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Abstract: Transferrin (Tf) is a liver-derived iron transport protein whose plasma concentration increases following exposure to hypoxia. Here, we present a cell culture model capable of expressing Tf mRNA in an oxygen-dependent manner. A 4-kilobase pair Tf promoter/enhancer fragment as well as the 300-base pair liver-specific Tf enhancer alone conveyed hypoxia responsiveness to a heterologous reporter gene construct in hepatoma but not HeLa cells. Within this enhancer, a 32-base pair hypoxia-responsive element was identified, which contained two hypoxia-inducible factor-1 (HIF-1) binding sites (HBSs). Mutation analysis showed that both HBSs function as oxygen-regulated enhancers in Tf-expressing as well as in non-Tf-expressing cell lines. Mutation of both HBSs was necessary to completely abolish hypoxic reporter gene activation. Transient co-expression of the two HIF-1 subunits HIF-1alpha and aryl hydrocarbon receptor nuclear translocator (ARNT)/HIF-1beta resulted in enhanced reporter gene expression even under normoxic conditions. Overexpression of a dominant-negative ARNT/HIF-1beta mutant reduced hypoxic activation. DNA binding studies using nuclear extracts from the mouse hepatoma cell line Hepa1 and the ARNT/HIF-1beta-deficient subline Hepa1C4, as well as antibodies raised against HIF-1alpha and ARNT/HIF-1beta confirmed that HIF-1 binds the Tf HBSs. Mutation analysis and competition experiments suggested that the 5' HBS was more efficient in binding HIF-1 than the 3' HBS. Finally, hypoxic induction of endogenous Tf mRNA was abrogated in Hepa1C4 cells, confirming that HIF-1 confers oxygen regulation of Tf gene expression by binding to the two HBSs present in the Tf enhancer.

DOI: https://doi.org/10.1074/jbc.272.32.20055

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-1444

Originally published at:
DOI: https://doi.org/10.1074/jbc.272.32.20055
Oxygen-regulated Transferrin Expression Is Mediated by Hypoxia-inducible Factor-1*

(Received for publication, February 3, 1997, and in revised form, May 12, 1997)

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Transferrin (TF) is a liver-derived iron transport protein whose plasma concentration increases following exposure to hypoxia. Here, we present a cell culture model capable of expressing TF mRNA in an oxygen-dependent manner. A 4-kilobase pair TF promoter/enhancer fragment as well as the 300-base pair liver-specific TF enhancer alone conveyed hypoxia responsiveness to a heterologous reporter gene construct in hepatoma but not HeLa cells. Within this enhancer, a 32-base pair hypoxia-responsive element was identified, which contained two hypoxia-inducible factor-1 (HIF-1) binding sites (HBSs). Mutation analysis showed that both HBSs function as oxygen-regulated enhancers in TF-expressing as well as in non-TF-expressing cell lines. Mutation of both HBSs was necessary to completely abolish hypoxic reporter gene activation. Transient co-expression of the two HIF-1 subunits HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT)/HIF-1β resulted in enhanced reporter gene expression even under normoxic conditions. Overexpression of a dominant-negative ARNT/HIF-1β mutant reduced hypoxic activation. DNA binding studies using nuclear extracts from the mouse hepatoma cell line Hepa1 and the ARNT/HIF-1β-deficient subline Hepa1C4, as well as antibodies raised against HIF-1α and ARNT/HIF-1β confirmed that HIF-1 binds the TF HBSs. Mutation analysis and competition experiments suggested that the 5′ HBS was more efficient in binding HIF-1 than the 3′ HBS. Finally, hypoxic induction of endogenous TF mRNA was abrogated in Hepa1C4 cells, confirming that HIF-1 confers oxygen regulation of TF gene expression by binding to the two HBSs present in the TF enhancer.

