Perivenous expression of the mRNA of the three hypoxia-inducible factor α-subunits, HIF1α, HIF2α and HIF3α, in rat liver

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The cDNAs of three hypoxia-inducible factor (HIF) α-subunits were cloned from RNA of primary rat hepatocytes by reverse transcriptase PCR. All three cDNAs encoded functionally active proteins, of 825, 874 and 662 amino acids. After transfection they were able to activate luciferase activity of a luciferase gene construct containing three HIF-responsive elements. The mRNAs of the rat HIF α-subunits were expressed predominantly in the perivenous zone of rat liver tissue; the nuclear HIFα proteins, however, did not appear to be zonated.

Key words: glycolysis, hepatocyte, metabolic zonation, oxygen.

INTRODUCTION

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors consisting of an α- and a β-subunit, both belonging to the basic-helix-loop-helix (bHLH)-PAS [Per/arylhydrocarbon receptor nuclear translocator (ARNT)/Sim] protein superfamily. The β-subunit was found to be identical to the ARNT, whereas the α-subunit, first cloned from the human hepatoma cell line Hep3B, was identified as a novel protein and named HIF1α [1]. HIF1α is a member of the PAS superfamily 1 (MOP1) [2]. Besides HIF1α, two other HIF α-subunits were cloned from human and mouse sources, HIF2α [2] and HIF3α [3]. They also belong to the bHLH-PAS superfamily of transcription factors and form heterodimers with ARNT. HIF2α, the term first introduced by Wenger and Gassmann [4], is also known as endothelial PAS domain protein 1 (EPAS1) [5], HIF1α-like factor (HLF) [6] and HIF-related factor (HRF) [7], and it is a member of the PAS superfamily 2 (MOP2) [2]. HIF3α is also referred to as MOP7 [2]. Recently, a quail HIF2α homologue was cloned [8]. In the rat, there has only been a hint of the existence of HIF1α [9].

HIF1 has been shown to activate a number of genes under low O_2 tensions, including those encoding glycolytic enzymes like phosphofructokinase or enolase [10,11]. Transcriptional activation was achieved by the binding of HIF1 to the hypoxia-response element (HRE) located in either the 5′ or the 3′ regions of the genes [12,13].

In the liver, due to the unidirectional bloodflow from the portal vein and hepatic artery to the central vein and due to the oxygen-consuming metabolic processes of the cells along the sinusoids, an oxygen gradient is formed. Thus the O_2 tension drops from 65 mmHg in the periportal area to 35 mmHg in the perivenous area. This O_2 gradient was proposed to be an important regulator for the expression of genes encoding the heterogeneously distributed key enzymes of the carbohydrate metabolism [14]. Glycolytic enzymes like glucokinase or pyruvate kinase are expressed predominantly in the perivenous zone of the liver acinus, where the pO_2 is low, whereas gluconeogenic enzymes like phosphoenolpyruvate carboxykinase are expressed predominantly in the periportal zone, where the pO_2 is high [15]. The transcription factors mediating the zonated expression have not been identified yet, but the HIFs appear to be attractive candidates.

It was the aim of this study to clone, sequence and functionally express the HIF1α, HIF2α and HIF3α cDNAs from RNA of primary rat hepatocytes to establish whether the different α-subunits of HIF are expressed in rat liver. Furthermore, the possible zonated expression of the rat HIF (rHIF) 1α, rHIF2α and rHIF3α was to be studied by *in situ* hybridization and immunohistochemistry.

It was found that rat hepatocytes expressed all α-subunits of the HIFs. The rHIF1α cDNA encodes a protein of 825 amino acids, the rHIF2α cDNA a protein of 874 amino acids and the rHIF3α cDNA a protein of 662 amino acids. The *in situ* hybridization showed that the rHIF1α, rHIF2α and rHIF3α mRNAs were expressed mainly in the perivenous area of the liver acinus. Immunohistochemistry, however, indicated that the nuclear proteins were not zonated.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and purchased from commercial suppliers. Collagenase, Superscript™ II reverse transcriptase, oligo-dT_{12-18}, hexanucleotides, digoxigenin-UTP, the digoxigenin nucleic acid detection kit, T3 and T7 polymerases, digoxigenin (DIG) RNA-labelling mixture and fetal calf serum were obtained from Roche (Mannheim, Germany), and medium M199 was from Gibco-BRL (Eggenstein, Germany).

Abbreviations used: bHLH, basic helix loop helix; EPO, erythropoietin; HIF, hypoxia-inducible factor; rHIF, rat HIF; HRE, hypoxia-response element; LUC, luciferase; ARNT, arylhydrocarbon receptor nuclear translocator; PAS, Per/ARNT/Sim; MOP, member of the PAS superfamily; TAD, transactivation domain; SV40, simian virus 40.

