (*lane 2*) but not in the presence of labeled 29-ntMut probe (*lane 4*), suggesting the formation of specific DNA-protein complexes that were not present in normoxia (*lane 1*). Hypoxia-induced DNA-protein complexes were supershifted in the presence of an antibody against HIF-1β (*lane 3*, indicating that HIF-1 can bind to the *pfkfb3* promoter sequence 5′-GCATGCGGG**ACGTG**ACGC**ACGTG**TGGCAG-3′). As a positive control, HIF-1-overexpressing cell extracts were analyzed by electrophoretic mobility shift assay using a probe containing the wild type HIF-1-binding site from the erythropoietin gene (28). As expected, a slow migrating doublet was also detected (*lane 5*) and HIF-1 binding was supershifted in the presence of anti-HIF-1β antibody (*lane 6*). Second, another approach consisted of an oligonucleotide pull-down assay using T98G cell extracts obtained after 6-h normoxia (basal), hypoxia, and $CoCl_2$ treatments. As shown in Fig. 6*b*, Western blot showed the presence of HIF-1 complexes in the streptavidin-Sepharose beads incubated with hypoxic and $CoCl_2$ extracts, whereas no complexes were observed in the normoxic extracts. Taken together, these results show that HIF-1α does bind to the HRE consensus sequence from −1297 to −1269 of *pfkfb3* promoter under hypoxia.

pfkfb3 Expression Analysis on an HIF Knockout Model—The importance of HIF-1 in the hypoxic response of *pfkfb3* gene promoter was also studied in the mEF cell line with a deletion of the HIF-1α gene (31). In hypoxic conditions an increase of *pfkfb3* mRNA was detected in the HIF (+/+) cells, whereas no changes were seen in the HIF-1 (−/−) cells (11). Similar results were obtained by Western blot (data not shown). Furthermore when the *PFKFB3*/−3566 reporter construct was transiently transfected into mEF/HIF-1 (−/−) and mEF/HIF (+/+) cell lines, and the cells were then exposed to hypoxia or maintained in normoxia. Luciferase activities measured 16 h later indicated a substantial induction in hypoxic mEF/HIF (+/+) cells, whereas no significant increase was observed in hypoxic mEF/HIF (−/−) cells (Fig. 7). Thus, HIF-1 is necessary to activate the transcription of the *pfkfb3* gene in response to hypoxia.

DISCUSSION

The ability to respond to differential levels of oxygen is important to all respiring cells. The most ancient adaptation to hypoxia is the Pasteur effect, which includes decreased oxidative phosphorylation and an increase in glycolysis (13). One of the best known mechanisms that switches induction of different glycolytic isozymes is through HIF-1α stabilization (30). HIF-1 is a critical integrator of cellular adaptation to hypoxia, and HIF-1α null cells show physiologically significant alterations in energy metabolism (31). It is likely that HIF-1, because of its role in regulating glycolysis, is also a primary mediator of the Warburg effect, in which tumor cells show increased glycolytic activity under physiological oxygen conditions (12).

Previous studies have provided evidence for the induction of glycolytic enzyme gene expression via *cis*-acting DNA sequences containing putative HIF-1-binding sites (30). Sequence analysis revealed the presence of several putative HREs within the −3566 nucleotides of the human *pfkfb3* promoter, which could explain the described effect of hypoxia on the induction of *pfkfb3* gene (11, 29). Luciferase expression showed that the two longest constructs (*PFKFB3*/−3566 and *PFKFB3*/−1407) exerted the maximal hypoxic response, pointing out the major contribution of nucleotides over −1407 bp. Preceding studies on HRE-binding sites of glycolytic genes such as enolase-1, lactate dehydrogenase A, and phosphoglycerate mutase-1 revealed that the hypoxia response elements contained a pair of contiguous HIF-1 binding sites separated by 4–10 bp (30). Also our results indicate that putative HRE at