Iron is an essential trace metal in all living organisms. Both iron overload and iron depletion can severely affect physiological processes such as development, erythropoiesis, or biochemical metabolism (reviewed in Refs. 1 and 2). The liver represents the major organ of iron storage in the body and is most susceptible to injuries due to iron overload (1). Thus, iron hemostasis has to be tightly balanced, and, as a consequence, free iron occurs only transiently in the serum. When iron is absorbed from the small intestine into the blood, it immediately binds apotransferrin to form transferrin (TF)1, which is then transported by the plasma to all tissues of the vertebrate's body. Delivery of iron occurs by binding of TF to the TF receptor followed by endocytosis. In erythroblasts, iron is primarily required for heme synthesis in mitochondria. Tissue-specific expression of the TF gene is controlled by distinct positive and negative regulatory elements located 5′ to the transcription initiation site. Apart from the promoter, the best studied element within this region is the −3600/−3300 enhancer (hereafter referred to as the TF enhancer). This cis-acting element enhances the activity of the TF promoter in human Hep3B hepatoma cells in a tissue-specific manner (3, 4). Studies in Hep3B and HeLa cells revealed that multiple liver-enriched and ubiquitous factors interact with the TF enhancer (3, 5, 6). The TF enhancer, however, is inactive in TF-expressing neuronal and Sertoli cells (4, 5).

Hypoxia, a reduction in oxygen concentration, is increasingly recognized as an important regulator of gene expression (reviewed in Ref. 7). The best established example of oxygen-regulated gene expression is provided by the erythropoietin growth factor erythropoietin (Epo, reviewed in Ref. 8). The two human hepatoma cell lines HepG2 and Hep3B are so far the only permanent cell culture models available to investigate oxygen-regulated Epo expression (9). Apart from Epo, we recently demonstrated hypoxic induction of several acute phase genes in HepG2 cells (10). Acute phase reactants are liver-derived serum proteins whose production is induced by proinflammatory cytokines (reviewed in Ref. 11). TF expression was of particular interest since this protein is one of the rare examples of acute phase reactants that are down-regulated during the acute phase response in both human serum and HepG2 cells (11). In contrast, we found a marked increase in TF transcription following hypoxic (1% O2) culture of HepG2 cells (10), suggesting that different signaling pathways are mediating the effects of these two stimuli. Given that the erythroid marrow uses more than 80% of plasma iron (2), and considering that hypoxia increases erythropoiesis, it is conceivable that an increase in plasma iron transport capacity is required for hypoxia-induced Epo-mediated erythropoiesis. Indeed, hypoxia was shown to increase iron absorption (12), and hypoxic up-regulation of TF serum protein concentrations has previously been found in mice (13) and rats (14, 15) exposed to hypobaric hypoxia (0.5 atm) for 1–3 days. Although some of these experiments were established some 40 years ago, the molecular mechanisms leading to hypoxically enhanced TF expression have not been unraveled so far, mainly due to the lack of a suitable cell culture model.

The hypoxia-inducible factor-1 (HIF-1) was originally iden-

* This work was supported in part by Swiss National Science Foundation Grant 31-47111.96, and grants from the Julius Klaus-Stiftung, the Olga Mayenfisch-Stiftung, and the Stiftung für wissenschaftliche Forschung an der Universität Zürich. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TF, transferrin; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; HBS, HIF-1 binding site; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; bp, base pair(s); kb, kilobase pair(s).
tified by its ability to bind to a hypoxia-responsive cis-element located 3' to the Epo gene (16). HIF-1 is a heterodimer consisting of an α and a β subunit, both belonging to the basic-helix-loop-helix-Per-AhR/ARNT-Sim family of transcription factors (17). Whereas the α subunit is a novel member of this family, the β subunit is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) known to heterodimerize with the aryl hydrocarbon receptor/dioxin receptor (AhR) following ligand binding (reviewed in Refs. 7 and 18).

We have established cell culture models to study oxygen-dependent Tf expression and have subsequently analyzed the regulation of the Tf enhancer. Our results demonstrate the presence of two HIF-1 binding sites (HBSs) within the Tf enhancer and show that binding of HIF-1 to these sites confers oxygen-regulated Tf gene expression.

