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Role of the HBx oncoprotein in carbonic anhydrase 9 induction

Shortened title: HBx and CA9 induction

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Abstract

Carbonic anhydrase 9 (CA9), as one of the most hypoxia-responsive genes, has been associated almost exclusively with hypoxic tumours. Its principal role is in pH regulation which helps tumour cells overcome intracellular acidosis and survive extended periods of time with low oxygen. Hypoxia-inducible factor 1 (HIF-1) is the main transcriptional activator of CA9. Hepatitis B virus X protein (HBx) has been shown to increase the transcriptional activity of HIF-1. HBx is often expressed from the gene integrated in the hepatocytes infected persistently and contributes significantly to alterations in host gene expression that can lead to the development of hepatocellular carcinoma (HCC) associated with HBV. The aim of this study was to determine the effect of HBx on expression of CA9. Transient transfection of HBx led to an increase in the expression of CA9 as assessed by RT-PCR and Western blotting. HBx was able to increase CA9 promoter activity significantly in several cell lines. The effect was mediated via HIF-1 and a functional HRE element located –10/–3 bp upstream of the CA9 transcription initiation site. These data suggest that CA9 may be involved in the development of HCC by contributing to the survival of hepatocytes infected with HBV in liver tissue with fibrosis.

Key Words

Hypoxia; hepatitis B virus; hypoxia-inducible factor 1; hepatocarcinogenesis; hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV) infection is one of the most common viral infections. It is self-limiting in 90-95% of adults infected acutely, while the majority of neonatal infections become persistent. Similarly, horizontal transmission to children or immunocompromised adults will result in a significant risk of developing persistent infection. Perinatal transmission accounts for most infections in highly endemic areas. Over 350 million people worldwide are infected chronically with HBV, with one million deaths per annum resulting from the complications of chronic infection [Ganem and Prince, 2004; Guidotti and Chisari, 2006].

HBV is an enveloped hepatotropic virus. It is a noncytopathic virus and most of the liver damage occurs as a result of immune responses, especially the cytotoxic T lymphocyte (CTL) response, with contributions from antigen-nonspecific inflammatory cells and possibly platelets facilitating the intrahepatic accumulation of CTLs [Iannacone et al., 2007]. The 3.2-kb-long, partially double-stranded circular DNA genome of HBV encodes the surface, core, polymerase, and X (HBx) genes. HBx is a portion of HBV DNA integrated most frequently into hepatocyte chromosomes during the development of hepatocellular carcinoma (HCC), which is a highly vascularized and malignant solid tumour. Such a tumour phenotype is often associated with an insufficient supply of oxygen, which creates selective pressure in favour of tumour cells that can adapt to this microenvironmental stress. Hypoxia-inducible factor 1 (HIF-1) is the main mediator of hypoxic regulation facilitating the transcription of a battery of genes implicated in angiogenesis, erythropoiesis, anaerobic glycolysis, pH control, and more [Semenza et al., 1991; Semenza et al., 1994; Ke and Costa, 2006; Brahimi-Horn and Pouyssegur, 2007].

HIF-1 is a heterodimer that consists of an oxygen-sensitive α subunit and a constitutively expressed β subunit [Wang et al., 1995]. Under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) on specific proline residues localized within an oxygen-dependent degradation domain (ODDD) and by Factor inhibiting HIF-1 (FIH-1) on an asparagine residue in the C-terminal transactivation domain (C-TAD) [Masson et al., 2001; Epstein et al., 2001; Lando et al., 2002]. Von Hippel Lindau protein (pVHL) recognizes the prolyl hydroxylated form of HIF-1α and recruits ubiquitin ligase for its ubiquitination [Hon et al., 2002]. HIF-1α is then destroyed in

the proteasome. Hydroxylated asparagine residue in the C-TAD domain on the other hand cannot recruit the p300 transcription co-activator. In hypoxia, the HIF-1α subunit is not hydroxylated efficiently by PHDs and FIH-1 because of shortage of oxygen, which serves as the hydroxylation substrate, and thus HIF-1α is able to escape degradation mediated by pVHL in the proteasome. It is then accumulated in the cytoplasm and translocates to the nucleus where it dimerizes with the constitutive β subunit to form the HIF-1 transcription factor that binds to hypoxia-response elements (HREs) in the promoters of hypoxia-inducible genes and induces their transcription [Wang et al., 1995].

Although HIF-1 is regulated mainly by oxygen tension, other factors also modulate HIF-1 expression and consequent function, such as nitric oxide, cytokines, growth factors, etc. [Stroka et al., 2001]. HBx stimulates HIF-1α stability by blocking the proteasomal degradation mediated by pVHL and also increases its transcriptional activity [Moon et al., 2004]. The C-terminus of HBx binds the bHLH/PAS, ODDD and C-TAD domains of HIF-1α [Yoo et al., 2004]. By binding to bHLH/PAS, HBx potentiates HIF-1 interaction with its target promoters. Through the C-TAD domain, HBx can block the association of FIH-1 with HIF-1α, and inhibit Asn^{803} hydroxylation, thus facilitating binding of the p300/CBP co-activator.

Several hypoxic tumour markers are direct targets of HIF-1, such as vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1) or carbonic anhydrase IX (CA IX). However, there is variability between expression patterns of these markers in tumours, and out of these three genes, CA IX is the most reliable marker of hypoxia [Rafajova et al., 2004]. CA IX is one of the most hypoxia-responsive genes and was proposed to serve as an intrinsic marker of hypoxia [Potter and Harris, 2003]. It catalyzes the reversible conversion of CO_2 to bicarbonate ion and proton like other active carbonic anhydrases, and participates in ion transport and pH control [Svastova et al, 2004]. Additionally, CA IX also reduces E-cadherin-mediated adhesion by binding to β-catenin [Svastova et al, 2003]. CA IX is a transmembrane isoform associated mainly with tumours, in contrast to other CAs that are present mostly in differentiated cells of normal tissues [Pastorek et al., 1994]. CA IX in the normal human liver is expressed on the basolateral surfaces of all ductal epithelial cells in the portal tracts and is absent from hepatocytes, while

some hepatocellular carcinomas exhibit positive immunostaining for CA IX [Saarnio et al, 2001]. Expression pattern of CA IX is determined principally by the strong activation of the CA9 gene transcription via HIF-1 that binds to the HRE localized in the CA9 promoter proximal to the transcription start site at the -10/-3 position [Wykoff et al., 2000]. CA IX expression contributes actively to adaptation to a hypoxic microenvironment, and is crucial during carcinogenesis.

