Rapid Communication

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Identification and Characterization of the Hypoxia-Responsive Element of the Human Placental 6-Phosphofructo-2-Kinase/ Fructose-2,6-Bisphosphatase Gene

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The placenta-type isozyme of human 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (HP2K, identical to PFKFB3) is expressed in a variety of cells and tissues such as placenta, brain, testis, liver, kidney, skeletal muscle, primary blood mononuclear cells and cancer cells. We observed previously that the enhancer region of the HP2K gene, which has been identified in the 5′-flanking region between –1265 and –1329, could respond to serum stimulation following the transfection of human choriocarcinoma BeWo cells with HP2K promoter-luciferase constructs. The HP2K enhancer region also contains two copies of the hypoxia-inducible factor-1 (HIF-1) binding motif (5′-ACGTG-3′). In this study we performed characterization of the HP2K gene expression in response to hypoxic conditions. Both electrophoretic mobility shift and co-transfection assays of the HP2K promoter-luciferase reporter with HIF-1 expression vectors indicated that HIF-1 binds to the hypoxia-responsive element (HRE) of HP2K, thereby upregulating its gene expression. In addition, we demonstrated using site-directed mutagenesis that a complete tandem repeat of the HIF-1 binding motif with a 4-bp interruption is required for full induction of HP2K expression (up to 22-fold) under hypoxic conditions, and that this response is much stronger than that of the erythropoietin (EPO) gene. These results suggest that the sequence 5′-ACGTGNNNNACGTG-3′ in the HP2K enhancer is the authentic HRE consensus motif that mediates increased transcription, under hypoxic conditions, via HIF-1.

Key words: bifunctional enzyme, gene expression, glycolysis; HIF-1, hypoxia, isozyme.

The expression of a number of glycolytic isozymes (*e.g.* PFK-L, ALD-A, ALD-C, PGK-1, ENO-1, and LDH-A) is induced by hypoxia in a tissue-specific manner (*1*, *2*). Analysis of the cis-acting sequences of these genes has revealed that hypoxia-induced activation requires binding sites for hypoxia-inducible factor-1 (HIF-1). HIF-1 was identified originally as an erythropoietin-inducible factor (*3*), and the mechanism underlying hypoxic induction has been well characterized (*4*). The bifunctional enzyme 6-phosphofructo-2-kinase [EC2.7.1.105]/fructose 2,6-bisphosphatase [EC3.1.3.46] (PFK-2/F2,6BPase) catalyzes the synthesis and hydrolysis of fructose 2,6-bisphosphate (F-2,6-P_2), which is the most potent activator of phosphofructokinase (*5*, *6*), a key regulatory enzyme in glycolysis. HP2K, one of the isozymes of PFK-2/F2,6BPase, was originally identified as a placenta-type enzyme but has now been shown to be present in a variety of other cell types and has also been well characterized, both genetically and enzymatically (*7*–*9*). In addition, the high level of kinase activity, and the lack of F2,6BPase activity, implicates HP2K as an essential regulator in the maintenance of high levels of F-2,6-P_2 in highly glycolytic tissues where it is specifically expressed (*10*, *11*).

Because of the particularly high expression levels of HP2K in highly glycolytic cells and tissues, we are interested in understanding its transcriptional regulation. By the use of a luciferase reporter system, containing the promoter/enhancer region of the HP2K gene, we previously demonstrated that the upstream region, between bases –1265 and –1329, contains a gene enhancer that responds to serum in BeWo cells (*12*). In another previous report by Minchenko *et al*. (*13*) it was shown that the expression of PFKFB3, which is identical to HP2K, is closely linked to HIF-1 activation, but they did not find any HREs in the gene promoter region. In this communication, we show that the HP2K gene promoter contains a HRE that responds to hypoxic conditions.