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The sequence data reported here have been deposited in the EMBL Nucleotide Sequence Database under the following accession numbers: rHIF1α, Y09507; rHIF2α, AJ277828; rHIF3α, AJ277827.

The Bluescript vector (pBS) was purchased from Stratagene (Heidelberg, Germany) and the vectors pcDNAI/AMP, pcDNA6, pCR2.1, pCRII and pCRIITOPO were from Invitrogen (Leek, The Netherlands).

Cloning and sequencing of rHIF1α, rHIF2α and rHIF3α

The rHIF1α, rHIF2α and rHIF3α cDNAs were cloned from RNA of cultured rat hepatocytes by reverse transcriptase PCR. Total RNA was extracted from the cells using the NucleoSpin RNA purification kit (Macherey-Nagel, Düren, Germany). Then, RNA was denaturated for 10 min at 68 °C, followed by incubation on ice for 10 min. First-strand cDNA was synthesized in a total volume of 25 μl containing 1 μg of RNA with 1 μl of random hexanucleotides (10×, Roche), 1 μl of oligo-$dT_{12\text{-}18}$ (500 μg/ml) as primers, 2.5 μl of dithiothreitol (0.1 M), 1.5 μl of dNTPs (10 mM each), 1 μl of RNasin (40 units/μl), 5 μl of reaction buffer (5×) and 1 μl of Superscript™ II (200 units/μl) by incubation for 90 min at 42 °C, 30 min at 52 °C and 15 min at 95 °C.

The HIF cDNAs were then amplified using specific primers (see below) from 1 μl of the cDNA template solution by PCR with an initial denaturation for 3 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 48 °C (rHIF1α), 55 °C (rHIF2α) or 61 °C (rHIF3α), polymerization for 3 min at 72 °C and a final polymerization step (10 min at 72 °C).

The rHIF1α cDNA was amplified with the sense primer HIF-90, 5′-GTCTCGAGATGCAGCCAGATCTCG-3′, corresponding to positions 91–114, and the antisense primer HIF-868, 5′-GGTCAGATGATCAGAGTCCAAAGC-3′, corresponding to positions 869–892 of the human HIF1α sequence (GenBank accession number U22431). The resulting 800 bp rHIF1α cDNA fragment was cloned into pCRII. Using the sense primer HIF-90 and the antisense primer HIF-2560, 5′AGGTAGTGAGCCAC-CAGTGTC-3′, corresponding to positions 2539–2559 of the human HIF1α sequence, a 2470 bp rHIF1α cDNA fragment was obtained and cloned into pCR2.1. The 5′ end of the rHIF1α cDNA was amplified using the rapid amplification of cDNA ends (RACE) method with 1 μg of poly(A)$^+$-rich RNA from rat hepatocytes as template and HIF-868 as primer for first-strand cDNA synthesis. An anchor with the sequence 5′-(ph)CTA-TCGATTCTGGAACCTTCAGCG-3′ (where ph means phosphorylated) was ligated to the 3′ end of the generated single-stranded cDNA in a reaction mixture composed of 7.5 pmol of anchor, 2.5 μl of single-stranded cDNA solution and 5 μl of Hepes/KOH buffer (50 mM, pH 8.3), containing 10 mM $MgCl_2$, 5 mM ATP and 14 units of T4 RNA ligase (MBI Fermentas). After ligation a semi-nested PCR reaction was carried out in a 100 μl reaction volume containing 0.1 μl of the ligation mixture, 200 μM of each dNTP, 0.3 μM of the HIF1α reverse primer 5′-GCTTTATCAAGATGGGAGCTCACG-3′, generated from the 800 bp rHIF1α cDNA fragment amplified previously, 0.3 μM of the forward anchor primer and 0.5 units of *Taq* DNA polymerase. A total of 35 PCR cycles were performed at 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min, followed by 10 min at 72 °C. A 230 bp cDNA fragment was obtained and cloned into pCRII.

In order to obtain a rHIF1α cDNA which encodes the whole open reading frame, the pCR2.1-rHIF1α-2470 bp plasmid was linearized with *Sac*I, dephosphorylated and ligated with the 230 bp rHIF1α cDNA fragment excised from pCRII-HIF800 with *Sac*I. The resulting plasmid contained then a 2700 bp rHIF1α cDNA fragment with the entire open reading frame. The 2700 bp rHIF1α cDNA fragment was subcloned into the *Eco*RI/*Bam*HI sites of the eukaryotic expression vector pcDNAI/AMP.