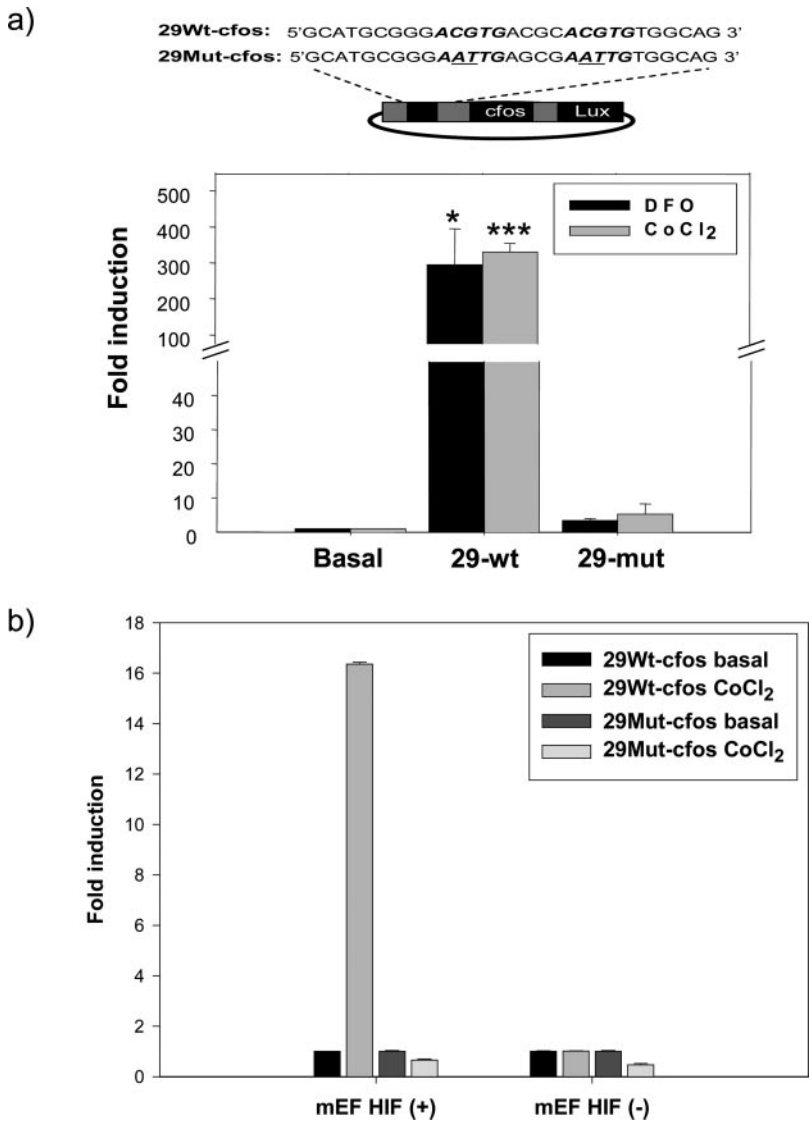


FIG. 5. **Enhancer activity of the region containing two HRE consensus sequences of the *pfkfb3* gene promoter.** *a*, scheme of the wt and mutated 29-nt region (−1269/−1297) subcloned as an enhancer in a luciferase reporter pGL2-basic containing the c-*fos* minimal promoter unit (cfos-pGL2-basic). 29Wt-cfos and 29Mut-cfos resulting constructs are identical except that the latter contains a mutation in two base pairs in each of the HIF-1-binding sites (nucleotides underlined). HRE sequences are indicated in bold. T98G cells were incubated with DMEM or DMEM supplemented with 200 μM of $CoCl_2$ or 200 μM of DFO after transfection. Transfections were performed at least in triplicate, and the individual values were averaged to give the result of one experiment. In each experiment, the individual data were calculated as the means of at least triplicates and expressed as the ratio of luciferase to β-galactosidase activity measured in the same cell lysate. The results are the means ± S.E. for at least three independent experiments. The results are expressed in folds of induction compared with basal condition. *b*, 29Wt-cfos and 29Mut-cfos constructs were transiently transfected in mEF cells. Four hours after transfection cells were maintained for 24 h with DMEM, 10% fetal bovine serum and split into a 12-well plate. Luciferase activity of the wild type HIF (+) or knockout HIF (−) cells was measured following incubation with 200 μM of $CoCl_2$ or with DMEM during 16 h. Transfections were performed at least in triplicate, and the individual values were averaged to give the result of one experiment. In each experiment, the individual data were calculated as the mean of at least triplicates and expressed as the ratio of luciferase to β-galactosidase activity measured in the same cell lysate. The results are the means ± S.E. for at least three independent experiments. Luciferase activity is expressed as fold of induction compared with basal condition.

−1902 is not significant for hypoxic response because *PFKFB3*/−3566 and *PFKFB3*/−1407 folds of induction were not statistically different. Having in mind these data, we have specially focused attention on the region around −1269 to −1297 because it contains two HRE sequences adjacent and separated by 4 nt (Fig. 2). This area could be particularly interesting to establish the *cis*-acting DNA sequences (HREs) required for HIF-1 binding and transcriptional response to hypoxia. Confirmation of the direct implication of HIF consensus binding sites spanning from −1269 to −1297 comes from transfection experiments utilizing 29Wt-cfos and 29Mut-cfos constructs of the human *pfkfb3* promoter in T98G cell line, showing a high hypoxia response in the wild type construct, whereas the 29Mut-cfos had no effect. Furthermore, similar transfections of 29Wt-cfos construct in a wild type mouse embryo fibroblast, mEF/HIF (+) cell line or one with a deletion of the HIF-1α gene, mEF/HIF(−) (31), resulted in lost of induction in mEF/HIF (−), indicating that this 29-nt sequence is essential for the *pfkfb3* hypoxic response.