Human choriocarcinoma BeWo cells were grown in Waymouth's medium supplemented with 40% Gey's balanced salt solution and 10% FCS, and human hepatocellular carcinoma Hep3B cells were cultured in D-MEM supplemented with 10% FCS. Cells were cultured at 37°C

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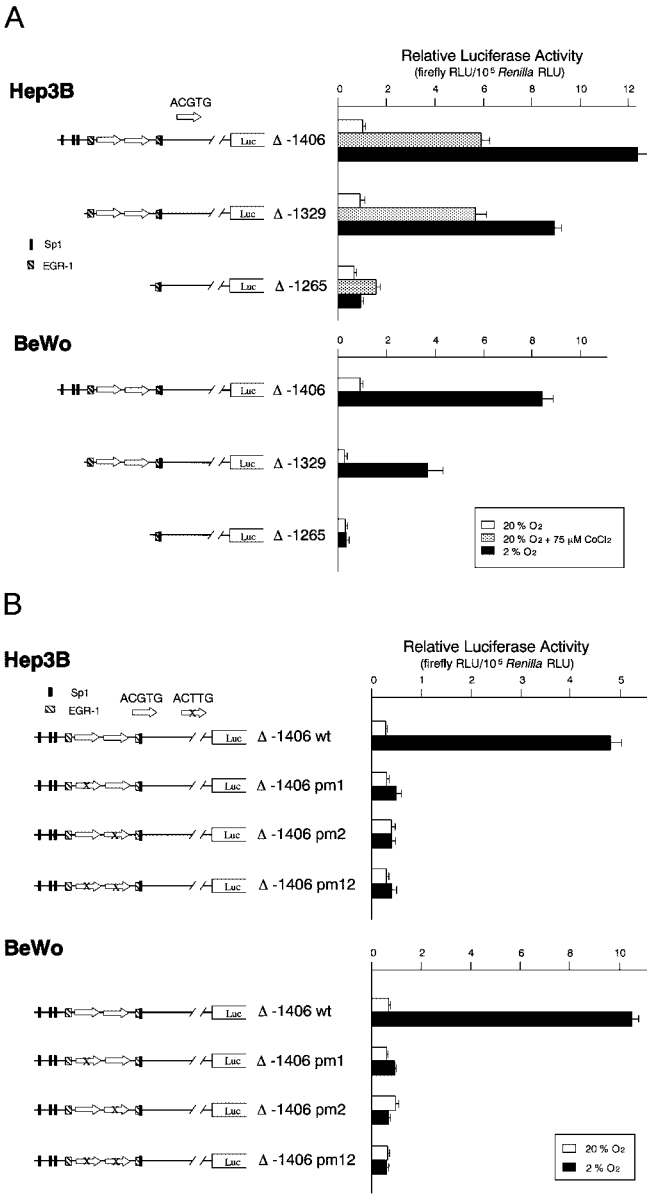

Fig. 1. **Functional analysis of the hypoxia-responsive HP2K promoter region.** Transient transfection of truncated (A) or point-mutated (B) HP2K promoter-reporter constructs into Hep3B and human choriocarcinoma BeWo cells. A schematic representation of the regulatory elements in a portion of the HP2K promoter is shown on the left. Open-boxed arrows indicate HIF-1 binding consensus sequences, the symbols for other transcriptional elements being indicated in the figure. The Δ –1406 wt construct is identical to Δ–1406 in (A). The sequence information for mutant vectors Δ –1406 pm1, Δ –1406 pm2 and Δ –1406 pm12 is identical to that for pGV-HP-E.pm1, pGV-HP-E.pm2 and pGV-HP-E.pm12 shown in Fig. 2A, respectively. The results for cell lysates, 18 h after stimulation, are shown as the firefly luciferase activity per *Renilla* luciferase activity, which was used as an internal control. Data are expressed as means ± SD for duplicated experiments, which were repeated at least three times.

under a humidified atmosphere of 5% CO_2 in air (normoxia). Hypoxic conditions were subsequently generated by exposure of the cells to a 2% O_2, 93%N_2 and 5% CO_2 environment. Construction of 5′-truncated promoter-luciferase reporter plasmids was previously reported (*12*). For the generation of Δ –1406 pm1, Δ –1406 pm2 and Δ –1406 pm12 constructs, point mutations were introduced into the HRE sequence of Δ –1406 wt by PCR-mediated site-directed mutagenesis. The cells were transfected with the promoter-luciferase vectors (0.4 μg) and then co-transfected with *Renilla* luciferase vector pRL-SV40 (0.2 μg), as an internal control, with Effectene Transfection Reagent (QIAGEN) according to the manufacturer's instructions, and then cultured for 8–18 hours. For stimulation assays, transfected cells were subjected to stimulation with 75 μM $CoCl_2$, or treated under hypoxic conditions (2% O_2) for 8–18 h. The cells were then harvested to prepare lysates for the luciferase assay by using a Pika-Gene Dual system (TOYO Ink, Tokyo, Japan). Luciferase activity was assayed using a Lumat LB9501 luminometer (Berthoid).