Since HIF-1 is the main transcriptional activator of CA9, and HBx possesses the ability to stimulate HIF-1α, the reports cited above were the rationale behind the interest to investigate the influence of HBx on CA9 expression.

Materials and methods

Cell culture

Rat2 thymidine kinase-deficient immortalized fibroblasts, BP6 fibrosarcoma cells (provided kindly by Dr. J. Zavada, Institute of Molecular Genetics, Prague, Czech Republic), H9c2 rat embryonic cardiomyocytes (provided kindly by Prof. I. Morano, Charité University Medicine, Berlin, Germany), rat liver epithelial stem-like WB-F344 cells (provided kindly by Dr. M. Machala, Veterinary Research Institute, Brno, Czech Republic), HIF-1β-deficient mouse Hepa 1c4 hepatoma cells derived from the wild-type Hepa 1c1c7 cell line (provided kindly by Prof. L. Poellinger, Karolinska Institute, Stockholm, Sweden), immortalized HIF-1α knock-out mouse embryonic MEF HIF-$1\alpha^{-/-}$ fibroblasts (also provided kindly by Prof. L. Poellinger), and human hepatocellular carcinoma cell lines HepG2 (provided kindly by Prof. L. Poellinger) and PLC/PRF/5 (HPA Culture Collections, Porton Down, UK, Cat No 85061113) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% FCS (BioWhittaker, Verviers, Belgium) and 40 μg/ml gentamicin (Lek, Ljubljana, Slovenia) in a humidified 5% CO_2 incubator at 37°C. Hypoxic treatments were performed in a hypoxic workstation (Ruskinn Technology, Bridgend, UK) in 2% O_2, 5% CO_2, 2% H_2 and 91% N_2 at 37°C.

Constructs

As a source of the HBx nucleic acid sequence, we used the pREP4D/HBV991A plasmid containing the complete HBV genome (provided kindly by Dr. G. Alexander, Cambridge University Hospitals, NHS Foundation Trust, Cambridge, UK). HBx was amplified by Phusion polymerase (Finnzymes, Espoo, Finland) with primers flanking the HBx ORF (F – caaaactcatcgggactgacaa; R – acagcttggaggcttgaacag). The resultant 565-bp PCR product was ligated into the pBKS- vector (Stratagene, La Jolla, CA) linearized by EcoRV (Invitrogen, Carlsbad, CA). The insert was subcloned into pcDNA3.1 (Invitrogen) eukaryotic expression plasmid using EcoRI and XhoI (both from Invitrogen). The pcDNA3.1 HBx plasmid was sequenced to ensure that the HBx open reading frame was unaltered.

The pGL3 PR5 and pGL3 PR1 plasmids containing the human CA9 core and minimal promoters were generated by insertions of –173/+37 (PR5) and –50/+37 (PR1) CA9 genomic regions amplified by PCR upstream of the firefly luciferase gene in pGL3-Basic luciferase reporter vector (Promega, Madison, WI) [Kopacek et al., 2005]. The pGL3 PR5 mutHRE plasmid coding the human core CA9 promoter with a mutated HRE upstream of the firefly luciferase gene was created from the original pBMN5HREmut [Kaluz et al., 2002]. Renilla pRL-TK vector (Promega) served as a transfection efficiency control. Human HIF-1α cDNA cloned in pcDNA1 expression plasmid was provided kindly by Prof. P. Maxwell, Imperial College of Science, Technology and Medicine, London, UK [Wood et al., 1998]. HIF-1α dominant negative mutant (mut HIF-1α) was generated from pcDNA1-HIF-1α by a deletion of a large part of ODDD and N-TAD (aa 402-564) [Holotnakova et al, 2008]. Empty pcDNA3.1 plasmid (Invitrogen) was used as a negative control for mock transfection. Plasmid containing full-length HIF-1β (pARNT/GEM7) was provided kindly by Prof. L. Poellinger, Karolinska Institute, Stockholm, Sweden [Lindebro et al., 1995]. Empty pCMV (Genlantis, San Diego, CA) vector was used as a negative control.

Reverse transcription PCR

Rat2 and HepG2 cells were transfected transiently with 1 μg of pcDNA3.1 HBx or an empty pcDNA3.1 vector using GenePorterII reagent (Genlantis) according to the manufacturer's recommendations, and split the following day. The parallel transfectants were incubated in normoxia and hypoxia for 24 or 48 h followed by RNA isolation using InstaPure (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. Reverse transcription was performed with M-MuLV reverse transcriptase (Finnzymes) and random heptameric primers (400 ng/μl). The mixture of 3 μg total RNA and random primers was heated for 10 min at 70°C, cooled on ice and supplemented with dNTPs (each at 0.5 mM concentration, Finnzymes), M-MuLV reverse transcriptase buffer (Finnzymes), and with 200 U of reverse transcriptase M-MuLV, incubated for 1 h at 42°C, heated to 70°C for 15 min and stored at -20°C until further use. Reverse transcription PCR (RT-PCR) was performed with Taq polymerase (Promega) in an automatic DNA thermal cycler (Eppendorf AG, Hamburg, Germany) using gene-specific primers

and primers for ß-actin that served as internal standards (Table 1). The PCR protocol consisted of 94℃ for 3 min followed by 30-35 cycles of: denaturation at 94℃ for 30 s, annealing for 40 s (Tm in Table 1) and extension at 72℃ for 40 s, followed by final extension at 72℃ for 5 min. The intensity of bands corresponding to individual PCR products was evaluated with ImageJ 1.34s software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007). Amount of gene-specific PCR products was expressed semiquantitatively as the ratio of the intensity of each band to the intensity of the related β-actin internal standard.

