Die Bedeutung von zellulären Wirtsfaktoren für die Hepatitis B Virus Replikation

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meinen Eltern,
meinem Mann Olaf

Directory

List of abbreviations

Alas	delta-aminolevulinate synthetase
ApoB	apolipoprotein B
AL	adult liver
BSEP	bile salt exporting pump
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
C/EBP	CAAT enhancer-binding protein
CK8/18	cytokeratine 8/18
CO	carbon monoxide
CoPP	cobalt protoporphyrin-IX
COUP-TF	chicken ovalbumin promoter transcription factor
CYP1A2	cytochrom p450 family member 1A2
DNA	desoxyribonucleic acid
DMSO	dimethyl sulfoxid
ED	embryonic day
FCS	fetal calf serum
FL	fetal liver
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HBV	hepatitis B virus
HBeAg	hepatitis B virus e antigen
HBV M protein	hepatitis B virus middle envelope protein
HBV L protein	hepatitis B virus large envelope protein
HBsAg	hepaitis B virus s antigen
HBx protein	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HMG-CoA	hydroxymethylglutaryl-CoA-reductase
HNF	hepatocyte nuclear factor
HO	heme oxygenase
HPRT	hypoxantin-phosphoribosyl-transferase
IL	interleukin

IFN	interferon
IP-10	IFN gamma inducible protein-10
LRH-1	liver receptor homolog-1
LSA	liver specific antigen
mRNA	messenger RNA
OATP-C	organic anion transporting polypeptide C
2’5’-OAS	2’5’ – oligoadenylate synthetase
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated-receptor-γ coactivator 1 alpha
pgRNA	pregenomic RNA
PHH	primary human hepatocytes
RNA	ribonucleic acid
ROS	reactive oxigen species
siRNA	small interfering RNA
SREBP-2	steroid regulatory element binding protein 2
SVP	subviral particle
TDO	2’3’-tryptophan dioxygenase

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1 Introduction

1.1 Replication of Hepatitis B Virus (HBV) and hepatocyte differentiation

1.1.1 HBV is an important public health problem

Hepatitis B Virus (HBV) is a noncytopathic enveloped deoxyribonucleic acid (DNA) virus. It belongs to the family of hepadnaviridae (hepatotrop associated DNA viruses) [1]. The virus is also classified as a pararetrovirus because DNA synthesis of HBV occurs via reverse transcription of a ribonucleic acid (RNA) intermediate.

The human HBV is highly species specific [2] and infects only humans and humanoid primates, such as chimpanzees [3], orang utans [4], gibbons [5] and gorillas [6]. HBV is also highly organ-specific. It transiently and persistently infects the liver, which may result in an inflammatory liver disease – acute or chronic hepatitis B [7].

HBV is an important health public problem worldwide. To date, out of 2 billion people, infected with HBV, 350 million are chronically infected [8, 9]. Prevalence of HBV infection varies greatly. It is endemic in Southeast Asia, China and Africa with about 10% of the population infected. Intermediate infection rates of 1 to 8% are found in the Middle East, Russia, India and Brazil. HBV is least prevalent in developed countries (Europe, Australia, North America). Despite the availability of a potent vaccine, infection levels continue to increase, predominantly in the developing world [8, 10].

HBV is transmitted by perinatal, percutaneous, and sexual exposure. The virus is also transmitted by close person-to-person contact presumably by open cuts and sores, especially among children in hyperendemic areas. The ability of HBV to survive outside the body for prolonged periods facilitates the transmission [9].

The natural history of hepatitis B is complex and not well defined. Primary infection is followed by a six week to four month long incubation period. At the onset of HBV infection, only one-third of adults experience symptoms of acute hepatitis, while the majority (65%) have subclinical disease [11]. The course of the disease depends on age and immune status of the patient. An excessive immune response may result in a severe form of acute hepatitis - fulminant hepatitis. It is unusual, occurring in approximately 0.1 to 0.5 percent of patients [12]. It is generally assumed that patients after an acute hepatitis B develop life-long immunity. After complete recovery from acute hepatitis B, despite high levels of serum antibodies and HBV-specific cytolytic T lymphocytes, traces of HBV are often detectable in the blood

for many years [13]. So, sterilizing immunity to HBV frequently fails to occur, and HBV-specific cytolytic T lymphocytes keep the virus under control, perhaps for life [14]. During immunosuppression, HBV infection can reactivate, and in some cases even develop a fulminant course [15, 16, 17].

About 5% of adults do not clear the virus and develop chronic hepatitis B. The rates of chronicity are higher in newborns (90%), children (30%), and in immune deficient individuals [11]. 15% to 40% of individuals chronically infected with HBV will develop cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) during their life time [9]. Importantly, 25% of patients infected as neonates die prematurely from cirrhosis or liver cancer [18].

At present, there is no treatment capable of eradicating HBV. The aims of current treatment of chronic hepatitis B are to achieve sustained suppression of HBV replication and remission of liver disease. The ultimate goal is to prevent cirrhosis, hepatic failure and HCC. The efficacy of currently available treatment (interferons: standard interferon, peginterferon and nucleoside analogues: lamivudine, telbivudine, adefovir dipivoxil, and entecavir) is still very low [11, 9]. Prognosis of patients with HBV-related HCC is poor. Curative approaches, such as surgical resection and liver transplantation, are possible in a very limited cohort of patients, and the recurrence rate of HCC is high. Other therapeutic options, such as systemic and local chemotherapy, external radiation, administration of octreotid, tamoxifen, interferon, and antiandrogenics, do not improve survival of patients [19].

Taken together, the overall prognosis of patients with chronic hepatitis B, even when under treatment, is unacceptable. Therefore, development of new therapeutic approaches and, especially, improvement of prophylaxis of vertical HBV infection are urgently required.

1.1.2 Intrauterinal transmission of HBV: Epidemiology and possible mechanisms

The active-passive immunization against HBV is reported to effectively protect infants at high-risk against viral transmission [20, 21]. However, perinatal transmission of HBV still occurs. There are three possible routes of perinatal transmission of the virus: transplacental transmission of HBV *in utero*, transmission during delivery, or postnatal transmission during care or through breast milk.

Since transplacental transmission occurs antenatally, hepatitis B vaccine and anti-HBV immunoglobulin cannot block this route. Epidemiological studies on HBV intrauterine infection in China showed that intrauterine infection occurs in 3.7-9.9% pregnant women with positive Hepaitis B virus s Antigen and in 9.8-17.39% with positive Hepaitis B virus s Antigen (HBsAg) / Hepatitis B virus e Antigen (HBeAg) [22]. Other studies suggest higher rates of HBV intrauterine infection: up to 40.1% [23].

The main risk factors for intrauterine HBV transmission are: history of threatened premature labor, HBeAg positivity [24, 25], low titers of maternal anti-HBV core antibodies [26], presence of HBV DNA in placenta [27, 28, 24], and maternal viremia [29, 30, 31]. *Del Canho et. al* reported that high maternal serum HBV DNA concentration is the most important factor associated with failure of passive-active hepatitis B immunoprophylaxis [32]. However, even very low levels of viremia in a mother are sufficient for transmission of HBV to the fetus [33, 34].

The studies on transplacental transmission of HBV suggested two possible mechanisms: first, a hematogenous route. Certain factors, such as threaten abortion, can break the placental microvasculature, thus the high-titer maternal blood leaks into fetus circulation. Second, a cellular transfer is also possible. The placental tissue is infected by a high-titer of HBV in maternal blood from the mother's side to the fetus step by step, and, finally, HBV reaches fetus circulation through the villous capillary endothelial cells [22]. As support of the second mechanism, HBsAg and HBcAg were detected in placentas from HBsAg-positive mothers, whereas the concentration of both antigens decreased from the mother's side to the fetus side of placenta: maternal decidual cells > trophoblastic cells > villous mesenchamal cells> villous capillary endothelial cells [23]. *Bhat and Anderson* have shown that trophoblast-derived cells were able to transcytose cell-free HBV, hereby the transcytosed virus remains infectious [35]. Since transcytosis of HBV was reduced with syncytiotrophoblast formation, transplacental viral transmission is most probable in the first trimester of pregnancy.

However, it is still unknown, at what stage of the liver development exclusively hepatotropic HBV can infect and replicate in hepatocyte precursors. The lack of knowledge on the infectious and replicative potential of the virus in the developing liver represents a major obstacle for the development of prophylaxis for intrauterine HBV transmission.

1.1.3 Structure and genomic organization of HBV

HBV is an enveloped virus with a partially double-stranded (ds) DNA genome. The intact virion, also referred to as a Dane particle, has a spherical structure and is 42 nm in diameter [36] (Fig.1). The envelope consists of a host derived lipid bilayer with three integrated viral surface proteins: the small protein (S), the middle protein (M), and the large protein (L) [37]. Homodimers of the viral core protein, arranged with triangulation numbers of three or four, build the 27 nm icosahedral capsid inside the envelope [38]. The capsid encloses the 3.2 kilo base pairs (kb) viral genome, with the viral polymerase (P) covalently linked to the negative strand via its terminal protein (TP) domain [39].

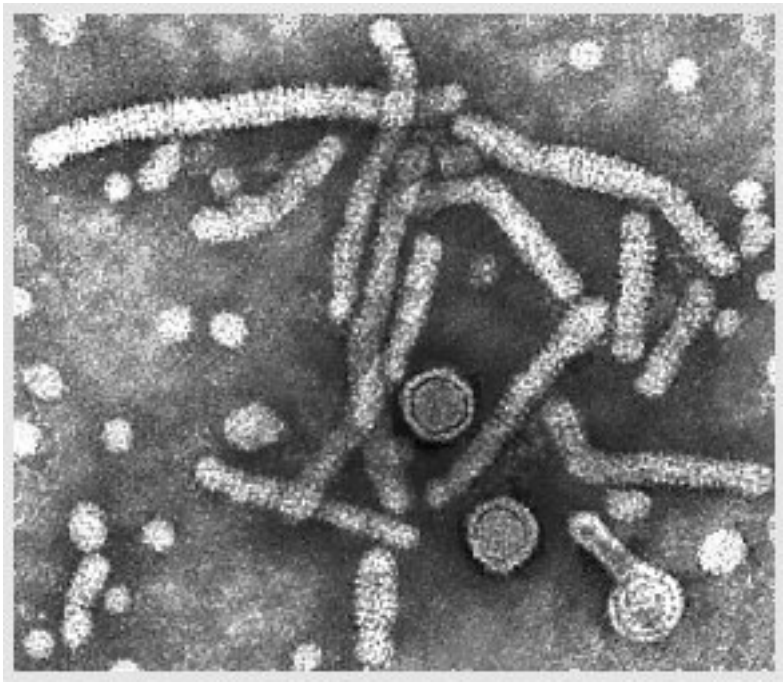


Fig. 1: Electron micrograph of HBV: Dane particles, spheres and filaments
Adopted from www.bact.wisc.edu

The 3.2 kb DNA genome present in the virion is a partially double-stranded (ds) relaxed circular (rc) molecule. The minus strand is of unit length and linked to the viral polymerase via the terminal protein at the 5` end. The 5` end of the plus strand is of variable length so that part of the minus strand is single stranded (ss). The small genome is organised compactly with four partially overlapping open reading frames (ORF) and does not contain non coding regions [37] (Fig. 2). The open reading frames include:

1) Product of ORF P is the viral polymerase (P).
2) ORF C encodes the core protein (C) and the precore protein (HBeAg).
3) ORF S/pre-S products are the surface proteins S, M and L.
4) ORF X codes for the X protein (X).

Four promoters in concert with enhancers I and II [40, 41, 42] control transcription of the independent mRNAs: *preC/C*, *preS1, preS2/S* and *X* (summarized in *Ganem and Schneider* [37]). Enh1 increases transcription from all four promoters, in contrast to enh2, which only up-regulates transcription from the preS1 promoter. HBV RNA transcription starts at the different promoters and stops at one common polyadenylation signal [43].

3.5 kb *preC/C* RNA, the longest transcript, consists of a subset of more than genome length RNAs, which are transcribed from two physically overlapping, but functionally distinct, promoters [44, 45]. *C* RNA contains complete genomic information of the virus and can serve as mRNA for translation of HBV core and polymerase proteins or as pregenomic RNA (pgRNA), which is encapsidated and reverse transcribed in the viral capsid [37]. *preC* mRNA is only translated into the hepatitis B e antigen (HBeAg) [46].

The core protein is the structural component of the viral capsid. It possesses self-assembly properties, first building homo-dimers, which then aggregate to form capsids [47].

The protein product of the polymerase gene encompasses three functional domains, the RNAseH domain, the reverse transcriptase (RT) domain, and the TP domain. The polymerase drives packaging of the pgRNA into the capsids by binding to the encapsidation signal (ε) of the RNA. As soon as the RNA-polymerase complex is encapsidated, the polymerase reverse transcribes the pgRNA into DNA and removes the RNA with its RNAseH activity.

PreC protein, also referred to as HBeAg, contains identical amino acids (aa) as the core protein plus additional 29 aa at the N-terminus. It is a non structural protein that is secreted in high amounts from infected cells [48]. The function of HBeAg is yet unknown.

The 0.7 kb *X* (X protein), 2.1 kb *preS2/S* (M and S protein) and 2.4 kb *preS1* (L protein) are subgenomic RNAs and serve as mRNAs.

Subgenomic RNAs of 2.4 kb and 2.1 kb encode the L protein, and the S and M envelope proteins, respectively. All surface proteins share the S-protein sequence. HBV L protein is considered to be essential for viral entry [49, 50].

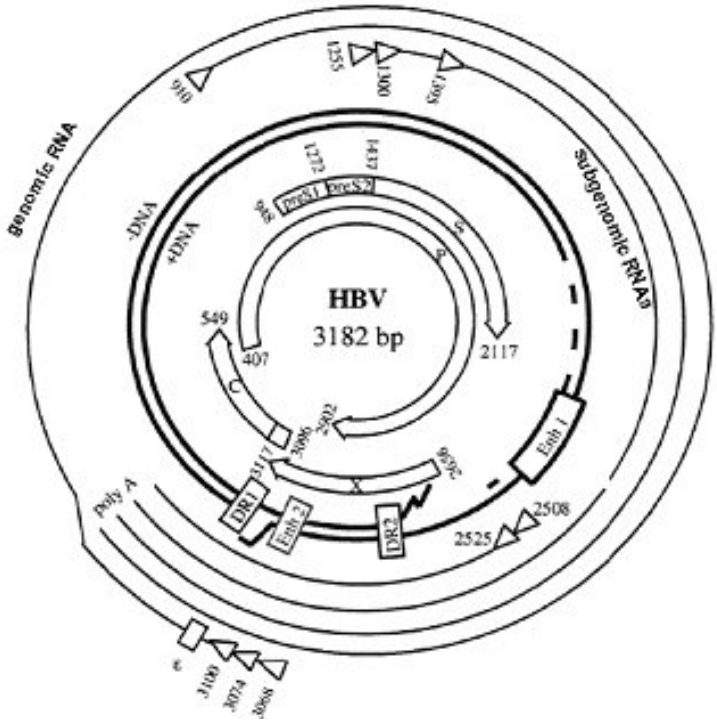


Fig. 2: Genome organisation of HBV

Outer circles depict the transcripts (genomic and subgenomic RNAs). The triangles indicate the transcription starts, the square symbols the encapsidation signal (ε). The bold black lines represent the partially ds genome. Enhancers (Enh1, Enh2) and direct repeats (DR1, DR2) are outlined in squares. The arrows illustrate the four open reading frames (ORF) with their protein products X, C, P and the surface proteins S, M and L. (modified from Zedler U., dissertation, 2008).

Small subgenomic 0.7 kb HBV RNA gives rise to HBV X protein. This protein is reported to be a prerequisite for productive viral infection *in vivo* [51] and a positive regulator of HBV replication *in vitro* and *in vivo* [52]. HBV X protein may also be involved in liver cancerogenesis [53].

HBV is highly organ specific, reproducing only in the liver. The target cell of HBV is the hepatocyte.

1.1.4 Liver – the target organ of HBV

The liver is the largest internal organ in mammals with numerous endocrine and exocrine functions, which serve to maintain homeostasis within an organism.

Liver functions include: (1) production of serum proteins, including clotting factors and transport proteins e.g. albumin, transferrin; (2) removal and breakdown of serum proteins, red blood cells and microbes; (3) gluconeogenesis and glycolysis; (4) processing of fatty acids and triglycerids; (5) synthesis and catabolism of cholesterol; (6) synthesis and interconversion of non-essential amino acids; (7) breakdown of toxic endogenous compounds e.g. ammonia, bilirubin; (8) production and excretion of bile components; (9) detoxification of xenobiotic agents; and (10) storage of numerous substances, e.g. glycogen [54].

The liver anatomy is favourable for carrying out these functions. First, the liver receives blood from two sources, the portal vein and the hepatic artery. Second, the hepatic biliary system enables the liver to transport bile into the intestines. Finally, the hepatic architecture facilitates the exchange of materials between blood and hepatocytes and prevents leakage of bile components into blood.

The adult liver is comprised of repeating structural units termed lobules. The lobule is a hexagonal structure consisting of plates of anastomosing hepatocytes. At the center of the lobule is the central vein, whereas six portal triads ring the hypothetical edge of each lobule. Each portal triad is comprised of an intrahepatic bile duct, portal vein and hepatic artery [55]. This lobular organization is of physiological significance. Some liver functions can be carried out by all hepatocytes, but other functions are limited to hepatocytes located around the central vein or in the periportal regions [56]. This compartmentalization of function within the liver lobule is called positional (zonal) heterogeneity or metabolic zonation. Zonal heterogeneity may be of the “gradient” type, with gradual changes in levels of expression across the portocentral axis, or of the “compartment” type, in which a highly defined boundary of expression is observed. The example of the later is the expression of carbamoyl phosphate synthetase I and glutamin synthetase, which are present only in periportal and pericentral regions, respectively [54]. Certain zonal expression patterns are “dynamic”, changing in response to hormonal or metabolic changes in the liver, while others are “stable”, in which adaptive changes do not occur [57].

1.1.4.1 Liver cell types

The adult liver is comprised of numerous cell types.

Hepatocytes carry out most liver functions and are the predominant cell type in this organ. These polygonal, metabolically active, parenchymal cells comprise 60% of the cells and 80% of the cellular mass within the liver [58, 55]. Hepatocytes are polarized cells with apical and basolateral surfaces separated by intercellular domains [59]. The apical surface, or canalicular membrane, of hepatocytes, form small channels called bile canaliculi and is involved in the unidirectional transfer of components into the bile. The basolateral surface, or the sinusoidal membrane, interfaces with the space of Disse and is involved in the bidirectional exchange of materials between hepatocytes and the bloodstream [60]. The intercellular domains are regions of hepatocyte-hepatocyte contact and contain junctions involved in intercellular communication. In particular, the tight junctions, occurring between canalicular and intercellular domains, ensure that components of the bile do not leak into the bloodstream [61].

The other cell types in the liver are bile epithelial cells or cholangiocytes, oval cells, liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate-, DCs, NK-, NKT-cells, conventional and unconventional T and B-lymphocytes.

The cholangiocytes line the bile ducts and have multifaceted functions. Their capability to secrete a range of different pro-inflammatory mediators, cytokines, and chemokines, indicates a major role of cholangiocytes in the inflammatory reaction. Furthermore, paracrine secretion of growth factors and peptides mediates an extensive cross-talk with other liver cell types, including hepatocytes, stellate, endothelial and inflammatory cells [62, 63].

The transitional region between the intrahepatic bile ducts and bile canaliculi, lined by cholangiocytes and hepatocytes, respectively, is called the canals of Hering. These regions contain a population of cells called oval cells [64]. Oval cells serve as resident stem cells in the adult liver and can serve as progenitors for both hepatocytes and cholangiocytes [65].

Liver sinusoidal endothelial cells (LSEC) surrounding the sinusoids are crucial for exchange of materials between blood and the space of Disse. These cells also produce proteins of the extracellular matrix and factors, regulating blood coagulation

and vessel tone. By secreting cytokines and presenting antigens, LSEC play an important role in liver immunity [66, 67, 68, 69].

Kupffer cells are hepatic macrophages, accounting for 80% to 90% of the total population of fixed tissue macrophages in the body. Comprising a part of the sinusoidal lining of Disse spaces, Kupffer cells eliminate microbes, LPS, aged red blood cells, cell debris and immune complexes out of the blood flow. They also exert metabolic functions including degradation of LDL, and synthesis of erythropoietin, insulin-like growth factor, proteoglycans and apolipoprotein E. Kupffer cells present antigens and produce a number of cytokines and paracrine factors, thereby modulating innate and adaptive immune responses and functions of other liver cells [70].

Hepatic stellate cells are the major reservoir of vitamin A in the body. They also produce a variety of extracellular matrix proteins as well as a number of cytokines and chemokines [55].

NK, NKT, conventional and unconventional T- and B-lymphocytes in the liver carry out numerous immunological functions [71].

1.1.4.2 Liver development and hepatocyte differentiation

In mammals, the liver is formed from the developing ventral foregut endoderm. By approximately embryonic day (ED) 8.0, the ventral foregut endoderm initiates its development towards a hepatic fate [72, 73]. The cells of the ventral foregut endoderm start to express HNF3β and GATA4 [74, 75, 76], and mRNA transcripts of albumin and AFP become detectable [77]. Between ED 8.5 and ED 9.5, cells of the ventral gut endoderm – hepatoblasts - start to proliferate and generate the primary liver bud [73]. By ED 9.5, the basement membrane surrounding the liver bud is lost and cells delaminate from the bud and invade the septum transversum mesenchyme as cords of hepatoblasts. Between the migrating hepatic cords, angioblasts, the primitive sinusoidal endothelial cells, form capillary-like structures [78]. At ED 10-11 hematopoietic stem cells colonize the fetal liver, and the liver becomes the major site of hematopoiesis [79]. Beginning at approximately ED 10-11 of mouse embryogenesis, the caudal part of the liver bud gives rise to the extrahepatic bile ducts, the cystic duct, and the gallbladder, which remain in continuity with the foregut and connect the liver hilum with the digestive tract. By ED 12.5, the liver is a relatively large,

differentiated organ composed of various cell types, of which up to 60% are haematopoietic cells [79].

Prior to ED 12 in mice, hepatoblasts remain morphologically undifferentiated. They have an irregular shape, a large nucleus to cytoplasmic ratio and relatively few organelles when compared to the mature hepatocyte. Around ED 12, rough endoplasmic reticulum and Golgi apparatus increase that reflects the onset of the synthesis of secreted proteins. Between ED13.5 and ED15.5, hepatoblasts start to differentiate towards either hepatocytes or bile duct cells. Around ED18, differentiating hepatocytes gain a spherical shape and depositions of glycogen and the presence of peroxisomes become identifiable. Just prior to birth, on ED 20, hepatocytes become polygonal and begin to establish the polarized epithelium [78]. During the perinatal period, the zonal heterogeneity of the liver develops and a large number of metabolic enzymes are induced within the hepatocytes. The hematopoietic cells migrate elsewhere and the liver prepares to control metabolite and serum protein levels in the blood, store glycogen, and detoxify [54].

1.1.4.3 Markers of hepatocyte differentiation

At present, there is no consensus in defining a differentiated hepatocyte. Since the hepatocyte is a metabolically active cell, the evaluation of specific enzymatic properties, such as glycogen metabolism and gluconeogenesis, is crucial for assigning the cell as a hepatocyte [80].

Many key enzymes of hepatocytes, such as phosphoenolpyruvate carboxykinase (PEPCK) [81, 82, 83] and 2'3'-tryptophan dioxygenase (TDO) [84, 85, 86], are first expressed at birth and therefore considered to be markers of adult differentiated hepatocytes.

Transcellular bile [87] and xenobiotic [88] secretion from sinusoidal blood plasma into bile canaliculi is also an important function of differentiated hepatocytes in all mammalian species. This vectorial secretory process is driven by the polarized expression of distinct transport systems at the basolateral (sinusoidal) and canalicular (apical) surface domains of hepatocytes [87]. Expression of several members of these transport systems, such as organic anion transporting polypeptide - C (OATP-C) and bile salt exporting pump (BSEP) mRNA in the liver occurs first in the late phase of rat gestation [89] and increases during the postnatal

period of liver development [90]. Moreover, expression of OATP-C displays a strong positive correlation with the morphological differentiation of HCC [91]. Therefore, the expression levels of OATP-C and BSEP can also be considered as markers of differentiated hepatocytes.

The differentiation of hepatoblasts into hepatocytes during liver development as well as the maintenance of liver-specific gene transcription in the adult liver are governed by the coordinated interaction of hepatocyte-enriched transcription factors with the ubiquitous transactivating factors [92, 93, 78].

1.1.4.4 Hepatocyte-enriched transcription factors: role in liver development and hepatocyte differentiation

A number of hepatocyte-enriched transcription factors including HNF3αβγ, HNF1α, C/EBPα, HNF6 and HNF4 are known. On the basis of homology within DNA-binding domains, the hepatocyte-enriched transcription factors are grouped into related protein families.

HNF3α (Foxa1), HNF3β (Foxa2), and HNF3γ (Foxa3) bind to DNA as a monomer and activate transcription of target genes using a winged helix DNA-binding domain. HNF3α, HNF3β and HNF3γ share greater than 90% homology in amino acid sequence in this domain and therefore bind to similar DNA target sequences within hepatocyte-specific regulatory regions and exhibit functional redundancy in hepatocytes [94].

During development of definitive endoderm, HNF3β is the first to be activated, followed by HNF3α and subsequently HNF3γ. HNF3β -/- embryos die in utero prior to liver development because of severe defects in gastrulation resulting in abnormalities of the node, notochord, gut and visceral endoderm. In contrast, postnatal conditional deletion of HNF3β had no apparent effect on liver gene expression [95, 94]. Overexpression of HNF3β leads to 50% reduction in the levels of Pepck, glycogen synthase, Glut-2, and UDP-glucoronosyltransferase, and decreased expression of NTCP as well as accumulation and increased β-oxidation of lipids [94]. HNF3α and HNF3γ are dispensable for early liver development. However, HNF3α -/- mice are hypoglycaemic and show severe postnatal growth retardation followed by death

between days 2 and 12 postnatally [96]. HNF3γ-deficient mice displayed merely a 50% reduction in hepatic expression of tyrosine aminotransferase, Pepck, and TNF with compensatory increases in levels of HNF3α and β genes, suggesting that disruption of HNF3γ gene is not sufficient to cause severe defects in hepatic function [94].

The HNF6 or ONECUT-1 binds to its DNA recognition site as a monomer through the cut-homeodomain [94]. HNF6 is essential for regulating expression of HNF1β and stimulates HNF3β-induced gene transcription. HNF6 largely contributes to differentiation of hepatoblasts into cholangiocytes [97] and is involved in gluconeogenesis [93, 98] as well as hepatocyte regeneration [99].

The C/EBPα and β proteins are coexpressed in hepatocytes and are able to form either homodimers or heterodimers for DNA-sequence-specific binding through the bZIP protein motif [94].

C/EBPα is already expressed in endodermal cells of liver primordium on ED 9.5, whereas the expression C/EBPβ is first observed between ED 13.5 and 14.5 [100]. C/EBP-/- mice die from hypoglycemia within the first hours post partum due to a complete absence of hepatic glycogen storage and a failure to store lipids in hepatocytes and adipocytes [101]. Even postnatally induced C/EBPα knock-out leads to the rapid and severe impairment of glucose and lipid metabolism and death of mice [102]. C/EBP is involved in the regulation of hepatocyte proliferation [103, 104] The knock-out of C/EBP gene in mice results in increased hepatocyte proliferation and disruption of the normal liver architecture [104]. Thus, C/EBP is involved in the regulation of hepatocyte glucose, lipid homeostasis and hepatocyte proliferation.

The HNF1α uses a POU-homeodomain sequence and a myosine-like dimerization domain in the amino terminus of the protein to bind its DNA recognition sequence as a dimer. In the liver, HNF1α is coexpressed with the isoform HNF1β (vHNF1) and forms heterodimers with the HNF1β-related family member [94].

HNF1α is dispensable for mammalian liver development and specification of the hepatocyte cell lineage [105]. HNF1α is an important regulator of glucose and amino acid homeostasis [106]. HNF1α-/- mice die at the time of weaning due to a severe wasting syndrome with massive glucosuria, phosphaturia, and aminoaciduria from renal tubular dysfunction. Hepatic expression of phenylalanine hydroxylase is completely extinguished. HNF1α-/- mice also exhibit a partial reduction in hepatic expression of albumin, A1-antitrypsin, and fibrinogen [107]. HNF1α plays an important

role in the formation of tight and adherens junctions [108, 109]. It is a key transcription factor that regulates expression of transporters of apical and basolateral hepatocyte membranes [110, 111]. HNF1α binds 6% of RNA-polymerase II-enriched promoters in hepatocytes [112]. Expression levels of HNF1α correlate with differentiation of hepatoma cell lines [113] as well as hepatocellular carcinoma and hepatoblastoma [114, 115]. Thus, HNF1α contributes to the development and maintenance of a differentiated and polarized phenotype of hepatocytes and normal liver structure [116].

HNF1β plays an important role in the development of the gallbladder and intrahepatic bile ducts [94].

The orphan nuclear receptor HNF4α protein utilizes the zinc finger DNA-binding domain to recognize DNA while both the DNA- and ligand-binding domain are used to form homodimers or heterodimers with retinoic X receptor α. HNF4α is critical for transcriptional regulation of the orphan receptor - pregnane-X-receptor (PXR) and for crossregulation of the HNF1α [117].