The rHIF2α cDNA was amplified using the primer mEPAS-F, 5′-AGCTGCGGAGGGCCACAGCAAA-3′, corresponding to positions 9–30, and primer mEPAS-R, 5′-GGAAGTGAAGC-AGGCAGGTCAAGAC-3′, corresponding to positions 2768–2792 of the mouse HIF2α sequence (GenBank accession number U81983). The resulting 2700 bp rHIF2α cDNA fragment was cloned into pCRIITOPO and then subcloned into pcDNAI/AMP with *Eco*RI/*Xba*I. The primers hEPAS-F, 5′-GGCCCC-CAGATCCACCATTACAT-3′, corresponding to positions 2065–2087, and hEPAS-R, 5′-CGGGCACGTTCACCTCACA-GTCAT-3′, corresponding to positions 2674–2697 of the human rHIF2α sequence (GenBank accession number U81984), were used to generate a 650 bp rHIF2α cDNA fragment that was cloned into pCR2.1 and then subcloned into the *Bam*HI site of pBS.

The rHIF3α cDNA was amplified using the sense primer MOP7-F5, 5′-CACATGGACTGGGACCAAGACAGG-3′, corresponding to positions 81–104, and the antisense primer MOP7-R, 5′GTGTAGGCTGCTGGTGTGGAGTGT-3′, corresponding to positions 2122–2145 of the mouse HIF3α sequence (GenBank accession number AF060194), leading to a 2000 bp cDNA fragment that was cloned into pCRIITOPO and subcloned into the *Eco*RI site of pcDNA6.

Two independent clones of all rHIFα cDNA clones obtained were sequenced in both directions using the dideoxy chain-termination method and the DNA 373A sequencer (Applied Biosystems, Weiterstadt, Germany) according to the protocols provided by the manufacturer. Those sequences of rHIF1α, rHIF2α and rHIF3α that contained the complete open reading frames were submitted to GenBank.

Cell-culture experiments

Hepatocytes were isolated by collagenase perfusion [16]. Cells (1×10^6 per dish) were co-transfected with 2 μg of a pGL3-EPO-HRE_3-SV40-LUC gene construct (where EPO is erythropoietin, SV40 is simian virus 40 and LUC is luciferase), and 0.5 μg of the expression vectors pcDNAI-rHIF1α, pcDNAI-rHIF2α or pcDNAI-rHIF3α, containing the whole open reading frames of the cDNAs under the control of the cytomegalovirus promotor. The pGL3-EPO-HRE_3-SV40-LUC gene construct was generated by ligation of the double-stranded and phosphorylated oligonucleotide 5′-cgc(CCCTACGTGCTGTCTCACACAGG)$_3$-gagct-3′, having *Kpn*I/*Sac*I overhangs (in lower case) and which contained three copies of the 3′-flanking region of the human *EPO* gene (in parentheses) with the HRE (underlined) and the supporting sequence 5′-CACAG-3′, into the *Kpn*I/*Sac*I sites of pGL3prom (Promega). This placed three HREs in front of a SV40 promoter and the *LUC* gene. Co-transfections were performed by calcium phosphate precipitation [17] and the cells were maintained for the initial 5 h of culture under standard conditions under an atmosphere of 16% O_2, 79% N_2 and 5% CO_2 (by vol.) in medium M199 containing 0.5 nM insulin added as a growth factor for culture maintenance, 100 nM dexamethasone required as a permissive hormone and 4% fetal calf serum. After 5 h cells were cultured in serum-free M199 under the same atmosphere with 16% O_2, mimicking arterial O_2 tensions. These O_2 values take into account the O_2 diffusion gradient from the media surface to the cells [18]. After an additional 24 h of incubation the LUC activity was measured with the LB 953 luminometer (Berthold, Pforzheim, Germany) as described previously [19].