We next searched for any *cis*-elements in the HP2K promoter region that was responsive to hypoxia. As shown in Fig. 1A, the Δ –1329 construct, containing HRE-like motifs, showed approximately 14- and 10-fold activation under hypoxic conditions in BeWo and Hep3B cells, respectively. However, this hypoxic induction was completely abrogated in deletion mutant Δ –1265, indicating that the HRE of HP2K is located between –1265 and –1329, and contains a tandem repeat of the hypoxia-inducible factor-1 (HIF-1) responsive element (HRE)-like motif (5′-ACGTG-3′). Interestingly, we had previously determined that this was the site of the HP2K serum-responsive enhancer in BeWo cells (*12*). In order to further assess the importance of the HRE-like tandem repeat, we introduced mutations into these motifs. The results shown in Fig. 1B demonstrate that each of the resulting mutants exhibited severely reduced hypoxia sensitivity, indicating that both copies of the HRE core sequence (ACGTG) are necessary for hypoxia-induction. Similar results were obtained using either BeWo or Hep3B cells.

In order to then characterize the HRE motif of the HP2K gene, we constructed an SV40 promoter driven luciferase vector, pGV-P2 (TOYO Ink), that harbored various enhancer sequences related to HP2K HRE, and used the resulting plasmids to determine the effect of each enhancer element upon reporter expression. The enhancer inserts were blunt end-ligated into the *Sma* I site of the pGV-P2 vector and the direction of each cloned insert was confirmed by sequencing. As shown in Fig. 2B, a vector containing wild-type HRE sequences derived from the HP2K gene, pGV-HP-E, clearly exhibited the characteristics of a hypoxia-responsive enhancer. In addition, an inverted wild type enhancer, pGV-HP-E.R, showed comparable activity to pGV-HP-E. Furthermore, a vector containing three copies of HRE (pGV-HP-3E) significantly increased both basal and hypoxia-induced activity. The hypoxia-induced activity of HP2K HRE (22.6-fold for pGV-HP-E) was also much greater than the level induced by the enhancer of erythropoietin (EPO) (4.5-fold for pGV-Epo-E). These effects were not due to the copy number of the 5′-ACGTG-3′ motif in the HRE, as individually these motifs (pGV-HP-E.pm1 and pGV-HP-E.pm2) did not show any enhancer activity (Fig. 1). Interestingly, alterations in the distance between the two 5′-ACGTG-3′ motifs (pGV-HP-E.dm1 and pGV-HP-E.im1) resulted in a complete loss of enhancer activity. These

A

Reporter vector	Enhancer sequence
pGV-Epo-E	AATTGCCCT**ACGTG**CTGTCTCACACAGCCTGTTCGA
pGV-HP-E	ATGCGGG**ACGTG**AGCG**ACGTG**TGGCAGCCTCGA
pGV-HP-E.R	TCGAGGCTGCCA**CACGT**CGCT**CACGT**CCCGCAT
pGV-HP-3E	ATTGG**ACGTG**AGCG**ACGTG**TGG**ACGTG**AGCG**ACGTG**TGG**ACGTG**AGCG**ACGTG**TT
pGV-HP-E.pm1	ATGCGGGACtTGAGCG**ACGTG**TGGCAGCCTCGA
pGV-HP-E.pm2	ATGCGGG**ACGTG**AGCGACtTGTGGCAGCCTCGA
pGV-HP-E.pm12	ATGCGGGACtTGAGCGACtTGTGGCAGCCTCGA
pGV-HP-E.pm34	ATGCGGGACGTcAGCGACGTcTGGCAGCCTCGA
pGV-HP-E.dm1	ATGCGGG**ACGTG**AG_G**ACGTG**TGGCAGCCTCGA
pGV-HP-E.im1	ATGCGGG**ACGTG**AGtCG**ACGTG**TGGCAGCCTCGA