In mouse development, HNF4α is expressed in the primary and extraembryonic visceral endoderm prior to gastrulation and in epithelial cells at the onset of liver, pancreas, and intestine formation [118]. HNF4α-/- embryos exhibit a severe visceral endoderm defect preventing gastrulation and causing a failure to develop past ED 6.5 [119]. Heterozygous mice have a diminished expression of albumin, AFP, transferrin, several distinct apolipoproteins, L-type fatty acid binding protein, erythropoietin, and the retinal-binding protein. The prenatal conditional knock-out of HNF4α results in a failure to store glycogen due to the decreased expression of glycogen-synthase, Pepck, and glucose-6-phosphatase. Since HNF4α regulates the developmental expression of a myriad of proteins required for cell junction assembly and adhesion [120], prenatal conditional knock-out of the transcription factor also leads to the marked disruption of the liver architecture with loss of organized hepatic cords and sinusoids [121]. Mice with postnatal conditional knock-out of HNF4α in hepatocytes accumulate lipids in the liver, exhibit greatly reduced serum cholesterol and triglyceride levels and increased serum bile acid concentrations [105] as well as severely impaired amino acid metabolism [122].

Thus, HNF4α is a key regulator of carbohydrate, lipid, cholesterol, amino and bile acid homeostasis and is essential for formation of epithelial phenotype of hepatocytes and normal liver structure.

HNF4α was shown to bind to 12% of genes represented on the Hu13K microarray from HepG2 cells, which was significantly higher than either HNF1α (1.6%) or HNF6 (1.7%). Moreover, 42% of hepatocyte genes are regulated by HNF4α [112].

The siRNA against HNF4α have been shown to abrogate hepatocyte differentiation [116]. In contrast, overexpression of HNF4α in dedifferentiated hepatoma cells restores many important functions of differentiated hepatocytes [123, 124]. Furthermore, forced expression of HNF4α in either NIH3T3 fibroblasts or in E9 embryonal carcinoma cells induces a mesenchymal to epithelial transformation that includes expression of cell junction proteins [109].

HNF4α is lost during the progression of slow-growing, non-invasive mouse HCC, to a fast-growing invasive form. Forcible expression of HNF4α in the fast-growing invasive hepatocellular carcinoma cells leads to reduced proliferation and suppression of tumor formation [125]. The expression levels of HNF4α also correlate with differentiation of human HCCs [115, 92, 113].

Thus, HNF4α is the central regulator of hepatocyte differentiation and liver function.

Transcriptional regulation of target genes by HNF4α is closely associated with PGC-1α acting as a positive co-factor and COUP-TF [126, 127, 128] often working as an antagonist of HNF4α. PGC-1α synergises with HNF4α in the regulation of carbohydrate [129, 98] and lipid [130] metabolism and coordinates the process of metabolic adaptation in the liver [130].

1.1.5 Replication cycle of HBV

The early steps of HBV infection are still obscure, but current data suggest that L protein is responsible for attachment and binding to still unknown receptors on hepatocyte surface [131, 49] (Fig. 4).

After uncoating, the capsid translocates in a microtubule-dependent manner to the nucleus. Within the nucleus the gap in the rcDNA is repaired by a host-specific polymerase, generating a fully duplex genome. This form of the hepatitis B viral genome exists only inside the host-cell nucleus and is termed covalently closed circular DNA (cccDNA). It serves as a template for the transcription of viral RNAs [132].

The transcription of HBV genes by host RNA-polymerase II is finely regulated by hepatocyte enriched transcription factors: CCAAT/enhancer binding protein

(C/EBP) [133, 134] and hepatocyte nuclear factors (HNF), such as HNF1 [135], HNF3 [136] or HNF4 [137, 138] in concert with nuclear receptors PGC-1α [139] and COUP-TF [137, 140] (Fig. 3). These factors confer hepatocyte specific activity of the viral *preS1* [133, 134, 141, 142] and *preC/C* promoters [143, 133, 134, 135, 137, 139, 138, 144, 145], and the viral enhancer elements [42, 146] and determine, to a great extent, the hepatocyte tropism of HBV.

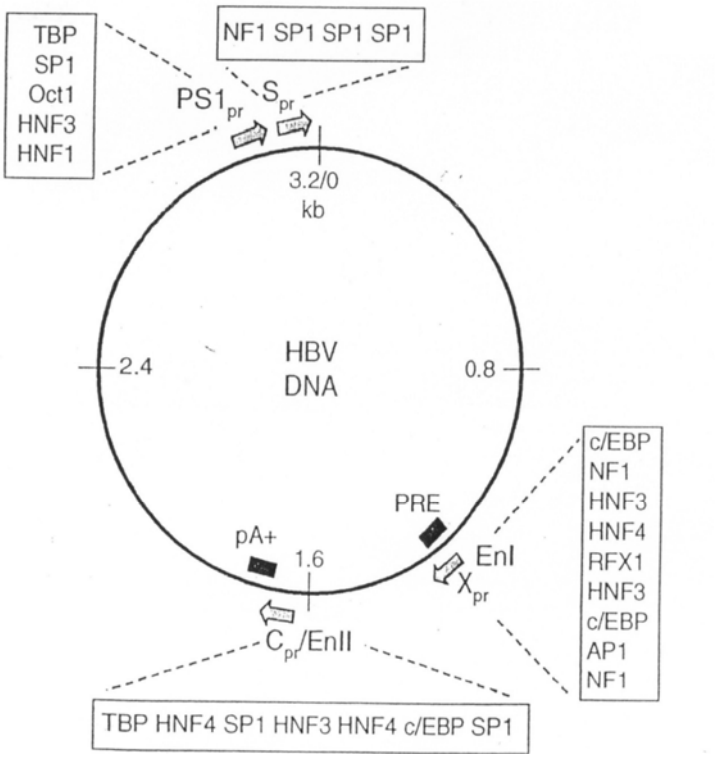


Fig. 3: Regulation of HBV gene transcription by different liver-enriched transcription factors
Adopted from Ganem D and Schneider RJ, Fields, Virology, 2001

All HBV RNAs are transported into the cytoplasm without splicing. Translation of the viral surface proteins S, M and L takes place at ribosomes of the ER, where they integrate into the ER membrane. The products of the pregenomic (core and polymerase) and precore (HBeAg) RNAs are synthesised at free ribosomes [37].

As soon as a sufficient amount of core protein has been produced, it self-assembles into capsids. The TP domain of the polymerase recognises a stem-loop-formation at the 5` end of the pgRNA (ε) [37] and the pgRNA-protein-complex is packaged into the capsids, where the polymerase starts to reverse transcribe the DNA minus strand. Simultaneously, the pgRNA is eliminated by the RNAseH activity of the viral polymerase. Then, synthesis of the DNA plus strand initiated at direct repeat 2 (DR2) occurs, resulting in the rcDNA genome with an overlapping minus

strand segment. Complementary sequences at the 5` end of the plus strand and the 3` end of the minus strand lead to circularisation of the DNA molecule [37].

The newly developed mature nucleocapsids can now follow two different pathways. Some translocate to the nucleus and re-import the DNA to refill the cccDNA pool. Others bud into the ER, thereby receiving their envelope, consisting of the viral surface proteins S, M and L integrated into the ER lipid bilayer. They traverse the Golgi before being exocytosed. Little is known about the transport and sorting mechanisms, which HBV uses to be efficiently exported in order to infect neighbouring cells and new hosts. Recently, the role of cell polarity for egress of DHBV [147] and HBV [35] has been shown.

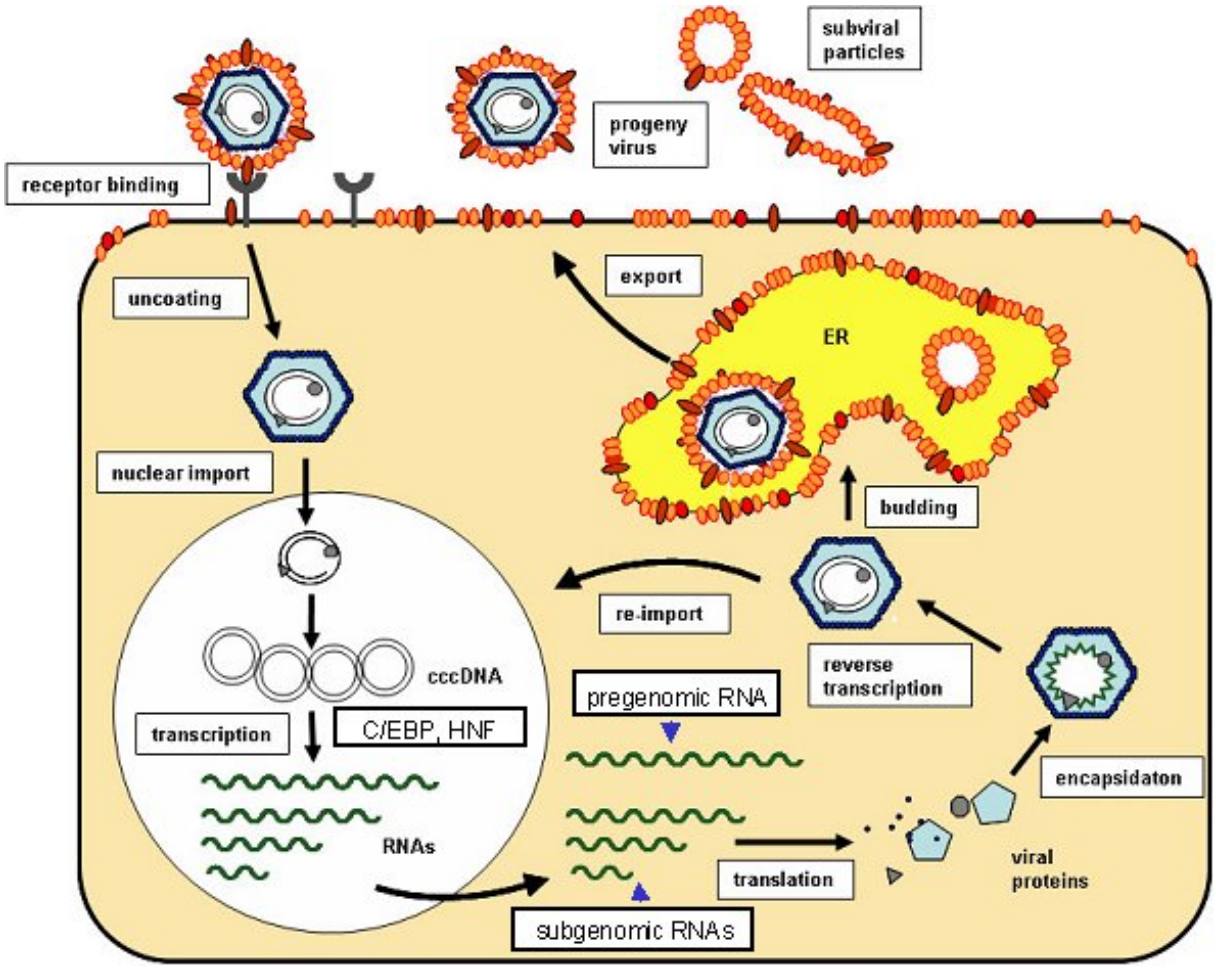


Fig. 4: Replication cycle of HBV

Virions bind to a receptor on the cell surface and enter. The nucleocapsids uncoat and the partially ds DNA genome is imported into the nucleus. DNA reparation leads to cccDNA formation and transcription of viral RNAs. Transcription of viral RNAs is finely regulated by C/EBP and HNFs. After translation and capsid formation, the pgRNA is encapsidated. Reverse transcription takes place within the capsids. Nucleocapsids are either re-imported into the nucleus or bud into the ER, obtaining their envelope. Virions, as well as budded SVP, are transported to the cell surface and are secreted.

1.1.6 Models of HBV infection

The establishment of an animal model of HBV is hampered by the species specificity of the virus and the fact that the animal species, which can be infected with a human HBV, are not characterised and difficult to maintain.

To date, HBV transgenic mice serve as an *in vivo* model for chronic HBV infection.

Guidotti et. al. generated HBV-trangenic mice with a terminally redundant viral DNA construct (HBV 1.3) that starts just upstream of HBV enhancer I, extends completely around the circular viral genome, and ends just downstream of the unique polyadenylation site [148]. These transgenic animals replicate the virus at levels comparable to that seen in the infected livers of patients with chronic hepatitis without any evidence of cytopathology [148].

Using mutations in the HBV genome construct, various types of HBV-transgenic mice were produced. By introducing a 3' and 5' frameshift mutation into the ORF X of the HBV 1.3 construct, which results in a premature stop codon, HBV 1.3 xfs transgenic mice were generated. These mice lack expression of the HBV X protein [149, 150].

Transduction of mice with adenoviral vectors containing the complete HBV genome mimics many aspects of an acute HBV infection [151]; *John von Freyend M et. al., in preparation*.

The repertory of cells that can serve as a cell-culture model is limited by the hepatotropism of the virus. Human hepatoma cell lines, such as HuH7 or HepG2, are useful to study intracellular steps of the viral replication cycle. However, they cannot be infected with HBV. The viral genome has to be transferred into the cells via transfection or transduction with viral vectors. HepG2.2.15 cells, an established cell line replicating HBV from four intergrated dimeric HBV genomes [152], and HepG2-H1.3 cells, a cell line containing one copy of a 1.3-fold overlength HBV genome and establishing HBV cccDNA as additional transcription template [153, 154] are also suitable to study the late steps of the viral replication cycle.

To gain insight into the complete replication cycle of HBV, the culture of primary human hepatocytes (PHH) provides a necessary and valuable tool [155]. Recently, HepaRG, a new hepatoma cell line, has been established from differentiated human HCC. Upon differentiation, HepaRG cells exhibit hepatocyte-like morphology, express hepatocyte-specific functions and can be infected with HBV [156].

1.1.7 HBV replication and hepatocyte differentiation: current research and unresolved questions

Studies with the transfection of HBV genomes in hepatoma cells have demonstrated that HBV replication takes place in differentiated, but not in undifferentiated human hepatoma cells [157] and is more efficient in quiescent hepatocytes as compared to proliferating hepatocytes [158]. The efficiency of HBV replication can be increased by cultivating primary hepatocytes [159, 160] or stable HBV-producing hepatoma cell lines [156, 161, 162] under differentiation conditions (usage of collagen-coated dishes, low FCS-content in cultivation medium, addition of dexamethasone and DMSO). In addition, the activity of preC/C- and especially preS1- promoters seems to depend on the hepatocyte differentiation state and is enhanced in quiescent hepatocytes [163, 164, 158, 135, 165].

However, none of these studies thoroughly analyzed, which hepatocellular factors link HBV transcription and replication to hepatocyte differentiation. Considering hepatocyte-enriched transcription factors as putative candidates, it is not known whether only one or a combination of these transcriptional regulators is responsible for differentiation-dependent activity of HBV promoters.

Moreover, it is still unclear, whether hepatocyte-enriched transcription factors act on HBV promoters in a dose-dependent manner.

Thus, the essential link between the efficiency of HBV replication and the degree of hepatocyte differentiation remains to be elucidated. A study of the exact dependence of HBV replication on hepatocyte differentiation and identification of the link factor would help to define the stage of the liver development, at which exclusively hepatotropic HBV can infect and replicate in hepatocyte precursors.

1.2 Heme oxygenase I

1.2.1 Heme oxygenase I, its function and induction

Heme oxygenase (HO) is the small molecule that catalyzes the initial and rate limiting step in the oxidative degradation of heme acting together with NADPH-cytochrome p450 reductase as a reducing agent [166] (Fig. 5).

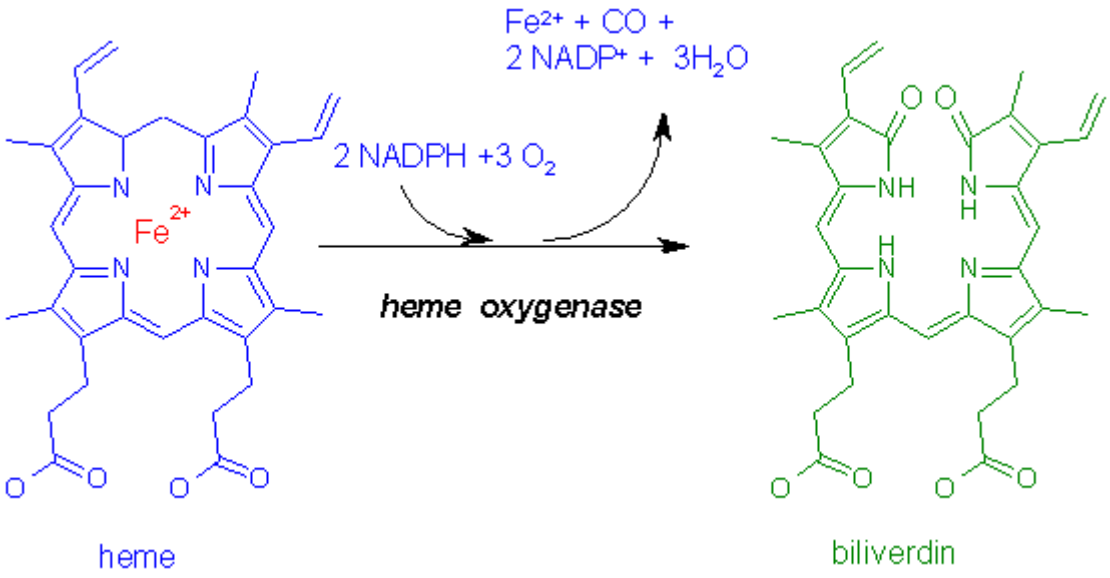


Fig. 5: Reaction, catalized by heme oxygenase
Adopted from http://www.med.unibs.it/~marchesi/heme.html

Heme released from oxidized free haemoglobin of senescent erythrocytes, myoglobin or cytochromes, constitutes a potentially harmful molecule and may generate reactive oxygen species (ROS), which cause oxidative stress [167]. HO catabolizes heme and yields equimolar amounts of carbon monoxide, biliverdin, and iron. In mammalian cells, biliverdin is subsequently converted to bilirubin by biliverdin reductase and the released iron is used in intracellular metabolism or sequestered into ferritin [168, 169].

Until now, three isoforms of the HO protein have been identified, which are encoded by separated genes. HO1, also known as heat shock protein 32, is the only inducible isozyme [170, 171]. Under basic conditions, the 32 kD protein is expressed at low levels in the liver, kidney, endothelial cells, bone marrow and most prominently in the spleen, where senescent erythrocytes are disposed and red blood cell haemoglobin is degraded. Beside the major substrate heme, HO-1 synthesis is known to be upregulated by a multitude of non-heme inducers, including heavy metals, cytokines e.g. IL-6, hormones, endotoxins, oxidants and a heat shock [172, 173]. Induction of HO-1 is also highly sensitive to exposure to agents causing oxidative stress, such as UV irradiation, sodium arsenite, hypoxia and glutathione depletion, which indicates that HO1 induction may be a protective strategy to defend cells against oxidative and inflammatory damage [174, 175, 176, 177]. Cobalt protoporphyrin-IX, a potent inducer of HO-1 is an important experimental tool that is widely used for

different *in vivo* and *in vitro* studies to examine effects derived from inducible HO-1 [178, 179, 180, 181]. Activation of HO-1 expression by most stimuli is controlled at the transcriptional level and involves gene activation and de novo enzyme protein synthesis.

1.2.2 Effects of HO-1 induction

Accumulating evidence suggests that HO-1 induction in addition to its role in heme degradation might confer a large spectrum of cytoprotection that is mostly associated with different end products of heme catabolism [167].

Bilirubin derived from biliverdin possesses potent free radical scavenging and antioxidant properties [182-184]. Along with potent antioxidant poperties, bilirubin also exerts anti-inflammatory effects. So, bilirubin mediates inhibition of leukocyte adhesion and rolling after induction of HO-1 in a model of ischemia-reperfusion injury of mesenterial tissues [185].

Free iron, a strong pro-oxidant, upregulates an iron-transporter ATPase pump that removes free iron from the cell and induces the expression of ferritin, a multimeric iron-chelating protein. Ferritin limits the generation of free radicals by binding free iron and making it unavailable for catalytic reactions [186].

CO, the third catalytic product of HO-1 activity, possesses anti-thrombotic, anti-apoptotic, anti-inflammatory and anti-proliferative effects and also promotes vasodilation. For example, CO is able to suppress platelet activation or aggregation [187]. In addition, CO has also been reported to protect endothelial cells [181, 188] fibroblasts, hepatocytes and beta-cells of the pancreas from undergoing apoptosis. Furthermore, CO downmodulates the expression of plasminogen activator inhibitor type I and markedly inhibits the proinflammatory response in macrophages, whereas it stimulates production of IL-10 [189]. Moreover, CO decreases antigen-presenting capacity in antigen-presenting cells [190], inhibits T-cell proliferation [191, 192], promotes activation-induced cell death in T-cells [193] and increases numbers and function of CD4+CD25+FoxP3+ regulatory T-cells [194, 195]. Induction of HO-1 and its cytoprotective potential have been shown to contribute to therapeutic implications in the treatment of diseases associated with oxidative stress, apoptosis or inflammation [177, 196, 197, 198, 199]. In particular, HO-1 is reported to protect the

liver from ischemia-reperfusion [200], hemorrhage/resuscitation [201], and immune-mediated [202] and inflammation-related [203] liver damage.

HO-1 deficiency in animals results in a high mortality rate after 25 weeks which is associated with a macromolecular oxidative damage, tissue injury, chronic inflammation, significant weight reduction and anemia [204, 205]. HO-1 deficiency in humans leads to severe growth retardation, persistent endothelial damage and haemolytic anemia characterized by marked erythrocyte fragmentation [206].
In contrast to the beneficial properties of inducible HO-1, the protein has been shown to contribute to the protection of solid tumors [207, 208, 209].

However, induction of HO-1 by non-stressful stimuli appears to be a promising target and might become a novel therapeutic approach for diseases associated with oxidative stress, apoptotic and inflammation-related tissue damage.

1.2.3 HO-1 in the pathogenesis of viral infections

HO-1 is involved in the pathogenesis of several viral infections and its suppression or induction might be implemented in the treatment of viral diseases.
Thus, induction of HO-1 in monocytes by hemin administration substantially inhibited HIV replication in a dose-dependent manner. In addition, hemin treatment significantly suppressed infection of both monocytes and T cells inoculated with R5, X4, R5X4 tropic HIV strains and reverse transcriptase-resistant, azidothymidine-resistant, ddC/ddL-resistant, nivirapine-resistant, and other clinical isolates. Moreover, induction of HO-1 exerted anti-HIV effects *in vivo*. Intraperitoneal administration of hemin 4 days after HIV infection reduced viral load in the serum of human PBMC-reconstituted non-obese diabetic SCID mice by more than 6-fold [210].
The impact of hepatitis C virus (HCV) proteins on HO-1 expression seems to be ambiguous. HCV core protein inhibits HO-1 expression in hepatocytes [211] and hepatoma cells [212], thereby sensitizing cells for oxidative injury [212]. However, when the HCV proteins core, E1, E2, p7, NS2, and the aminoterminal domain of NS3 were simultaneously expressed in hepatoma cells, HO-1 expression was upregulated. Furthermore, non-structural proteins of HCV induced HO-1 expression [213]. The final effect of HCV replication on HO-1 expression *in vivo* seems to be negative as shown with biopsy samples from chronically infected patients [211].

In histological samples with chronic hepatitis B, HO-1 seems to be upregulated [211]. However, *Protzer et. al.* did not show any up-regulation of HO-1 in HBV-producing compared to parental hepatoma cells and suggested that HO-1 induction in HBV infection is most likely caused by pro-inflammatory cytokines [154]. The authors also demonstrated a pronounced antiviral effect of CoPP associated with HO-1 induction in HepG2.215 cells, in the mouse model of acute HBV infection (mice transduced with an adenoviral vector encoding HBV genome) and in HBV-transgenic mice. In a model of HIV infection, *Vzorov et. al.* showed that porphyrins display their own antiviral activity [214]. Therefore, it remains unclear, whether an antiviral effect of CoPP against HBV was mediated by HO-1 or was merely caused by porphyrins. Although, *Protzer et. al* showed antiviral activity of HO-1 overexpression using an adenoviral vector, proof that knocking out HO-1 expression abolishes the antiviral activity was lacking. Also, *Protzer et. al* showed amelioration of liver injury in the model of acute HBV infection, when HO-1 was induced by CoPP prior to the onset of HBV replication. Considering a therapeutic application of HO-1 induction in acute hepatitis B or during flares of chronic hepatitis B, it is important to study the effect of enzyme overexpression on the liver injury on ongoing HBV replication. Here, considering a therapeutic potential of HO-1 in acute and flares of chronic hepatitis B, it is important to study the effect of enzyme induction at the peak of inflammation.

1.3 Interleukin-6 and its role in HBV infection

1.3.1 IL-6 is a pleotropic cytokine

Interleukin-6 (IL-6) is a 26-kDa glycoprotein encoded on chromosome-7, produced by macrophages, T-cells, endothelial cells and osteoblasts. IL-6 has a wide range of biological functions: (1) stimulates antibody production by activated B-cells; (2) is involved in T-cell activation, growth, differentiation, and expression of cytotoxic cell function; (3) induces the proliferation of pluripotent hematopoietic progenitors and acting synergistically with MCSF and GM-CFS, stimulates differentiation of macrophages and granulocytes; (4) induces maturation of megakaryocytes; (5) regulates the function of macrophages and neutrophiles; (6) induces acute-phase response; (7) stimulates secretion of adrenocorticotropic hormone by the pituitary gland; (8) stimulates expression of nerve growth factor; (9) directly or indirectly affects osteoclast development and plays a role in postmenopausal osteoporosis;

(10) is essential for regeneration of hepatocytes; (11) and is involved in the regulation of carbohydrate and lipid metabolism and insulin sensitivity [215]. IL-6 knock-out mice are viable and fertile and do not exhibit any overt phenotypic abnormality. Females demonstrate higher rates of bone turnover. Also, T-cells in both genders are reduced in number by 20-40% compared with controls. In addition, the transgenic animals have impaired responses to viruses and bacterial infection but an almost normal response to lipopolysaccharide [216]. Moreover, IL-6-deficient mice exhibit impaired liver regeneration and develop mature-onset obesity due to disturbed carbohydrate and lipid metabolism [215].

1.3.2 IL-6 signaling

The interaction of IL-6 with its target cells is mediated by IL-6 receptor (IL-6R). It consists of two polypeptide chains, a 80 kDa IL-6R and a 130 kDa signal transducer (gp130). The 80 kDa receptor exists in two forms, the transmembrane form and a soluble form. The transmembrane form has a short intracytoplasmic region and, upon stimulation by binding of the IL-6 molecule, triggers an association with gp130. The soluble receptor can form a stimulatory complex with IL-6, which can associate with gp130 and trigger cellular events called trans-signaling. The gp130 has a transmembrane domain and is responsible for transducing the signal across the membrane [217]. IL-6 and its receptor interact to form a complex consisting of two IL-6 molecules plus two IL-6 receptor proteins and two gp130 proteins. The dimerized gp130 then transduces the signals.

Activation of gp130 leads to the activation of the intracytoplasmic JAK tyrosine kinases (Janus family tyrosine kinases). These kinases cause induction of tyrosine phosphorylation and recruitment of STAT3, which dimerizes and is translocated to the nucleus and leads to gene expression. Furthermore, formation of the IL-6-IL-6R complex leads to the activation of the Ras-mitogen-activated protein (MAP) kinase pathway. Moreover, IL-6R activates a number of non-receptor tyrosine kinases, such as Btk, Tec, Fes and Hck, although the biological significance of these signal transduction pathways remains to be clarified. All signalling pathways activated by IL-6R may interact with each other and contribute to a variety of biological activities [215]. IL-6 signaling systems are regulated by negative feedback by the suppressors of

cytokine signalling (SOCS) and the protein inhibitors of activated STATs (PIAS) [218, 217].

1.3.3 IL-6 and HBV pathogenesis

The role of IL-6 in the pathogenesis of HBV infection is being actively studied. In humans, serum levels of IL-6 closely correlate with the severity of tissue injury and clinical course of HBV disease [219, 220, 221]. IL-6 activity was reported to be significantly enhanced during acute exacerbation of the illness, followed by clearance of HBeAg [219]. The levels of sIL-6R correlated with response to IFN-alpha therapy [222]. Therefore, IL-6 may contribute, at least in part, to the elimination of HBV by the immune system.

The data concerning the role of human IL-6 for infection of cells with HBV and virus replication are controversial. *Galun et al.* observed that simultaneous incubation of human liver tissue with HBV serum and human IL-6 for 24 h before transplantation into SCID mice leads to a higher rate of HBV DNA positive animals than incubation of liver tissue with HBV serum only [223]. *Waris and Siddiqui* incubated HBV-replicating HepG2.2.15 cells with human IL-6 and demonstrated a positive influence of IL-6 on HBV transcription [224]. However, *in vivo,* in the HBV-transgenic mice model, administration of the recombinant human IL-6 led to the suppression of HBV 2.1 kb-steady state mRNA expression 16 to 20 h after injection of the cytokine [225].

Recently, Hoesel M et. al (submitted) have shown that non-parenchymal liver cells, such as Kupffer cells or LSECs, recognize the HBV pattern in inoculum containing virions as well as secretory HBsAg and HBeAg prior to HBV replication in hepatocytes. The authors emphasized that recognition of HBV envelope proteins and, possibly other HBV-patterns, but not HBV replication leads to NF-$\kappa\beta$ activation and the rapid secretion of the NF-$\kappa\beta$-regulated cytokines: IL-6, IL1β and TNFα. In contrast, recognition of the HBV pattern does not induce any interferon response and interferon-regulated genes are even down-regulated. Importantly, secretion of NF-$\kappa\beta$-regulated cytokines was correlated with a decrease of HBV progeny release in the cell culture media. Application of IL-6-blocking antibody and recombinant IL-6 (rIL-6) confirmed a causative role of the cytokine in the inhibition of HBV replication. Further experiments by Hoesel M et. al (submitted) indicate that inhibition of HBV replication by IL-6 occurs at the transcriptional level.