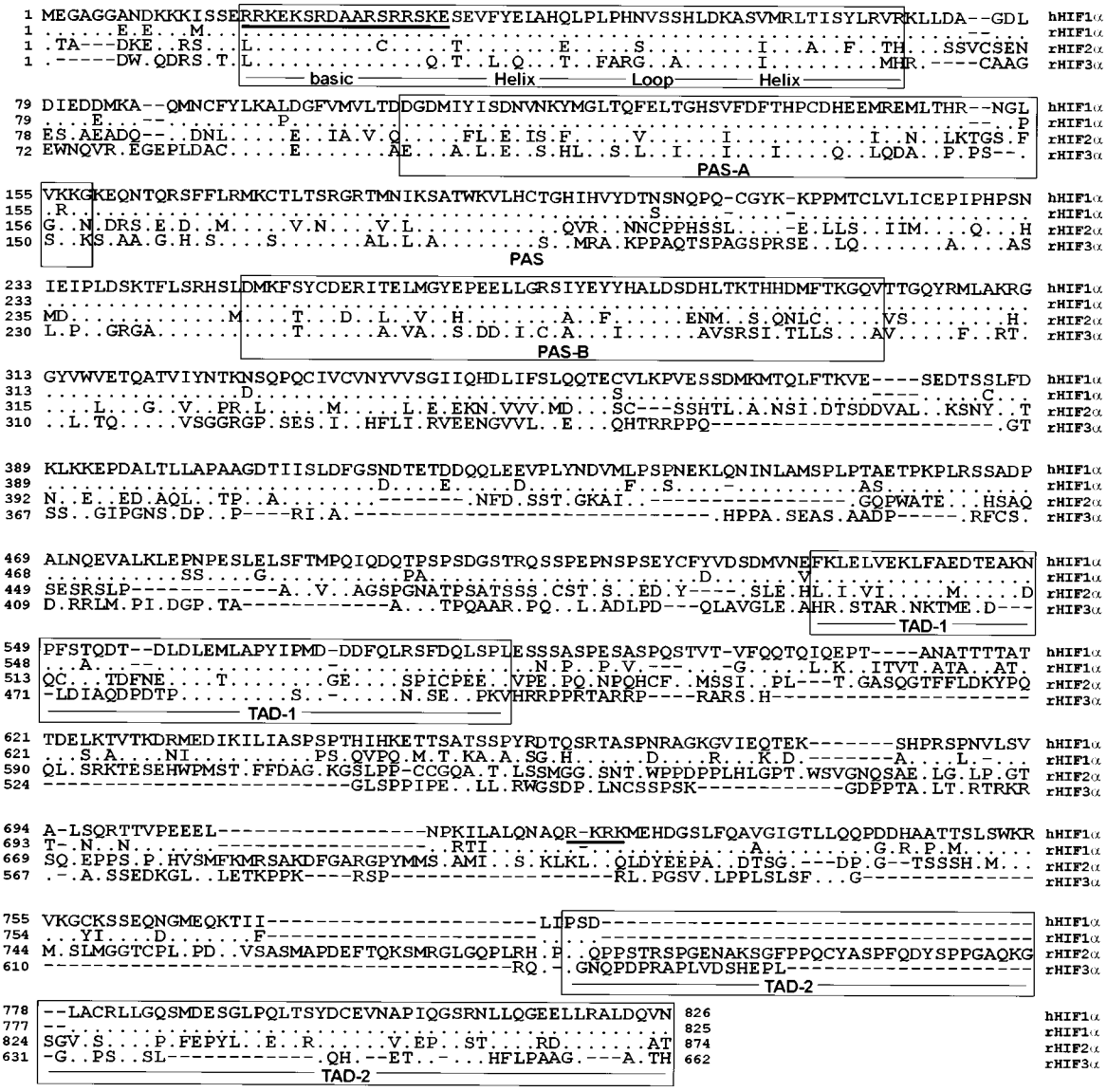

Figure 1 Alignment of the rHIF1α, 2α and 3α subunits with the human HIF1α

The cDNA-derived protein sequences of rHIF1α (825 amino acids), HIF2α (874 amino acids) and HIF3α (662 amino acids) are shown aligned with the human (h) HIF1α protein sequence. The alignment was performed using the Clustal Alignment algorithm. Dots indicate the positions of sequence identity, and dashes reflect gaps introduced into the amino acid sequence to obtain optimal sequence homology. The putative functional domains, bHLH, PAS, TAD-1 and TAD-2, are boxed, and the sites of the nuclear-localization signals are underlined. The amino acid positions are shown on the left.

Preparation of antisense RNA and *in situ* hybridization

Antisense RNA probes were generated with the digoxigenin RNA-labelling mixture. *In vitro* transcription of the following plasmids was performed: pCRII-rHIF1α-800 and pCRIITOPO-rHIF3α-2000, using T7 RNA polymerase, and pBS-rHIF2α-650, using T3 RNA polymerase.

In situ hybridization was essentially performed as described in [20].

Immunohistochemistry and Western blotting

Paraffin-embedded liver of normal fed rats was cut into 5 μm sections. Paraffin was removed from the liver sections by xylene, followed by rehydration in decreasing ethanol concentrations. After incubation in 1 × TBS (Tris-buffered saline, 50 mM Tris/HCl/150 mM NaCl, pH 7.5), the sections were permeabilized by pepsin digestion (0.45 % pepsin/0.01 M HCl; 100 μl/section) and washed in TBS. In order to avoid unspecific antibody binding, sections were blocked in TBS containing 20 % fetal calf serum for 30 min at 37 °C. The primary antibody against rHIF1α was generated in rabbits against a 233 amino acid peptide as described in [8]. The antibodies against rHIF2α and rHIF3α were generated by conjugating a 15 amino acid rHIF2α peptide (amino acids 507–521) and a 15 amino acid rHIF3α peptide (amino acids 507–521), respectively, with keyhole-limpet protein and injecting these conjugates into rabbits. The primary antibody against glutamine synthetase, raised in mice, was obtained from Affinity (Exeter, Devon, U.K.). Incubation with the primary antibody was performed overnight in TBS containing 0.1 % BSA at 37 °C. The antibody against rHIF1α was applied