B

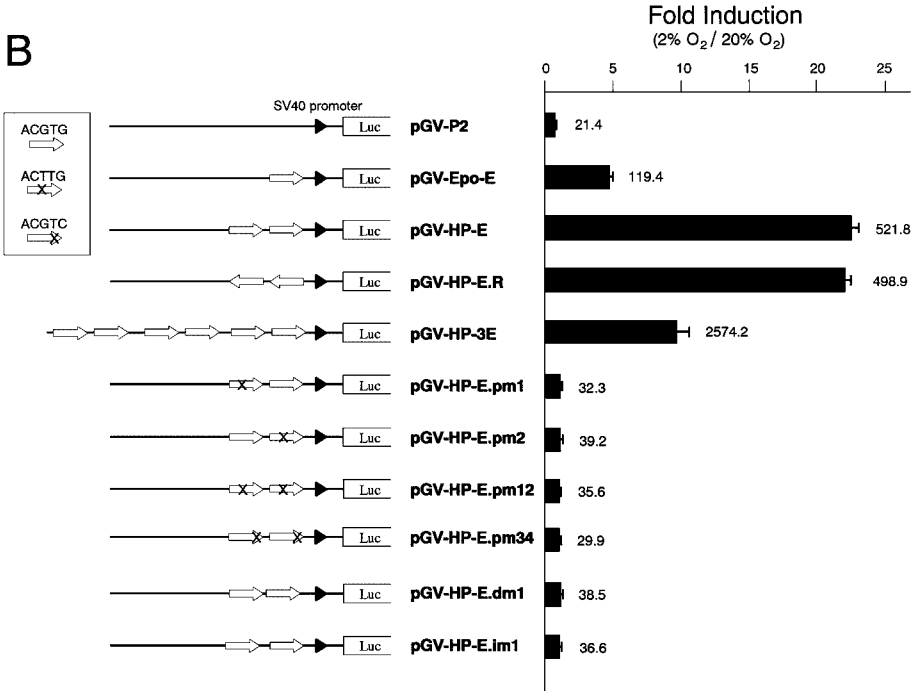

Fig. 2. **Functional analysis of the HRE of the HP2K gene.** (A) Sequences of the oligonucleotides used for constructing enhancer-SV40 promoter–reporter vectors. Only the coding strand sequence of each double-stranded oligonucleotide is shown, the HIF-1 binding core sequence also being indicated (underlined bold). Substituted or inserted bases are shown in lowercase letters. (B) Transient transfection of the enhancer vector constructs into Hep3B cells. A schematic representation of regulatory elements in a portion of the HP2K promoter is shown on the left. Open-boxed arrows indicate the HIF-1 binding consensus sequence. pGV-P2 was transfected as a negative control. The results are shown as fold induction of relative luciferase activity (firefly luciferase activity per *Renilla* luciferase activity) on hypoxic stimulation for 18 h. Relative luciferase activity of 2% O_2 is also indicated on the right of each column. Duplicate experiments were repeated at least three times and the results of a representative experiment are shown as means ± SD.

results indicate that both the 5′-ACGTG-N_4-ACGTG-3′ consensus sequence and the four nucleotides separating the ACGTG repeats within the HP2K HRE are critical for the hypoxic response of the promoter.