MATERIALS AND METHODS

Cell Culture and Hypoxic Induction—The human hepatoma cell lines Hep3B and HepG2 were obtained from American Type Culture Collection (ATCC numbers HB-8064 and HB-8065, respectively). The mouse hepatoma cell lines Hepa1 (also termed Hepa1c1c7) and Hepa1C4 (19) were kind gifts of L. Poellinger (Karolinska Institute, Stockholm, Sweden). All cells were cultured in Dulbecco’s modified Eagle’s medium (high glucose, Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 × non-essential amino acids, 2 mM l-glutamine, and 1 mM sodium pyruvate (all Life Technologies, Inc.) in a humidified atmosphere containing 5% CO2 at 37 °C. Oxygen tensions described elsewhere (20). Briefly, HeLaS3 cells were incubated at a concentration of either 20% O2, 5% CO2, and 75% N2 (normoxia), or 1% O2, 5% CO2, and 94% N2 (hypoxia). Cells were subjected to hypoxic induction at a cell density of 2 × 10^6 cells/cm^2. The human epithelial carcinoma cell line HeLaS3 (ATCC CCL-22) was cultured in suspension in Ham’s F-12 medium (Life Technologies, Inc.) supplemented as described above. Hypoxic induction was achieved as described above (20). Briefly, HeLaS3 cells were incubated for 10 min at a density of 1 × 10^6 cells/ml in an IL 237 tomometer (Instrumentation Laboratory) under continuous stirring for 4 h at 37 °C using gas mixtures of either 20% O2, 5% CO2, and 75% N2 (normoxia), or 1% O2, 5% CO2 and 94% N2 (hypoxia) at a flow rate of 500 ml/min.

RNA Blot Analysis—Immediately following stimulation, RNA was isolated as described by Chomczynski and Sacchi (21). Total RNA (10 μg) was denatured in formamide/formaldehyde, and ethanol precipitated through a 1% agarose gel containing 6% formaldehyde as described (22). Following pressure blotting (Stratagene) to nylon membranes (Biodyne A, Pall) and UV cross-linking (Stratalinker, Stratagene), the filters were hybridized to cDNA probes labeled with [α-32P]dCTP to a specific activity of 1 × 10^9 dpm/μg using the random-primed DNA labeling method (22). Hybridization was performed in 50% formamide, 10% dextran sulfate, 5 × Denhardt’s solution, 200 μg/ml sonicated salmon sperm DNA, 1% SDS, 0.9 mM NaCl, 60 mM NaHPO4, 6 mM EDTA (pH 7.0) for 14 h at 42 °C. The filters were washed to a final stringency of 55 °C in 0.1 × SSC, 0.2% SDS and the signals recorded using a PhosphorImager (Molecular Dynamics). The TF, α1-antitrypsin, β-actin, ribosomal protein L28, and 28 S ribosomal RNA cDNA probes were obtained as described previously (10, 23). All cDNA probes were labeled by the random primer method using [α-32P]dCTP (Hartmann) and run on 4% non-denaturing polyacrylamide gels. Electrophoresis was performed at 200 V in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA) at 4 °C, the gels were autoradiographed. For supershift analysis, each 1 μl of rabbit polyclonal antisera directed against HIF-1α or ARNT/HIF-1β (kind gift of L. Poellinger) was added to the completed EMSA reaction mixture and incubated for 15 min at 4 °C prior to loading. For competition experiments, a 4–500-fold molar excess of unlabeled antisense strands was added to the binding reaction prior to addition of labeled probes.