Immunoblotting

Rat2 and HepG2 cells were transfected transiently with 1 μg of pcDNA3.1 HBx or an empty pcDNA3.1 vector with GenePorterII reagent and split the following day. The parallel transfectants were incubated in normoxia and hypoxia for 24 or 48 h followed by protein extraction with RIPA buffer (1% Triton X-100 and 0.1% sodium deoxycholate in PBS) containing inhibitors of proteases Complete mini (Roche Applied Science, Mannheim, Germany) for 15 min on ice. The extracts were then centrifuged for 15 min at 13000 rpm in an Eppendorf microcentrifuge at 4°C, and total protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Total protein extracts (20 μg/lane – Rat2, 50 μg/lane – HepG2) were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After separation, the proteins were blotted onto polyvinylidene fluoride membrane (Immobilon™, Millipore, Billerica, MA, USA). For CA IX detection, the membrane was incubated with the M75 primary antibody and secondary anti-mouse peroxidase-conjugated antibody (Sevapharma, Prague, Czech Republic). For loading control, the membrane was probed with anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal rabbit anti-goat IgG-HRP (horseradish peroxidase; Dako, Glostrup, Denmark). After repeated washing, the membranes were developed with the ECL detection system. The intensity of bands was evaluated with ImageJ 1.34s software and

expressed as the ratio of the intensity of each CA IX band to the intensity of the related actin internal standard.

Luciferase assay

Cells were plated into 35-mm Petri dishes to reach ~70% density on the following day. Transient transfection was performed with 1 μg of luciferase construct containing promoter and 50 ng of pRL-TK plasmid DNA using GenePorterII reagent. To analyze the effect of HBx either with or without transcription factors (HIF-1β, HIF-1α mut HIF-1α), the plasmids containing corresponding cDNAs were co-transfected. Cells transfected with control empty vectors served as negative controls. The day after transfection, cells were trypsinized and plated in triplicates into 24-well plates. Cells were allowed to attach for 24 h, and then they were either transferred to hypoxia or maintained in normoxia for additional 24 h. Reporter gene expression was assessed 72 h after transfection using Dual-Luciferase Reporter Assay System (Promega), and luciferase activity was normalized against renilla activity. Results were analyzed by two-tailed unpaired *t* test (Student`s test) with $P < 0.05$ considered significant.

Results

HBx increases CA9 expression

It was demonstrated that the expression of VEGF and the formation of new blood vessels were increased in hepatoma cells transfected with HBx [Lee et al., 2000]. VEGF and CA9 are both hypoxia-regulated genes, and their main transcriptional activator HIF-1 has been reported to be influenced by HBx. The HBx ORF was cloned into pcDNA3.1 under the control of a constitutive CMV promoter, and Rat2 immortalized fibroblasts were transfected transiently with HBx or an empty pcDNA3.1 vector. After splitting the cells the following day, the parallel transfectants were incubated in normoxia or hypoxia for 48 h. Afterwards, the expression of hypoxia-inducible genes was analyzed by RT-PCR and immunoblotting (Fig.1). Increased normoxic transcription can be seen only with VEGF and slightly with GLUT4 in cells transfected with HBx (Fig.1A). In hypoxia, HBx was able to increase the transcription of *Car9* (more than 1.5-fold), GLUT4, PDK1, and slightly of VEGF. HBx had no effect on expression of iNOS or GLUT1 in Rat2 cells. As expected, the levels of HIF-1α transcript were roughly the same in all the samples. At the protein level, HBx was also able to increase CA IX expression significantly in Rat2 cells (Fig.1B). Each of the examined hypoxia-regulated genes showed different magnitude of induction, most probably as a consequence of different molecular mechanisms involved in their regulation. For example, the regulation of VEGF, GLUT1 and CA9 has several common features but there are also clear differences. The CA9 promoter contains HRE at a position close to the transcription initiation site, serving as its main transcriptional regulator, whereas VEGF possesses HRE almost 1 kb upstream of the transcription start and utilizes it more as an enhancer element [Levy et al, 1995], as well as the GLUT1 promoter, which contains HRE in the enhancer 1 region, more than 2 kb upstream of the transcription site [Ebert et al, 1995]. On the other hand, VEGF mRNA is regulated at the level of posttranscriptional stability, and GLUT1 expression is additionally influenced by intracellular glucose levels.

HBx induces CA9 promoter activity

After realization that HBx was able to increase CA9 expression in Rat2 cells, the effect of HBx on CA9 promoter activity was studied by employing dual luciferase reporter assay (Promega) (Fig.2). Rat cells of different origin were transfected transiently with the pGL3 PR5 construct containing the core human CA9 promoter linked to luciferase reporter. They were also co-transfected with renilla plasmid used as a transfection efficiency control and either with HBx or empty pcDNA3.1. Two days later, sparse (Fib.2A) or dense (Fig.2B) cell cultures were incubated in normoxia and hypoxia for 24 h. HBx elevated CA9 promoter activity significantly in Rat2, BP6 and WB-F344 cell lines under hypoxia (Fig.2A). The promoter activity increased slightly also in hypoxic H9c2 cardiomyocytes. Under normoxic conditions, HBx was able to induce CA9 promoter activity only in WB-F344 cells. In dense Rat2 cell culture, HBx increased the CA9 promoter activity also under normoxia (Fig.2B) probably due to pericellular hypoxia. In this experiment, the effects of HBx on the core (pGL3 PR5) and minimal (pGL3 PR1) CA9 promoters were tested. The effect was more prominent on the minimal promoter most likely because the core promoter contains a silencer element [Kaluz et al., 1999]. Since HBx also increased the activity of the minimal promoter, it can be assumed that the effect is mediated via the functional HRE contained within this region. Indeed, the effect was abolished on the core promoter with mutated HRE (pGL3 PR5 mutHRE). Empty pGL3 vector served as negative control.