However, which cellular pathways are involved in the control of HBV replication by this cytokine remained unclear. Thus, to further understand the complex role of IL-6 in the pathogenesis of HBV infection, a detailed study of mechanisms underlying the effect of IL-6 on HBV replication is required.

For better understanding of the virus-cell host interactions, the study of the replicative potential of HBV in the developing liver and factors linking efficient HBV replication to hepatocyte differentiation; detailed analysis of the antiviral and cytoprotective activity of HO-1 and the effect of IL-6 on HBV replication should be performed. The results of such studies may contribute to the development of new therapeutics against HBV and to the improvement of prophylaxis of intrauterine HBV infection.

2 Aims of the thesis

Hepatitis B is still an important health problem. In 10% of adults and 90% of infected newborns, HBV causes a chronic infection with increased risk of liver cirrhosis, hepatic decompensation and hepatocellular carcinoma. Intrauterine HBV transmission contributes to 8-40% of mother to child transmission. Prophylaxis of intrauterine infection has not been established so far. The important obstacle for development of a prophylaxis of intrauterine HBV transmission is the lack of knowledge on infectious and replicative potential of HBV in the developing liver. In this context, it seemed essential to know to what extent HBV replication depends on hepatocyte differentiation and which hepatocellular factors are responsible for that.
The purpose of this study therefore was:

- to determine the replicative potential of HBV in the developing liver;
- to study the changes of HBV replication efficiency along with hepatocyte maturation;
- to understand in detail, to what extent HBV replication depends on hepatocyte differentiation;
- to pinpoint factors responsible for the dependence of HBV replication on hepatocyte differentiation and, in particular, influencing the replicative potential of HBV in the developing liver

Heme oxygenase-1 (HO-1), a heme degrading enzyme, is involved in the pathogenesis of several viral infections. We observed a pronounced antiviral effect of HO-1 induction in stable HBV-producing cell lines, in a mouse model of acute HBV infection and in HBV-transgenic mice. However, lacking was:

- a proof that knocking out HO-1 expression abolishes the antiviral activity
- whether and how HO-1 activity affects the viral persistence form HBV cccDNA
- considering a therapeutic potential of HO-1 in acute and flares of chronic hepatitis B, it is important to study the effect of enzyme induction at the peak of HBV-induced inflammation in the liver

Besides hepatocellular factors, extracellular mediators can also affect HBV replication and are therefore important for a detailed understanding of the virus-host

interactions. In this term, there are contrary data on the influence of IL-6 on HBV replication. Previous study in our working group indicated that IL-6 inhibits HBV replication at the transcription level. So far, it has not been studied, which cellular pathways are involved in the control of HBV replication by this cytokine.
Therefore, the third purpose of this study was:

- to further analyze, which step of HBV replication cycle is influenced by IL-6;
- to find out hepatocellular factors mediating the effect of IL-6 on HBV replication.

3 Material and Methods

3.1 Material

3.1.1 Expendable items

Centricon Plus-70, Biomax 100	Millipore Corp., Billerica, MA, USA
Chamber Slides LabTekII, RS Glass	Nunc, Wiesbaden, Germany
Cuvettes	Sarstedt, Nümbrecht, Germany
Freezing Container Nalgene™	Nunc, Wiesbaden, Germany
Nylon membrane, positively charged	Roche Diagnostics, Mannheim, Germany
Nitrocellulose membrane	Whatmann GmbH, Limburg, Germany
Hyperfilm ECL	GE Healthcare, Buckinghamshire, UK
Tissue paste Histoacryl®	Braun, Melsung, Germany
Whatman 3MM	Biometra, Göttingen, Germany
Ultra centrifuge-tubes, polyallomer	Beranek Laborgeräte, Weinheim, Germany
Dialysis chamber (PERBIO* 66453 Slide-A-Lyser 10 kDa)	PERBIO Sciences, Bonn, Germany

3.1.2 Equipment

Centrifuges:	
Centrifuge 5417C / 5417R	Eppendorf, Hamburg, Germany
Megafuge 1.0 / 1.0 R	Heraeus Holding GmbH, Hanau, Germany
Ultra centrifuges:	
Sorvall RC 50 Plus	Kendro, Langenselbold, Germany
XL 70	Beckman, München, Germany
Scales Kern 440-47	Sartorius AG, Göttingen, Germany
Fraction recovery system	Beckman, München, Germany
Biocycler Thermocycler T3	Biometra, Göttingen, Germany
Blot chamber MiniProtean®3 Cell	BIO-RAD Laboratories, Hercules, USA

Dot blot apparatus Minifold I	Schleicher & Schuell, Dassel, Germany
ELISA Reader MRX Revelation	Dynex, Gaithersburg, USA
Film processor Curix 60	Agfa Geveart NV, Mortsel, Belgium
Gel chambers	BIO-RAD Laboratories, Hercules, USA
Heating block	Eppendorf, Hamburg, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Incubator	Heraeus Holding GmbH, Hanau, Germany
Light Cycler System	Roche Diagnostics, Mannheim, Germany
Microscopes	
Fluorescence microscope IX81	Olympus, Hamburg, Germany
Confocal microscope FluoView1000	Olympus, Hamburg, Germany
pH-Meter	WTW, wissenschaftlich technische Werkstätten
Phosphoimager, Molecular Imager FX	BIO-RAD Laboratories, Hercules, USA
Photometer Smart Spec 3000	BIO-RAD Laboratories, Hercules, USA
Photo system for agarose gels Gel-doc 2000	BIO-RAD Laboratories, Hercules, USA
Power Supplies Pack300	BIO-RAD Laboratories, Hercules, USA
Sterile hood (cell cultur)	Heraeus Holding GmbH, Hanau, Germany
UV-Oven GS Gene Linker™	BIO-RAD Laboratories, Hercules, USA

3.1.3 Software

Autoradiography	Quantity One, 4.2.1, BIO-RAD Laboratories, Hercules, USA
Data processing	Windows 2000, MS Office 2000, Word and Excel, Microsoft, Redmont, USA
Fluorescence microscopy	Cell P, AnalySIS, Soft Imaging System GmbH, Münster, Germany
FV10-ASW, Version 1.6a	
Graphic programmes	Adobe Photoshop 5.5, Adobe, San Jose, USA Power Point 2000, Microsoft, Redmont, USA
Light Cycler	Probe Design Analysis and Rel Quant, Roche Diagnostics, Mannheim, Germany

3.1.4 Chemicals

All solutions were prepared with deionised water from the Ultra Pure Water System Easy Pure UV/UF (Werner Reinstwassersysteme, Wilhelm Werner GmbH, Leverkusen, Germany).

Acidic acid	Roth, Karlsruhe, Germany
Acryl amide	Sigma, Deisenhofen, Germany
Agarose SeaKem LE	Cambrex Bio Science, Rockland, USA
Ammonium acetate	Merck, Darmstadt, Germany
Bromphenol blue	Merck, Darmstadt, Germany
Caesium chloride	Roth, Karlsruhe, Germany
Chlorophorm	Roth, Karlsruhe, Germany
Chlorophorm/Isoamylalcohol 24:1	Roth, Karlsruhe, Germany
Cobalt protoporphyrin-IX	Alexis Deutschland GmbH, Grunberg, Germany
Collagen IV	Serva, Heidelberg, Germany
Developer G153 A + B	Agfa Geveart NV, Mortsel, Belgium

1,4- Diazabicyclo[2,2,2]octane 98% (DABCO)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
4',6-Diamidino-2-phenylindol (DAPI)	Molecular Probes, Inc, Eugene, USA
Ethylenedinitrilotetraacedic acid (EDTA)	Roth, Karlsruhe, Germany
Ethidium bromide	Merck, Darmstadt, Germany
Ethanol	Roth, Karlsruhe, Germany
Glycerol	Roth, Karlsruhe, Germany
HEPES	Biochrom AG
Hydrochloric acid	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
MAPK inhibitors: PD98059 for pERK SP600125 for pJNK	Calbiochem, La Jolla, USA
Magnesium Chloride ($MgCl_2$)	Roth, Karlsruhe, Germany
Methanol	Roth, Karlsruhe, Germany
Milk powder, low fat	Sigma, Deisenhofen, Germany
Mowiol 4-88 reagent	Calbiochem, La Jolla, CA, USA
NaOH	Roth, Karlsruhe, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Phenol	Roth, Karlsruhe, Germany
Polybed 812 and epoxy resin	Polysciences, Warrington, PA, USA
Ponceau S	Roth, Karlsruhe, Germany
Potassium	Roth, Karlsruhe, Germany
Potassium acetate	Merck, Darmstadt, Germany
Proteinase Inhibitor tablets	Roche Diagnostics, Mannheim, Germany
Rapid Fixer G354	Agfa Geveart NV, Mortsel, Belgium
Sodium acetate	Merck, Darmstadt, Germany
Sodium chloride	Roth, Karlsruhe, Germany
Sodium dihydrogenphosphate	Roth, Karlsruhe, Germany
Sodium hydroxide	Roth, Karlsruhe, Germany
Sucrose	Sigma, Deisenhofen, Germany
Tetramethylethylendiamine (TEMED)	Sigma, Deisenhofen, Germany
Tris base	Roth, Karlsruhe, Germany

Triton X-100	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany
Water, DNAse-, RNAse-free	Promega, Mannheim, Germany

3.1.5 Cell lines and primary cells

HepG2	Human hepatoma cell line, ATCC no. HB-8065™
HuH7	Human hepatoma cell line, JCRB0403
PHH	Primary human hepatocytes, isolated from liver resections
Pop10	Hepatocyte cell line established by immortalization of primary human hepatocytes using Cre-excisable lentiviral vectors coding for SV40 T antigen, telomerase, and/or Bmi-1 [226]
HepaRG	Human hepatoma cell line, HepaRG cells can be differentiated and become permissive for HBV if cultivated with corticosteroids and dimethyl sulfoxide (DMSO) [50, 156]
293	Human embryonic kidney cell line, stably producing adenovirus E1 Protein, ATCC no. CRL-1573 [227]
HepG2.2.15	Human hepatoma cell line replicating HBV from four integrated dimeric HBV genomes [152]
HepG2-H1.3	Human hepatoma cell line containing one copy of a 1.3-fold overlength HBV genome, which establishes HBV cccDNA as an additional transcription template [153, 154]

3.1.6 Experimental animals (mice)

Eight to ten week old male C57BL/6 wild-type mice (purchased from Charles Rivers, Sulzfeld, Germany) or HBV or HBVxfs transgenic mice (kindly provided by H. Schaller, Heidelberg, Germany) selected for comparable levels of HBV replication were used.

HBV transgenic mice were established with a terminally redundant viral DNA construct (1.3-fold overlength of HBV genome) that starts just upstream of HBV

enhancer I, extends completely around the circular viral genome, and ends just downstream of the unique polyadenylation site in HBV [148].

HBVxfs transgenic mice were produced with a similar HBV construct, in which a 3' and 5' frameshift mutation, resulting in a premature stop codon, was introduced into the open reading frame X. Therefore, these mice lack HBV X protein [149, 150]. Legal requirements for biosafety and animal care were met. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the US Academy of Sciences and published by the National Institutes of Health.

3.1.7 Patient samples

Human HCC or surrounding, non-tumorous (peritumor) HBV infected liver tissue samples were selected from the tissue bank of the Institute of Pathology, University Hospital Cologne, established after informed consent from patients. Selection criteria were: active HBV infection (HBsAg, anti-HBc and / or HBV DNA positive in patient's serum), absence of any other obvious cause for HCC (e.g. HCV infection, hematochromatosis) and availability of snap frozen tumor and peritumor tissue. Tumors were graded according to the American Joint Commission on Cancer. Healthy liver tissue was obtained from human liver grafts (HBV, HCV, HIV negative) not suited for transplantation.

Staging and grading of hepatocellular carcinoma samples

patient number	serum HBsAg/ antiHBc	tumor staging and grading	peritumor, fibrosis stage	peritumor, inflammation grade
1	+	pT3 N0 Mx G2 R0	3	3
2	+	pT1 N0 Mx G2-4 R0 areals of mixed (high and no) differentiation	2-3	2
3	+	pT1 N0 Mx G2 R0	2-3	2
4	+	pT1 N0 Mx G2 R0	nd	nd
5	+	pT2 N0 Mx G2 R0	2-3	2
6	+	pT1 N0 Mx G1 R0	4	2
7	+	pT3 N0 Mx G3 R0	4	1
8	+	pT1 N0 Mx G1 R0	4	1

3.1.8 Media and supplements

Ampicillin	Sigma, Deisenhofen, Germany
Collagenase Worthington	Biochemical Corporation, Lakewood, NJ, USA
Dulbeccos MEM	Gibco, BRL, Eggenstein, Germany
Dimethylsulfoxid (DMSO)	Merck, Darmstadt, Germany
Ethyleneglycolbis (2-aminoethyl) -tetraacetic acid (EGTA)	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	Biochrom AG, Berlin, Germany
Gentamycin	Gibco BRL, Eggenstein, Germany
Glutamine	Gibco, BRL, Eggenstein, Germany
HBSS	Gibco BRL, Eggenstein, Germany
Heparin Liquemin N 25000	Roche, Mannheim, Germany
HEPES	Gibco BRL, Eggenstein, Germany
Hydrocortison	Sigma, Deisenhofen, Germany
Inosine	Serva, Darmstadt, Germany
Insulin	Serva, Darmstadt, Germany
Penicillin/ Streptomycin (P/S)	Biochrom AG, Berlin, Germany
Sodium pyruvate	Gibco BRL, Eggenstein, Germany
Non essential amino acids (NEAA)	Biochrom AG, Berlin, Germany
Polyethylenglycol (PEG) 6000	Serva Electrophoresis, Heidelberg, Germany
RPMI 1640	Gibco, BRL, Eggenstein, Germany

Williams Medium E	Gibco, BRL, Eggenstein, Germany

Primary human hepatocytes

Preperfusion medium	HBSS, Ca/Mg-free	500 ml
	EGTA, 100 mM	2.5 ml
	Heparin, 5000 U/ml	1 ml
Collagenase medium	Williams Medium E	250 ml
	$CaCl_2$, 1 M	0.9 ml
	Gentamycin, 10 mg/ml	2.5 ml
	Collagenase type IV	200 mg
Wash medium	Williams Med E	500 ml
	*Glutamine, 200 mM	5.6 ml
	*Glucose, 5%	6 ml
	*Hepes, 1 M, pH 7.4	11.5 ml
	*P/S, 5000 U/ml	5.6 ml
(*Solutions were mixed and stored as premix at –20°C)		
PHH medium	Wash medium	500 ml
	Gentamycin, 10 mg/ml	5 ml
	Hydrocortison	0.5 ml
	Insulin	0.45 mg
	DMSO	8.7 ml
	Inosine, 82.5 mg/ml	2 ml

HuH7 and HepG2

Cultivation medium	Dulbeccos MEM	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml
	NEAA, 100 x	5.5 ml
Freezing medium	Dulbeccos MEM	500 ml
	FCS	20%
	DMSO	10%

HepaRG

Cultivation medium	Williams E medium	500 ml
	FCS	5 ml (10 %)
	P/S, 5000 U/ml	5.5 ml
	Streptomycin, 5000 U/ml	5.5 ml
	Hydrocortison hemisuccinate	0.005mM
	Insulin	2.5 mg
Differentiation medium	Williams E medium	500 ml
	FCS 10 %	5 ml
	P/S, 5000 U/ml	5.5 ml
	Streptomycin, 5000 U/ml	5.5 ml
	Hydrocortison hemisuccinate	0.005mM
	Insulin	2.5 mg
	DMSO	10 ml (2%)

Pop10

Cultivation medium	Dulbeccos MEM-F12	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml
	NEAA, 100 x	5.5 ml
	NaPyruvate	5.5 ml
	Dexamethason	500µl (1 µM)
	Insulin	830µl (5µg/ml)

293 cells

Cultivation medium	Dulbeccos MEM	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml

HepG2.2.15

Cultivation medium	Dulbeccos MEM	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml
	NEAA, 100 x	5.5 ml

Virus production medium	PHH medium	250 ml
	Williams E medium	250 ml
	FCS	25 ml
	Glutamine, 200 mM	2.5 ml
	P/S, 5000 U/ml	2.5 ml
	NEAA, 100 x	2.5 ml

HepG2-H1.3

Differentiation medium	PHH medium	250 ml
	HepG2/HuH7 cultivation medium	
	w/o FCS	250 ml
	FCS	5 ml (1%)

3.1.9 Buffers and solutions

3.1.9.1 Solutions for purification and storage of adenoviral vectors

CsCl, 1.2 g/ml	CsCl	26.8g
	Tris-Cl, 10 mM, pH 8.0	92 ml
CsCl, 1.4 g/ml	CsCl	53g
	Tris-Cl, 10 mM, pH 8.0	87 ml
Dialysis buffer	Tris-Cl, pH 8.0	10 mM
	MgCl2	2 mM
	Sucrose 4%	w/v
	ddH_2O	
Storage buffer	Tris-Cl, pH 8.0	10 mM
	NaCl	100 mM
	BSA	0.1%
	Glycerol	50%
	ddH_2O	

3.1.9.2 Cytospins

modified HBSS (GibcoBRL without Ca and Phenolred):

HBSS (10x)	50 ml
$MgCl_2$ (1M)	400µl (0.8 mM)
HEPES pH 7.4 (1M)	10ml (20 mM)
Pen/Strep	5 ml
ddH_2O	ad 500 ml

The solution was sterile filtered using 22 µm filter and stored at 4^0C

modified HBSS+EGTA (5 mM)

HBSS modified	4.8 ml
EGTA (47.5µg/ml)	
pH 7.6	200µl (0.125M)

3.1.9.3 Transfer of nucleic acids and radioactive hybridization

Proteinase K buffer	Tris (100 mM), pH 8.5	10 ml (1 M)
	EDTA (5 mM)	1 ml (0.5M)
	SDS (0.2%)	1 ml (20%)
	NaCl (200 mM)	4 ml (5M)
	ddH_2O	ad 100 ml
TE-buffer	TrisHCl pH 8.0	10 mM
	EDTA	1 mM
STE-buffer	NaCl	100mM
	Tris HCl pH 7.5	20 mM
	EDTA	10mM
TBE 10x	Tris pH 8.0	
	adjusted with HCl	121.1g (100mM)
	Boric acid	47.8g (77 mM)
	EDTA 2.5 mM	5 ml (0.5M)
	add 1000ml H_2O	
TAE 50x	Tris base	242g
	glacial acetic acid	57.1g
	EDTA 0.5M	100 ml
	ddH_2O ad 1000ml	
	pH 8.0 was adjusted	

Denaturation solution	NaOH	20.0g (0.5 M)
	NaCl	58.55g (1 M)
	ddH_2O	ad 1000ml
Neutralisation solution	Tris, pH 7.4	60.6g (0.5 M)
	NaCl	175.3g (3 M)
	ddH_2O	ad 1000ml
10x Electrophoresis buffer (E-buffer)	$NaH_2PO_4x2\ H_2O$ (300 mM)	46.8 g
	EDTA (50 mM)	18.6 g
	DEPC-H_2O	ad 1000 ml
	pH 7.0 adjusted with NaOH	
RNA loading mix	Sample RNA 15µg	10µl
	E-buffer 10x	10µl
	Formaldehyd	15 µl
	Formamid (deionised)	40 µl
	heat to 65^0C for 10 min, then cool on ice and add 5µl loading buffer (see DNA loading buffer besides H_2O: DEPC-H_2O)	
Marker loading mix	RNA-Marker	6µl
	DEPC-H_2O	3µl
	E-buffer 10x	2µl
	Formaldehyd	3 µl
	Formamid (deionised)	8 µl
	heat to 65^0C for 5 min, then cool on ice and add 2.5µl loading buffer (see DNA loading buffer besides H_2O: DEPC-H_2O)	
NaOH 0.2M	200 ml (1M) + 800 ml ddH_2O	
NaOH 0.4M	400 ml (1M) + 600 ml ddH_2O	

SSC buffer

	1x	2x	10x	20x
NaCl , g	6	12	60	120
Na-citrate dehydrate, g	4.4	8.8	44	88
ddH_2O add 1000ml (for Northern blot DEPC-H_2O)				

Pre-hybridization / hybridization buffer	salmon sperm	1% (10µg/ml)
	Church buffer	1x
Church buffer	Phosphate buffer 0.5M, pH 7.2	250 ml (0.25M)
	EDTA 0.5M	1ml (1mM)
	BSA	5 g (1%)
	SDS	35 g (7%)
	ddH_2O	ad 500 ml
	store at -20^0C	
Phosphate buffer	$Na_2HPO_4x2H_2O$ (1M)	342 ml
	$NaH_2PO_4x2H_2O$ (1M)	158 ml
	ddH_2O	ad 500 ml
	pH 7.2 was adjusted with NaOH	
Wash buffer	SSC	1 x
	SDS	0.5%

3.1.9.4 Protein analysis

3.1.9.4.1 Immunofluorescence

3.1.9.4.1.1 Immunofluorescence staining of cell lines

Fixation solution	Paraformaldehyde	3.7%
	PBS	1 x
Saturation solution	Ammonium chloride	50 mM
Permeabilisation buffer	Saponin	0.5%
	PBS	1 x
Blocking solution	FCS	10%
	Saponin	0.1%
	PBS	1 x

Antibody incubation buffer	Saponin	0.1%
	PBS	1 x
Mounting medium	Mowiol	2.4 g
	Glycerol	12 g
	Tris, pH 8.5	0.2 M
	Dabco	50 mg
Donkey serum	Sigma	

3.1.9.4.1.2 Immunofluorescence staining of cytospins

BB (blocking buffer)	Donkey serum	5%
	PBS	1x
	Tween 20	0.05%
Antibody Blocking solution		
Primary Antibody	Donkey serum	1%
	PBS	1x
	Tween 20	0.05%
Secondary Antibody	Donkey serum	1%
	PBS	1x
	Tween 20	0.05%
	DAPI	0.01%

3.1.9.4.2 Protein isolation and Western blot

CHAPS, buffer for extraction of total cellular proteins	HEPES, pH 7.4 (10 mM)	500µl 1M HEPES
	NaCl 150mM	0.433 g
	CHAPS (1%)	0.5 g
	Protease inhibitor cocktail	1 tablette for 50 ml
SDS, buffer for extraction of total cellular proteins	Tris HCl, pH 6.8	15 mM
	Glycerol	2.5%
	SDS	0.5%
	EDTA	1 mM
TED, buffer for extraction of membrane proteins		

	Tris HCl (1M)	
	pH 7.5	5 ml (50mM)
	DTT 15.42 mg	1 mM
	EDTA	1 mM
	Protease inhibitor cocktail	1 tablette for 50 ml
APS	APS	2g
	ddH_2O	20 ml
	stored at -20^0C	
2 x Sample buffer	Tris HCl, pH 8.8	200 mM
	EDTA	5 mM
	SDS	3%
	Bromphenol blue	0.1%
	Sucrose	10%
	β-Mercaptoethanol	1.7%
1 x Running (Laemmli) buffer, 1l	Glycine	14.4 g
	Tris Base	3.03 g
	SDS	1.0 g
	ddH_2O ad 1000ml	
1x Transfer buffer, 1l	Tris Base (25 mM)	3.03 g
	Glycine (192 mM)	14.4 g
	Methanol 20%	200 ml
	ddH_2O ad 1000ml	
Blocking solution	Milk powder, non fat	0.5%
	PBS	1x
	Tween 20	0.05%

Resolving gel

	12.5%	10.0%	8.0%	6.5%
Acryl amide, 30%, ml	2.5	2.0	1.6	1.2
Tris, 1.5 M, pH 8.8, ml	1.5	1.5	1.5	1.5
H_2O, ml	1.92	2.42	2.82	3.22
SDS, 10%, µl	60	60	60	60
TEMED, µl	3	3	3	3
APS, 10%, µl	40	40	40	40

Stacking gel, 5%	Acryl amide, 30%	340 µl
	Tris, 0.5 M, pH 6.8	500 µl
	H_2O	1.12 ml
	SDS, 10%	20 µl
	TEMED	2 µl
	APS, 10%	20 µl
Antibody incubation buffer	Tris 10 mM	0.6 g
	Dry milk powder (5%)	25.0 g
	BSA (2%)	10.0 g
	Tween20 (0.1%)	0.5 g=0.5 ml
	H_2O	ad 500 ml
	pH 7.6 adjusted with HCl oder NaOH, stored at -20^0C	
Wash buffer	Tween20 (0.5 %)	5 ml
	PBS (10 x)	100 ml
	ddH_2O	ad 500 ml
Stripping buffer	Sodium hydroxide	0.2 M

3.1.10 Antibodies

3.1.10.1 Immunofluorescence and immunohistochemistry

3.1.10.1.1 Primary antibodies

Target	Source	Working dilution	Manufacturer
HBV core protein (H800)	rabbit	1:5000	polyclonal antiserum [46]
HNF4α	mouse	1:1000	Clone 6939, Abcam, Cambridge, UK
Pancytokeratin	mouse	1:1000	Clone C-11, Sigma, Deisenhofen, Germany
HBV core protein	rabbit	1:100	DAKO, Glostrup, Denmark

3.1.10.1.2 Secondary antibodies

Alexa Fluor 488 F(ab)2 fragment goat anti mouse IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 568 F(ab)2 fragment goat anti rabbit IgG (H+L)	Invitrogen, Karlsruhe, Germany

Alexa Fluor 594 F(ab)2 fragment goat anti rabbit IgG (H+L)	Invitrogen, Karlsruhe, Germany
Cy™-3-conjugated donkey-anti-mouse IgG (H+L)	Dianova, Hamburg, Germany

3.1.10.2 Western blot

3.1.10.2.1 Primary antibodies

Primary antibodies used for Western blot analysis

Target	Source	Working dilution	Manufacturer
liver specific antigen (LSA)	mouse	1:1000	Clone OCH1E5, DAKO
organic anion transporter polypeptide C (OATP-C)	mouse	1:1000	Clone mMDQ (provided by D. Keppler)
β-actin	mouse	1:4000	Clone AC-15, Sigma
Lamin B	goat	1:2000	polyclonal antiserum, Santa Cruz
hydroxyl-methylglutaryl-CoA-reductase (HMG-CR)	goat	1:400	polyclonal antiserum, Santa Cruz
steroid regulatory element binding protein –2 (SREBP-2)	mouse	1:5000	Clone IgG-1C6, BD Biosciences
cytochrome p450 family member (CYP1A2)	rabbit	1:1000	polyclonal antiserum, BD Biosciences
apolipoprotein B (ApoB)	mouse	1:1000	Clone 13, BD Biosciences
Albumin	rabbit	1:2000	polyclonal antiserum, DAKO
Ferritin	rabbit	1:1000	polyclonal antiserum, Santa Cruz
heme oxygenase-1 (HO-1)	rabbit	1:5000	Stratagene Biotechnologies Inc., San Diego, USA
hepatocyte nuclear factor (HNF)4α	rabbit	all 1:400	polyclonal antisera, Santa Cruz
HNF1	rabbit		
HNF3α	rabbit		
HNF3β	rabbit		
HNF3γ	goat		
CCAAT/enhancer binding protein (C/EBP)α	rabbit		
C/EBPβ	rabbit		
PGC-1α	rabbit		
ARP-1	goat		

Target	Source	Working dilution	Manufacturer
liver receptor homolog – 1 (LRH-1)	mouse	1:400	clone H2325, R&D
HBV Core and GFP	rabbit	1:2000	polyclonal antiserum [228]
HBV core protein (H800)	rabbit	1:10000	polyclonal antiserum [46]
HBV L and M (H863)	rabbit	1:1000	polyclonal antiserum [50]

3.1.10.2.2 Secondary antibodies

Goat anti rabbit, HRP-conjugated	Sigma, Deisenhofen, Germany
Goat anti mouse, HRP-conjugated	Sigma, Deisenhofen, Germany
Rabbit anti goat, HRP-conjugated	Sigma, Deisenhofen, Germany

3.1.10.3 Infection studies

Hepatect®	human hepatitis B immunoglobulin, Biotest Pharma GmbH, Dreieich, Germany

3.1.11 Enzymes

RNaseA 10 mg/ml	Roche Diagnostics, Mannheim, Germany
Proteinase K	Roth, Karlsruhe, Germany
RNAse free DNAse	Qiagen, Hilden, Germany

3.1.12 Kits

3.1.12.1 RNA isolation

RNeasy® total RNA extraction kit Qiagen, Hilden, Germany

3.1.12.2 RT-PCR

LC FastStart DNA Master^{Plus} SYBR Green1 mix	Roche Diagnostics, Mannheim, Germany
Superscript II Reverse Transcriptase	Invitrogen, Carlsbad, USA

3.1.12.3 DNA isolation

DNeasy® Blood and Tissue Kit	Qiagen, Hilden, Germany
QIAamp MinEluate Virus Spin Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction kit	Qiagen, Hilden, Germany

3.1.12.4 DNA labelling

Rediprime DNA Labeling System	Amersham, Buckinghamshire, England

3.1.12.5 Transfection

HiPerFect	Qiagen, Hilden, Germany

3.1.12.6 Western blot detection

ECL Western Blotting Detection Reagents	Amersham, Buckinghamshire, England
WestDura Enhanced Chemiluminescence	Pierce, Rockford, USA

3.1.13 Nucleic acids

3.1.13.1 Vectors

pAdGH1.3

Adenoviral vector for transduction of eukaryotic cells, containing a 1.3-fold HBV overlength genome and a GFP reporter cassette under CMV promoter control [151].

pAdHBV

Adenoviral vector for transduction of eukaryotic cells, containing a 1.3-fold overlength genome of HBV [154].

pAdHBVk/o

Adenoviral vector for transduction of eukaryotic cells that contains a 1.3-fold HBV overlength genome. Stop codons were introduced in all open reading frames so that translation of viral proteins does not occur [154].