RESULTS

Oxygen-regulated Tf mRNA Expression in Two Human Hepatoma Cell Lines—We previously reported on oxygen-regulated mRNA expression of several acute phase genes in the human hepatoma cell line HepG2 (10). Regulation of the Tf gene was of special interest since Tf transcription was down-regulated in response to proinflammatory cytokines (e.g. interleukin-6), but was up-regulated following exposure to low oxygen concentrations. To test whether hypoxic Tf induction observed in HepG2 cells might represent a general phenomenon in liver cells, we transfected Hep3B cells with a plasmid encoding an hypoxia-inducible Tf cDNA construct (Fig. 4, C). Upon hypoxia, the cells were split in two aliquots and incubated for 36 h at 20% or 1% O2, respectively. After washing twice with phosphate-buffered saline, the cells were lysed in reporter lysis buffer (Promega) and luciferase and β-galactosidase activities were determined according to the manufacturer’s instructions (Promega) using a Biocounter M1500 luminometer (Lumac) and a DU-62 spectrophotometer (Beckman), respectively. Differences in the transcription efficiency and extract preparation were corrected by normalization to the corresponding β-galactosidase activities. Luciferase activities were expressed relative to the empty parental vector (pGL3Basic or pGL3Promoter) transfectants. For transient over-expression assays in Hep3B cells, 10 μg of each expression vector was co-transfected together with equal amounts of the luciferase reporter construct pTHH89sw and the control plasmid pCMVlacZ. The unre-
which was included as positive control (Fig. 1, A and B). Specificity of hypoxic up-regulation was shown using L28 and 28 S control hybridizations since β-actin mRNA was also slightly up-regulated in both hepatoma cell lines and thus not suitable as a normalization probe (10).

The Tf Enhancer Is Hypoxia-responsive—In a first attempt to identify Tf regulatory sequences conveying hypoxia-inducible Tf transcription, a 2400 to 139 (numbering according to Ref. 3) Tf promoter/enhancer DNA fragment (Fig. 2A) was inserted upstream of a promoterless luciferase reporter gene vector. Following transient transfection into Tf-expressing Hep3B and HepG2 cells, as well as into non-Tf-expressing HeLa cells, this 4-kb Tf promoter/enhancer induced basal luciferase expression 10-, 26-, and 8-fold in Hep3B, HepG2, and HeLa cells, respectively (Fig. 2B, open bars). Hypoxia (1% O2) stimulated luciferase expression 4.1- and 5.6-fold in Hep3B and HepG2 cells, respectively, but no significant hypoxic induction could be observed in HeLa cells (Fig. 2B, filled bars). Thus, hypoxia responsiveness seems to be coupled to liver-specific cis-acting elements present within this Tf promoter/enhancer DNA fragment.

In analogy to the liver-specific enhancer and the hypoxia-responsive element residing in close vicinity in the Epo 3′ flanking region (16), we wondered whether the −3600/−3300 bp liver-specific Tf enhancer (Fig. 2A) might be responsible for oxygen responsiveness of the Tf gene. To test this, we subcloned the 300-bp Tf enhancer downstream of a luciferase reporter gene driven by a heterologous SV40 promoter. The Tf enhancer induced normoxic luciferase expression by 8.1-, 1.6-, and 2.0-fold in Hep3B, HepG2, and HeLa cells, respectively (Fig. 3, open bars). This expression level was further up-regulated by exposing the cells to hypoxia; luciferase activity in Hep3B, HepG2, and HeLa cells increased 3.1-, 6.8-, and 1.8-fold, respectively (Fig. 3, filled bars). The weak hypoxic inducibility (1.4-fold) of the pGL3Promoter plasmid itself has been reported previously (24). Thus, similar to the observations using the 4-kb Tf promoter/enhancer, the 300-bp Tf enhancer alone conferred hypoxia inducibility in Hep3B and HepG2 hepatoma cells, but was not significantly active in non-Tf-expressing HeLa cells.