HBx requires both HIF-1 subunits for CA9 promoter activity induction

The mechanism of HBx action in HIF-1 transactivation is understood incompletely. Its C-terminus binds the bHLH/PAS and C-TAD domains of HIF-1α and thus potentiates HIF-1 interaction with its target promoters [Yoo et al., 2004]. The aim of this series of experiments was to confirm that HBx mediates CA9 promoter induction through HIF-1 and to find out whether HBx, by binding to the bHLH/PAS domain of HIF-1α, could block HIF-1 subunit dimerization and whether HIF-1α/β and HBx alone could possibly induce activity of the CA9 promoter.

Mouse Hepa 1c4 hepatoma cells that lack natural expression of HIF-1β were co-transfected transiently with the pGL3 PR1 minimal CA9 promoter luciferase construct, renilla plasmid, and HBx (Fig.3A). They were also co-transfected with an empty pCMV vector or the

same amount of HIF-1β. As can be seen in Fig.3A, HIF-1β is indispensable to the induction of the CA9 promoter mediated by HBx.

Just like HIF-1α, HIF-1β also possesses the bHLH/PAS domain. A similar experiment on mouse embryonic fibroblasts knocked-out for HIF-1α (MEF HIF-1$\alpha^{-/-}$) was performed (Fig.3B). MEF HIF-1$\alpha^{-/-}$ cells were co-transfected transiently with the pGL3 PR1 construct, renilla plasmid, HBx, and an empty pCMV vector or the same amount of HIF-1α. From this experiment, it is evident that HIF-1α is crucial to HBx-mediated CA9 promoter induction (Fig.3B). It can thus be concluded that HBx does not block HIF-1 subunit dimerization and influences the CA9 promoter through a functional HIF-1 dimer.

HBx regulation of CA IX expression in human hepatocellular carcinoma cells

All the previous experiments were done on rat and mouse cellular models. The possible regulation of CA IX expression by HBx in human hepatocellular carcinoma cells was tested first on a human HepG2 HCC cell line, which was transfected transiently with HBx, or an empty pcDNA3.1 vector. After splitting the cells the following day, the parallel transfectants were incubated in normoxia or hypoxia for 24 h. Afterwards, the expression of hypoxia-inducible genes was analyzed by RT-PCR and immunoblotting (Fig.4). In contrast to Rat2 cells, CA IX expression was increased significantly in HepG2 cells even under normoxic conditions, at both mRNA and protein levels. The increase was significant also in hypoxia. The same pattern was seen with other hypoxia-regulated genes (Fig.4A). HBx stimulated the transcription of GLUT1 and PDK1 in normoxia, as well as hypoxia, and slightly of VEGF and MCT4 in hypoxia.

The hypoxic induction of the CA9 promoter activity was increased significantly by HBx in HepG2 cells as assessed by dual luciferase reporter assay (Fig.5A). The induction was even more prominent in the PLC/PRF/5 HCC cell line (Fig.5B). Apart from being transfected transiently with the pGL3 PR1 construct, renilla plasmid, HBx or empty pcDNA3.1, the cells were also co-transfected with the dominant-negative HIF-1α mutant (mut HIF-1α) or control pcDNA3.1. Mut HIF-1α can dimerize with HIF-1β and bind to DNA but it is not capable of transactivating HIF-target genes as it lacks the N-TAD domain [Holotnakova et al, 2008]. As can be seen in Fig.5,

mut HIF-1α decreased the CA9 promoter activity stimulated by HBx significantly in both HCC cell lines. In PLC/PRF/5 cells, the effect of HBx was completely abolished in cells transfected with mut HIF-1α (Fig.5B). This experiment provides further proof that HBx influences the CA9 promoter through HIF-1α.

Discussion

Apart from cancer and ischaemic diseases, the HIF system plays an important role in embryogenesis, wound healing, exercise, inflammatory diseases, anaemia, and many other situations. There are also viral proteins capable of stabilizing or transactivating HIF and promoting changes in cells reminiscent of hypoxic conditions. One such viral protein is HBx. It has a role in hepatocarcinogenesis because HCC incidence has been reported in animals infected with mammalian hepadnaviruses that have a conserved X-open reading frame in their genomes but not in birds infected with avian hepadnaviruses where the X-ORF is absent [Tang et al., 2006]. Knock-down of HBx reduces the tumourigenicity of HCC cells [Chan and Ng, 2006]. HBx has been regarded as a multifunctional viral regulator that modulates a wide variety of host functions in a rather promiscuous way. It is a trans-activating protein that stimulates HBV gene expression and replication, and also alters host gene expression important to the pathogenesis of chronic liver disease and the development of HCC associated with HBV [Feitelson, 1999].

This study showed for the first time that HBx stimulated the CA9 promoter activity significantly in several different cell types, and that this stimulation was mediated via the hypoxia-response element located in the CA9 promoter proximal to the transcription start site. It also demonstrated that HBx induces the CA9 promoter activity through a functional HIF-1 dimer. The previously proposed mechanisms include HBx-mediated HIF-1α stabilization in the cytoplasm [Moon et al., 2004] and increased transcriptional activity of HIF-1α in the nucleus [Yoo et al., 2004]. The C-terminus of HBx interacts physically with the bHLH/PAS, C-terminal ODDD, and C-TAD domains of HIF-1α [Moon et al., 2004; Yoo et al., 2004]. The interactions with ODDD and C-TAD constrain the access to proline and asparagine residues for the PHD and FIH-1 dioxygenases. The association with bHLH/PAS enhances HIF-1 DNA binding. In the experimental settings described here, the effect of HBx on the CA9 promoter was seen only in conditions of hypoxia and pericellular hypoxia (dense cultures); in normoxia, HBx increased the CA9 promoter activity only in the stem cell-like hepatic WB-F344 cells and hepatocellular carcinoma cells. This could mean that the reported HIF-1α stabilization by HBx in normoxic conditions is possible only in cells of hepatic origin, which might be connected to the

hepatotropism of HBV. It is not unreasonable to assume that a factor, which could help stabilize the interactions between HBx and HIF-1α in normoxia, is present in cells of hepatic origin. This possibility opens a new window of opportunity to explore this area for an important component in hepatocarcinogenesis induced by HBV.