in a 1:1500 dilution, the antibodies against rHIF2α and rHIF3α were used in a 1:2000 dilution, the antibody against glutamine synthetase was diluted 1:100. The rabbit preimmune sera of the antibody preparations against rHIF1α, rHIF2α and rHIF3α, used as a control, were applied in the same concentrations as the antibodies. Sections were then washed in TBS for 10 min. Peroxidase-conjugated anti-rabbit (rHIF1α, rHIF2α, rHIF3α) or anti-mouse (glutamine synthetase) IgGs, respectively, were used as a secondary antibody, applied in a 1:100 dilution in TBS containing 0.1% BSA for 30 min at 37 °C. The sections were washed in TBS for 10 min, followed by an incubation in diaminobenzidine concentrate diluted 1:10 in 1 × peroxidase buffer (Pierce, Rockford, IL, U.S.A.) for 10 min. The reaction was stopped by transferring the sections into TBS. After dehydration in increasing ethanol concentrations the sections were fixed in DePeX (Serva, Heidelberg, Germany).

Western-blot analysis was performed as described in [21]. A total of 100 μg of protein was loaded on to a 10% SDS/polyacrylamide gel and after electrophoresis it was blotted on to nylon membranes. The primary rabbit antibodies against rHIF1α, rHIF2α and rHIF3α were used in a 1:2000 dilution in 3% non-fat dry milk. The secondary antibody was a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and used in a 1:2000 dilution. The ECL Western-blotting system (Amersham, Freiburg, Germany) was used for detection. Under these conditions rHIF1α appeared as a band of about 120 kDa, rHIF2α as a band of about 110 and rHIF3α as a band of about 70 kDa.

RESULTS

Isolation and characterization of the rHIF1α, rHIF2α and rHIF3α cDNA sequences

The rHIF1α, rHIF2α and rHIF3α cDNAs were amplified by reverse transcriptase PCR using total RNA of primary rat hepatocytes as the templates. Primers were generated corresponding to the human HIF1α, the mouse and human HIF2α and the mouse HIF3α cDNA sequences. The open reading frames of the amplified cDNAs were subjected to sequence analysis; this allowed us to predict the sequences of 825 amino acids for rHIF1α, 874 amino acids for rHIF2α and 662 amino acids for rHIF3α (Figure 1). The HIFs of the same subtype are highly homologous between the different species: the overall identities of rHIF1α, rHIF2α and rHIF3α with the corresponding mouse homologues are 96, 92 and 93%, respectively, and those with the corresponding human homologues are 89 and 85%, respectively (the hHIF3α cDNA has not been completely cloned and sequenced yet; Figure 1). The three HIFα subtypes of one species, however, possess only a low homology: rHIF1α has an overall identity with rHIF2α of only 37% and with rHIF3α of only 35% (Figure 1); the corresponding values for the mouse homologues are 38 and 30% (results not shown). However, the identity of the three HIFα subtypes is much higher within most of the different functional domains derived from their human counterparts [22–25]. The bHLH domain of rHIF1α (positions 17–70) is 82% identical with that of rHIF2α (positions 14–67) and 74% with that of rHIF3α (positions 12–65; Figure 1). The PAS-A domain (positions 107–158) and PAS-B domain (positions 249–300) of rHIF1α have identities with the corresponding domains in rHIF2α (positions 105–157 and 251–302) and in rHIF3α (positions 102–150 and 246–297) of 63 and 58%, and of 73 and 58%, respectively. Finally, the transactivation domain (TAD)-1 domain (positions 532–582) and the TAD-2 domain (positions 775–825) of rHIF1α have lower identities with the corresponding domains of rHIF2α (positions 497–582 and 797–874) and rHIF3α (positions 458–506 and 614–662) of 54 and 37%, and of 32 and 28%, respectively.