3.1.13.2 Plasmids

pCH -9/3091

Contains a 1.3 overlength HBV construct under the control of the CMV promoter (HBV nucleotides 3091-3182, 1-3182, 1-84). It was used to establish an external standard of HBV DNA for LightCycler™ real-time PCR and HBV DNA probe for radioactive hybridization (from R. Bartenschlager; s. also *Nassal et al.* [46]).

3.1.13.3 Oligonucleotides

3.1.13.3.1 SiRNAs (target sequences)

HNF4α	(aacctagagattgttacagaa)
HNF1α	(caggacaagcatggtcccaca)
HNF3γ	(ttgatggatgttattggctaa)
non-silencing control	
labelled with AlexaFluor™ 488	(aattctccgaacgtgtcacgt)
all from	Qiagen, Hilden, Germany
human HO-1	(gagcctggaagacaccctaat)
	Eurogentec Deutschland, Cologne, Germany

3.1.13.3.2 Primers for quantitative real-time PCR

All primers were purchased from Invitrogen.

For gene expression analysis, appropriate exon-exon spanning primer pairs were selected whenever possible. To recognize gene DNA, primers were selected to bind the intron sequences.

Target gene	GenBank accession number	Primer forward	Primer reverse
organic anion transporter polypeptide C (OATP-C)	NM_006446	1939-1954	2038-2021
bile salt exporting pump (BSEP)	NM_003742	3577-3592	3901-3886
2'3'-tryptophan dioxygenase (TDO)	NM_005651	921-936	1130-1114
pterin-4 alpha-carbinolamine dehydratase (PDG)	NM_000281	316-332	519-503
glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	NM_002046	607-623	973-958
delta-aminolevulinic acid synthetase (ALAS)	NM_000688	2007-2026	2192-2177
cytosolic phosphoenolpyruvate carboxykinase (PEPCK)	NM_002591	1626-1643	1840-1824
hepatocyte nuclear factor (HNF) 4α	NM_000457	687-702	962-945
HNF 1α	NM_000545	980-995	1309-1292
HNF 3γ	NM_004497	327-344	571-550
2'5'-oligoadenylate synthetase (2'5' OAS)	NM_016816	377-392	601-585
interferon-gamma inducible protein –10 (IP-10)	NM_001565	145-160	310-294
C-reactive protein (CRP)	NM_000567	1224-1239	1554-1569
human HO-1	NM_002133	700-718	929-911
mitochondrial DNA	*Wieland et. al.* [229]		

mHNF4α	NM_008261	1297-1313	1481- 1496
mHNF1α	NM_009327	1721-1736	1980-1996
mouse peroxisome proliferator-activated receptor gamma coactivator-1α (mPGC-1α)	NM_008904	2141-2156	2329-2346
mouse HO-1	NM_010442	318-338	716-696
mouse GAPDH	NM_001001303	512-527	782-767
hypoxanthine guanine phosphoribosyl transferase 1 (mHPRT) DNA primers	NM_013556	756-776	1013-1032
HBV 3.5 RNA	HBV genotype D, subtype ayw, numbering from core AUG = 1	383-401	705-721
total HBV DNA		1745-1767	1844-1823
HBV ccc DNA		2251-2267	92-71

3.1.14 Ladders

3.1.14.1 DNA standards

SmartLadder, 0.2-10 kb	Eurogentec, Liege, Belgium
SmartLadder, 0.1-2 kb	Eurogentec, Liege, Belgium

3.1.14.2 Protein standards

Prestained Protein ladder	Invitrogen, Karlsruhe, Germany

3.2 Methods

All procedures were performed at room temperature (RT), if not indicated otherwise.

3.2.1 Cell culture

All procedures were carried out under sterile conditions using sterile solutions and equipment. All cells were cultivated in a humidified incubator at 37°C containing 5% CO_2.

3.2.1.1 Calculation of cell number and cell viability

To determine cell numbers, a Neubauer hemacytometer was used. The hemacytometer consists of a glass chamber, which is divided into 4 large B-squares, each consisting of 4 small C-squares. The C-squares are further divided into smaller D-squares, which possess a surface area of 1 mm^2 und a depth of 0.1 mm. Thus, each D-square has a volume of 0.1µl. Therefore, to calculate a cell number in 1 ml of suspension, a multiplication factor of 10^4 must be taken into account. A homogenous cell suspension was filled into the hemacytometer by capillary action. In all 4 large squares, cells were counted and the cell number was calculated as follows:

$$\text{Cell number/ml} = \frac{\text{total cell number x dilution factor x } 10^4}{4}$$

The cell viability was determined with trypan blue.

3.2.1.2 Freezing and thawing of cells

Cell pellets were re-suspended in freezing medium. 1 ml of cell solution with a density of approx. 1 to 3 x 10^6 cells was transferred to one cryo vial. The vials were slowly cooled to -80°C in a freezing container and then stored in liquid nitrogen. To re-cultivate frozen cells, they were quickly thawed and carefully re-suspended in 10 ml cultivation medium. After centrifugation for 5 min at 1200 rpm, the cells were re-suspended in cultivation medium.

3.2.1.3 Primary cells: Primary human hepatocytes

PHH were isolated from surgical liver specimen of patients undergoing partial hepatectomy. The procedure was approved by the local Ethics Committee, with obtained informed consent of the patients.

The protocol is based on collagenase perfusion with an additional pre-perfusion step using Ca^{2+} and Mg^{2+} free medium, followed by differential centrifugation [155].

A large branch of the port vein of a healthy liver tissue piece was canulated, and the canula was fixed with tissue paste (Histoacryl®, Braun, Melsung). The two-step collagenase perfusion started with 500 ml pre-perfusion medium, with a flow rate between 20 and 40 ml/min. At cut surfaces with high medium passage, the large vessels were occluded with tissue paste. The medium was discarded after traversing the liver tissue. After 15 to 20 min, perfusion was continued with 350 ml perfusion medium containing freshly added collagenase type IV (Worthington, Lakewood). Collagenase treatment was performed for 15 to 20 min, depending on liver section size. As soon as the tissue softened and liver cells appeared in the medium, the liver was cut into small pieces and the tissue was scratched off with a scalpel. If collagenase digestion was incomplete, the suspension was transferred to a sterile beaker and stirred for 10 min at 37°C. The cell suspension was filtered through double-layered gauze and a 70 µm cell strainer. After centrifugation in 50 ml Falcon tubes for 5 min at 50 x g at 10°C, the pellet was re-suspended in 40 ml wash medium. The wash step was repeated three times. The cells were re-suspended in PHH medium and the cell number and viability was determined. The cells were seeded on collagen IV-coated dishes at a density of 8×10^5 cells/ml of PHH medium supplemented with 10% FCS. After 3 h, the medium was exchanged to remove non-adherent cells. One day post seeding, the cells were kept in medium containing 5% FCS. From day two post seeding the cells were cultivated in FCS-free medium and used for experiments.

3.2.1.4 Treatment of cells with CoPP

CoPP solution	CoPP	10 mg/ml
	NaOH	0.2 mol/L
	H_2O	10 ml
	adjusted to neutral pH level	

CoPP was diluted in the PHH/DMEM 1:1 culture medium to obtain the final concentration of 10µg/ml, sterile filtered and added to the cells. Cell culture medium containing CoPP was changed every two days in order to achieve continuous induction of HO-1.

3.2.1.5 Transfection of cells with siRNAs

HepG2-H1.3 cells were transfected with indicated siRNAs using the HiPerFect transfection reagent according to the fast forward protocol supplied by the manufacturer. Briefly, $3.5x10^5$ HepG2-H1.3 cells per well were seeded onto collagen IV-coated six-well plates just prior to transfection. SiRNAs were mixed with serum-free Dulbecco's MEM without antibiotics to achieve the final concentration of 5 nM/ml in a well. Then, 12 µl of HiPerFect per well of six-well plate were added to siRNA solution. After 15 min incubation at RT, the solution was added drop wise to the cells. No medium exchange was carried out before harvesting. Transfection efficiency was controlled by fluorescent microscopy using Alexa Fluor 488™ labeled siRNA. Knock-down efficiency was determined by quantitative Western blot analysis (see below).

3.2.1.6 Production of wild type HBV

For the production of wtHBV, the HepG2.2.15 cell line was used. For virus preparation, the cells were cultivated in complete DMEM medium until they were 100% confluent. Then, the medium was exchanged to 50% PHH medium and 50% complete Williams E medium. Every three days, the virus-containing medium was collected. Cell debris was removed by centrifugation at 1000 rpm for 5 min. The supernatant was transferred to centrifugal filter devices (Centricon Plus-70, Biomax 100, Millipore Corp., Billerica) The first centrifugation was performed at 3500 x g for 1 h at 4°C to capture the virus particles in a filter. Due to the exclusion limit of 100 kDa,

serum proteins flow through the filter, while proteins larger than 100 kDa remained in the filter. Then, an invert centrifugation step with the filter system turned upside down was performed at 2600 x g for 10 min to elute the virus. The virus concentrate was supplemented with 10% glycerol and stored at -80°C. The titer of the produced wtHBV was determined by caesium chloride density-gradient, followed by dot blot analysis, as outlined below.

3.2.1.7 HBV infection of cells

Infection of cells with HBV was performed in medium containing 5% PEG 6000 [230]. The cells were incubated over night with HBV at a moi of 100 vp/cell. If indicated, neutralising antibodies (1 IU/10^6 cells; Hepatect, Biotest Pharma, Dreieich) were added simultaneously [155]. After over night inoculation, cells were washed 3 times with PBS. Cells were further cultivated in fresh medium and harvested at indicated time points.

3.2.1.8 Treatment with IL-6

In order to examine the effect of endogenous IL-6 released after contact with HBV, 200 ng/ml of IL-6ab were added to PHH cultures prior to HBV infection.
To study the effect of IL-6 on HBV transcription and replication, 15 ng/ml recombinant IL-6 (rIL-6) were added to HBV infected cells on day 1 and 3 p.i., after the peak amount of endogenous IL-6 in HBV-infected cells had been removed by exchanging the culture medium. As a control, rIL-6 was pre-incubated with anti-IL-6 antibodies (1 µg/ml). Cells and supernatants were analyzed on day 5 p.i.

3.2.1.9 Production and purification of adenoviral vectors

Adenoviruses (Ad) are known to infect a broad range of cells and are therefore widely used as vectors. In this study, the adenoviral vectors of the first generation based on Ad type 5 and produced as described in *Sprinzl et al.* were used [151, 231]. The plasmids were linearised with Pac1 before transfection of 293 cells using the calcium phosphate method. The 293 cells provide the adenoviral E1 genes in *trans*,

which are necessary for virus production [227]. Three days after transfection, the medium, containing dead cells and virions, was collected and subjected to three freeze / thaw cycles in liquid nitrogen and a 37°C water bath. This breaks the cells and sets the intracellular virions free. Following a centrifugation step for 5 min at 2000 rpm to remove the cell debris, the virus containing supernatant was supplemented with 10% glycerol and stored at -80°C. The Ad vectors obtained are suitable for transduction of cell culture, however, these stocks are too diluted and not pure enough for *in vivo* applications.

To establish Ad stocks for *in vivo* applications, 9x175cm^2 flasks with 293 cells were seeded. After they become 80% confluent, cells were transduced with 1-2x10^8 IU of Ad vectors per flask (moi 5-10) and cultivated for 48 hours until all cells showed the cytopathic effect. Then, cells were taken up into suspension and pelleted at 2000 rpm for 5 min, RT. Ad vectors were set free from cells by three freeze / thaw cycles. After centrifugation for 5 min at 4000 rpm, RT, the supernatant containing Ad vectors was kept, and, if not clear enough, centrifuged again.

The purification of Ad vectors was carried out using a CsCl density gradient. 8 ml of CsCl solution with a density of 1.4 g/ml were added to the bottom of SW-28 polyallomer vials and carefully overlayed with 6 ml of CsCl solution with a density of 1.2 g/ml. After CsCl solutions were added, 10 ml of supernatant containing Ad vectors were layered, and the vials were filled up with 8 ml Tris-Cl (10 mM) and mineral oil. The first centrifugation step was performed in an SW 28 rotor at 100.000g for 1.5 hours without the brake. A band containing Ad vectors was taken up, dissolved using 10 mM Tris-HCl solution, and loaded up onto the similar CsCl density gradient. The second centrifugation step was performed under the same conditions. The band containing Ad was taken up. Since CsCl is toxic to cells, it must be removed before the adenoviral stock is used for *in vivo* applications. For this purpose, several dialysis steps were performed in a dialysis chamber with the virus dialysis buffer under agitation. The final dialysis was carried out using a virus storage buffer. Thereafter, the virus was taken up and stored at -80°C.

To determine the titer of the produced Ad vectors, 293 cells were plated on a 12 well cell culture dish. Serial dilutions of the vector stock were added to the confluent cells. The cytopathic effect and/or GFP fluorescence was monitored 48 h after transduction. Cells display a cytopathic effect if they are transduced with 3-5 adenoviral vectors. Since each well contains 10^6 cells, the well with 100% cytopathic

effect yields 3-5x10^6 adenoviral particles. Therefore, to calculate a number of Ad particles per ml, 5x10^6 was multiplied with a dilution factor of viral stock given onto the cells.

3.2.1.10 Transduction of cells with GFP-expressing adenoviral vectors

Prior to transduction of cells with adenoviral vectors, the medium was exchanged. The indicated multiplicity of infection (moi) of the vector was added to the cells to achieve 90-95% green fluorescent cells. For PHH, I used a multiplicity of infection of 5, for HuH7 and HepaRG, 30, and for HepG2 and Pop10 cells, 10 infectious units / cell. After 3 h, the inoculum was removed and fresh medium was added.

3.2.1.11 Caesium chloride (CsCl) density gradient centrifugation

During HBV preparation, different types of viral particles are obtained: naked DNA, unenveloped DNA-containing capsids, and enveloped virions. Their different densities enable their separation using a density gradient. DNA is centrifuged down to the bottom, due to the very small size. DNA-containing capsids sediment at a density of 1.3 g/ml, while intact virions can be found in the 1.22 g/l fraction.
The CsCl (Roth, Karlsruhe) density gradient ultra centrifugation was performed with the SW-60 swing bucket rotor. In SW-60 polyallomer vials (Beranek Laborgeräte) 500 µl of CsCl solutions with the following densities were carefully layered one upon the other: 1.4 g/ml, 1.3 g/ml and 1.15 g/ml. On top of the CsCl solutions, 500 µl of a 20% sucrose solution was layered, and the sample was applied. The vials were filled with PBS and tared on micro scales. Ultra-centrifugation was performed at 55000 rpm at 20°C for 4 h. The density fractions were collected with a Fraction recovery system (Beckman). Each fraction contained 6 drops, which approximates a volume of 175 µl. The fractions were subjected to quantitative dot blot analysis.

3.2.1.12 Determination the half-life of cccDNA

HepG2-H1.3 cells were seeded on collagen IV-coated dishes and cultivated until confluent. After changing the medium to PHH / DMEM 1:1, cells were cultivated for additional 10 days to establish the pool of cccDNA in the nucleus. Thereafter, the first treatment with either lamivudin (15 µM) or CoPP (10µg/ml) or both was performed. Every two days during the 10 day study, the treatment was repeated and cells were harvested for isolation of total cellular DNA. Amounts of cccDNA were determined relative to mitochondrial DNA using real-time PCR.

3.2.2 Molecular biological methods

3.2.2.1 Calculation of RNA or DNA concentrations

To calculate the concentration of a RNA or DNA preparation, the absorption at 260 nm and 280 nm was determined with a photometer (Smart Spec 3000, BIO-RAD, Hercules). This technique relies on the characteristic of nucleic acids to absorb UV light with a wave length of 260 nm. Absorption at 260 nm (OD_{260}) of one equals a DNA concentration of 50 µg/ml or an RNA concentration of 40 µg/ml. The ratio of the absorption at 260 nm and 280 nm gives an estimation of the purity of the RNA or DNA and should range between 1.8 and 2.0. The absorption was always normalised to ddH_2O.

3.2.2.2 Gel electrophoresis

DNA molecules are negatively charged, with the charge being proportional to the molecular weight. Therefore they can be separated according to their size in an electric field.

Electrophoresis was performed in 0.8 to 2% horizontal agarose gels. The agarose (Cambrex Bioscience, Rockland) was dissolved in 1 x TAE or TBE buffer and boiled. Ethidium bromide was added to a final concentration of 0.5 µg/ml after the agarose was cooled. Ethidium bromide intercalates into dsDNA. This results in a complex that fluoresces when exposed to UV light (254 nm to 366 nm). Emission of 590 nm light allows for the visualisation of the DNA, with a detection limit of approximately 20 ng dsDNA. The polymerised gel was covered with TAE or TBE buffer. The samples

were mixed with 1x DNA sample buffer to provide ballast to the DNA with glycerol and to mark the separation front with bromphenol blue. In addition to the samples, a DNA standard with defined DNA sizes was loaded on the gel. Electrophoresis was performed with a constant voltage of 50 to 120 V.

3.2.2.3 Phenol/Chloroform extraction of DNA

This method was used to isolate total DNA from cells for subsequent Southern blot analysis. First, to destroy DNA-protein complexes, cells were lysed using 1ml proteinase K buffer, and lysates were digested with 100µl (1µg/ml) proteinase K at 56^0C over night. In order to avoid RNA contamination, samples were then digested with RNAse A (10µg/ml) for 1 hour at 37^0C. During subsequent steps, samples were purified from proteins. First, samples were mixed with equal volumes of phenol and centrifuged at 3.500 g for 20 min. Second, the upper aqueous phase, which contains DNA, was transferred into a clean tube, mixed with an equal volume of phenol:chloroform (1:1) and centrifuged at 3.500 g for 20 min. Third, the aqueous phase was taken up into a clean tube, mixed with chloroform: isoamylalcohol (24:1) and centrifuged at at 3.500 g for 20 min. For DNA precipitation, the aqueous phase was transferred into a clean tube, mixed with 10% of the volume of 3M Na+ acetate, pH 5.0, and a 2.5-fold volume of 100% ethanol. Samples were then incubated over night at -20^0C. Thereafter, DNA was pelleted by centrifugation at 17000 g for 30 min at 4°C and washed using 70% ethanol. Purified DNA pellets were air dried for 5-10 minutes, re-suspended in DNAse-, RNAse-free water and stored at -20^0C.

3.2.2.4 DNA isolation with DNeasy Blood and tissue kit

High-purity DNA for real-time PCR was isolated from cells or mouse liver tissue using a DNeasy Blood and tissue kit according to the manufactor's instructions. The method allows for rapid purification of total DNA (genomic, mitochondrial and pathogen) and is based on the ability of DNA to selectively bind to silica-based membrane in the presence of high concentrations of chaotropic salts. Samples were lysed with proteinase K over night under vortexing. Addition of lysis buffers set DNA free from proteins and optimized DNA binding to the silica-based membrane. During centrifugation, DNA bound to the silica-based membrane as contaminants passed

through. Remaining contaminants and enzyme inhibitors were removed in two wash steps and DNA was then eluted with DNAse-, RNAse- free water.

3.2.2.5 DNA isolation from sera or supernatants

High-purity DNA from sera or cell culture media was isolated using a QIAamp MinElute Virus Spin kit. The method is based on the ability of DNA to selectively bind to a silica-gel-based membrane in the presence of high concentrations of chaotropic salt. Since the spin procedure is established for use with a starting volume of 200µl, the volume of all samples was adjusted to 200µl using 1xPBS. Samples were lysed under highly denaturating conditions at elevated temperatures. Optimal binding of DNA to the silica-based membrane was achieved by addition of an RNA-carrier and ethanol to the sample. During the centrifugation step, nucleic acid bound to the membrane as the lysate passed through. Salt and pH conditions ensured that protein and other contaminants were not retained on the QIAamp MinElute membrane. All contaminants were washed away during 3 wash steps. Purified DNA was then eluted with DNAse-, RNAse- free water equilibrated to RT.

3.2.2.6 Production of ^{32}P-probe

The pCH-9/3091 HBV construct was digested with *Nhe I* and *Sal I* and digestion products were loaded onto a 0.8% gel. 3054 bp long DNA fragment was purified using a QIAquick Gel Extraction kit. This fragment was amplified and radioactively labeled using the Rediprime DNA Labeling System. This kit contains oligonucleotides (random primers), nucleotides, polymerase and buffer. First, the HBV DNA probe was dissolved in 45µl TE-buffer and boiled for 10 min at 100°C in a water bath for DNA denaturation. Second, the denatured HBV DNA probe was added to a Rediprime reaction kit tube, and 5 µl radioactive [^{32}P] dCTP were added. For HBV DNA probe amplification and radioactive labeling, the reaction mix was incubated at 37°C for 30 min. Finally, the HBV DNA probe was purified a using purification column (Stratagene) and 1xSTE-buffer. For hybridization, the HBV DNA probe was dissolved in 25ml church buffer containing 10µg/ml salmon sperm DNA, boiled for 10 min at

100°C in a water bath to denature the HBV DNA probe, cooled and put on the membrane.

3.2.2.7 Southern blot analysis

Southern blot analysis was used to study the synthesis of HBV replicative intermediates.

15 µg of total cellular DNA were cut with 150 units of *Hind III* (10 enzyme units per 1 µg DNA) for 16-18 hours in order to excise the HBV integrate. Subsequently, DNA fragments were separated by electrophoresis using 0.8% agarose gel. For DNA depurination, the gel was incubated with 0.25 M HCl for up to 25 minutes until the bromophenol blue marker turned yellow. Then, to denature the DNA, the gel was incubated with 0.4 M NaOH. For transfer of the DNA fragments from the gel onto positively charged nylon membrane, a downward capillary transfer procedure was performed (Fig. 6). It is rapid and the intensity of signal is 30% greater than can be achieved by conventional upward transfer. The Whatman papers and positively charged nylon membrane were pre-wet with 0.4 M NaOH and ddH_2O, respectively, and the transfer tower was set up without air bubbles as shown on the picture below. The transfer was performed over night (16 hours) using a 0.4 M NaOH solution as the transfer buffer. The alkaline transfer buffer is drawn from reservoirs on the top of the gel through wicks and sucked through the gel by an underlying stack of paper towels. DNA fragments are thus carried through in a downward direction with the flow of buffer and are deposited onto the surface of a positively charged nylon membrane. After the transfer finished, the membrane was removed, neutralized for 5 minutes with 2x SSC and air dried. Further, DNA was twice cross linked to the membrane by UV irradiation at 254 nm and 125 kJ. The membrane was pre-hybridised using 0.1 ml of pre-hybridisation solution per cm² for 2 hours at 65^0C and then hybridised with a ^{32}P-labeled HBV DNA probe [151] over night at 65 °C in the hybridisation oven. Thereafter, the membrane was washed twice with 2xSSC for 5 min at 65 °C and twice with 2xSSC containing 0.1 % SDS for 15 min at 65 °C. Autoradiography was performed using a phosphor-screen for 24 hours and HBV replicative intermediates were quantified relative to the HBV integrates using autoradiography software.

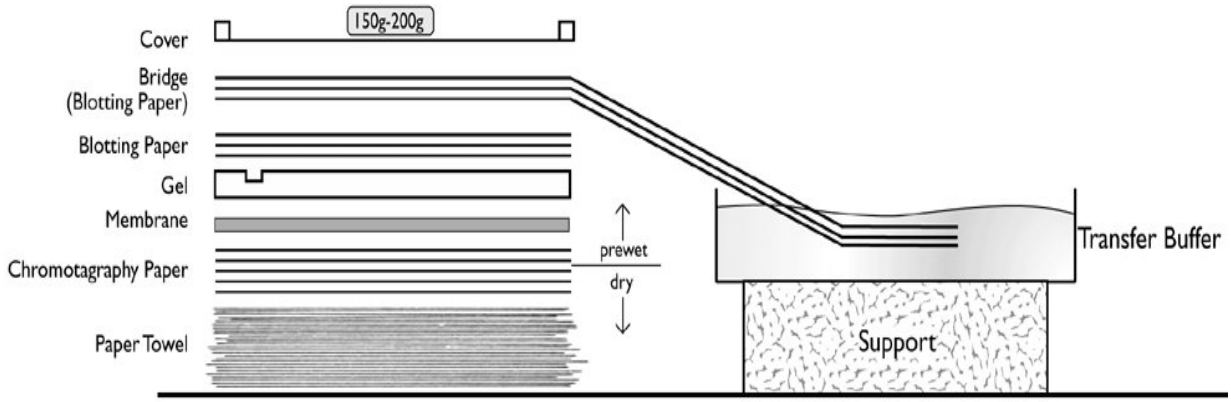


Fig. 6: Setup of downward capillary transfer
Adoped from www.ambion.com

3.2.2.8 Dot blot analysis

For quantitative and qualitative analysis of the produced wtHBV or secreted HBV particles, a DNA dot blot was performed. The DNA fractions, obtained by CsCl density centrifugation, were dotted to a nylon membrane (Roche Diagnostics, Mannheim) in a dot blot aperture (Schleicher & Schuell, Dassel). A HBV plasmid standard ranging from 8 pg to 1000 pg was added. The dotted samples and standards were washed with 200 µl PBS before the membrane was transferred to a 3 mm Whatman paper soaked with denaturation solution. Subsequently, the membrane was transferred to a 3 mm Whatman paper soaked with neutralisation solution. Then, the DNA was cross linked to the membrane by UV irradiation at 254 nm and 125 kJ. The membrane was hybridised with a ^{32}P-labelled HBV DNA probe at 65°C over night. After hybridization, the membrane was washed and autoradiography was performed (see Southern blot analysis).

3.2.2.9 RNA isolation using TRIzol reagent

A TRIzol reagent was used to isolate total RNA for Northern blot analysis.
The TRIzol reagent, a mono-phasic solution of phenol and guanidine-isothiocyanate, efficiently disrupts cells and dissolves cell components while maintaining the integrity of RNA. 1.3x10^6 cells were homogenized with 1 ml TRIzol reagent for 5min. 200µl chloroform were mixed with each sample, incubated for 2 min and centrifuged at 12.000xg for 15 min at 4^{0}C. The upper RNA-containing aqueous phase was taken up

and RNA was precipitated using 500µl of isopropyl alcohol at -20^{0}C for 1 hour. RNA was pelleted by centrifugation for 10 min at 12.000g and at 4^{0}C and washed using 75% ethanol (75% ethanol + 25% DEPC-water). The RNA pellet was air dried for 5 min and dissolved in DNAse-, RNAse- free water.

3.2.2.10 RNA isolation using RNeasy Mini Kit

To purify RNA for cDNA synthesis, an RNeasy Mini Kit was used. This method allows for purification of RNA molecules longer than 200 nucleotides. The procedure provides an enrichment for mRNA since most RNAs < 200 nucleotides (5.8S rRNA, 5S rRNA and tRNAs) are selectively excluded. The principle is based on the ability of RNA to selectively bind to a silica-gel-based membrane in the presence of high concentrations of a chaotropic salt.
First, samples were lysed and homogenised in the presence of a highly denaturing guanidine-thiocyanate- and β-mercaptoethanol-containing buffer. Guanidine-thiocyanate and β-mercaptoethanol immediately inactivate RNAses and thereby ensure the purification of intact RNA. After sample lysis, ethanol was added to provide appropriate binding conditions. Samples were then applied to an RNeasy Mini spin column where the total RNA bound to the membrane, but contaminants were washed away. To avoid the contamination of purified RNA with DNA, DNAse digestion was performed on the column. After subsequent wash steps, high-quality RNA was eluted with DNAse-, RNAse- free water.

3.2.2.11 Northern blot analysis

Norhern blot analysis was performed to study the expression of HBV RNAs.
15µg of total cellular RNA were separated by size via electrophoresis in an vertical 1.5% agarose gel (1.5g agarose + 105 ml DEPC-H_2O+15 ml 10x E-buffer) under denaturing conditions (30 ml formaldehyde) and constant voltage (60 – 90V). To transfer RNA from the gel onto a positively charged nylon membrane, the downward capillary transfer was performed over night (16 hours) with 10xSSC as transfer buffer. Thereafter, the membrane was rinsed with 2xSSC and the transferred RNA was twice cross linked to the positively charged nylon membrane by UV irradiation at

254 nm and 125 kJ. The membrane was pre-hybridised using 0.1 ml of pre-hybridisation solution per cm² for 2 hours at 65^0C and then hybridised with a ^{32}P-labeled HBV DNA probe [151] or ^{32}P-labeled GAPDH DNA probe over night at 65 °C in the hybridisation oven. The membrane was washed and autoradiography was performed (see Southern blot analysis). HBV RNAs were quantified relative to GAPDH using autoradiography software.

3.2.2.12 Reverse Transcription

Reverse Transcription (RT reaction or first strand cDNA synthesis) is a process of complementary DNA (cDNA) synthesis by a reverse transcriptase (RTase) enzyme using single-stranded RNA as a template. In this study, SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen), including RTase Enzyme Mix, 2xRT Reaction Mix and RNase H was used. RTase Enzyme Mix is a version of MMLV RTase with reduced RNAse H activity and increased thermal stability. 2xRT Reaction Mix contains RNAseOUT™ (RNase inhibitor), oligo(dT)20, random hexamers, MgCl2, and dNTPs in a buffer formation. The reaction was performed as followed:

Reagent	Vol.
2xRT Reaction Mix	10 µl
RT Enzyme Mix	2 µl
RNA (1µg)	x µl
DEPC-treated water	to 20 µl

The reaction mix was incubated at 25°C for 10 min to allow primers to anneal to the RNA. Then, cDNA synthesis was performed at 50°C for 30 min. To inactivate the enzyme, samples were heated for 5 min at 85°C. To remove the RNA template from the cDNA:RNA hybrid molecules, 1µl (2 U) of RNAse H was added and samples were incubated at 37°C for 20 min. cDNA was stored at -20°C.