The effect of HBx that was manifested in hypoxia in all the cell lines could be explained by its ability to stimulate activated transcription [Lin et al., 1998]. Since HBx cannot bind double stranded DNA directly, protein-protein interactions are crucial for HBx transactivation. Lin et al. (1998) proposed that HBx could act as a co-activator in transcription through modulation of the transcriptional machinery and distal binding activators. They suggested that HBx might bridge the activators (in our case HIF-1) and the transcription machinery or release co-repressors from the machinery. HBx has also been reported to enhance transcriptional activity of HIF-1α through activation of the MAPK pathway [Yoo et al., 2003], which has been implicated previously in CA9 expression induced by density and hypoxia [Kopacek et al., 2005].

Clearly, HIF target genes must be important in hepatocarcinogenesis induced by HBV since HBx has an additive effect on HIF-transactivation. One proof is the hypervascularity of HCC correlating with expression of VEGF [Lee et al., 2000]. Angiogenesis has also been observed in chronic hepatitis and in liver and biliary cirrhosis [Medina et al., 2004], all preceding HCC. Most chronic liver diseases are characterized by fibrosis and inflammation. Cirrhosis is the end stage of liver fibrosis and is related highly to the development of HCC. HBx expression in hepatocytes induces paracrine activation of surrounding cells and contributes actively to the development of fibrosis in patients infected by HBV chronically [Martin-Vilchez et al., 2008]. Fibrotic tissue offers resistance to blood flow and to the delivery of oxygen, thus becoming ischaemic. The activation of acid buffering systems in response to intracellular acidosis in hypoxia causes an irreversible accumulation of Na^+ that precipitates osmotic hepatocyte lysis by altering cell volume regulation [Alchera et al., 2008]. Expression of CA IX induced by HIF-1 thus increases hepatocyte tolerance to hypoxia by preventing the alteration of intracellular pH and Na^+ homeostasis that lead to ischaemic cell death [Alchera et al., 2008]. Silencing of anion exchanger 2 (AE2), a member of the chloride-bicarbonate transporter family which has an

important function in pH regulation and is overexpressed in HCC tissues, indicated its role in the survival of poorly differentiated HCC cells [Hwang et al., 2009]. CA IX is also capable of interacting directly with AEs and improving their bicarbonate transport activity [Morgan et al., 2007]. CA IX in the normal human liver is expressed on the basolateral surfaces of all ductal epithelial cells in the portal tracts and is absent from hepatocytes [Saarnio et al., 2001]. In the study of Saarnio et al. (2001), 33% of HCCs exhibited positive immunostaining for CA IX. The role of CA IX in hepatocarcinogenesis is yet unknown, and in vivo data about CA IX expression in chronic hepatitis and liver fibrosis is missing completely.

Jin et al. (2001) found that cells positive for HBx were localized preferentially in the periportal region of liver from patients with chronic hepatitis or on the periphery of cirrhotic nodules where high necroinflammatory activity was observed. Only 4% of HCC showed HBx positivity, whereas 30% of surrounding non-tumour tissue was still HBx-positive. They suggested that HBx might play a major role at the promotion stage of carcinogenesis because it was related to the progression of chronic hepatitis and accumulated preferentially in the active pathological stages. Therefore, it would be interesting to see the correlation between HBx and CA IX expression, which might be overlapping in the periportal regions during chronic hepatitis. Upregulated HIF-1α in HCC is correlated well with portal vein invasion, lymph node metastasis, and is an adverse prognostic factor for HCC patients [Xie et al., 2008].

The significant co-activating effect of HBx on CA9 expression was also prominent in cell lines of non-hepatic origin. That means that its effects are more general and not solely tissue-specific. Many patients with HBV infection experience a spectrum of extrahepatic disorders (e.g. dermatological disease, arthritis, glomerulonephritis, aplastic anaemia, neuropathy, vasculitis, myocarditis), and the virus has extensive reservoirs of extra-hepatic replication (e.g. lymph nodes, spleen, bone marrow, kidney, colon, stomach, skin, pancreas, brain, heart, lung) [Rong et al., 2007]. It is therefore conceivable that chronic HBV infection could upregulate the expression of hypoxia-inducible genes even in tissues other than the liver. The extrahepatic disorders associated with HBV have all been linked to hypoxia before.

This study demonstrated that HBx increased the CA9 promoter activity efficiently in several cell lines. This could have implications in chronic hepatitis and during the development of HCC, where CA IX could help increase hepatocyte tolerance to ischaemia caused by fibrosis. Targeting CA IX, as well as other hypoxia-regulated genes that favour tumour cells able to adapt to this microenvironmental stress, might help decrease the risk of developing HCC. Early detection still remains the most important factor in prevention.