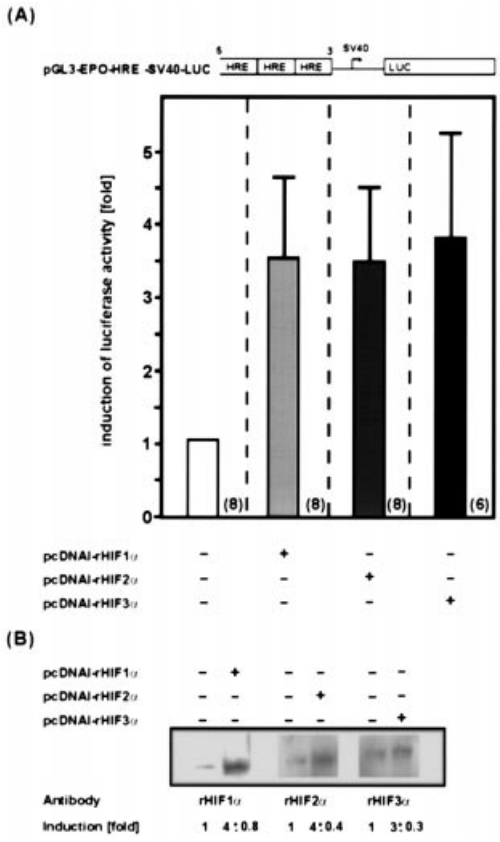

Figure 2 Induction of LUC activity of the transfected pGL3-EPO-HRE$_3$-SV40-LUC gene construct by co-transfected rHIF1α, rHIF2α or rHIF3α expression vectors in primary rat hepatocytes

(**A**) Primary rat hepatocytes were co-transfected with pGL3-EPO-HRE$_3$-SV40-LUC and pcDNAI-rHIF1α, pcDNAI-rHIF2α or pcDNAI-rHIF3α. After 5 h of incubation the cells were cultured in serum-free medium under 16% O_2, mimicking arterial O_2 tensions, and 24 h later LUC activity was measured. The numbers of transfections are shown in parentheses. (**B**) Representative Western blots of rHIF1α, rHIF2α and rHIF3α expression after transfection of the pcDNAI-rHIF1α, pcDNAI-rHIF2α or pcDNAI-rHIF3α expression vectors. Total cellular protein (100 μg) was subjected to Western-blot analysis (see the Materials and methods section) with antibodies against rHIF1α, rHIF2α or rHIF3α. Blots were scanned by video densitometry and in each experiment the level measured in the non-transfected cells was set equal to 1. Values represent the fold induction of four independent experiments.

Activation of transfected EPO-HRE$_3$-SV40-LUC gene constructs by rHIF1α, rHIF2α and rHIF3α in primary rat hepatocyte cultures

To test the ability of the cloned rHIFs to activate a gene construct containing three HREs of the human *EPO* gene [12] in front of the SV40 promotor and the *LUC* gene (pGL3-EPO-HRE$_3$-SV40-LUC), the rHIF1α, rHIF2α and rHIF3α cDNAs were cloned into the eukaryotic expression vector pcDNAI/AMP. Primary rat hepatocytes were co-transfected with pGL3-EPO-HRE$_3$-SV40-LUC and pcDNAI-rHIF1α, pcDNAI-rHIF2α or pcDNAI-rHIF3α. The LUC activity obtained in hepatocytes co-transfected with pGL3-EPO-HRE$_3$-SV40-LUC and the empty pcDNAI/AMP vector was considered as basal expression (Figure 2). Compared with this basal expression, co-transfection of pcDNAI-rHIF1α resulted in a 3.5-fold increase of LUC activity; the same enhancement of LUC activity was