3.2.2.13 Real-time polymerase chain reaction

PCR is a method used to amplify specific DNA sequences located between two primers complementary to the 5` and 3` ends of the sequence. Real-time PCR detection techniques make a kinetic quantification of amplified sequence possible.
The Light Cycler© instrument from Roche Diagnostics, Mannheim, is a thermocycler combined with a fluormeter. For fluorescence detection, the SYBR Green I Dye was used. This fluorescent dye intercalates only in dsDNA. The fluorescence intensity of this DNA-SYBR green complex is much higher than the intensity of the dye alone. The signal intensity is directly proportional to the DNA amount and, therefore, the highest at the end of the elongation phase. The fluorescence intensity is measured at the end of each elongation phase, at a certain temperature, and in a single optical unit. The quantification occurs in the log-linear phase of constant amplification since only in this phase the dependence between the amounts of PCR end product and starting material is linear. The cycle number, at which the increase in fluorescence is exponential, is measured and called the crossing point.
Different quantification methods are available with the LightCycler© System. Absolute quantification is performed with an external standard curve of known concentrations of the target DNA. The relative quantification expresses the target gene concentration in relation to a reference gene, usually a house keeping gene. The efficiency of amplification reactions of target and reference genes is normalized using a dilution series of calibrator DNA or cDNA. Standard curves of both target and reference gene are used to obtain the concentrations.
The identification of specific DNA products is possible using melting curve profiles. The melting temperature of dsDNA depends on length, sequence, and GC content. Thus, every sequence has a specific melting curve profile. Within the PCR reaction, the melting curve is obtained by steadily increasing temperatures while the fluorescence is monitored.
The LightCycler PCR is very sensitive with a detection limit of one to ten copies for plasmid DNA per sample. For genomic DNA the detection of a single-copy gene in 3 pg DNA is estimated.
This sensitive PCR technique was used to detect low amounts of HBV rcDNA and cccDNA, to clearly distinguish between the two DNA forms, for precisely quantify the

expression of the HBV pgRNA as well as of differentiation markers and transcription factors.

The reaction mix for real-time PCR contains the Taq-DNA-polymerase, reaction buffer, $MgCl_2$ and dNTPs. The following master-mix was prepared for each reaction:

Reagent	Vol.
LightCycler FastStart DNA Masterplus SYBR Green I	4 µl
Primer fw	1 µl
Primer rev	1 µl
ddH_2O	12 µl

2 µl of template DNA or ddH_2O were added to the mix. The LightCycler instrument was programmed as shown:

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	10 sec
4. Elongation	72°C	30 sec
5. Detection	at the end of the elongation phase	

For some target genes the detection temperature and time were changed.

Human oligoadenylate synthetase

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	10 sec
4. Elongation	72°C	30 sec
5. Detection	87°C	1 sec

HBV pgRNA

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	5 sec
4. Elongation	72°C	20 sec
5. Detection	83°C	2 sec

HBV rcDNA

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	5 sec
4. Elongation	72°C	20 sec
5. Detection	at the end of the elongation phase	

HBV cccDNA

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	5 sec
4. Elongation	72°C	45 sec
5. Detection	88°C	2 sec

mito DNA

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	5 sec
4. Elongation	72°C	30 sec
5. Detection	at the end of the elongation phase	

3.2.3 Protein analysis

3.2.3.1 Protein isolation

3.2.3.1.1 Isolation of total cellular proteins

For extraction of total cellular proteins, cells were lysed with CHAPS or SDS- buffer and incubated for two hours at room temperature. For total protein extraction from the human tissue, human tissue pieces of approximately 25 mg were chopped up using plastic chops and liquid nitrogen. Then, chopped tissue was homogenized by passaging 10 times through a 24 gauge needle and incubated with SDS- buffer for two hours on ice. To discard cell or tissue debris, samples were centrifuged at 13.000 rpm for 30 min at $+4^0$C. The supernatant containing total proteins was kept and the pellet containing cell or tissue debris was discarded. Isolated proteins were stored at -20^0C. Prior to the Western blot analysis, samples were mixed with the Western blot probe buffer, boiled at 95^0C for 5 min under reducing conditions for denaturation of secondary and tertiary structures and loaded onto SDS-page.

3.2.3.1.2 Isolation of membrane proteins

Cells were homogenized in TED buffer, and cell lysates were centrifuged for 15 min at 500g and 4^0C for isolation of total proteins. One volume of supernatant was mixed with one volume of KCl (1M) and incubated for 15 min at RT. Then, samples were centrifuged for 45 min at 100.000g and 4^0C. Pellets, which contained membrane proteins, were re-suspended in 150 µl TED buffer and stored at -80^0C.

3.2.3.2 Calculation of protein concentrations

To determine total protein concentrations, the BCA™ Protein Assay Kit (Pierce, Rockford) was used. This assay is based on the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium, combined with the colorimetric detection of Cu^{+} with a reagent containing bicinchonic acid (BCA). The complex formed by the chelation of 2 BCA molecules with one Cu^{+} molecule exhibits a strong absorbance at 562 nm. The absorbance is linear with increasing protein concentrations over a working range of 20 to 2000 µg/ml. The reaction was performed according to the manufacturers'

instructions, and dilution series of bovine serum albumin served as the protein standard.

3.2.3.3 SDS-page gel electrophoresis

15 µg or 30µg or 80µg of total cellular or membrane proteins from cells, human or mouse tissue, respectively, were mixed with probe buffer containing β-mercaptoethanol and boiled for 5 min at 95°C. Samples were loaded onto a 6.5-12.5% SDS polyacrylamid gel according to the expected protein size. As a standard for protein size a pre-stained marker (Fermentas) was added. The proteins were electrophoresed using a blot chamber (MiniProtean®3 Cell, BIO-RAD, Hercules) with constant voltage (100V per gel).

3.2.3.4 Western blot

The proteins were transferred from the SDS gel onto a nitrocellulose membrane (Amersham, Buckinghamshire) using a semi-dry transfer cell (BIO-RAD, Hercules). The amperage was calculated with the following formula: 1.2 mA/cm² of the gel for a 1 h transfer.

After protein transfer, the membrane was blocked in blocking solution for 1 h and probed for 1.5 h at RT or over night at 4°C with the indicated antibody. After three washing steps of 10 min each, the secondary antibody was added in a 1: 2000 dilution for 1h. The detection was performed with the WestDura or WestFemto enhanced chemiluminescence detection kit (Amersham, Buckinghamshire) using the Gel Doc 2000 System (BioRad Laboratories, Munich, Germany).

If re-probing of the membrane with another antibody was necessary, the membrane was stripped with 0.2 M NaOH for 10 min, rinsed twice for 5 min with ddH_2O, blocked using blocking solution and then incubated with the appropriate primary and secondary antibody.

3.2.3.5 Immuno-fluorescence staining

HepG2-H1.3 cells were plated onto collagen IV-coated 8-well glass slides and transfected with either siRNA against HNF4α or with non-silencing control siRNA. 5 or 7 days post transfection, cells were washed 3 times with PBS and fixed in 3.7% PFA for 10 min. Immediately after fixation, cells were incubated with 50 mM ammonium acetate to saturate the free PFA residues. Following three washes with PBS, cells were permeabilised with 0.5% saponin/PBS for 10 min. Unspecific antibody binding sites were blocked with 10% serum in 0.1% saponin/PBS for 1 h. The primary antibody was added over night. Three washing steps with 0.1% saponin/PBS for 10 min each followed, before the cells were incubated for 1 h with the secondary antibody, diluted 1 : 2000 and supplemented with Dapi (0.5 µg/ml). The cells were washed 3 times with 0.1% saponin/PBS. All incubation and washing steps were performed on a slowly rocking platform. After the staining procedure, the 8-well chambers were removed from the slide, and cells were mounted with Mowiol/Dabco. A cover slip was sealed on the slide with nail polish. The slides were stored at 4°C. Immunofluorescence analysis was performed on an Olympus FluoView1000 confocal microscope (Olympus, Hamburg).
For staining of cytospins, cytospins were first air dried for 30 min at RT and subsequently fixed with a mix of acetone and methanol 1:1. Second, cytospins were re-hydrated using 1xPBS for 10 min at RT. Finally, after blocking for 60 min at RT, the cytospins were incubated with primary antibody over night at 4°C, and after two wash steps, with secondary antibody for one hour at RT. The stained cytospins were mounted and stored at -20°C. Immunofluorescence analysis was performed on an Olympus IX81 microscope (Olympus, Hamburg).

3.2.3.6 Immunohistochemistry

Liver tissues were formalin fixed for 24 h, embedded in paraffin and sectioned. Three µm sections were stained with hematoxylin/eosin using a standard protocol and liver histology was analyzed by light microscopy. Immunohistochemical detection of HBV core antigen was carried out using a polyclonal rabbit anti-HBcAg antibody. Tissue

embedding, staining with hematoxylin/eosin and immunohistochemistry were performed by the Institute of Pathology, University Hospital Cologne.

3.2.4 Animals

For the study of HO-1 influence on HBV replication, C57Bl6 mice were used.
Adenoviral vectors encoding a 1.3-fold overlength HBV genome genotype D (AdHBV) or an HBV-genome with all knocked out open reading frames (AdHBVk/o) were diluted with 1xPBS to obtain 10^9 infectious units per inoculum (200 µl) and injected intravenously into the tail vein.
CoPP (10 mg/kg body weight) was dissolved in 0.2 mol/L NaOH, adjusted to neutral pH level, and administered intraperitoneally in a final volume of 200µl/20g of mouse.
For the study of onset of HBV replication during the liver development and hepatocyte differentiation, HBV 1.3 and HBV xfs transgenic mice were used.
Breeding pairs were selected according to the high serum levels of HBeAg, (15 ng/ml) indicating efficient HBV replication (measurement of HBeAg was performed by the Institute of Virology, University of Cologne). Mice were killed using a CO_2-chamber at ED 12.5, 15.5, 18.5, day 0.5, 2; week 1, 2 and 4 postnatally.
Mouse blood was taken up by heart puncture and the liver was removed. Liver tissue was cut into pieces and distributed for different purposes. To obtain paraffin slices, liver pieces were fixed with formalin for 24 hours at 4^0C and, upon rinsing with 1xPBS, paraffin-embedded. For RNA, DNA or protein isolation, liver pieces were conserved with 600 µl RNAlater, incubated at $+4^0$C over night and stored at -20^0C.

3.2.4.1 Analysis of liver injury

Liver injury in mice was determined by measurement of serum ALT activity using specific bioreaction strips on a Reflovet® Plus reader (Roche Diagnostics, Mannheim, Germany). The measurement of GPT is based on the following reactions:

$$\alpha\text{-ketoglutarate} + \text{alanine} \xrightarrow{\text{GPT}} \text{glutamate} + \text{pyruvate}$$

$$\text{pyruvate} + PO_4^{3-} + O_2 + H_2O \xrightarrow{\text{PyOD}} \text{acetyl phosphate} + H_2O_2 + CO_2$$

$$\text{indicator (red.)} + H_2O_2 \xrightarrow{\text{POD}} \text{indicator (ox.)} + H_2O$$

The change in indicator colour was detected by a photodiode element.

3.2.4.2 Cytospins

The abdominal cavity of pregnant mice was opened and foetuses were removed from the uterus and placed in the modified HBSS solution. Livers of embryos were isolated using 40 gauge needles, scalpel and light microscope, homogenized in the modified HBSS+EGTA solution and centrifuged for 5 min at 400g and 4^0C. To perform single cell suspensions from adult mouse livers, mice were killed and the abdominal cavity was opened. The mouse liver was perfused using portal vein with pre-warmed calcium-free wash medium for 10 min, 80-100 ml, 5-9 ml/min and subsequently with collagenase medium for 10 min, 80-100 ml, 10-15 ml/min (for medium contents see PHH isolation). Softened mouse livers were taken out of the abdominal cavity. Hepatocytes were gently removed from of the liver capsule using forceps and a scalpel, homogenized in the modified HBSS+EGTA solution and filtered through a 70µm cell strainer. The cell suspension was centrifuged for 5 min at 400g and 4^0C.
Pellets were washed with the modified HBSS+EGTA solution, re-suspended in 10% FCS-DMEM and filtered using a 40µm cell strainer into a 50 ml falcon tube. Cells were washed with 10% FCS-DMEM. The cell number was counted and then adjusted to 1-2x10^7/ml. 100 µl of cell suspension were put into a cytospin camera and centrifuged at 1100 rpm for 2 min at RT. Then, cytospins were air dried for 1 hour and stored at –20^0C.

3.2.5 Statistical analysis

The results were analyzed using Student's t test, Kruskal-Wallis test and Pearson/Spearman correlation. All data are expressed as a mean ± standard deviation. A p value of 0.05 or less was considered significant.

4 Results

4.1 Hepatocyte differentiation and replicative potential of HBV

4.1.1 HBV replication starts after birth

Although the HBV prophylaxis for newborns from HBV-positive mothers is available and has proven to be efficient, the rate of perinatal HBV transmission is still high, especially in developing countries. Here, intrauterinal transmission of the virus plays an important role. A prophylaxis for intrauterinal transmission of HBV has not been established so far. Recently, HBV has been shown to be able to cross the placenta and remain infectious. However, it is not known whether HBV can infect hepatocyte precursors and replicate in the developing liver.

I aimed to analyze the starting point of HBV replication in the developing liver, changes in HBV replication efficiency during liver development, and the hepatocellular factors that are responsible.

To analyze the starting point of HBV replication, I took advantage of HBV-transgenic mice that replicate the virus from a 1.3-fold overlength of HBV genome integrated in the mouse chromosomal DNA. Thus, I could investigate HBV replication irrespective of the early steps of the viral replication cycle. I selected breeding pairs based on high concentrations of HBeAg (>15 ng/ml), which indicated efficient HBV replication. Markers of HBV replication, such as HBV pgRNA, core and L proteins, and intracellular HBV DNA were studied in fetal livers on embryonic day (ED) 12.5, 15.5, 18.5, day 0.5, 2 (dpn) and week 1, 2 and 4 (wpn) postnatally and compared with those in corresponding adult mice.

4.1.1.1 Synthesis of HBV DNA during liver development

Since HBV replication is defined by new synthesis of viral DNA, the starting point of HBV replication was analyzed with sensitive qPCR using a Relative Quantification Software™ algorithm (Roche Diagnostics) with a detection limit of 0.2 HBV DNA copies per 10^3 cells as determined previously [232]. In order to distinguish newly synthesized HBV DNA from integrates of HBV genome present in all cells of the transgenic mice, the highest levels of HBV DNA in tails of homozygous adult mice were taken as baseline. HBV DNA levels in liver samples of homozygous animals at ED 15.5 and ED 18.5 were equal to baseline and double that of heterozygous

animals at ED 15.5, which clearly indicates that only integrated HBV genome was detected (Figure 7). While at 0.5 dpn, intracellular HBV DNA levels remained almost at baseline, levels of HBV DNA slightly increased in 2 / 5 animals at 2 dpn and significantly increased at 1 wpn in 3 / 5 animals (Kruskal-Wallis, $p \leq 0.05$) (Figure 7). After 1 wpn, HBV DNA levels increased rapidly and achieved levels seen in adult mice between 2 and 4 wpn (Figure 7). However, at all stages of postnatal liver development, HBV DNA levels showed high variations among individual animals with some transgenic mice having very low and others having rather high HBV DNA levels.

Interestingly, kinetics of HBV replication in HBV 1.3 xfs mice, which carry an X-deficient HBV genome, were comparable to those in HBV 1.3 mice, but generally higher at corresponding time points (Figure 7). To achieve maximal sensitivity, HBV 1.3 xfs mice were used for most of my studies, while HBV 1.3 mice were used to control for an influence of the lacking expression of HBV X-protein.

From these analyses, I concluded that even if all hepatocytes carry the HBV genome, HBV replication does not start before birth. I determined that replication of the virus starts within the first week after birth.

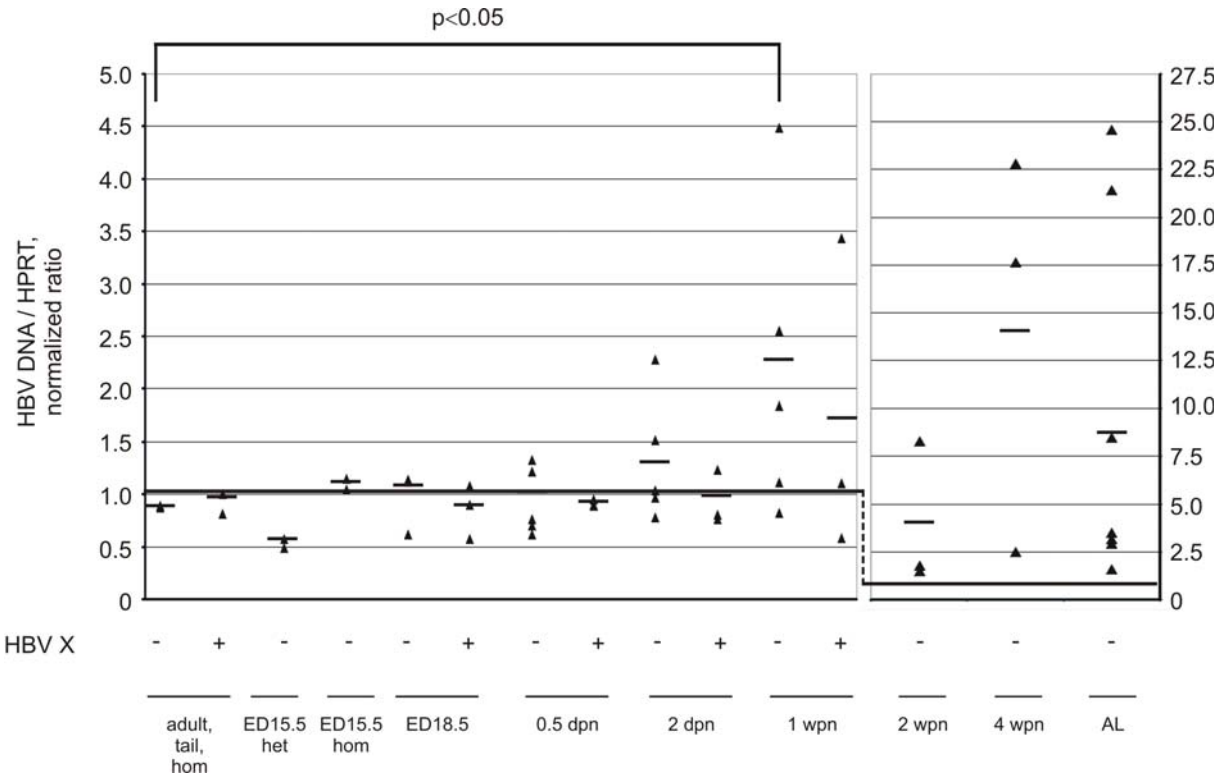


Fig. 7: Synthesis of HBV DNA during liver development

Total DNA from livers and tails of adult HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-), was isolated and compared to fetal liver DNA obtained at ED 15.5 and ED 18.5 and to newborn liver DNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. HBV DNA was quantified by LightCycler™ real time PCR relative to the house keeping gene HPRT and normalized to a dilution series of a calibrator DNA. Maximal levels of HBV DNA determined in tails of adult transgenic animals were set to 1 and used as baseline. Levels of HBV DNA from individual samples and mean values are given. At ED 15.5, heterozygous (het) and homozygous (hom) fetuses were analyzed.

4.1.1.2 Transcription of HBV pgRNA during liver development

To analyze why HBV replication started late during liver development, expression levels of HBV pgRNA, the template for reverse transcription and translation of HBV core and polymerase proteins, were determined by qPCR (Figure 8A&B). HBV pgRNA was barely detectable at ED 12.5 with levels at only 1.4 % of those found in the corresponding maternal adult liver (Figure 8A). Along with fetal and early postnatal liver development, amounts of HBV pgRNA increased continuously, although it still was significantly lower than in the AL until 1 wpn: 5.8%, 19.4%, 23% and 45.1% on ED15.5, ED 18.5, 0.5 dpn ($p \leq 0.01$) and 2 dpn ($p \leq 0.05$), respectively (Fig. 8A and B).

Interestingly, in HBV 1.3 mice, the dynamics of HBV pgRNA was comparable, but levels of HBV pgRNA were generally lower than in HBV 1.3 xfs mice at corresponding time points (Figure 8B). Irrespective of the mouse strain, levels of HBV DNA correlated closely with those of HBV pgRNA in individual animals (Spearman correlation: 0.78, $p \leq 0.05$).

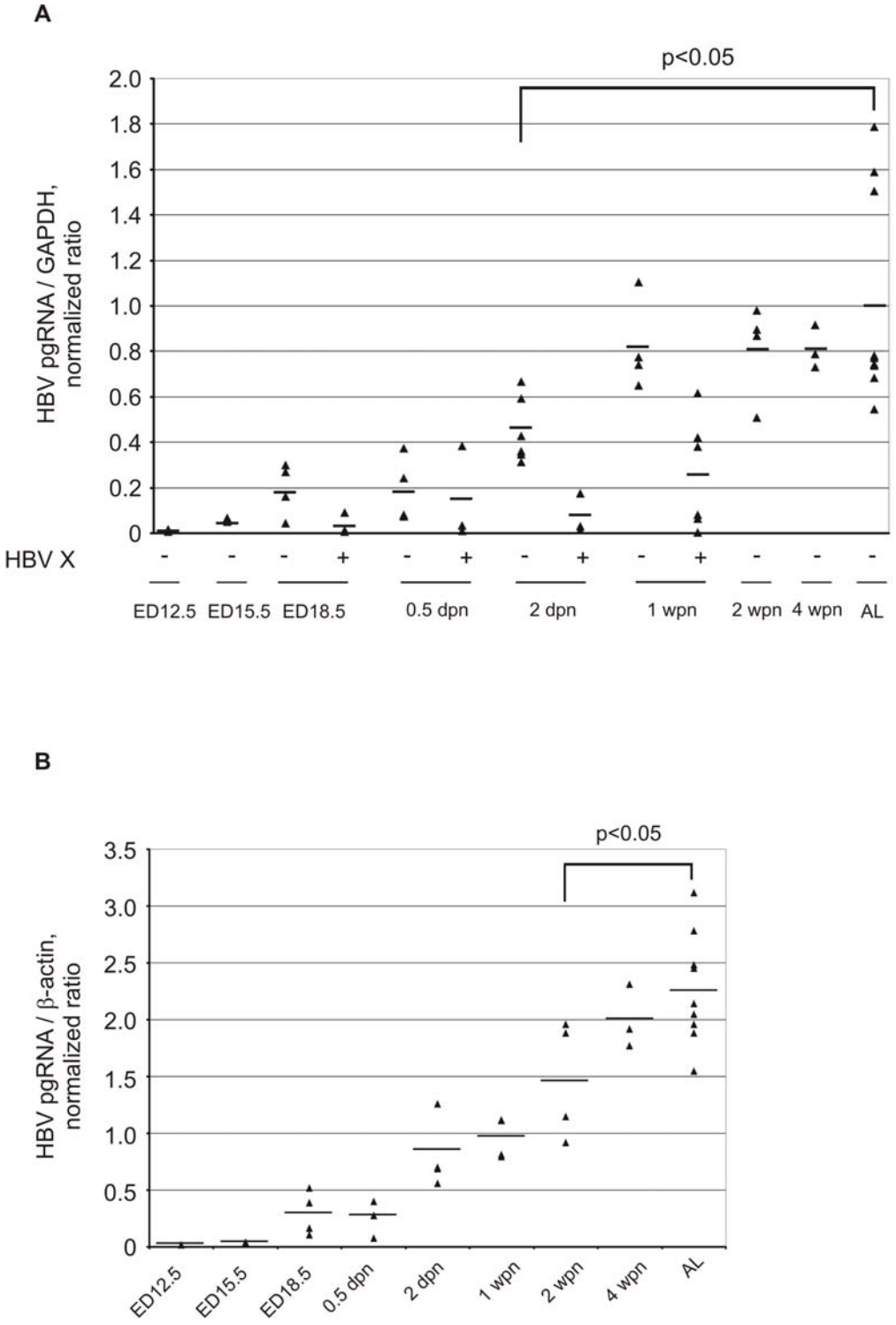


Fig. 8: Expression of HBV pgRNA during liver development

Total RNA from livers of adult HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-), which carry an X-deficient replication competent HBV genome, was isolated and compared to fetal liver RNA obtained at ED 12.5, ED 15.5 and ED 18.5 and to newborn liver RNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. Expression of HBV pgRNA was quantified relative to GAPDH ***(A)*** *or to β-actin* ***(B)*** *by LightCycler™ real-time PCR and normalized to a dilution series of calibrator cDNA using the Relative Quantification Software.*

4.1.1.3 HBV protein expression during liver development

HBV core protein builds the HBV nucleocapsid and is a prerequisite for HBV DNA replication. While HBV pgRNA was detected at very low level from ED 12.5 onwards, expression of HBV core protein was first detected by Western blot analysis at ED 18.5 (Figure 9A, B&C). At this time point, amounts of HBV core protein composed only 0.53% of those in AL. Levels of HBV core protein increased continuously during postnatal liver development (Figure 9A) irrespective of the presence of HBV X protein (Figure 9C), but did not reach levels detected in adult livers even at 4 wpn. HBV core protein comprised 2.2%, 3.3%, 28.4%, 32.5% and 37.8% of those in AL on 0.5 dpn, 2 dpn, 1, 2 and 4 weeks postnatally, respectively, as measured by Western blotting (Figure 9B).

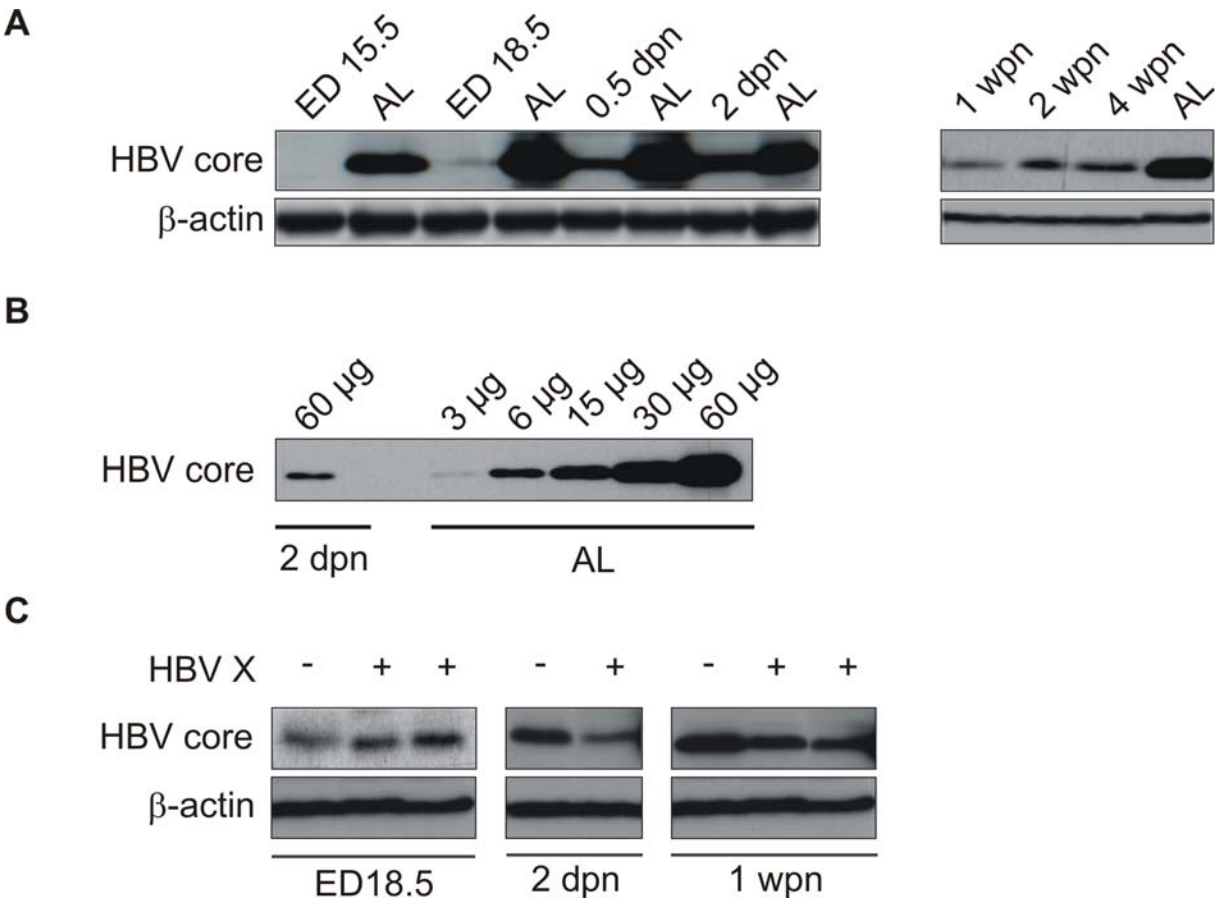


Fig. 9: Expression of HBV core protein during liver development

(A) *Total cellular proteins from livers of transgenic mice carrying replication competent, x-deficient HBV genomes were isolated at ED 12.5, ED 15.5 and ED 18.5, 0.5 and 2 days (dpn) as well as at 1, 2 and 4 weeks (wpn) after birth and from maternal adult livers (AL). 80 µg of proteins per sample were separated by SDS-PAGE, blotted and stained for HBV core protein and β-actin. Representative Western blots are shown.*

(B) *HBV core protein expression in 60 µg of total protein extracted from livers at 2 days postnatally (2 dpn) was compared to that in 3 to 60 µg of total protein in maternal livers.*
(C) *Expression levels of HBV core protein in HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-) were compared at different stages of liver development.*

To study the expression of HBV core protein at the single cell level and with higher sensitivity, I isolated fetal liver cells at ED 12.5 and ED 15.5 and subjected them to immunostaining. At neither time point, did fetal liver epithelial cells, as identified by co-staining with an epithelial-specific pancytokeratin marker, express HBV core protein, whereas hepatocytes isolated from adult mouse livers stained clearly positive (Figure 10A).