Two Tandemly Repeated HBSs Confer Hypoxia Responsiveness to the Tf Gene—A computer-assisted search using a HIF-1 consensus DNA-binding site (24) as query revealed the presence of two tandemly arrayed putative HBSs beginning at nucleotide positions 174 and 191, respectively (Fig. 4), within the 300-bp Tf enhancer (numbering according to Ref. 3). No other matches to the HIF-1 query were found in the published nucleotide sequences of the Tf gene. To test whether these two putative HBSs were functionally oxygen-responsive, we synthesized oligonucleotides containing both sites in either the wild type configuration (THBSww), or with one (THBSwm or...
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THBSnw) or both (THBSmm) HBS sites mutated (Fig. 4). Single copies of these oligonucleotides were inserted 3' to a luciferase reporter gene driven by a heterologous SV40 promoter. For comparison, a hypoxia-responsive luciferase construct (pGLEPOHBS.3) containing three concanamersized copies of the Epo HBS was included in this study (24). Luciferase activity was determined following transient transfection of Hep3B and HeLa cells. After 36 h of normoxic or hypoxic culture, luciferase activities were determined as described in Fig. 2. Means ± S.D. of three independent experiments are shown.

**NAME** | **SEQUENCE** | **PGL3Promoter** | **Luciferase** | **pGLTfenhancer** | **TF enhancer** | **Hep3B** | **HepG2** | **HeLa**
---|---|---|---|---|---|---|---|---
EPOHBS | tggagcAGCAGCTA gggc | 0.685 | 3082 | tggagcAGCAGCTA gggc | 3065 | | | |
THBSww | ttggc TGACAGCTA cacaacaag GCCAGCTA | 0.682 | 170 | TGACAGCTA cacaacaag GCCAGCTA | 201 | | | |
THBSwm | ttggc TGACAGCTA cacaacaag GCCAGCTA | 0.682 | 170 | TGACAGCTA cacaacaag GCCAGCTA | 201 | | | |
THBSmm | ttggc TGACAGCTA cacaacaag GCCAGCTA | 0.682 | 170 | TGACAGCTA cacaacaag GCCAGCTA | 201 | | | |

**FIG. 4. Oligonucleotides carrying wild type (w) and mutant (m) HBSs derived from the Epo (24) and Tf genes (3).** Note that for better comparison the complementary strand of EPOHBS is shown twice.

**FIG. 5. The Tf enhancer contains two functional HBSs.** Luciferase reporter gene constructs containing three copies of the EPOHBS (W) or a single copy of the two tandemly arrayed putative HBSs derived from the Tf enhancer (see Fig. 4) were transiently transfected into Hep3B and HeLa cells. Following normoxic or hypoxic exposure for 36 h, luciferase activity was determined as described in Fig. 2. Means ± S.D. of 3–10 independent experiments are shown.

**FIG. 6.** HIF-1 activates reporter gene expression via the Tf HBS. To investigate the involvement of the HIF-1 protein complex in Tf regulation, we performed transient expression experiments using the HIF-1α and/or ARNT/HIF-1β expression vectors pCMVhHIF-1α and pCMVhARN, respectively (19). They were co-transfected into Hep3B cells together with the reporter gene construct pGLTHBSww (depicted in Fig. 5), the normalization plasmid pCMVlacZ and the unrelated plasmid pBluescript used to equalize the total amount of DNA per transfection. As shown in Fig. 6 (open bars), transient overexpression, under normoxic conditions, of either of the two HIF-1 subunits weakly (about 2-fold) induced reporter gene expression, whereas expression of both HIF-1 subunits induced luciferase expression by 5.8-fold (Fig. 6, open bars). Co-expression with a reporter gene construct containing mutant HBSs (pGLTHBSmm) did not result in enhanced luciferase expres-
transfection efficiency and extract preparation yield according to the equalize the total amount of DNA per transfection. Following normoxic HBS-containing luciferase reporter gene construct pGLTfHBSww, the control vector pCMVlacZ, and the unrelated plasmid pBluescript to equalize the total amount of DNA per transfection. Following normoxic or hypoxic exposure, luciferase activity was determined, corrected for transfection efficiency and extract preparation yield according to the β-galactosidase activity, and normalized to the normoxic value of pGLT-

I-1 ARNT/HIF-1 1.8-fold. Interestingly, overexpression of a dominant negative ARNT/HIF-1 expression with HIF-1 needs to bind to the porter gene constructs.