HIF-1α usually acts as a heterodimer with HIF-1β (ARNT), which is expressed constitutively even under normoxic conditions. To study the interaction between HIF-1 and HP2K HRE, cells were co-transfected with HIF-1 expression vectors and the pGV-HP-E reporter vector. HIF-1 expression vectors were generated by PCR amplification of fragments of HIF-1α and HIF-1β, followed by subcloning into *Sfi* I sites in frame with the HA and Myc tags of pCMV-HA and pCMV-Myc (Clontech), respectively, to confirm their expression. Hep3B cells were transfected with HP2K enhancer/SV40 promoter-luciferase vectors (0.2 μg) and then co-transfected with appropriate combinations of pCMV-HA, pCMV-Myc, pCMV-HA-HIF-1α and pCMV-Myc-ARNT (0.2 μg each). The sense strand sequences of both the wild-type and mutant HP2K HRE oligonucleotides used in the EMSA experiments are identical to those of HP-E and HP-E.pm12, respectively. The results in Fig. 3A show that over-expression of HIF-1 results in increased luciferase activity, even under normoxic conditions, and that HIF-1α alone is more effective than HIF-1β alone. This suggests that HIF-1β is abundant and a small amount of HIF-1α is the limiting factor for HRE-driven gene expression in normoxic cells. Moreover, additive effects were also observed when both HIF-1α and HIF-1β were co-transfected, suggesting that the HIF-1 heterodimer may recognize HP2K HRE as a cis-element. The interaction of HIF-1 and HP2K HRE was also examined by means of an electrophoretic mobility shift assay (EMSA) using a DIG gel shift kit (Roche). Nuclear extracts of both normoxic and hypoxic Hep3B cells were prepared as described previously (*14*). Five μg aliquots of nuclear extracts of Hep3B cells, exposed to either normoxic (20% oxygen) or hypoxic (2% oxygen) conditions, were incubated with 1 μg of poly(dI-dC), 0.5 μg of sonicated herring sperm DNA (Sigma), and 1 ng of DIG-labeled HP-E probe in 25 mM

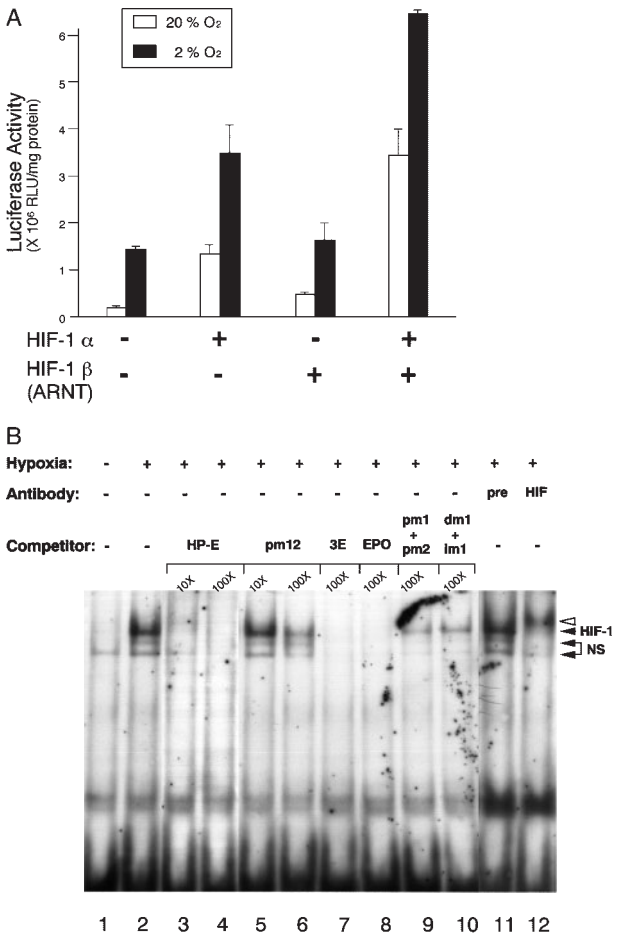

Fig. 3. **(A) Effects of over-expression of HIF-1α and/or HIF-1β in Hep3B cells under both normoxic and hypoxic conditions.** Cells were co-transfected with HP2K enhancer/SV40 promoter-luciferase vectors and with an appropriate combination of pCMV-HA-HIF-1α and pCMV-Myc-ARNT. (–) Transfection with pCMV-HA and pCMV-Myc control vectors. The results at 18 h after stimulation are shown as luciferase activity per mg total protein from each cell lysate. Each of the experiments was performed in triplicate and the data are expressed as means ± SD. **(B) Electrophoretic mobility shift assays.** Nuclear extracts of Hep3B cells, exposed to either normoxic (20% oxygen, lane 1) or hypoxic (2% oxygen, lanes 2–12) conditions, were incubated with DIG-labeled HP-E probe. For competition analysis, a 10 to 100-fold molar excess of unlabeled wild-type HRE (lanes 3, 4, 7, and 8) or mutant HRE (lanes 5, 6, 9, and 10) oligonucleotide was also added. The sequences of the sense strands of HP-E, pm12, 3E, EPO, pm1, pm2, dm1, and im1 are identical to those of HP-E, HP-E.pm12, HP-3E, Epo-E, HP-E.pm1, HP-E.pm2, HP-E.dm1, and HP-E.im1 in Fig. 2A, respectively. Supershift assays were performed with pre-immune rabbit sera (pre; lane 11) or HIF-1α polyclonal antibodies (HIF; lane 12). Arrows indicate either HIF-1 specific (HIF-1) or non-specific (NS) bands. The supershifted complex is indicated by the open arrowhead.