To analyze the distribution of HBV core protein within the hepatic lobule, immunocyto-/-histochemical stainings of liver samples at all stages of liver development were performed. In accordance with the results obtained using Western blot analysis, single hepatocytes first stained positive for HBV core protein at ED 18.5 (Figure 10B). During postnatal liver development, the number of HBV core positive cells, and in particular the number of cells with cytoplasmic HBV core staining, increased continuously (Figure 10B). Until 1 wpn, HBV core positive cells were distributed throughout the whole liver lobule (Figure 10B). The characteristic pericentral zonation of HBV core protein expression as seen in adult liver became obvious at 2 wpn and was readily formed at 4 wpn (Figure 10B).

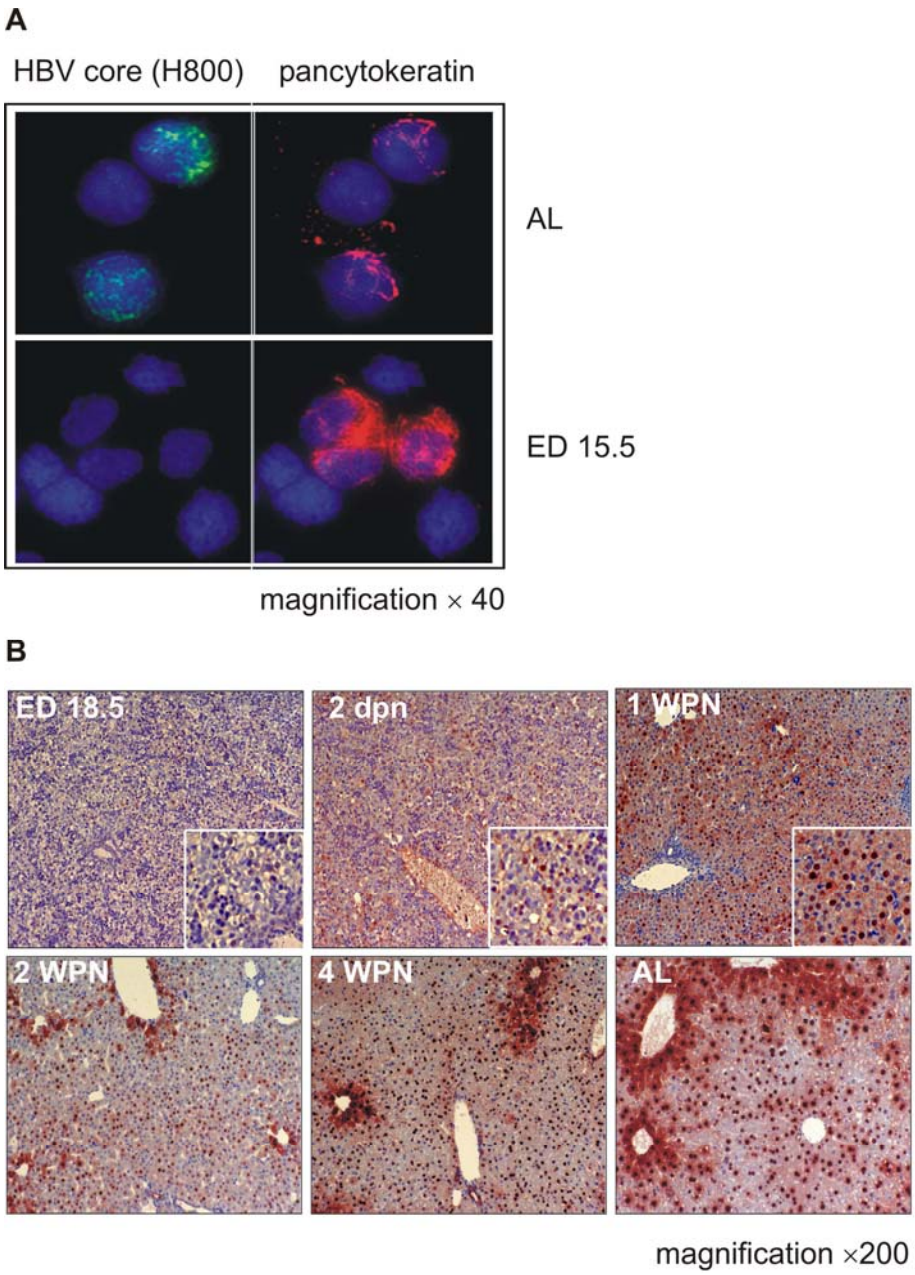


Fig. 10: Immunocyto-/-histochemistry for HBV core protein during liver development

(A) *Single cell suspension obtained from embryonic liver at ED15.5 or corresponding adult liver were spanned on cytospins and co-stained for HBV core protein (green) and pancytokeratine (red). Nuclei were stained with DAPI (blue).*
(B) *Livers of transgenic mice were fixed with formalin and paraffin-embedded. Slices of paraffin-embedded liver tissues were stained for HBV core protein and developed using the APC-system and horseradish peroxidase (brown) (performed by Dr. Uta Drebber).*

To investigate the time point when infectious HBV particles may start to be released, the expression of HBV L protein, an essential component of the viral envelope, was quantified by Western blot analysis. Low amounts of HBV L protein (15.5% of those in AL) were first detected at 0.5 dpn (Figure 11A). Postnatally, production of HBV L protein increased continuously, with levels at 23.3%, 35.5% and 50.7% of adult mice

on day 2, week 1 and 2 postnatally, respectively, and achieved levels seen in adult mice at 4 wpn (Figure 11A). In HBV 1.3 mice, HBV L protein was also first detected at 0.5 dpn, but measured amounts of this viral protein were lower than in HBV 1.3 xfs mice at corresponding time points (Figure 11B).

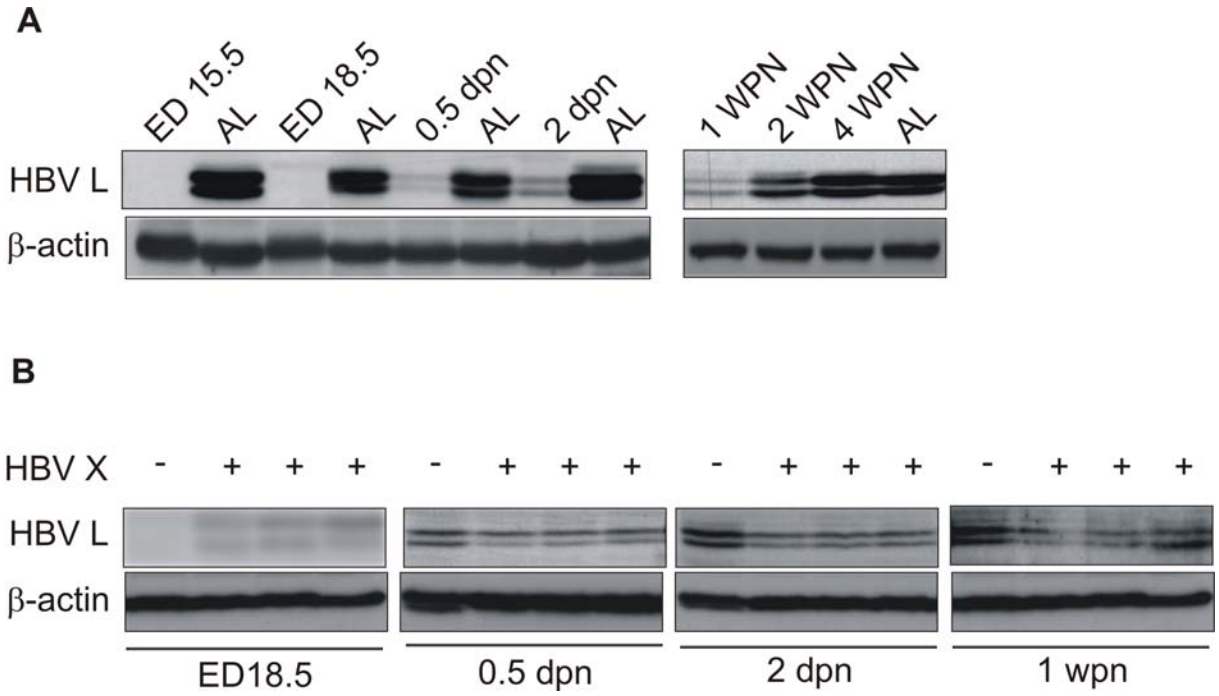


Fig. 11: Expression of HBV L protein during liver development

(A) *Total cellular proteins from livers of transgenic mice carrying replication competent, x-deficient HBV genomes were isolated at ED 12.5, ED 15.5 and ED 18.5, 0.5 and 2 days (dpn) as well as at 1, 2 and 4 weeks (wpn) after birth and from maternal adult livers (AL). 80 µg of proteins per sample were separated by SDS-PAGE, blotted and stained for HBV L protein and β-actin. Representative Western blots are shown.*

(B) *Expression levels of HBV L protein were compared in HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-) at different stages of liver development.*

4.1.2 High expression levels of HNF4α in concert with HNF1α determine the efficiency of HBV replication

Then, I aimed to identify cellular factors determining the replicative potential of HBV in the developing liver and changes in the efficiency of HBV replication along with hepatocyte differentiation.

Previous works suggested that the efficiency of HBV replication [159, 160, 157, 158, 156, 161, 162] and the activity of HBV promoters, especially the preS1-promoter, [163, 164, 158, 135, 165] depends on the degree of hepatocyte differentiation.
However, none of these studies throughly analyzed, which hepatocellular factors link HBV transcription and replication to hepatocyte differentiation. Considering hepatocyte-enriched transcription factors as putative candidates, it is not known whether only one or a combination of these transcriptional regulators is responsible for differentiation-dependent activity of HBV promoters. Moreover, it is still unclear whether hepatocyte-enriched transcription factors act on HBV promoters in a dose-dependent manner. Thus, the essential link between the efficiency of HBV replication and the degree of hepatocyte differentiation remains to be elucidated.

4.1.2.1 Markers of hepatocyte differentiation

To identify hepatocellular factors determining the efficiency of HBV replication during hepatocyte differentiation, I switched to cell culture experiments. I first analyzed how strong the dependence of HBV replication efficiency on hepatocyte differentiation is.
For this purpose, I chose primary human hepatocytes (PHH), the hepatoma cell lines HepG2, HuH7 and HepaRG and the hepatocyte cell line Pop10. HepaRG cells, which differentiate and become permissive for HBV if cultivated with corticosteroids and dimethyl sulfoxide (DMSO) [50, 156], were compared in an undifferentiated and a differentiated state.
To prove the differentiation status of my cells, I characterized the expression of hepatocyte specific differentiation markers.
All cells stained positively for albumin and ferritin, whose expression levels (Fig. 12A) did not correlate with the expected level of hepatocyte differentiation. Therefore, I analyzed key proteins of metabolic pathways in hepatocytes. Expression levels of cytochrome p450 family member CYP1A2, steroid regulatory element binding protein 2 (SREBP-2) (Fig. 12A), 2'3'-tryptophandioxygenase (TDO), and cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 12B) were markedly higher in primary hepatocytes than in HepG2 or HuH7 cells and increased upon differentiation in HepaRG cells. All cells expressed comparable levels of hydroxymethylglutaryl-CoA-reductase and pterin-4 alpha-carbinolamine dehydratase. In addition, I determined expression of mitochondrial cytochromes (liver specific antigen, LSA).

Only in primary hepatocytes and differentiated HepaRG cells expression of LSA was detected (Fig. 12A).

Since polarization is an important feature of hepatocytes and other epithelial cells, I analysed the organic anion transporter polypeptide C (OATP-C) and bile salt exporting pump (BSEP) localized in the basolateral and apical hepatocyte plasma membranes, respectively. Western blotting detected OATP-C only in primary hepatocytes (Fig. 12A), in which basolateral distribution and thus polarization was confirmed by immunofluorescence staining. Levels of OATP-C and BSEP mRNA were highest in PHH and increased upon differentiation in HepaRG cells, which indicates regulation mainly at the level of transcription (Fig. 12B). Pop10 cells expressed none or little of the studied markers of hepatocyte differentiation.

Taken together, differentiated hepatocytes expressed a set of proteins that indicate high metabolic activity as well as hepatocyte polarization. Expression of TDO, PEPCK, BSEP and OATP-C proved most appropriate as indicators of hepatocyte differentiation, which were regulated primarily at the transcriptional level.

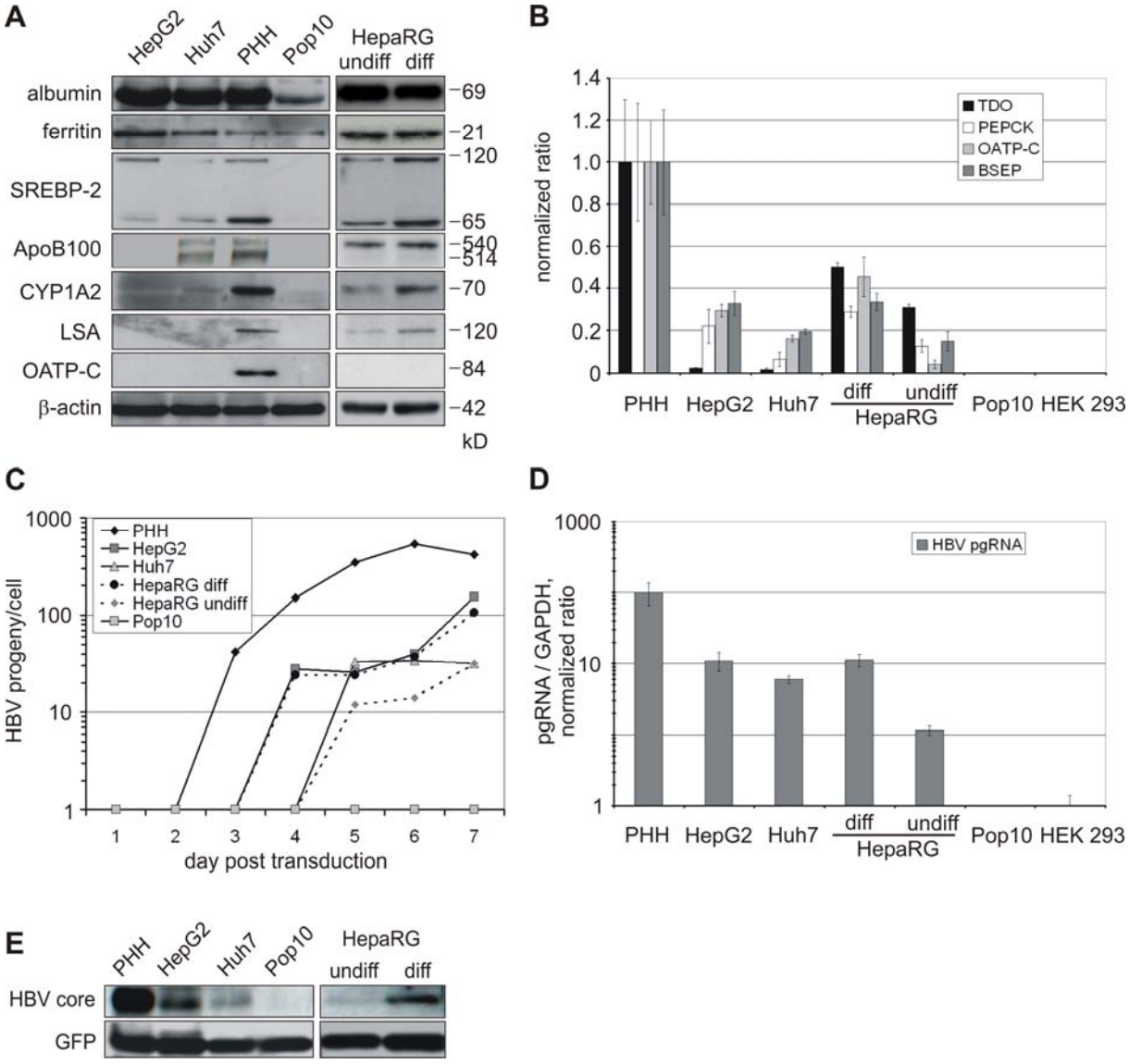


Fig. 12: Differentiation status of cells of hepatocyte origin and HBV replication

(A) *Western blot analysis of OATP-C, CYP1A2, LSA, albumin, ferritin, SREBP-2, ApoB100 and β-actin. Molecular weights of corresponding proteins are given in kD. A representative experiment is shown.*

(B) *Expression levels of TDO, PEPCK, OATP-C and BSEP were determined relative to GAPDH by LightCycler™ real-time RT-PCR. The normalized expression level in PHH was set to 1. Mean values ± SD from three different samples of each cell line are given. PHH from four different donors were included.*

(C) *PHH, HepG2, Huh7, Pop10 and differentiated and undifferentiated HepaRG cells were transduced with Ad-HBV to induce HBV replication. Enveloped HBV progeny released from transduced cells was quantified by DNA dot blot analysis following CsCl gradient sedimentation. Numbers of enveloped HBV particles released per cell were calculated. A representative experiment is shown.*

(D) *HBV pgRNA at day 7 after transduction with Ad-HBV was determined relative to GAPDH by LightCycler™ real-time RT-PCR. The normalized expression level in PHH was set to 1. Mean values ± SD from two independent experiments are given.*

(E) *Western blot analysis of HBV core protein and GFP. A representative experiment is shown.*

4.1.2.2 Efficiency of HBV replication in different hepatocytes

To study the efficiency of HBV replication independent of the early steps of virus uptake and to enable the transcription of HBV genes from an extrachromosomal template, I transduced PHH, HepG2, HuH7, differentiated and undifferentiated HepaRG and Pop10 cells with an HBV genome and a GFP cDNA using an adenoviral vector Ad-HBV. Adenoviral vectors have been demonstrated to deliver genes in a wide range of cell types and allow efficient transcription of genes from an episomal template.

I achieved comparable (90-95%) transduction rates of cells by Ad-HBV as confirmed by fluorescence microscopy and Western blot analysis of GFP expression (Fig. 12E).

I monitored the release of HBV progeny in cell culture media daily and harvested cells on day 7 post transduction. To distinguish enveloped HBV virions from HBV capsids, I performed CsCl density gradient centrifugation with subsequent fractionation that allowed separation of different HBV progeny forms based on their density. To quantify enveloped HBV progeny, I performed DNA dot blot analysis.

As shown in Fig. 12C, PHH started to release enveloped progeny HBV particles at day 3, HepG2 and HepaRG cells at day 4, and HuH7 cells at day 5 after transduction with Ad-HBV. Pop10 cells did not release progeny HBV at all (detection limit: one HBV particle / cell). PHH secreted > 500 virions per cell per day, which was the highest amount and markedly more than HepG2 cells (13.6-, 13.4-, 2.7-fold, at day 5, 6 and 7 post transduction, respectively), differentiated HepaRG cells (14.4-, 14.3-, 3.9- fold) or HuH7 cells (10.5-, 15.7-, 13-fold). Differentiated HepaRG cells released 2- to 3.3-fold more HBV progeny than respective undifferentiated cells.

By analysing the HBV replication cycle, I found expression levels of HBV pgRNA to be the limiting step. Although transduced to equal levels, HepG2, differentiated HepaRG and HuH7 cells expressed only 9, 8.5 and 6% of the amount of HBV pgRNA detected in PHH at day 7, respectively, and Pop10 cells expressed no HBV pgRNA at all (Fig. 12D). Upon differentiation, HBV pgRNA increased 5.7-fold in HepaRG cells. Accordingly, expression of HBV core protein was highest in primary hepatocytes and increased upon differentiation of HepaRG cells (Fig. 12E).

Taken together, primary hepatocytes, the highest differentiated cells in my study, replicated HBV better than hepatoma cells and released more progeny HBV. Pop10 cells, which express none or little of studied hepatocyte differentiation markers, did not replicate HBV at all. The differences in the efficiency of HBV replication

depended largely on the expression level of the HBV pgRNA. Thus, efficiency of HBV replication was regulated at the transcriptional level.

4.1.2.3 Expression of hepatocyte-enriched transcription factors in cells of hepatocyte origin with different degree of differentiation

Since HBV replication as well as hepatocyte differentiation was primarily regulated at the transcriptional level, I analyzed the hepatocyte-enriched transcription factors HNF1α/β, HNF3α/β/γ, HNF4α, C/EBPα/β, LRH-1, PGC-1α and COUP-TF II as candidate common denominators.

PHH expressed the highest levels of HNF1α, HNF3γ and HNF4α. Upon differentiation, HepaRG cells expressed increasing amounts of HNF1α, HNF3γ, HNF4α and C/EBPα and β, but decreasing amounts of HNF3α. In addition, PHH as well as HepaRG cells expressed different isoforms of C/EBPβ as compared to the other analyzed cells. Pop10 cells, in contrast, only contained trace amounts of HNF3γ and HNF4α and no detectable HNF1α (Fig. 13A&B). Interestingly, the expression of PGC-1α and COUP-TF II, the positive and negative co-factors of HNF4α, respectively, did not differ to a large extent between all cells analysed and was relatively constant during differentiation of HepaRG cells.

To correlate transcription factor expression levels with HBV replication, I cultivated stable HBV-producing cell lines under conditions promoting differentiation: a collagen matrix and medium containing dexamethasone, DMSO and low FCS. Levels of OATP-C and BSEP mRNA rose 2.9- and 5.7-fold in HepG2.2.15 and 10.8- and 6.4-fold in HepG2-H1.3 cells, respectively, which indicates successful differentiation during long-term culture. In parallel, HNF4α (Fig. 14C, D), HNF1α and HNF3γ (Fig. 14C, 15A&G) levels rose 2.9-, 3.3- and 2.3-fold in HepG2.2.15 cells and 7.0-, 4.5- and 3.0-fold in HepG2-H1.3 cells, respectively, whereas C/EBPα/β, HNF3α, HNF3β, LRH-1, PGC-1α and COUP-TF expression did not show a clear trend in both cell lines (Fig. 15A-J and table 1).

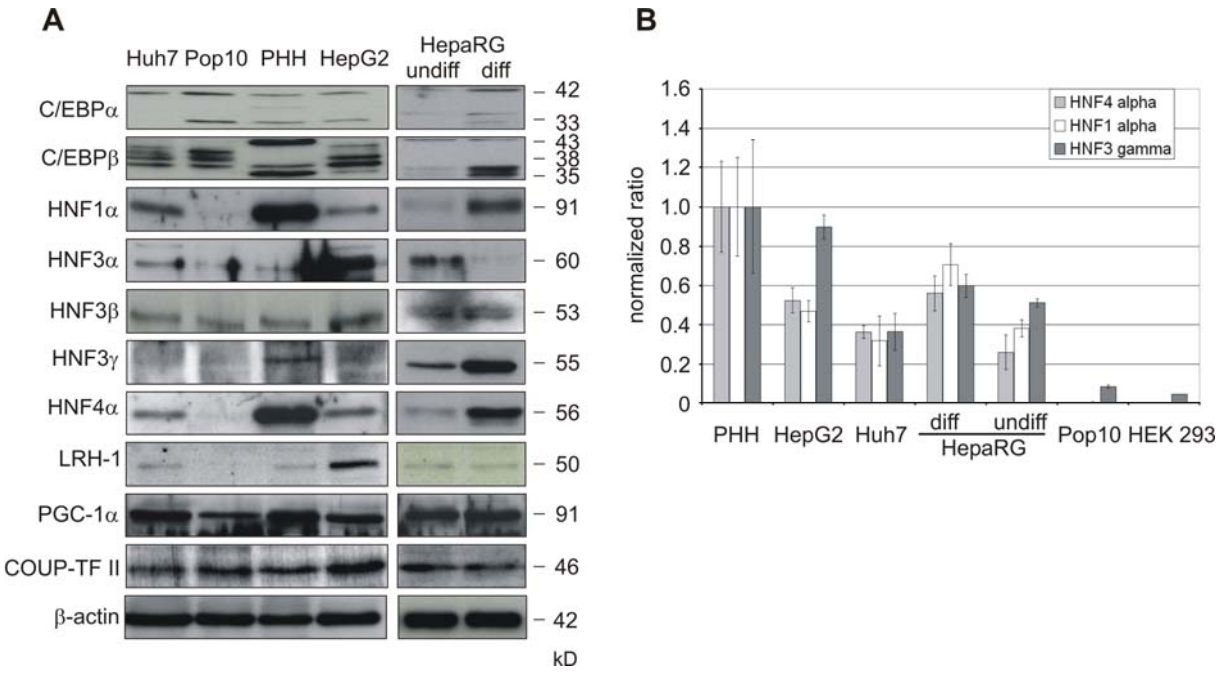


Fig. 13: Expression patterns of hepatocyte-enriched transcription factors
(A) *Western blot analysis of HNF1α, 3α, 3β, 3γ and 4α, C/EBP α and β, LRH-1, PGC-1α and COUP-TF II expression in primary hepatocytes (PHH), HepG2, Huh7, Pop10, differentiated and undifferentiated HepaRG cells. Molecular weights of corresponding proteins are given in kD.* ***(B)*** *LightCycler™ real-time RT PCR analysis of HNF1α, 3γ and 4α expression. Normalized expression levels in PHH were set to 1. Mean values ± SD from three different samples of each cell line are given (except HepaRG: two samples each). PHH from four different donors were included.*

Accordingly, levels of HBV pgRNA increased 8.1-fold in HepG2.2.15 and 8.6-fold in HepG2-H1.3 cells. The amount of progeny HBV released into the cell culture medium increased from day 0 to 16 from 2.3±0.14 to 217.4±43.38 (HepG2.2.15 cells) and from 1.79±0.1 to 1281.8±149.4 HBV-DNA copies / cell (HepG2-H1.3 cells) (Fig. 14A). HBV core- and L-protein also increased over time: 10- and 7.1-fold in HepG2.2.15 cells and 14- and 8.8-fold in HepG2-H1.3 cells, respectively (Fig. 14B).

Taken together, differentiation of the two HepG2 based cell lines resulted in increased expression of HNF1α, HNF3γ and HNF4α and raised HBV replication. In both cell lines, HBV replication depended on transcription of HBV pgRNA which correlated most closely with rising levels of HNF4α (Pearson correlation 0.93, $p \leq 0.01$) and also HNF1α and HNF3γ (see table 1). This led us to the hypothesis that HNF1α, HNF3γ and/or HNF4α provide the essential link between hepatocyte differentiation and HBV replication.