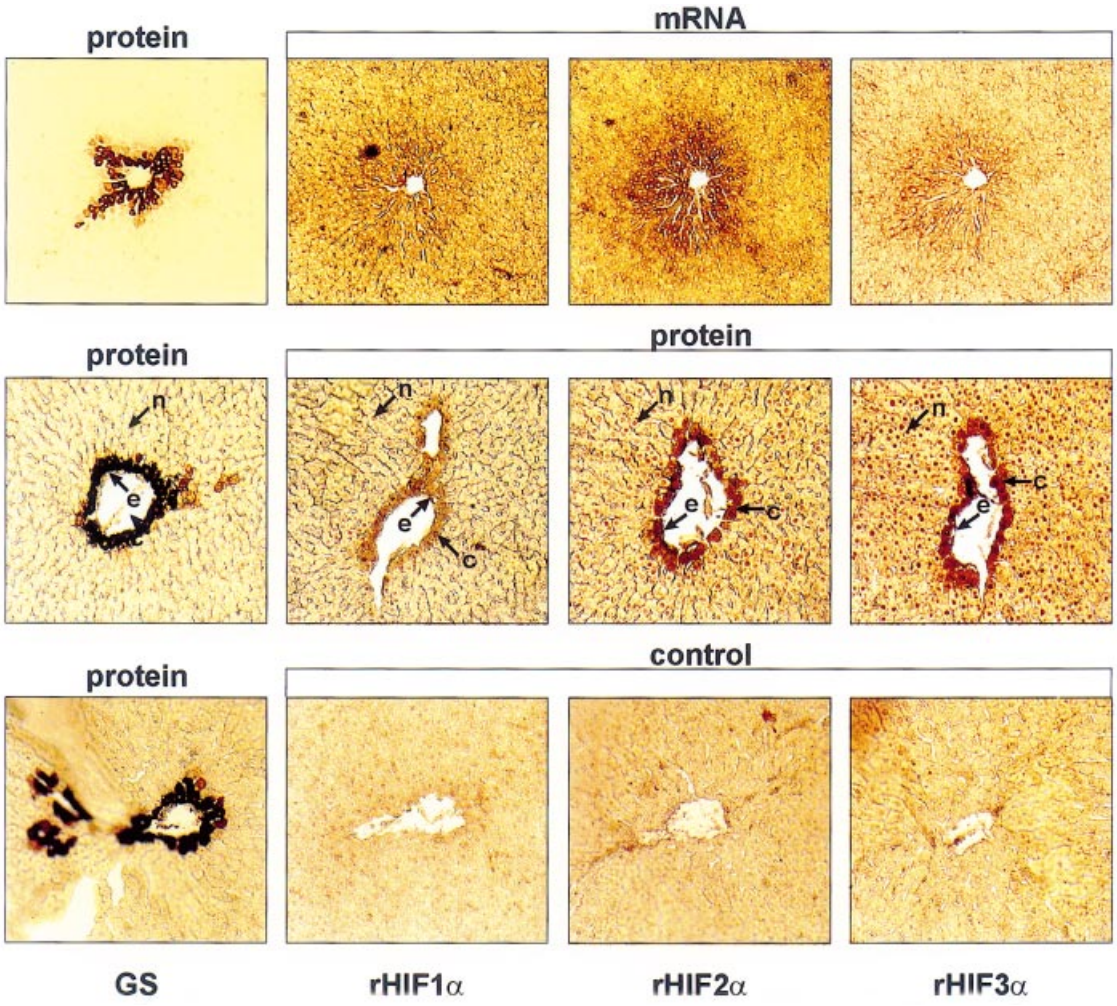

Figure 3 Perivenous zonation of rHIF1α, rHIF2α and rHIF3α mRNAs, even distribution of nuclear proteins but distal perivenous localization of cytosolic rHIF1α, rHIF2α and rHIF3α proteins in rat liver

Parallel sections (5 μm) were prepared from livers of rats kept under a normal daily feeding cycle. mRNAs were localized by *in situ* hybridization using digoxigenin-labelled rHIF1α, rHIF2α and rHIF3α antisense RNA probes. Immunohistochemistry was performed with antibodies against rHIF1α, rHIF2α, rHIF3α and glutamine synthetase (GS) as a distal perivenous marker. Preimmune sera were used as controls. Dark precipitates indicate high levels of mRNA or protein, respectively. Arrows point to the nuclei of the hepatocytes (n), the cytoplasm of the distal perivenous hepatocytes (c) and the endothelial cells of the central vein (e).

obtained after co-transfection with pcDNAI-rHIF2α. A 3.8-fold induction of LUC activity was observed in cells co-transfected with pcDNAI-rHIF3α (Figure 2).

Perivenous localization of the rHIF1α, rHIF2α and rHIF3α mRNAs and homogeneous distribution of the nuclear proteins

To test whether the expression of the rHIF1α, rHIF2α and rHIF3α mRNA and the distribution of the HIF1α, HIF2α and HIF3α proteins correlated with the O_2 gradient in the liver sinusoids, *in situ* hybridization and immunohistochemical analysis with parallel sections of paraffin-embedded rat livers were performed, respectively. The mRNAs of rHIF1α, rHIF2α and rHIF3α were located predominantly in the perivenous zone of the liver acinus (Figure 3). However, the proteins of HIF1α, HIF2α and HIF3α were found in the nuclei of all periportal and perivenous hepatocytes. In the distal perivenous zone, the proteins were also detected in the cytoplasm of the hepatocytes (Figure 3). Surprisingly, mRNAs and proteins of all three rHIFα subtypes were found in the endothelial cells of the central vein (Figure 3).

DISCUSSION

The cDNAs of three rHIF α-subunits were generated from RNA of primary rat hepatocytes. After transfection, all three cDNAs were able to activate LUC activity of the EPO-HRE_3-SV40-LUC gene construct to nearly the same extent. The mRNAs of the three rHIF α-subunits were expressed predominantly in the perivenous zone in rat liver; however, the nuclear HIFα proteins showed an even distribution.

Analysis of the rHIF α-subunit amino acid sequences

The rHIF α-subunits rHIF1α, rHIF2α and rHIF3α are 825, 874 and 662 amino acids long, respectively. In mice and humans the size ratio is the same [5]. The three rHIFα subtypes displayed the highest identities of 74–82 % within the N-terminal functional bHLH domain, intermediate identities of 58–73 % within the

central PAS-A and PAS-B domains and lower identities of 23–54 % within the C-terminal TAD-1 and TAD-2 domains (Figure 1). Similar findings were also described for the mouse and human HIFα subtypes [3,5]

Although there is no functional evidence yet, the amino acid alignment implicates at least one TAD-1 within the C-termini of rHIF1α, rHIF2α and rHIF3α (Figure 1). The 100 % amino acid identity between rHIF1α and human HIF1α within TAD-2 is a strong indication for a second TAD, at least in HIFα subtype 1. Due to the lower identities and the differences in length of the TAD-2 of HIFα subtypes 2 and 3, it is impossible to predict whether these domains represent true TADs.