Tris-HCl (pH 7.6), 80 mM KCl, 0.2 mM EDTA, 5 mM dithiothreitol, and 20% glycerol, for 20 min at 4°C. For competition analysis, a 10 to 100-fold molar excess of unlabeled oligonucleotide was also added just prior to the addition of the labeled probe. The sequences of the oligonucleotides are shown in Fig. 2A. For supershift analysis, 1 μl of a rabbit polyclonal antibody against HIF-1α was added after the initial incubation, and then the mixture was further incubated for 30 min at 4°C. As a negative control for supershifts, pre-immune rabbit sera (pre) were added to the solution. DNA-binding complexes were resolved on a 5% polyacrylamide gel with 0.25× TBE, electroblotted onto a nylon membrane, and then subjected to DIG-chemiluminescence detection according to the manufacturer's instructions. The results in Fig. 3B demonstrate the direct binding of HIF-1 to HP2K HRE. Furthermore, the results of the competition analysis did not contradict to reporter gene assay, although the difference in binding affinity of HIF-1 to HRE between HP-2K and EPO was indistinct.

In the present study, we clearly identified a tandem repeat of the HRE core sequence, 5′-ACGTG-3′, located between –1290 and –1274, upstream of the HP2K gene, and confirmed that this region functions as a HRE by reporter analysis. At present, the known HREs of hypoxia-inducible genes contain either a single copy or two copies of the 5′-ACGTG-3′ sequence (*1*, *15*–*18*), and a single copy of the HRE motif has been shown to be sufficient for hypoxic induction of human transferrin, even though this gene contains two HRE motifs (*15*). However, for the HRE of the HP2K gene to function, both 5′-ACGTG-3′ sequences are essential. In addition, the 4-bp distance separating these HRE elements is also critical, although base substitutions within these 4-bp did not affect the promoter function (data not shown). A comparison with other HRE-containing genes, such as erythropoietin (EPO), indicated that HP2K HRE exhibits a much stronger response to hypoxic stimuli, although the basal expression levels were comparable. Hence, we propose the sequence 5′-ACGTGNNNNACGTG-3′ as the basic HRE consensus sequence that is effective for the response to hypoxia via HIF-1.

Kimura *et al.* previously proposed a common structure for the HREs of hypoxia-inducible genes (*19*). They described the consensus sequence of the HRE as consisting of an imperfect inverted repeat, containing both a HBS (HIF-1 binding site) and a HAS (HIF-1 ancillary sequence) motif, 8nt apart. In comparison with other HREs, however, the HP2K HRE sequence does not conform to this arrangement and seems to lack a HAS, which has been shown to be necessary for the full induction of other hypoxia-inducible genes. HP2K gene expression, however, may be enhanced via the two copies of HBS alone, which implies either that HIF-1 is the only key transcriptional factor that interacts with HP2K-HRE, or that one of the HBS motifs can act as a HAS. The differences between the binding of HIF-1 to HP2K HRE and to other known HREs that contain both HBS and HAS sequences remains unclear.

Recently, Coulet *et al.* found the HRE of the endotherial nitric-oxide synthase (eNOS) gene to be responsive to HIF-2, rather than to HIF-1 (*20*). It is interesting that the tissues in which HP2K expression is highest *in vivo*, including placenta, brain, liver and kidney, also express both HIF-1 and HIF-2 (*10*, *21*, *22*). In our preliminary studies involving co-transfection assays of the HIF-2α expression vector, HP2K-HRE was found to be also responsive to HIF-2, which may account for the wider distribution of HP2K expression *in vivo* in combination with the high induction by HP2K HRE.

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