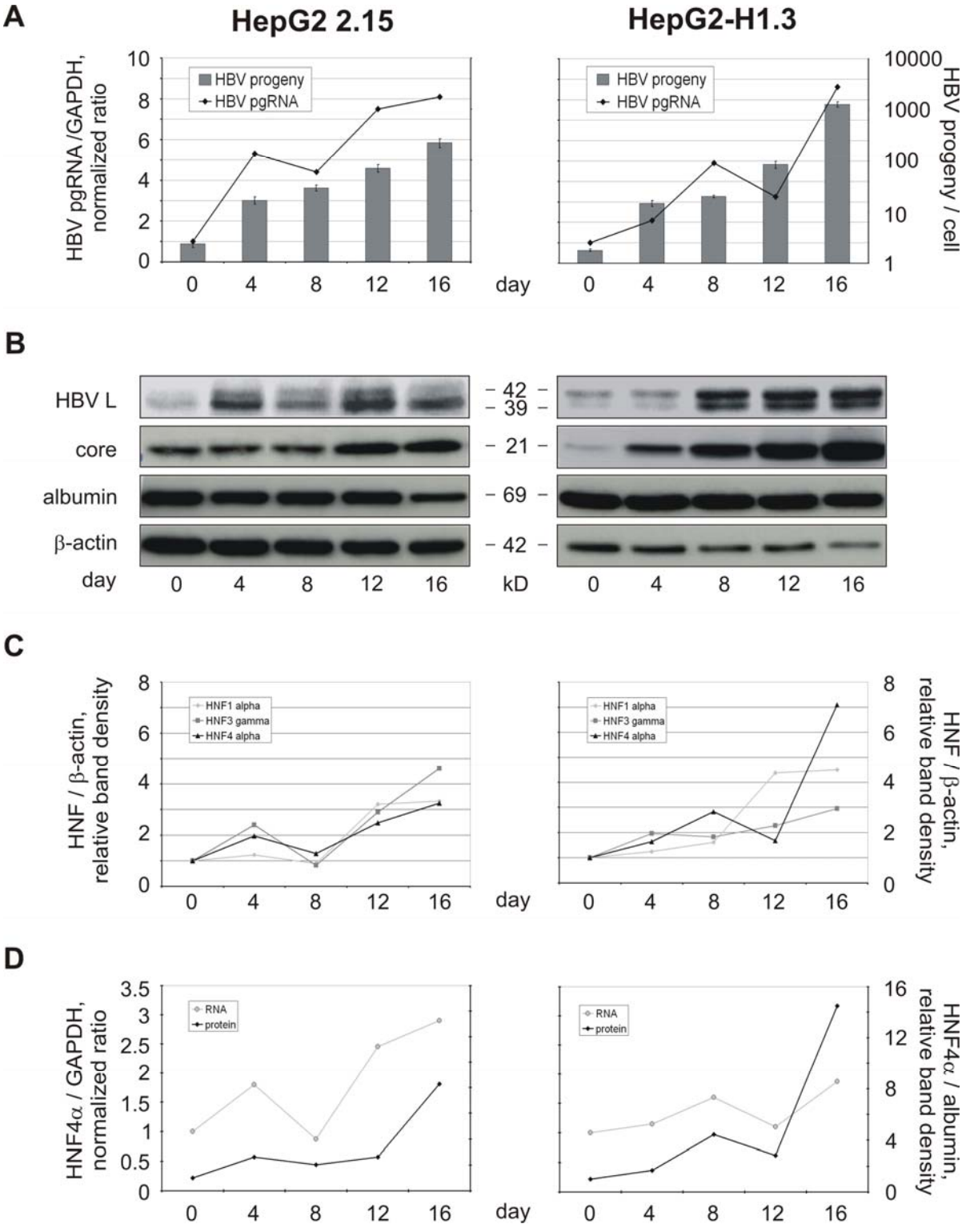
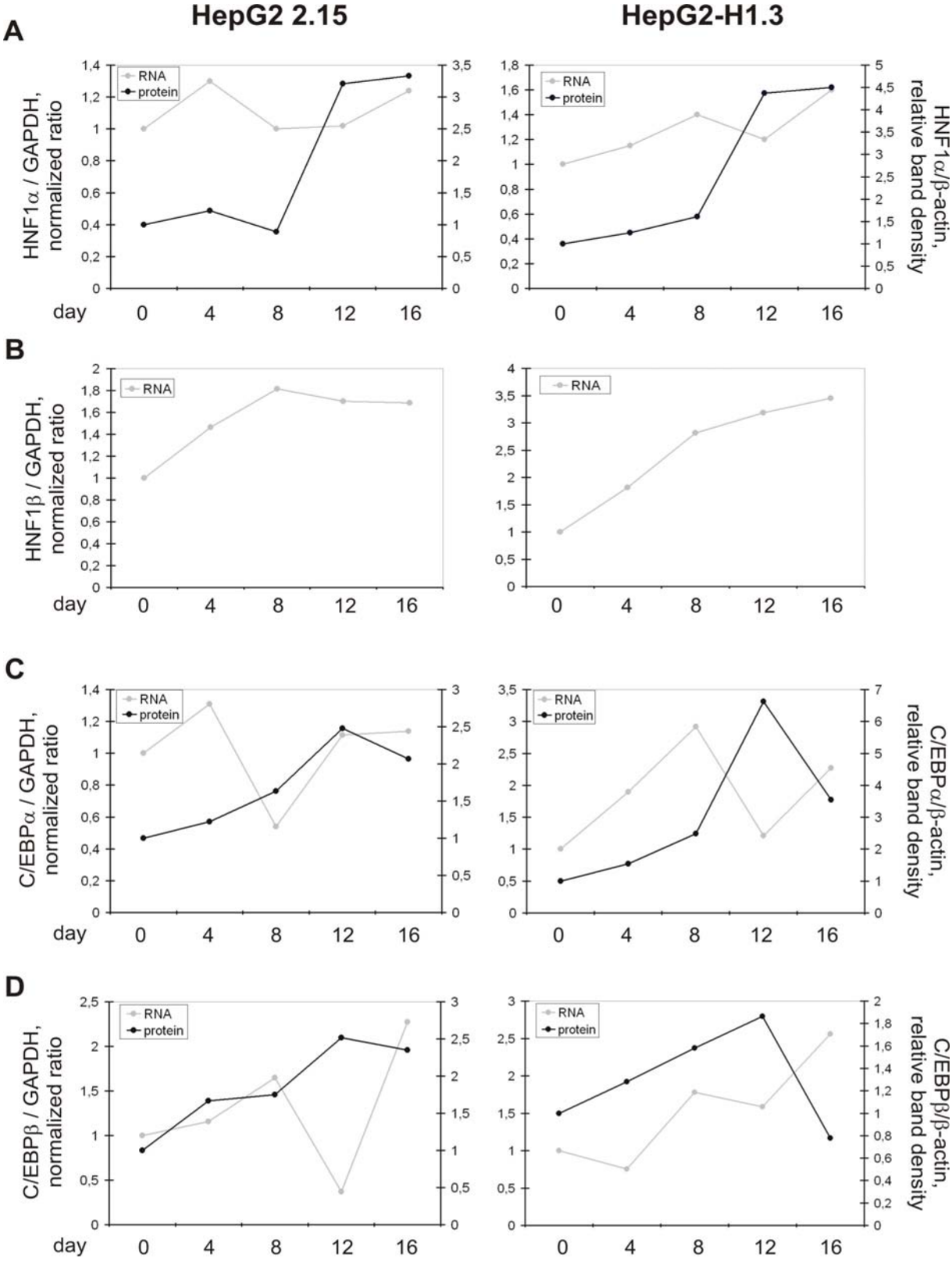
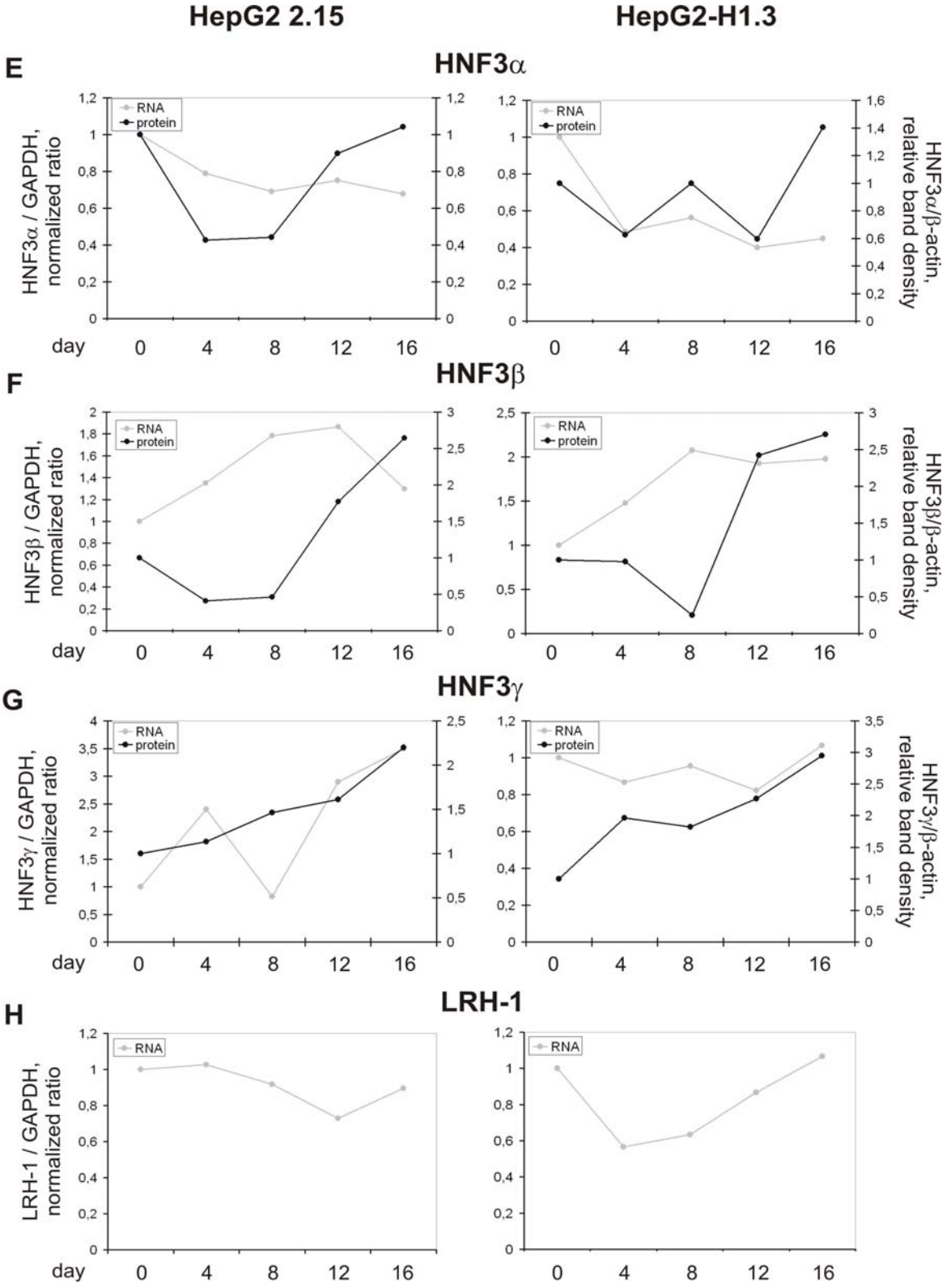


Fig. 14: HBV replication and hepatocyte differentiation in stable HBV-producing cell lines

Stable HBV-producing cell lines HepG2 2.15 (left panel) and HepG2-H1.3 (right panel) were cultured under differentiating conditions and lyzed at indicated time points. ***(A)*** *HBV-DNA was quantified by LightCycler™ real-time PCR in 200µl of cell culture medium relative to an external standard. The amount of HBV progeny per cell was calculated (given as mean ± SD) (grey bars). HBV pgRNA was determined*

relative to GAPDH by LightCycler™ real-time RT PCR (black line). ***(B)*** *Western blot analysis of HBV- core and L-protein as well as albumin and β-actin. Molecular weights are given in kD.* ***(C)*** *HNF4α, HNF1α and HNF3γ content was quantified relative to β-actin by chemiluminescence imaging of Western blots.* ***(D)*** *HNF4α content was quantified relative to albumin by chemiluminescence imaging of Western blots. HNF4α mRNA was quantified by LightCycler™ real-time RT-PCR. Normalized expression levels on day 0 were set to 1.*




HepG2 2.15
HepG2-H1.3
E
HNF3α
HNF3α / GAPDH, normalized ratio
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RNA
protein
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0
4
8
12
16
F
HNF3β
HNF3β / GAPDH, normalized ratio
HNF3β/β-actin, relative band density
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LRH-1
LRH-1 / GAPDH, normalized ratio

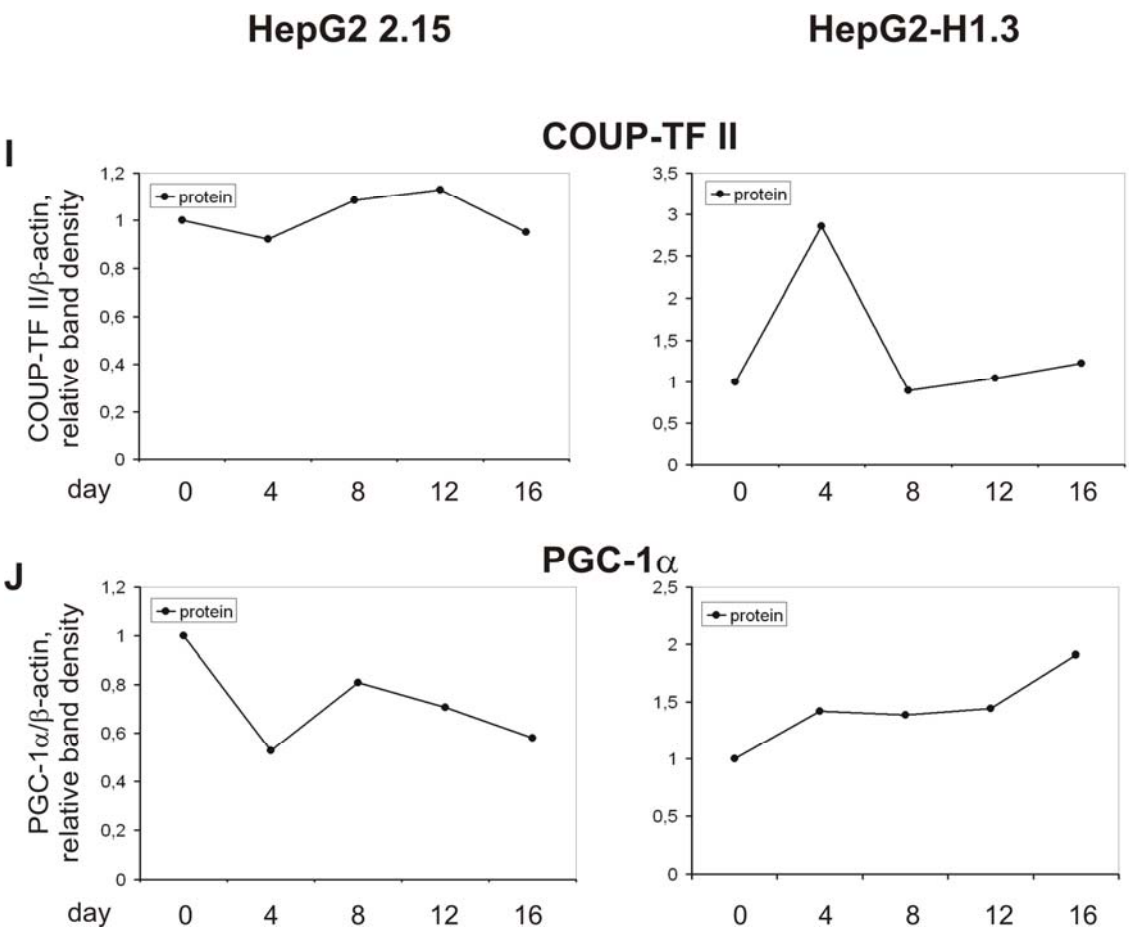


Fig. 15: Expression of hepatocyte-enriched transcription factors in stable HBV- producing cell lines

Stable HBV-producing cell lines HepG2 2.15 (left panel) and HepG2-H1.3 (right panel) were cultured under differentiating conditions and lyzed at indicated time points. ***(A-J)*** *Protein content of hepatocyte-enriched transcription factors was quantified relative to β-actin by chemiluminescence imaging of Western blots. Relative band intensity is given. mRNA of hepatocyte-enriched transcription factors was quantified by LightCycler™ real-time RT-PCR relative to GAPDH and normalized to serial dilutions of calibrator. Normalized expression levels on day 0 were set to 1.*

Table 1

Pearson correlation: HBV pg RNA and transcription factor					
	HNF1α	HNF1β	C/EBPα	C/EBPβ	HNF3α
HepG2.2.15	0.82	0.51	0.71	0.75	0.21
HepG2-H1.3	0.87	0.78	0.46	-0.42	0.56
	HNF3β	HNF3γ	LRH-1	PGC-1α	COUP-TFII
HepG2.2.15	0.57	0.94	-0.73	0.43	-0.30
HepG2-H1.3	0.61	0.93	0.48	0.87	-0.017

4.1.2.4 High expression levels of HNF4α and HNF1α are required for efficient HBV replication

To test whether efficient HBV replication depends on high expression levels of HNF4α, HNF1α or HNF3γ, I performed knock-down experiments in HepG2-H1.3 cells using specific siRNAs for these transcription factors. As control, I used HepG2-H1.3 cells transfected with AlexaFluor488-labeled, non-silencing siRNA to which all effects of specific siRNAs were related.

The transfection efficiency was approximately 80% as measured by fluorescent microscopy of cells transfected with AlexaFluor488-labeled, non-silencing siRNA.

I achieved a long-lasting 50%, 67% and 82% knock-down of HNF1α, HNF3γ and HNF4α, respectively (Fig. 16A).

On day 5, after the knock-down of HNF4α and HNF1α, mRNA levels of OATP-C decreased 2.55±0.23- and 2.30±0.13-fold, BSEP decreased 1.50±0.16- and 1.30±0.07-fold, TDO decreased 2.60±0.01- and 1.75±0.19-fold, and PEPCK decreased 2.80±0,19 and 2.30±0.13-fold (Fig. 16B).

Transcription of HBV pgRNA was significantly reduced in three independent experiments after knock-down of HNF4α (4.7±0.3 – fold on day 3; 2.2±0.1 - fold on day 5 post transfection) and HNF1α (4.05±0.25 – fold on day 5) (Fig. 16C). HBV core protein was diminished after the knock-down of HNF4α and HBV L protein was diminished after the knock-down of HNF1α (Fig. 16 D&E), both leading to a

significant decrease in HBV progeny release (Fig. 16F). Unlike HNF1α and HNF4α, HNF3γ knock-down did not have any inhibitory effect (Fig. 16A-F).

Southern blot analysis showed a 70 and 75% reduction in HBV replication after the knock-down of HNF1α and HNF4α, respectively (Fig. 16G). Concomitantly, the accumulation of HBV cccDNA in the nucleus of HepG2-H1.3 cells was inhibited (HNF1α 12% and HNF4α 23%) (Fig. 16H).

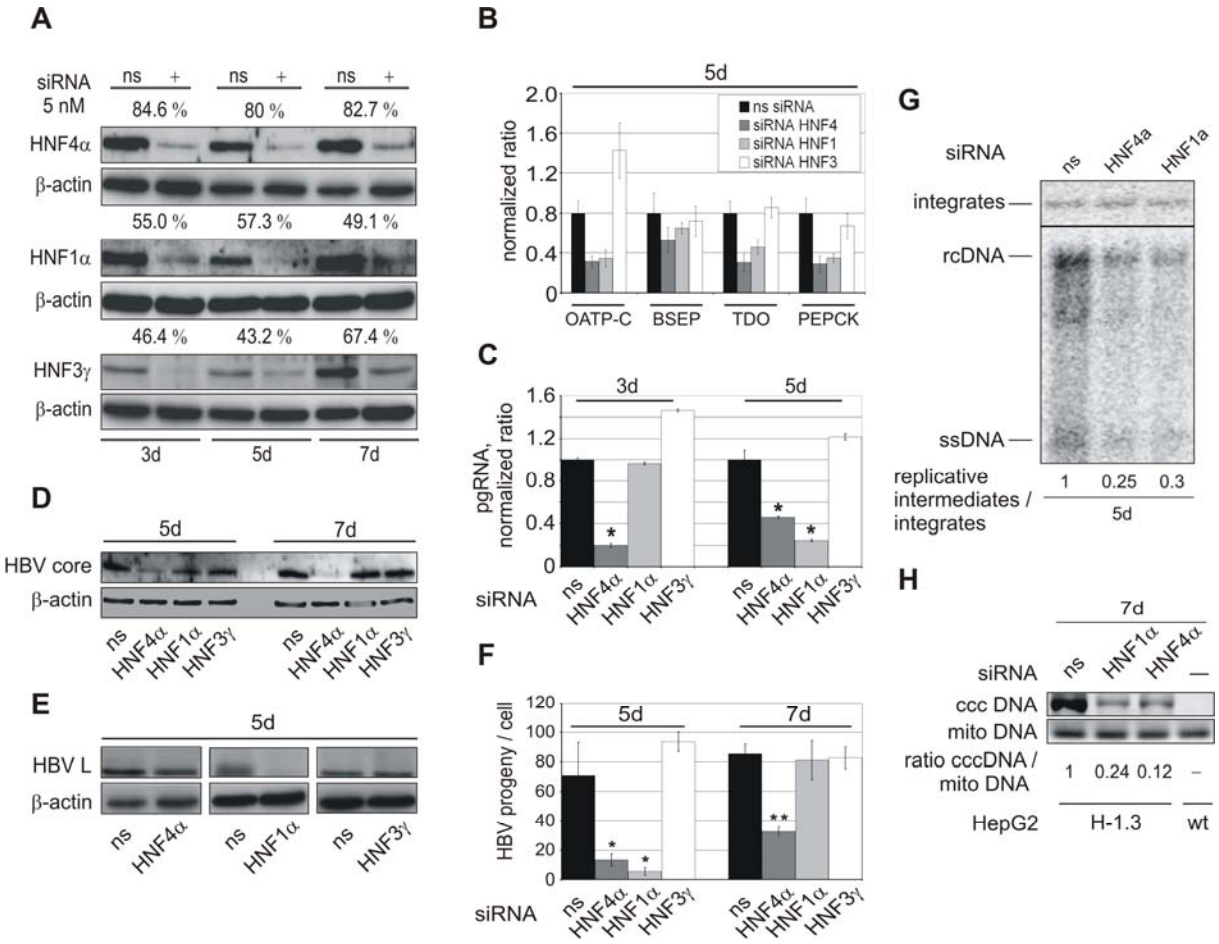


Fig. 16: Expression of hepatocyte differentiation markers and HBV replication after knock-down of HNF4α, HNF1α or HNF3γ

*HepG2 H-1.3 cells were transfected with 5 nM siRNA (+) either specific to HNF4α, HNF1α or HNF3γ or non-silencing (ns). Cells were harvested on day (d) 3, 5 and 7 after transfection. (**A**) Western blot analysis of HNF4α, HNF1α and HNF3γ. Knock-down efficiency of specific siRNAs are given in % (relative to ns siRNA). (**B**) Normalized expression levels of OATP-C, BSEP, TDO and PEPCK were determined by LightCycler™ real-time RT PCR relative to delta aminolevulinate synthase (ALAS). (**C**) Normalized expression levels of HBV pgRNA. (**D**) Western blot analysis of HBV-core protein at day 5 and 7 and **(E)** HBV L protein at day 5. **(F)** HBV-DNA was quantified by LightCycler™ real-time PCR relative to an external standard. The amount of HBV progeny per cell was calculated. (**G**) Southern blot analysis of Hind III digested total cellular DNA using a ^{32}P labelled HBV DNA probe. HBV replicative intermediates were normalized to HBV integrates following phosphoimager quantification. (**H**) PCR amplification products of HBV cccDNA and*

mitochondrial DNA. Ratios were determined from real-time PCR analysis. Parental HepG2 cells were used as control. In B, C and F ns siRNA transfected cells were set to 1. In A, D, E, G and H representative experiments are shown. In B, C and F, mean values ± SD of three independent experiments are given; (∗) p<0.05, (∗∗) p<0.01, student T test.

Since neither HNF1α nor HNF4α siRNA induced interferon-γ-inducible protein-10 (IP-10) or 2'-5'-oligoadenylate-synthetase, I excluded that induction of interferon type I, as a possible side effect of siRNAs, affected HBV replication (Fig. 17).

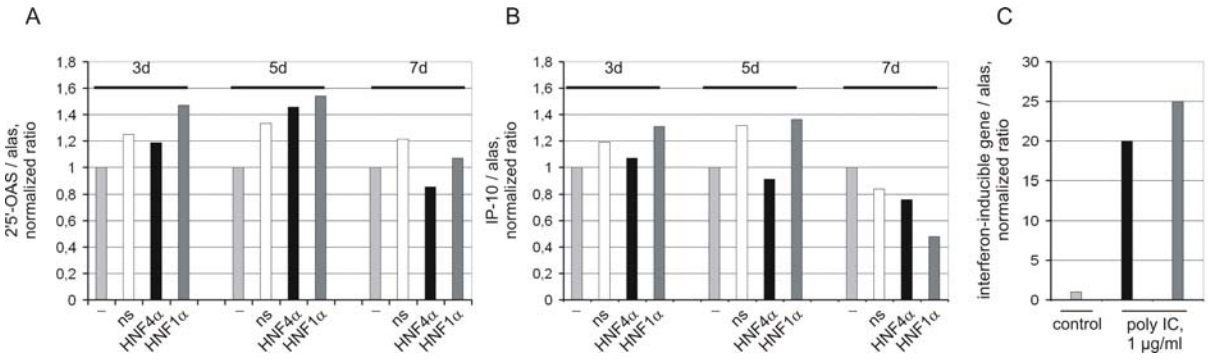


Fig. 17: Expression of interferon-inducible genes after transfection of HepG2 - H1.3 cells with siRNAs

HepG2-H1.3 cells were transfected with 5 nM siRNA (+) either specific to HNF4α, HNF1α or HNF3γ or non-silencing (ns). Cells were harvested on day (d) 3, 5 and 7 after transfection. Normalized expression levels of 2',5'-oligoadenylate synthetase (2',5'-OAS) ***(A)*** *and interferon-γ-inducible protein-10 (IP-10)* ***(B)****, LightCycler™ real-time RT-PCR.* ***(C)*** *HepG2-H1.3 cells were transfected with 1 µg/ml polyIC and used as a positive control for induction of 2',5'-oligoadenylate and interferon-γ-inducible protein-10 (IP-10)*

To show the direct effect of HNF4α on HBV replication in a given cell, I performed immunostaining for HNF4α and HBV core protein on day 7 after transfection with siRNA for HNF4α or with non-silencing siRNA (Fig. 18). In cells with reduced or undetectable HNF4α, HBV core protein was also strongly reduced.

From these experiments, I concluded that high expression levels of HNF4α and HNF1α are needed for efficient HBV replication as well as expression of hepatocyte differentiation markers.

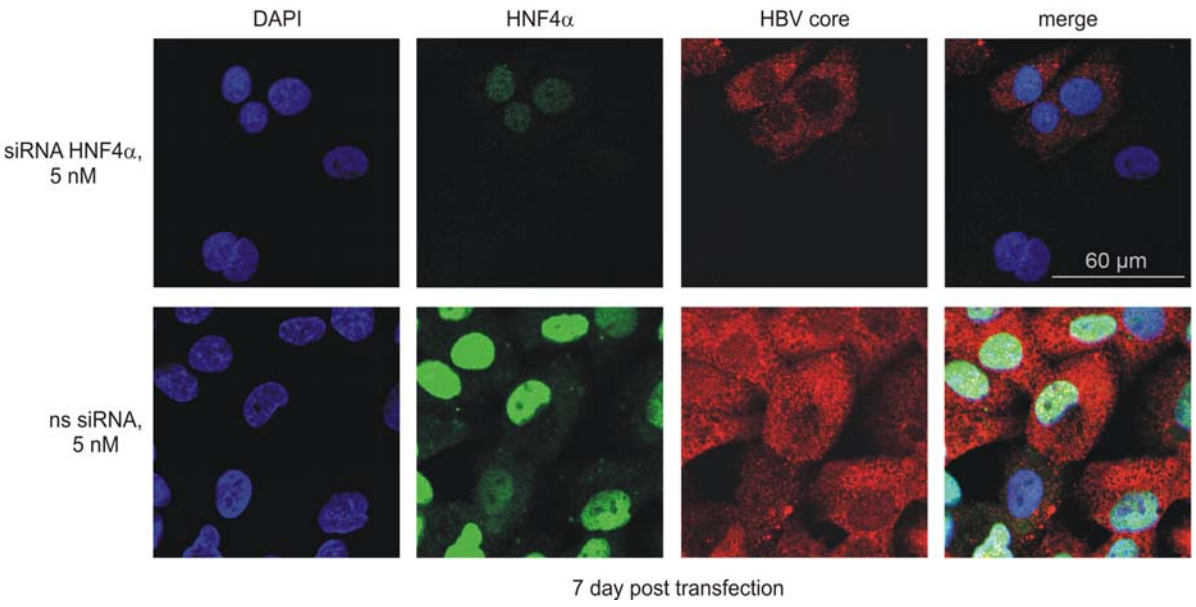


Fig. 18: Analysis of HBV core and HNF4α expression in single cells

Immunofluorescence staining of HNF4α (AlexaFluor™ 488, green), HBV-core protein (AlexaFluor™ 568, red) or cell nuclei (DAPI, blue) in cells transfected with either HNF4α-specific or ns siRNA at day 7 post transfection. Laser scanning confocal microscopy, scale bar 60µm.

The inhibitory effect of HNF1α on the expression of HBV pgRNA was unexpected since HNF1α does not bind HBV core promoter unless it is mutated. HNF1α is reported to regulate the expression of HNF4α, which is one of transcription factors regulating activity of the HBV core promoter. I therefore investigated the expression of HNF4α after transfection of HepG2-H1.3 cells with siRNA against HNF1α. Indeed, after the knock-down of HNF1α, expression of HNF4α was diminished by 75% on day 3 and 62% on day 5 (Fig. 19). Expression levels of HNF4α were restored on day 7 after knock-down of HNF1α, which was consistent with the disappearance of down regulation of HBV pgRNA levels (Fig. 19). Thus, inhibition of HBV pgRNA expression after knock-down of HNF1α was in first line mediated by decreased levels of HNF4α.

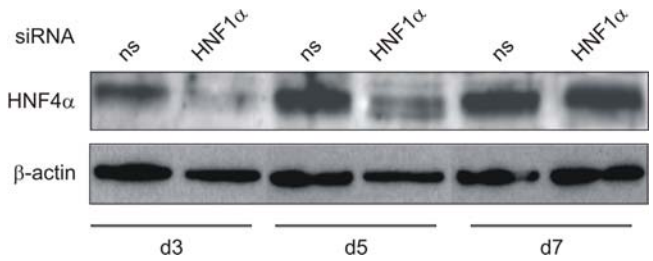


Fig. 19: Expression of HNF4α after the knock-down of HNF1α.
Western blot analysis with 15 µg of total cellular proteins from HepG2-H1.3 cells transfected with either ns siRNA or siRNA against HNF1α.

The knock-down of HNF3γ did not show any inhibitory effect on transcription of HBV genes and viral replication, but levels of this transcription factor correlated well with expression of HBV pgRNA in previous experiments. Since HNF3α and HNF3β possess structural and functional homology with HNF3γ, I hypothesized that they substituted for decreased amounts of HNF3γ in transfected hepatoma cells. In fact, after the knock-down of HNF3γ, expression levels of HNF3α and HNF3β increased 3.2- and 2.5-fold on day 5 and 4.3 and 2.6-fold on day 7, respectively (Fig. 20).