Hep3B cells were transiently co-transfected with HIF-1α and ARNT/HIF-1β expression vectors together with the Tf HBS-containing luciferase reporter gene construct pGLTfHBSww, the control vector pCMVlacZ, and the unrelated plasmid pBluescript to equalize the total amount of DNA per transfection. Following normoxic or hypoxic exposure, luciferase activity was determined, corrected for transfection efficiency and extract preparation yield according to the β-galactosidase activity, and normalized to the normoxic value of pGLT-

FIG. 6. HIF-1 subunits transactivate Tf HBS-containing reporter gene constructs. Hep3B cells were transiently co-transfected with HIF-1αs and ARNT/HIF-1β expression vectors together with the Tf HBS-containing luciferase reporter gene construct pGLTfHBSww, the control vector pCMVlacZ, and the unrelated plasmid pBluescript to equalize the total amount of DNA per transfection. Following normoxic or hypoxic exposure, luciferase activity was determined, corrected for transfection efficiency and extract preparation yield according to the β-galactosidase activity, and normalized to the normoxic value of pGLT-

Means ± S.D. of three independent experiments are shown.

DNA Binding of HIF-1 to the Two HBS Sites of the Tf Enhancer—To directly identify the endogenous transcription factor(s) binding to the HBS of the Tf enhancer, EMSAs were performed using the THBS oligonucleotides shown in Fig. 4 as probes. Following incubation of the THBSww probe with nuclear extracts derived from normoxic or hypoxic Hep3B cells, nonspecific, constitutive, and hypoxia-inducible factors were detected (Fig. 7A). Using Hep3B nuclear extracts, mutation of the 5′ HBS site (oligonucleotide THBSww) did not greatly affect binding of the hypoxia-inducible factor. In contrast, mutation of the 5′ HBS site present in the Tf enhancer (oligonucleotide THBSww) strongly reduced but (as could be seen after prolonged exposure, data not shown) did not completely abolish protein binding. Only the double mutation (oligonucleotide TfHB-

expression is hypoxically induced in HepG2 hepatoma cells (10).

DISCUSSION

Tf mRNA Is Not Induced in ARNT/HIF-1β-deficient Hepa1C4 Cells—To investigate whether HIF-1 is capable of hypoxically inducing the endogenous Tf gene, we made use of the ARNT/HIF-1β-deficient Hepa1C4 cell line, which was cultured at 20% or 1% O2 and analyzed by mRNA blotting and hybridization. In previous experiments, we have demonstrated a lack of hypoxic aldolase mRNA induction and a reduction in hypoxic VEGF mRNA induction in this cell line (19). Whereas in the parental ARNT/HIF-1β-positive Hepa1 cell line hypoxia reproducibly induced Tf mRNA by a factor of 1.5 over the normoxic control, Hepa1C4 cells did not show induction of Tf mRNA (Fig. 9). This is in agreement with the lack of HIF-1 DNA-binding activity to the THBSww probe in these cells (shown in Fig. 6B) and, despite the rather low Tf mRNA expression levels and hypoxic inducibility in this particular hepatoma cell line, confirms that HIF-1 is critically involved in the oxygen responsiveness of the Tf gene.

To demonstrate that the hypoxia-inducible factor binding to the THBSww probe is indeed identical with the previously identified HIF-1 (17), nuclear extracts were prepared from normoxic and hypoxic Hepa1C4 cells and analyzed by EMSA. The cell line Hepa1C4, a subline of Hepa1 cells, is deficient in functional ARNT/HIF-1β expression (19) and devoid of DNA binding activity to the EPOHBS probe as well as of reporter gene induction with Epo HBS luciferase constructs (19). As shown in Fig. 7B, this cell line also lacked hypoxia-inducible THBSww DNA binding activity, whereas the constitutive and nonspecific factors were still present. Moreover, rabbit polyclonal antibodies derived against HIF-1α and ARNT/HIF-1β supershifted the hypoxia-inducible factor binding to the EPOHBS probe as well as to the THBS wild type and single mutated probes in Hepa1 cells (Fig. 7C), suggesting that this factor is functionally and immunologically indistinguishable from HIF-1.