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Tables

Table 1

List of primers used for RT PCR analysis

Rat mRNA	GenBank® accession no.	Forward primer	Reverse primer	Tm (°C)	Product size (bp)
β-actin (r)	NM_031144	atcgtgggccgccctaggcac	gtacatggctggggtgttgaagg	61	300
β-actin (h)	NM_001101	tcctccctggagaagagcta	acatctgctggaaggtggac	60	367
Car9 (r)	XM_233380	atcacccaggctcagaacacac	gctggtggcagcaaagagaa	64	587
CA9 (h)	NM_001216	ccgagcgacgcagcctttg	ggctccagtctcggctacc	62	253
HIF-1α (r,h)	NM_024359 (r) NM_001530 (h)	gcttggtgctgatttgtgaacc	gcatcctgtactgtcctgtggtg	64	267
VEGF (r)	NM_031836	ggtgaagttcatggacgtctacc	ctctccgacgtgggcacgcac	61	180
VEGF (h)	NM_001025366	cagcacggtccctcttggaa	cctcctcttccctgtcagga	61	313
GLUT1 (r)	NM_138827	cagaaggtaattgaggagttctaca	acaaaggccaacaggttcatcatc	61	206
GLUT1 (h)	NM_006516	ctcctttctccagccagcaatg	ccagcagaacgggtggccatag	64	355
GLUT4 (r)	NM_012751	ttccttctatttgccgtcctcctg	tttccttcatcctggaggtaaggg	66	280
iNOS (r)	NM_012611	cgcaccaccctccttgttcaac	acaaggcctccaacctctgcct	67	340
MCT4 (h)	NM_004207	ctcaccatcctgggcttcat	agaagaagttgcccagcagca	62	427
PDK1 (r,h)	NM_053826 (r) NM_002610 (h)	attggaagcataaatccaaactg	cggtcactcatcttcacagtc	58.5	311

Car9, rat carbonic anhydrase 9; CA9, human carbonic anhydrase 9; GLUT, glucose transporter; (h), human gene; HIF, hypoxia-inducible factor; iNOS, inducible nitric oxide synthase; MCT4, monocarboxylate transporter 4; PDK1, pyruvate dehydrogenase kinase 1; (r), rat gene; VEGF, vascular endothelial growth factor

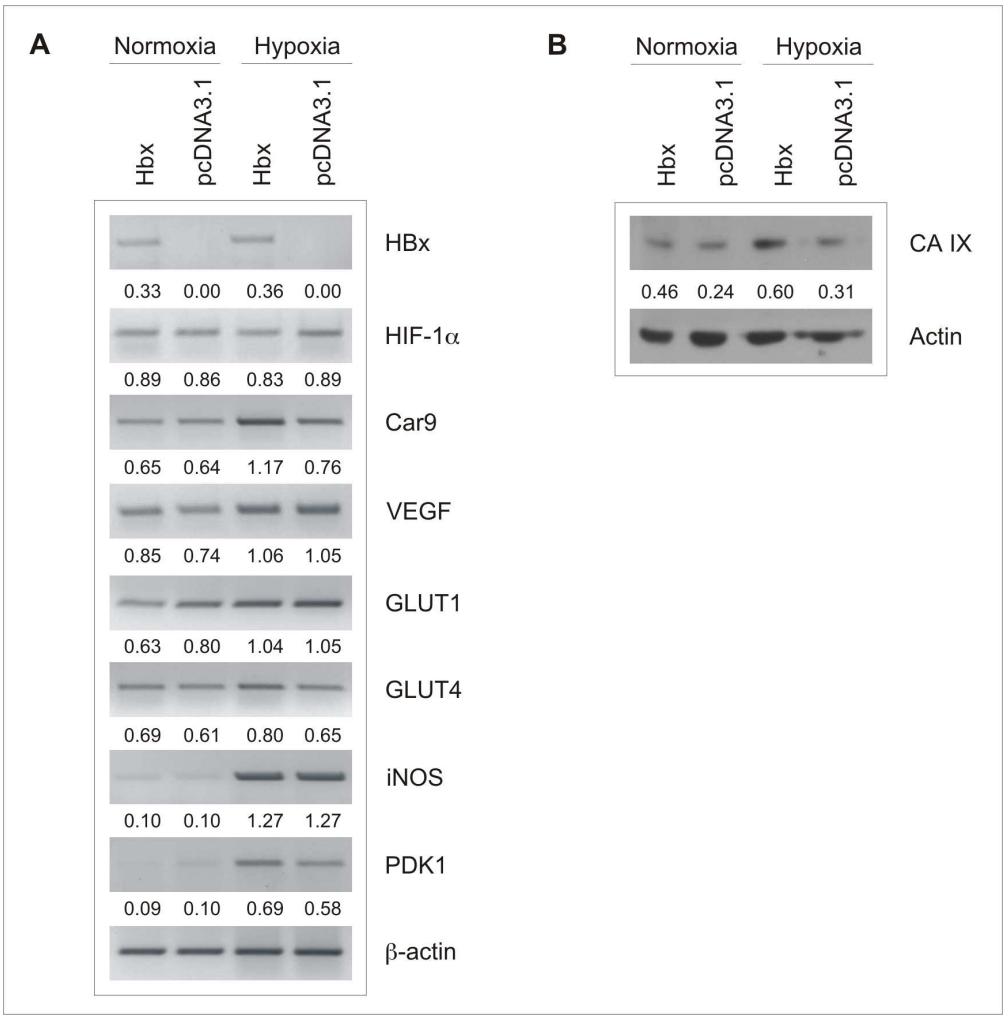

Figure 1
Effect of HBx on expression of hypoxia-inducible genes. Rat2 cells were transfected transiently with HBx or an empty pcDNA3.1 vector and incubated for 48 h in normoxia or hypoxia. A Expression of hypoxia-inducible genes was analyzed by RT-PCR. Numbers given below the figures represent relative levels of PCR products normalized against the internal standard β-actin. B Expression of CA IX protein was analyzed by Western blotting using the specific M75 monoclonal antibody. Anti-actin antibody was used for loading controls. Relative levels of CA IX protein, calculated relative to actin, are shown below the blot.