The implication of the HRE sequence in the binding to HIF-1 complex was corroborated by electrophoretic mobility shift assay and biotinylated oligonucleotide pull-down. Shifted bands were detected in the HIF-1α(401Δ603) and HIF-1β overexpressed whole cell extracts, and a supershifted band was also detected after the incubation of the probe with an anti-HIF-1β antibody. Moreover, HIF-1 binding to the same sequence was also detected by Western blot after precipitation with streptavidin-Sepharose beads. Altogether, these results demonstrate that *pfkfb3* is a hypoxia-inducible gene that is stimulated in highly transformed cell lines through HIF factor interaction with the consensus HRE sites located at −1279 and −1288 of the promoter region.

To confirm unequivocally the importance of HIF-1 complex, *pfkfb3* gene expression was induced with the use of the transactivating factors HIF-1α(401Δ603) and HIF-1β. No significant differences in luciferase activities were observed when transfecting HIF-1α(401Δ603) alone or co-transfected with HIF-1β. Thus, T98G cells demonstrate sufficient endogenous HIF-1β to fully complement overexpressed HIF-1α(401Δ603) (the non-oxygen-dependent HIF-1α subunit), agreeing with results previously published on enolase-1 (30). Furthermore, mEF cells knockout for HIF-1α were analyzed with luciferase responses to transient transfection experiments. The use of mEF/HIF (−/−) cells let us demonstrate not only the lack of *pfkfb3* protein induction in the absence of HIF-1α subunit but also the need of an active HIF-α factor to achieve the *pfkfb3* promoter regulation. The small differences found in hypoxia *pfkfb3* induction using nested deletions of the 5′-flanking region (constructs larger than −1407) (Fig. 3) and in transfected cells with *PFKFB3*/−3566 promoter (mEF/HIF (−/−)) (Fig. 6) could be