To analyze in more detail the binding of HIF-1 to the two functional Tf HBSs, competition experiments were performed using the labeled THBSww probe and increasing amounts of unlabeled type or mutant oligonucleotides (depicted in Fig. 4). As shown in Fig. 8, using nuclear extracts derived from normoxic or hypoxic Hepa1 cells, the 3′ single mutant oligonucleotide (THBSwm) competed as efficiently for HIF-1 DNA binding to the THBSww probe as the wild type THBSww oligonucleotide itself (i.e. competition was observed using a 20-fold molar excess). The reduction in HIF-1 band intensity with 20- and 100-fold molar excesses of the 5′ single mutant oligonucleotide (THBSwwm) was somewhat less prominent, confirming that although both Tf HBSs can bind HIF-1, the 3′ site has a lower affinity for HIF-1. The double mutant THBS-

expression levels and hypoxic inducibility in this particular hepatoma cell line, confirms that HIF-1 is critically involved in the oxygen responsiveness of the Tf gene.

Tf Is a New Member of the HIF-1-regulated Gene Family—HIF-1 was originally defined by its capability of binding to a site required for hypoxic induction of Epo gene transcription (16, 17). Other examples of HIF-1-dependent oxygen-regulated genes include those encoding for glycolytic enzymes (27–30), vascular endothelial growth factor (VEGF, Refs. 31–33), inducible nitric oxide synthase (34), and glucose transporter-1 (Glut-1, Ref. 35). We have previously found that Tf gene expression is hypoxically induced in HepG2 hepatoma cells (10).
In this paper, we demonstrate that this effect is also mediated by HIF-1 via binding of two HBSs present in the Tf enhancer. We and others recently characterized a previously obtained mouse hepatoma cell line (Hepa1C4), which lacks functional ARNT expression, and demonstrated that it is also devoid of functional HIF-1 expression in terms of DNA binding activity and reporter gene transactivation (19, 33, 36, 37). By using these Hepa1C4 cells, we found that ARNT/HIF-1 is necessary for formation of the hypoxia-inducible complex binding to the Tf HBSs. Moreover, hypoxic induction of the endogenous Tf gene is also abrogated in Hepa1C4 cells, suggesting that HIF-1 is critically involved in oxygen-regulated Tf gene expression.

**Architecture of the Tf HBS**—An interesting feature of the Tf enhancer is the presence of two functional HBSs in close vicinity to each other. Such an architecture was not found in the Epo 3' HBS. The finding that a single HBS derived from the Epo HBS was not sufficient to convey oxygen responsiveness to a heterologous promoter driving expression of a reporter gene, although the HIF-1 complex bound to this site in vitro (24), raised the question as to whether additional functional cis-acting elements are necessary for full activation. Such an additional element (CACA) was found close to the HBSs in the Epo (16) and VEGF genes (33), as well as in the genes encoding several glycolytic enzymes (30). The transacting factors binding to this element, however, still remain to be identified. As suggested by the hypoxic induction of reporter gene expression by concatamerized HBSs, such an additional element could also be the HIF-1 site itself (Refs. 24 and 27 and this report). In addition, an activator protein (AP-1) site in the vicinity of the HIF-1-binding HBS in the 5' flanking region of the VEGF gene (31) and a cAMP-responsive element close to the two consensus HBSs in the LDH gene (28) have been implicated in full hypoxic induction of gene expression. Protein-DNA interactions of cAMP-responsive element-binding transcription factors (ATF-1 and CREB-1) were also observed within the HIF-1 site of the Epo HBS itself (24). In conclusion, it seems to be a common feature of an HBS that a single HBS in isolation is not sufficient to convey full hypoxic activation of gene expression. The additional factors required for full activation, however, might differ between the oxygen-regulated genes.
Protein-DNA Interactions at the Tf HBS—As mentioned above, the hypoxia-inducible Tf enhancer region is composed of two adjacent HBSs, both of which are capable of conveying hypoxic induction to reporter gene expression. The two 8-bp HBSs in the Tf enhancer are spaced by 9 bp only (Fig. 4), raising the question whether two HIF-1 complexes could bind simultaneously to these two sites or whether due to steric hindrance only one complex can bind at once. As shown by the equal migration properties of TfHBS (wild type and single mutant) and EPO HBS probes in our EMSAs, the predominant protein-DNA complex might consist of only one HIF-1 heterodimer bound to the TfHBS. Although EMSA analysis is probably not the ideal method to determine molecular mass differences, we would predict that an increase of more than 200 kDa (the molecular mass of an additional HIF-1 heterodimer) should be detectable.