Function of the cloned rHIF α-subunit cDNAs

The cloned rHIF α-subunit cDNAs were functional, since LUC activity was clearly increased in co-transfections with a *LUC* reporter-gene construct that contained three hypoxia-inducible elements (HREs) of the human *EPO* gene [12] (Figure 2). In similar transfection studies with the HEK-293 cell line it was shown that the human HIF2α [4] and the mouse HIF3α [3] together with ARNT activated *LUC* gene constructs at the HRE of EPO.

Cellular distribution of the rHIFα proteins

The rHIFα proteins were expressed mainly in the hepatocytes but they were also found in the endothelial cells surrounding the central vein of the rat liver. This is remarkable and may be linked to the stabilization of the HIFα proteins by the low oxygen tensions there (see below). Moreover, it was surprising that the rHIF2α mRNA was expressed in hepatocytes since it was proposed that HIF2α is a transcription factor specific for endothelial cells [5]. However, in the meantime HIF2α mRNA expression has also been found in neurons of adult mice [7] and in the chromaffin cells of the organ of Zuckerkandl [26], indicating that HIF2α does not appear to be specific for endothelial cells.

Zonal expression of the rHIFα genes in the hepatocytes of the liver acinus

The mRNAs of rHIF1α, rHIF2α and rHIF3α were expressed predominantly in the perivenous zone of the liver acinus (Figure 3). This is in line with expectations, since *in vivo* [27,28] and *in vitro* [29] HIF1α mRNA levels were found to be enhanced by low oxygen tensions. But the HIFα proteins were found essentially evenly distributed in the cytosol at low levels and in the nuclei at higher levels of periportal and perivenous hepatocytes and, in addition, in the cytosol at very high levels of the distal perivenous cells around the central vein (Figure 3).

This lack of concordance between the zonal distribution of HIFα mRNA and protein may be surprising. However, a concordant zonation of mRNA and protein can only be expected if gene expression is regulated mainly at the pre-translational level of transcription and/or mRNA degradation, as has been shown for the periportal enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase and the perivenous enzyme glucokinase [14,15]. Yet, a discordant zonation of mRNA and protein has to be expected, if gene expression is controlled primarily at the translational or even post-translational level of protein degradation, as has been observed for the enzyme pyruvate kinase with an even distribution of the mRNA and a mainly perivenous localization of the protein [14,15]. The lack of a strong concordance in the zonal pattern of HIFα mRNA and proteins would indicate that the HIF α-subunit gene expression in rat liver is also regulated mainly at a translational or post-translational level. Accordingly, high levels of the HIFα proteins were found in the cytoplasm of the distal perivenous cells (Figure 3). Since the level of the human HIF1α protein is also known to be regulated by protein stabilization under hypoxic and protein degradation under normoxic conditions [30], the high cytoplasmic presence of the HIFα proteins in the distal least-aerobic perivenous cells appears to be the result of enhanced protein stabilization.

Maybe, under normal conditions, periportal and perivenous cells need the same levels of HIF proteins, the activity of which is regulated by the pO_2; but under abnormal conditions like haemorrhage or impaired lung function both leading to hypoxaemia or inflammation the perivenous cells need more HIF proteins within short periods of time. Therefore, they contain higher levels of HIFα mRNA so that the HIFα proteins can be elevated rapidly by *de novo* protein synthesis using pre-existing mRNA. Whether in addition the import of the HIFα proteins into the nuclei [31], which for HIF1α has been shown to be ARNT-independent [32], might also play a role during these processes is not known yet. However, the surprising even distribution of all three HIFα proteins in the nuclei may indicate that the proteins have functions other than those related to O_2-dependent gene regulation. Indeed, HIF proteins might have a role during inflammatory processes, since treatment of HepG2 cells with interleukin-1β and tumour necrosis factor-α stimulated the DNA binding of HIF1α [33]. Moreover, HIF proteins appear to have a key role during cellular proliferation [34]. In the liver, cellular proliferation during regeneration after partial hepatectomy starts from the periportal areas [35]. In addition, growth factors such as platelet-derived growth factor or hormones such as angiotensin II induced HIF1α activation and nuclear translocation in vascular smooth-muscle cells independent from the pO_2 [36]. Thus the details of the role of HIFα proteins in the zonation of gene expression and in other cellular functions remain to be determined.

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