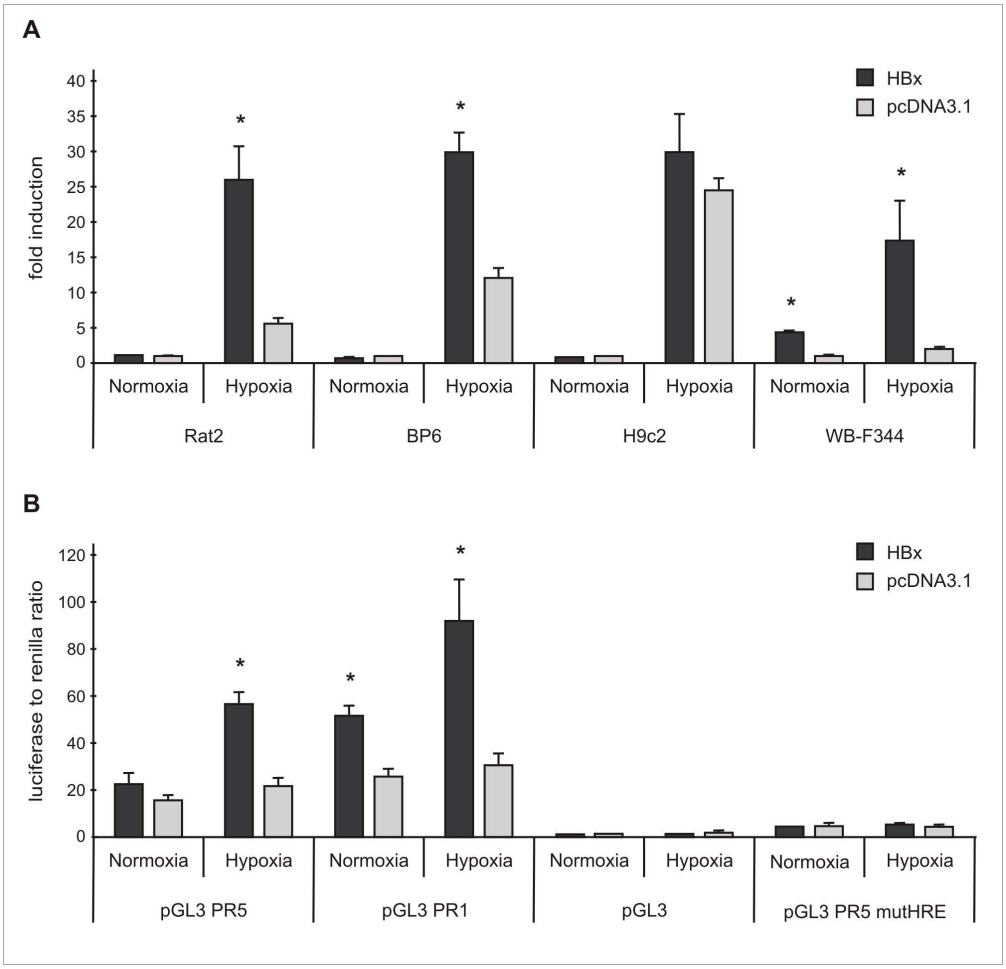

Figure 2

Effects of HBx on CA9 promoter activity. A Sparse Rat2, BP6, H9c2, and WB-F344 cells were co-transfected transiently with pGL3 PR5, renilla plasmid and either a plasmid containing HBx or an empty vector (pcDNA3.1). The cells were incubated in normoxia and hypoxia for 24 h. Luciferase activity was normalized by renilla activity and expressed as fold induction. B Dense Rat2 cells were co-transfected transiently with HBx or empty pcDNA3.1 and pGL3 PR5, pGL3 PR1, empty pGL3 vector or pGL3 PR5 mutHRE and renilla plasmid. The cells were incubated in normoxia and hypoxia for 24 h. Luciferase activity was normalized by renilla activity. *P < 0.05 (HBx vs. pcDNA3.1)

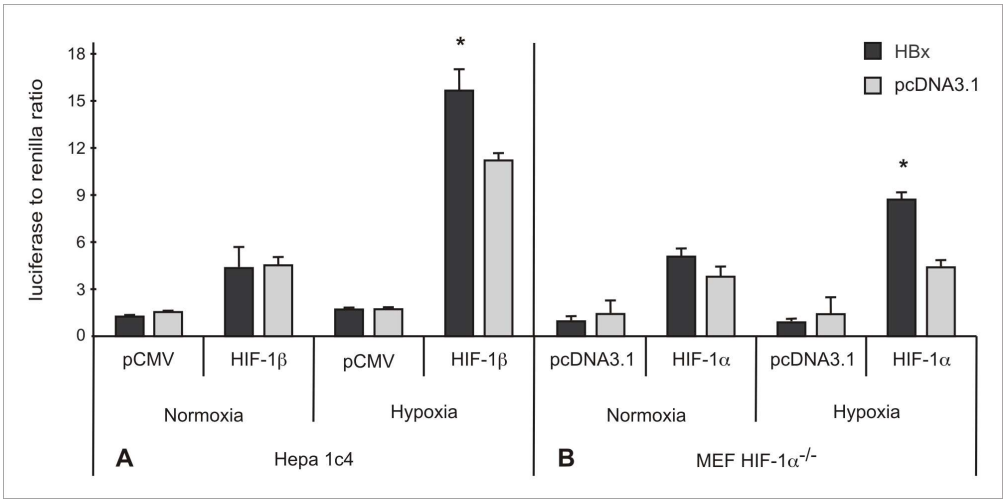

Figure 3
Effect of HIF-1β and HIF-1α on CA9 promoter activity induced by HBx. A Sparse mouse Hepa 1c4 hepatoma cells that lack natural HIF-1β expression and B mouse embryonic fibroblasts knocked-out for HIF-1α (MEF HIF-1α-/-) were co-transfected transiently with pGL3 PR1, renilla plasmid and either a plasmid containing HIF-1β (A) or HIF-1α (B) or an empty vector (pCMV) (A, B). They were also co-transfected with HBx or an empty pcDNA3.1 vector. The cells were incubated in normoxia and hypoxia for 24 h. Luciferase activity was normalized by renilla activity. *P < 0.05 (HBx vs. pcDNA3.1)