The two core sequences TACGGTCA and TACGTGCC (not that the complementary strand to that shown in Fig. 4 is given) conform well with a previously published (33) consensus HBS (BACGGTSK, where B is C or G or T, S is C or G, and K is G or T). Strikingly, the presence of an adenosine residue at position 8 of the 8-bp consensus sequence (found in the 5′ THBS) has never been reported in any of the so far published genes carrying HBSs (18). Moreover, positions 9 and 10 also did not contain an adenosine residue in these HBSs (not shown). Thus, one would predict that this lack of adenosine residues in the 3′ part of the HBS is of functional relevance and that the DNA binding affinity of HIF-1 for the 5′ THBS might be decreased. Surprisingly, as shown with mutated oligonucleotide probes and competition experiments, the 3′ rather than the 5′ HIF-1 site (which contains the unexpected adenosine residue) constantly produced lower HIF-1 band intensities in EMSAs. On the functional level, only mutation of the 5′ THBS significantly reduced (but did not completely abolish) hypoxic induction of reporter gene expression, confirming that the 5′ HBS is more effective in HIF-1 binding than the 3′ HBS. Thus, despite the considerable number of so far identified HBSs, a conclusive consensus sequence still remains to be determined.

How Is HIF-1 Itself Activated?—Having established that HIF-1 is mediating hypoxic induction of Tf gene expression, the question arises of how HIF-1 itself is activated. So far, little is known about the mechanisms of oxygen sensing and subsequent conditional regulation of HIF-1. Initially, it has been reported that HIF-1α (and to a lesser extent also ARNT/HIF-1β) is regulated at the level of mRNA expression (17), but work from our and other laboratories could not confirm this result (19, 38, 39). Hence, HIF-1 must be regulated at the post mRNA level. Possible mechanisms include translational up-regulation, post-translational protein stabilization (39) or protein modifications such as phosphorylation (40) or redox modifications (41). There is good evidence for all of these putative mechanisms and more than one might turn out to be involved in hypoxic HIF-1 activation (reviewed in Ref. 18). Interestingly, our transient overexpression experiments using the Tf HBS demonstrated that forced expression of the two HIF-1 subunits is sufficient to convey induction to reporter gene transcription even under normoxic conditions. This observation is in agreement with recent work using the Epo HBS (19, 42) and VEGF HBS (33). Since conditional regulation thus does not seem to be of primary importance for HIF-1 function, we favor the model(s) of translational up-regulation and/or protein stabilization. However, hypoxia represented a stronger stimulus than overexpression of HIF-1, indicating that for a full response conditional regulation of HIF-1 is required. Further investigations of HIF-1 regulation is crucial for the elucidation of the signal transduction pathway(s) involved in the expression of Tf as well as other oxygen-regulated genes.

Acknowledgments—We are grateful to L. Poellinger, M. M. Zakin, and S. Kozlov for the generous gift of cell lines, antibodies, and plasmid. F. Spielmann and W. Bauer-Kustermann for excellent technical assistance; J. Silke for critically reading the manuscript; C. Gasser for the artwork; and C. Bauer for support.

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