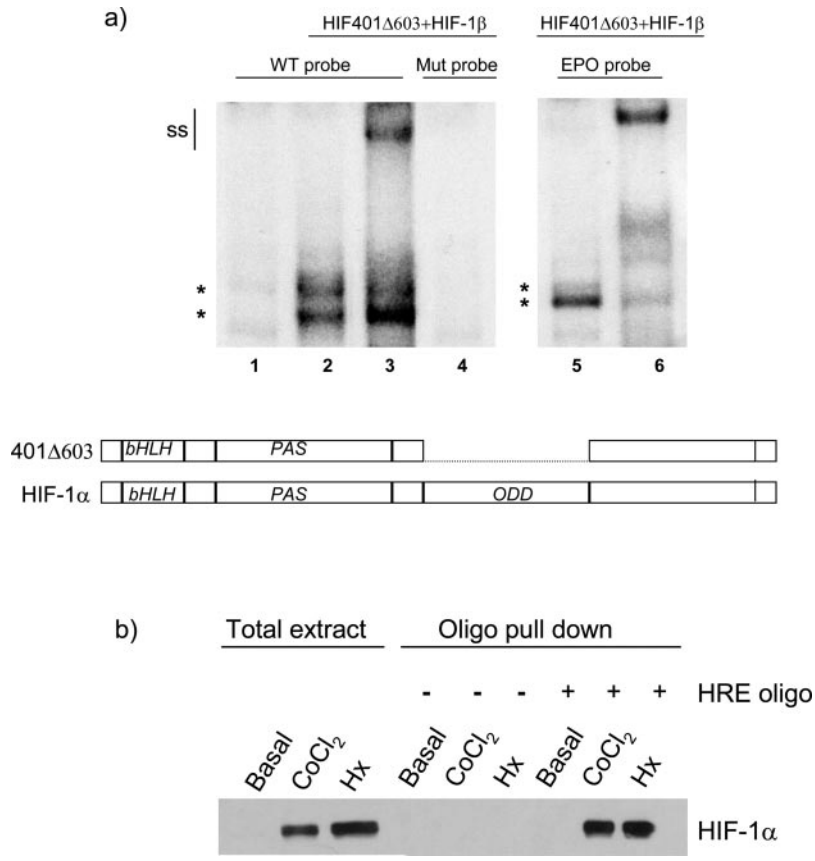


FIG. 6. **Specificity of HIF-1 binding to the *pfkfb3* promoter sequence.** *a*, T98G cells were transfected with pHA-HIF-1α(401Δ603) and pARNT (HIF-1β) (*lanes 2–6*) or cultured in DMEM supplemented with 10% fetal bovine serum (*lane 1*). 4 h after transfection, the cells were maintained for 48 h with DMEM, 10% fetal bovine serum. Whole cell extracts were then prepared and analyzed with electrophoretic mobility shift assay using ^{32}P-labeled oligonucleotides containing the putative HRE of the *pfkfb3* promoter (5′-GCATGCGGG**ACGTG**ACGC**ACGTG**TGGCAG-3′; −1269/−1297). Supershift assays were performed using anti-HIF-1β antibody (*lanes 3* and *6*). The 29 Mut oligonucleotide was used as a probe in *lane 4* (5′-GCATGCGGGAATTGAGCGAATTGTGGCAG-3′). EPO-1 was used as a probe in *lanes 5* and *6* (5′-GCCCTACGTGCTGTCTCA-3′). *b*, oligoprecipitation of a HRE complex containing HIF-1α. Biotinylated HRE (5′-GCATGCGGG**ACGTG**ACGC**ACGTG**TGGCAG-3′) (HRE-oligo) was incubated with normoxia (basal), $CoCl_2$, or hypoxia-induced (*Hx*) T98G cell extracts for 16 h. DNA-bound protein was precipitated using streptavidin-Sepharose beads for 1 h. The collected pellets were analyzed by Western blot using an antibody against HIF-1α.

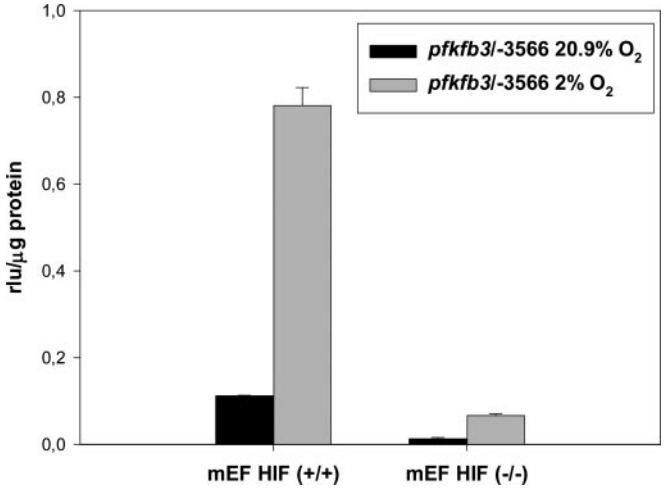


FIG. 7. ***pfkfb3* promoter induction in wild type (HIF (+/+)) or knockout (HIF (−/−)) mouse embryo fibroblast cells.** *pfkfb3*/−3566 luciferase expression vector was transiently transfected in mouse embryo fibroblast cells, and luciferase activity of the wild type HIF (+/+) or knockout HIF (−/−) cells was measured following incubation at 20.9% O_2, (normoxia) or 2% O_2, (hypoxia) for 16 h. Luciferase activity is expressed as relative units/μg of protein.

the consequence of binding to other sequences of other transcription factors, which could cooperate to achieve a high level of expression.

It seems clear that the coordinate induction of glycolytic enzymes occurs in hypoxic cells, and it is mediated at the transcriptional level by HIF-1. This could imply an increase in the flux of glycolytic pathway such that ATP generation is maximized. The specific role of induction of *pfkfb3* by hypoxia must be related with its key function on PFK-1 stimulation, one of the few multimodulated enzymes (32). PFK-1 is mainly inactive in the cell in the absence of allosteric modulators, and the main role of Fru-2,6-P_2 is to relieve its ATP inhibition, allowing glycolysis to proceed (33). The enzyme responsible of its synthesis and breakdown, PFK-2, is regulated, in addition to transcription, by phosphorylation through AMP-dependent protein kinase at Ser-461, increasing its V_{max} without changing the K_m (34). As a consequence, Fru-2,6-P_2 increases. Other putative phosphorylation sites for protein kinase A and C have been described (18), although these covalent modifications have not been reported *in vivo*. The *pfkfb3* gene product is present in proliferative (19–24) and transformed cells (19, 25–26) and various tumors (27). The high kinase/bisphosphatase activity ratio of this isozyme can explain the high Fru-2,6-P_2 found in the cells where it is present, which in turn sustains high glycolytic rates (33). There is evidence of up-regulation of its expression, in addition to hypoxia, in response to different stimuli such as progesterone (20), serum (35), insulin (26), or proinflamatory molecules (19). The *pfkfb3* gene seems to play an important role sustaining the high glycolytic flux of hypoxic or proliferative cells (19, 31, 36–38).

In summary, we have performed a detailed analysis of the *pfkfb3* promoter demonstrating that oxygen-regulated function depends upon HIF-1-binding sites. Having in consideration that the activation of HIF-1 complex is a critical response in hypoxic conditions and that *pfkfb3* has been found overexpressed in many tumors (27), it may provide a novel approach as a target for the development of new therapeutic strategies.

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