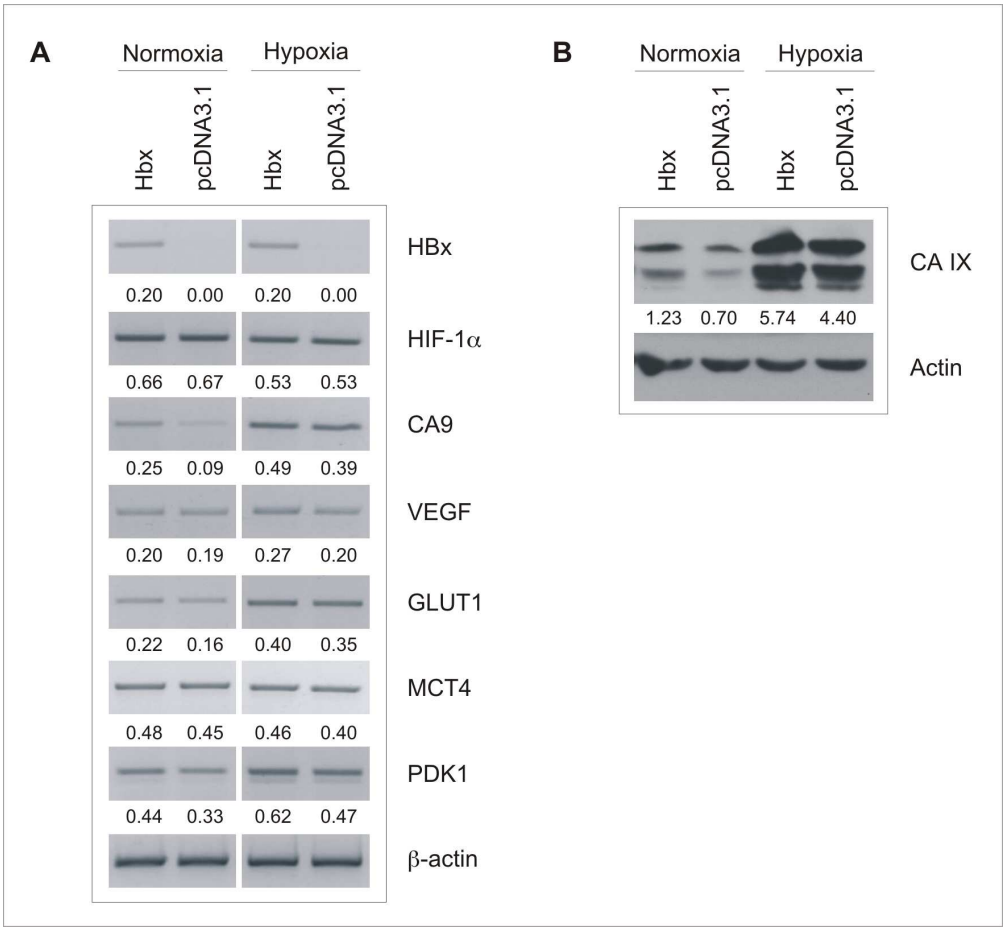

Figure 4
Effect of HBx on expression of hypoxia-inducible genes in HepG2 hepatocellular cell line. HepG2 cells were transfected transiently with HBx or an empty pcDNA3.1 vector and incubated for 24 h in normoxia or hypoxia. A Expression of hypoxia-inducible genes was analyzed by RT-PCR. Numbers given below the figures represent relative levels of PCR products normalized against the internal standard β-actin. B Expression of CA IX protein was analyzed by Western blotting using the specific M75 monoclonal antibody. Anti-actin antibody was used for loading controls. Relative levels of CA IX protein, calculated relative to actin, are shown below the blot.

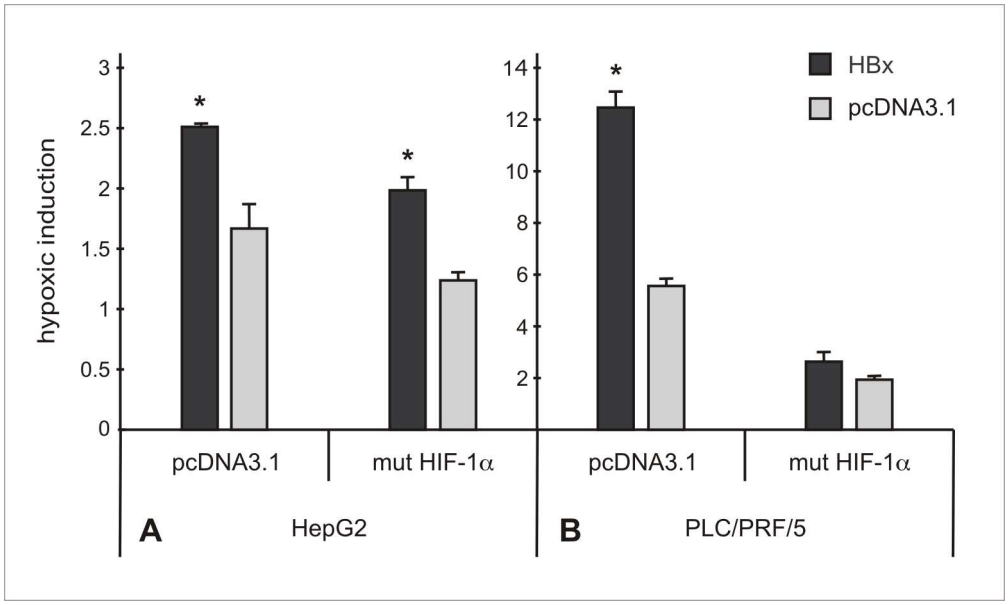

Figure 5
Effect of HBx on hypoxic induction of CA9 promoter in HCC cell lines. A Human HepG2 and B PLC/PRF/5 hepatocellular carcinoma cells were co-transfected transiently with pGL3 PR1, renilla plasmid and either a plasmid containing the dominant-negative mut HIF-1α or an empty vector (pcDNA3.1). They were also co-transfected with HBx or an empty pcDNA3.1 vector. The cells were incubated in normoxia and hypoxia for 24 h. Luciferase activity was normalized by renilla activity and expressed as fold induction. Mut HIF-1α vs. pcDNA3.1 values were all significant ($P < 0.05$). *$P < 0.05$ (HBx vs. pcDNA3.1)