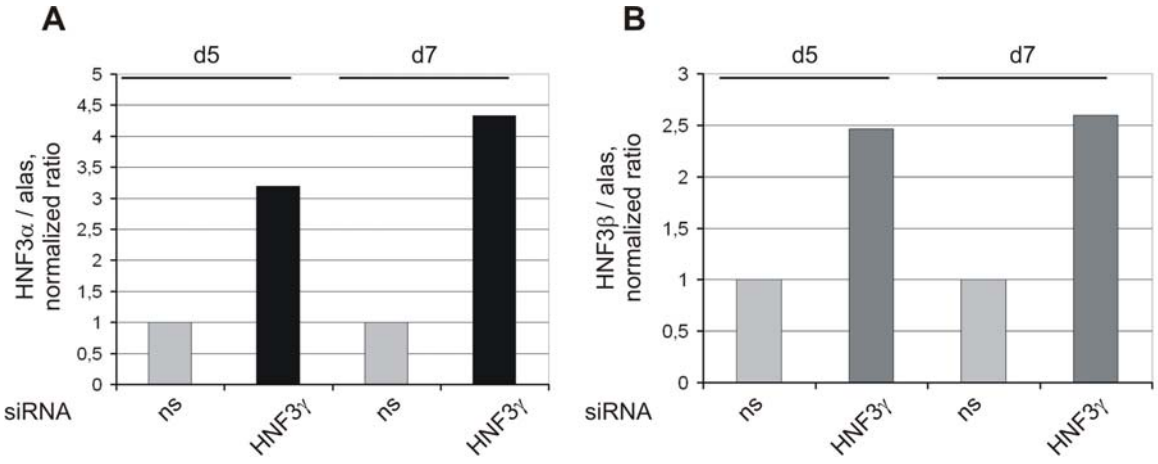


Fig. 20: Analysis of HNF3α and HNF3β expression after knock-down of HNF3γ
*LightCycler™ real-time RT-PCR with 1µg of total cellular RNA isolated from HepG2-H1.3 cells transfected with either ns siRNA or siRNA against HNF3γ. Expression of HNF3α and HNF3β was quantified relative to GAPDH and normalized to series dilutions of calibrator cDNA. Normalized expression levels of HNF3α **(A)** and HNF3β **(B)** are given. Expression levels of corresponding transcription factors in cells transfected with ns siRNA were set to 1.*

4.1.2.5 Levels of HNF4α determine HBV core protein expression and HBV replication *in vivo* in human tumor and non-tumor liver tissue

To test whether expression levels of hepatocyte-enriched transcription factors also determine the efficiency of HBV replication *in vivo,* in livers of infected patients, I analysed expression of hepatocyte-enriched transcription factors, production of HBV core protein and pgRNA in tumor as well as corresponding peritumor tissues of HCC patients chronically infected with HBV. Tumors were graded as shown in materials and methods. Minor differences of either HBV pgRNA or HBV core protein or hepatocyte-enriched transcription factors were shown between tumor and peritumor tissue samples of a given patient, whereas I found high interindividual variation. In all samples analysed, amounts of HNF4α significantly correlated with amounts of HBV core protein (Pearson correlation 0.82, $p<0.01$) (Fig. 21A). Also, levels of HBV pgRNA correlated with expression levels of HNF4α (Pearson correlation 0.57, $p=0.057$) (Fig. 21B). Thus, the data from patient material confirm that efficient HBV replication *in vivo* also relies on high expression levels of HNF4α.

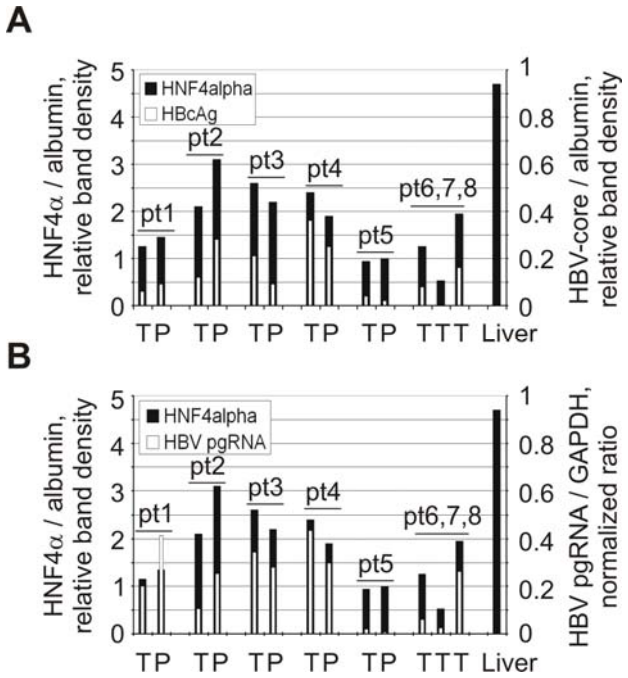


***Fig. 21:* Correlation of the amounts of HNF4α and HBV core protein or HBV pgRNA in tumor-peritumor tissues of HCC patients chronically infected with HBV**

***(A)** Western blot analysis for HNF4α, HBV core protein and albumin of lysates from tumor-peritumor tissue samples from five patients (pt 1 to 5), three unpaired HCC tissue samples (pt 6,7,8) and three normal liver tissue samples (pooled). HNF4α and HBV core protein, respectively, were quantified relative to albumin using chemiluminescence imaging. Relative band densities are given. **(B)** Normalized expression levels of HBV pgRNA were determined by LightCycler™ real-time RT PCR relative to GAPDH, normalized to a series dilutions of calibrator cDNA and are shown in comparison to HNF4α expression.*

4.1.3 Expression of hepatocyte-enriched transcription factors during liver development in transgenic mice

Next, I studied whether HNF4α and HNF1α correlate with the late onset of HBV replication and the changes in HBV replication efficiency during hepatocyte maturation. In addition, I examined expression of PGC-1α and COUP-TF, since they were reported to be an important co-activator and co-repressor of HNF4α-regulated transcription during fetal liver development, respectively. In accordance with the literature, expression of HNF4α, HNF1α, PGC-1α and COUP-TF was detected at the mRNA level on ED12.5, being 15.5%, 21.9%, 32.5% and 70% of those in AL, respectively (Fig. 22A).

In Western blot analysis, I first detected HNF1α, HNF4α and COUP-TF at ED 15.5 and PGC-1α at ED 18.5 (Fig. 22B). The protein levels of HNF1α, HNF4α and PGC-1α, which are required for transcription of HBV RNAs, increased from ED 15.5 onwards, being 12%, 5% and 7% on ED18.5, 33%, 30.5% and 38.4% on 0.5 dpn, 52%, 52% and 53.8% on 2 dpn, 79.9%, 81%, and 70.8% on week 1, 93.2%, 95% and 89.9% on week 2 postnatally, respectively, (Fig. 22B) achieving those in adult liver at 4 wpn (Fig. 22B). Expression of COUP-TF, which negatively regulates HBV pregenome transcription, readily increased until ED 18.5 and remained constant during further liver development (Fig. 22B). Among the studied transcription regulators, amounts of HNF1α and HNF4α correlated most closely with expression levels of HBV pgRNA (Spearman correlation 0.949, $p < 0.01$ and 0.889, $p < 0.01$, respectively).

I suggest that increase in expression levels of HNF4α in concert with HNF1α and PGC-1α is responsible for high-level transcription of HBV pregenomes and morpholological and metabolic differentiation of hepatocytes required for onset of HBV replication and formation and secretion of DNA-containing HBV particles.

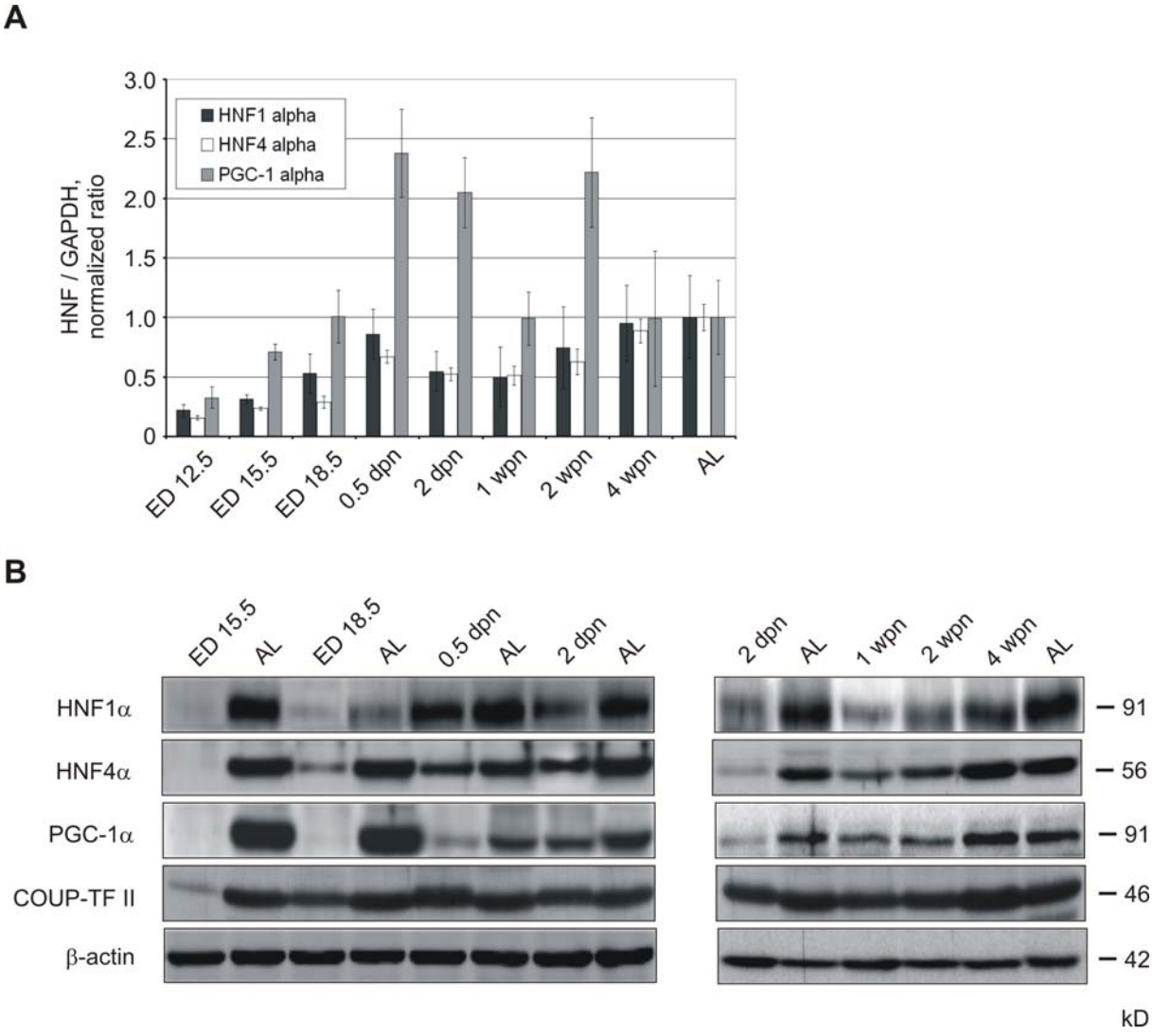


***Fig. 22:* Expression of hepatocyte-enriched transcription factors during liver development**

(A) *Total RNA was isolated from livers of HBV 1.3 xfs mouse mothers (AL) and compared to fetal liver RNA obtained at ED 12.5, ED 15.5 and ED 18.5 and to newborn liver RNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. Total cellular RNA was reverse transcribed into cDNA. Expression of HNF1α, HNF4α and PGC-1α was quantified relative to GAPDH by LightCycler™ real-time PCR and normalized to a dilution series of calibrator cDNA using the Relative Quantification Software. Medium ± SD of at least three animals per time point are given.*

(B) *Total cellular proteins from livers of transgenic mice carrying replication competent X-deficient HBV genomes (HBV 1.3 xfs) were isolated from maternal adult (AL) as well as fetal livers at ED 12.5, ED 15.5 and ED 18.5 and newborn livers obtained 0.5 and 2 days (dpn) as well as livers at 1, 2 and 4 weeks (wpn) after birth. 80 µg of proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were stained for HNF1α, HNF4α, PGC-1α, COUP-TF and β-actin as loading control. A representative Western blot is shown.*

4.2 Inhibition of HBV replication by induction of HO-1 in hepatic cells and in a mouse model of acute HBV infection

Heme oxygenase I (HO-1) is known to be involved into the pathogenesis of various viral infections including hepatitis C and HIV. In the case of HIV infection, HO-1 has been reported to inhibit viral replication and protect cells from infection with the virus. Recently, *Protzer et. al.* have shown strong inhibition of HBV replication after administration of CoPP in HepG2.215 cells as well as in HBV-transgenic mice and in a mouse model of acute HBV infection [154]. However, porphyrins were reported to possess their own antiviral effect, at least in the model of HIV infection [214]. Therefore, it is important to investigate whether the antiviral effect of CoPP in HBV infection results from HO-1 induction or is merely caused by porphyrins. Although, *Protzer et. al* showed antiviral activity of HO-1 overexpression using an adenoviral vector, proof that knocking out HO-1 expression abolishes the antiviral activity was lacking. Also, *Protzer et. al* showed amelioration of liver injury in the model of acute HBV infection, when HO-1 was induced by CoPP prior to the onset of HBV replication and development of anti-HBV immune response [154]. Here, considering a therapeutic application of HO-1 induction in acute or flares of chronic hepatitis B, it is important to study the effect of enzyme overexpression on liver injury on ongoing anti-HBV immune response.

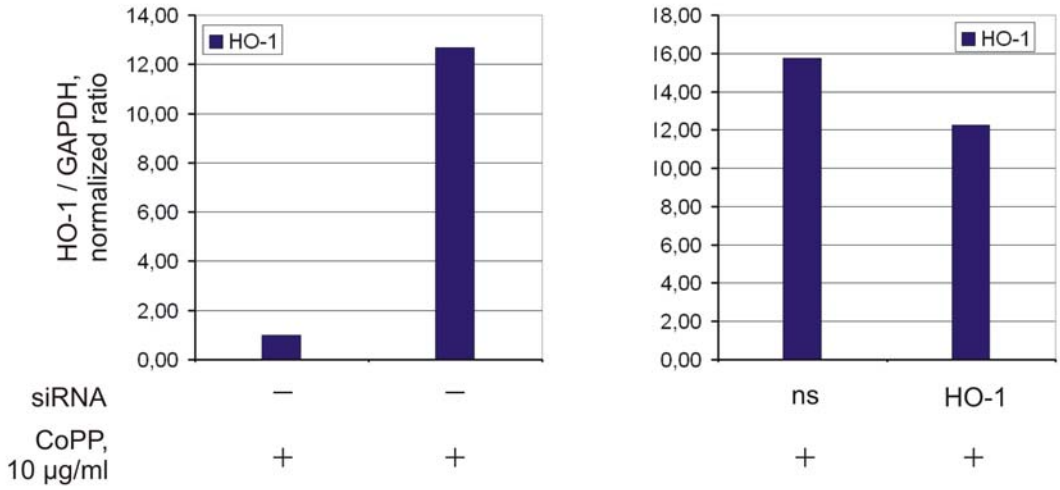
4.2.1 HO-1 mediates antiviral effect of CoPP on HBV replication in cell culture

To confirm that inhibition of HBV replication after CoPP administration is mediated by HO-1 induction and is not merely an antiviral effect of porphyrins, I performed knock-down experiments in HepG2-H1.3 cells using HO-1 specific siRNA or non-silencing siRNA as a control. I transfected HepG2-H1.3 cells directly after plating on collagen-coated dishes, treated the cells with 10 μg/ml CoPP at days 1 and 3 and harvested at day 5 post transfection.

In HepG2-H1.3 cells treated with CoPP, I detected 12.7-fold induction of HO-1 mRNA and 7.5-fold of the HO-1 protein as measured by real-time LightCycler PCR and Western blot analysis, respectively (Fig. 23A and B). After transfection of cells with specific siRNA, I observed a 30% decrease in levels of HO-1 mRNA (Fig. 23A).

Further, Western blot analysis proved that HO-1 specific siRNA inhibited CoPP induced expression of HO-1 protein by 74.1% (Fig. 23B).

A



B

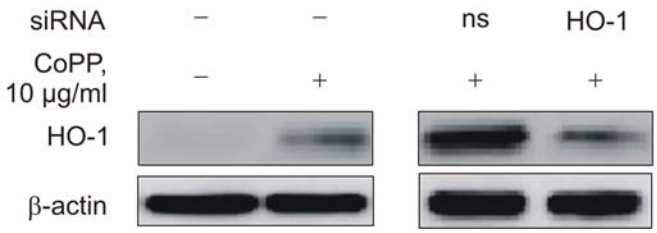


***Fig. 23:* HO-1-specific mRNA abolishes induction of HO-1 expression by CoPP**

HepG2-H1.3 cells were transfected with 5 nM of either HO-1-specific or scrambled siRNA and treated with 10 µg/ml CoPP.

***(A)** 1 µg of total cellular RNA was subjected to LightCycler™ real-time RT-PCR. Normalized expression level of HO-1 relative to GAPDH is given.*

***(B)** 20 µg of total cellular proteins were subjected to SDS-page and transferred to a nitrocellulose membrane. Membrane was stained for HO-1 and β-actin as a loading control.*

Then, I studied whether knock-down of HO-1 reversed inhibition of HBV replication caused by CoPP treatment.

First, I analyzed the effect of HO-1 knock-down on expression of viral RNAs by Northern blot analysis. Upon CoPP treatment, I found a 2.34-fold and a 1.95-fold increase in the expression of 3.5 kb and 2.4 kb HBV RNAs, respectively (Fig. 24A). Since the synthesis of HBV pregenomes is a critical step in the HBV replication cycle, I additionally quantified HBV pgRNA using LightCycler real-time PCR. Consistent

with results obtained by Northern blotting, levels of HBV pgRNA were increased 2.1-fold upon CoPP administration (Fig. 24B). However, when HO-1 was knocked down, levels of HBV RNAs remained unchanged (Fig. 24A&B).

Thus, HO-1 did not affect transcription of HBV RNAs.

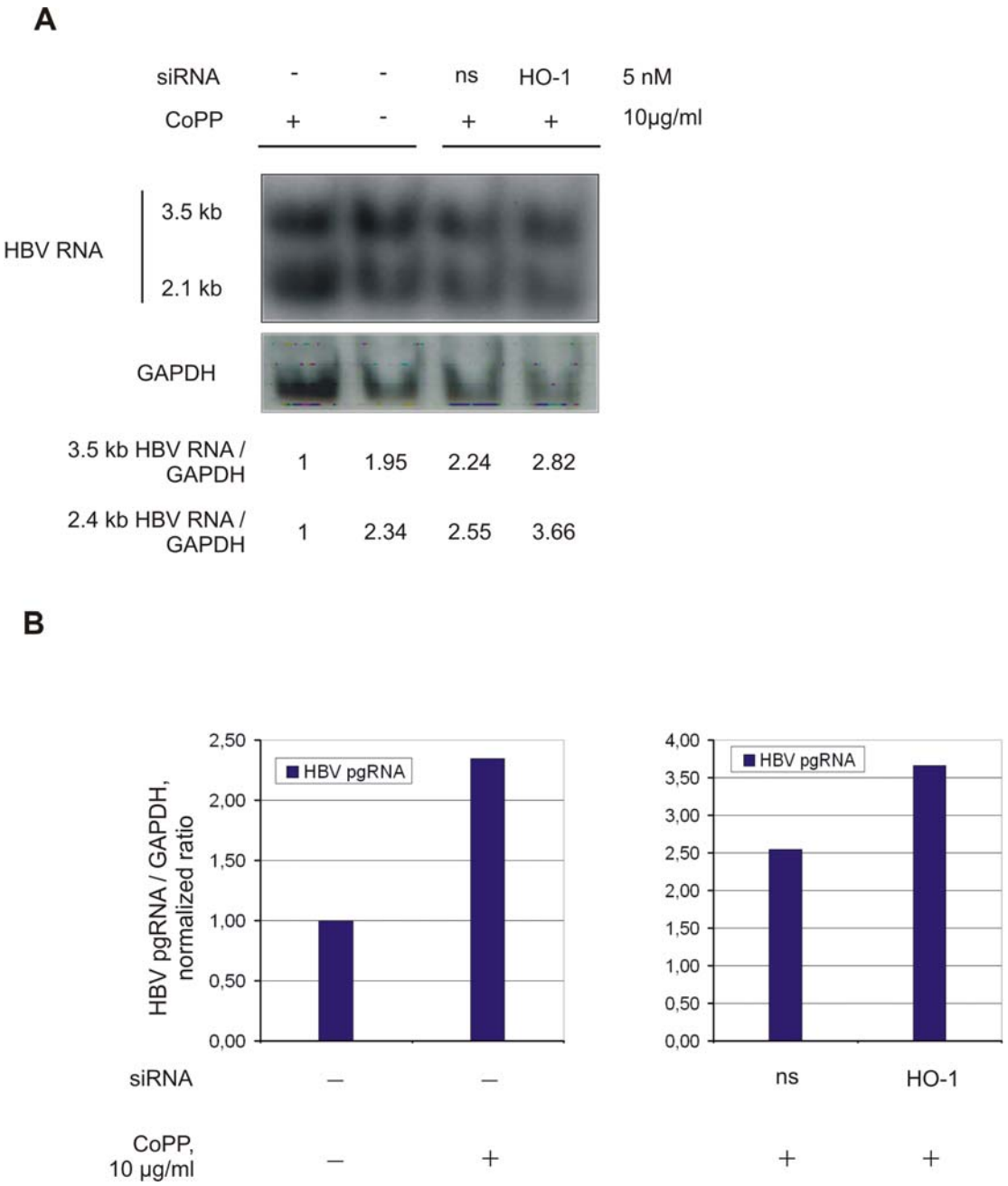


Fig. 24: ***Effects of HO-1 knock-down on expression of HBV RNAs***
HepG2-H1.3 cells were transfected with 5 nM of either HO-1-specific or scrambled siRNA and treated with 10 µg/ml CoPP. ***(A)*** *Northern blot analysis of 15 µg total cellular RNA using a ^{32}P labelled HBV DNA probe. Expression of HBV RNAs was quantified relative to GAPDH.* ***(B)*** *1µg of total cellular RNA was subjected to*

LightCycler™ real-time RT-PCR. Normalized expression level of HBV pgRNA relative to GAPDH is given.

Second, I looked for production of HBV core protein. Levels of HBV core protein were reduced 8.57-fold upon treatment of cells with CoPP (Fig. 25A). HO-1 specific siRNA reversed the reduction of HBV core protein by 27.3% (HO-1 siRNA vs. non-silencing siRNA, Fig. 25A). I also observed a 5.9-fold decrease in levels of HBV L protein upon CoPP treatment (Fig. 25B), which was not restored when HO-1 was knocked down.

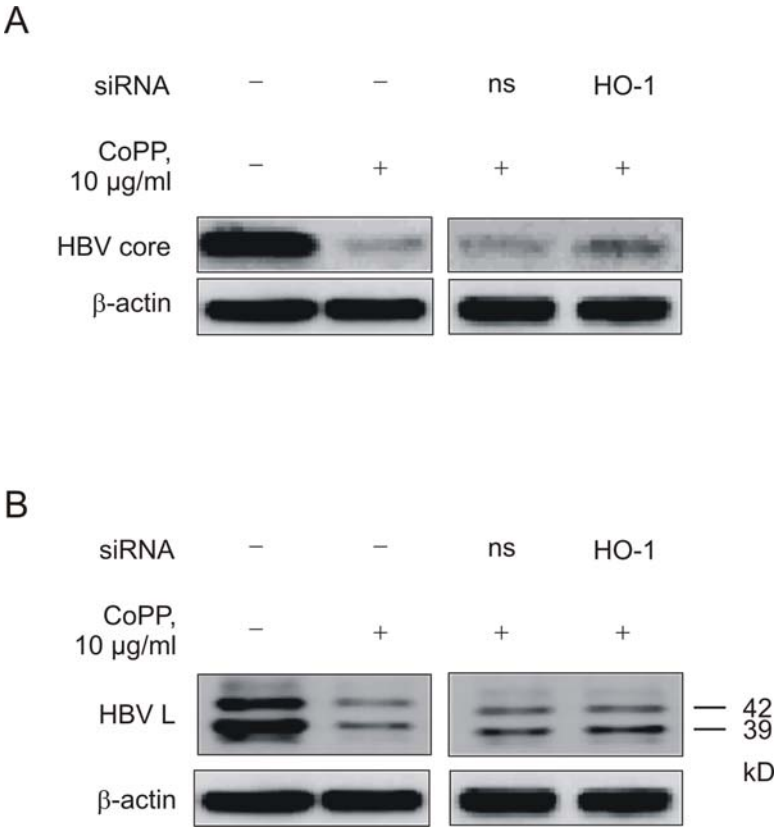


***Fig. 25:* Effects of HO-1 knock-down on expression of HBV proteins**

***(A, B)** 20 µg of total cellular proteins were subjected to SDS-page and transferred to a nitrocellulose membrane. Membrane was stained for HBV core **(A)** or HBV L protein **(B)** and β-actin as a loading control.*

Afterwards, I quantified HBV replicative intermediates relative to HBV integrates following Southern blot analysis (Fig. 26A). This revealed that HO-1 induction by CoPP reduced HBV replication in HepG2-H1.3 cells by 50%. In contrast, synthesis of HBV replicative intermediates recovered upon knock-down of HO-1 (52%).

Finally, I quantified HBV progeny in cell culture media. I found that application of CoPP to the HBV-producing cells decreased the number of HBV DNA in cell culture media by 11.2-fold as measured by LightCycler real-time PCR (Fig. 26B). This effect was reversed in cells transfected with HO-1 specific siRNA (59%) (Fig. 26B).

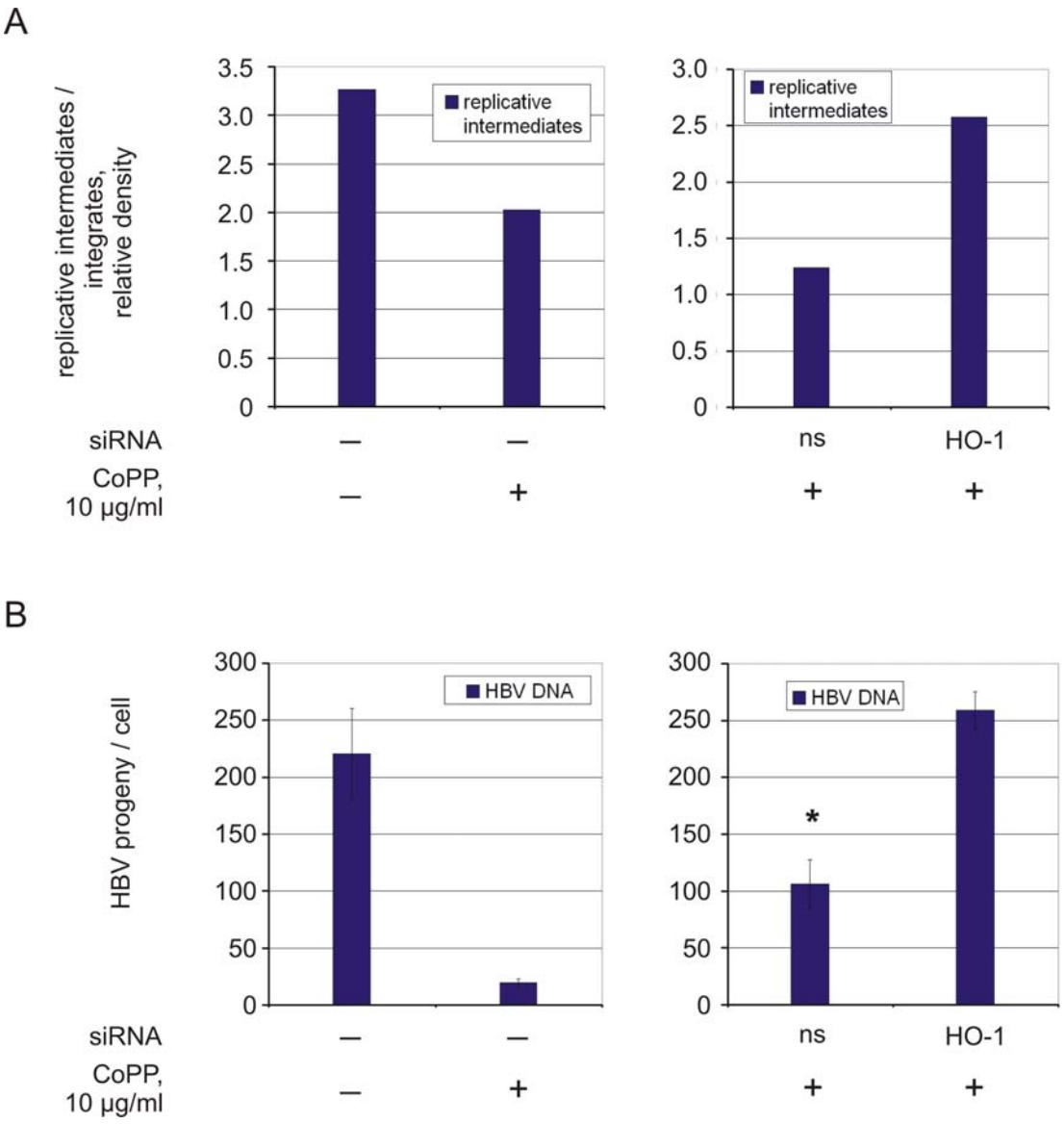


Fig. 26: Effects of HO-1 knock-down on HBV replicative intermediates and progeny HBV

HepG2-H1.3 cells were transfected with 5 nM of either HO-1-specific or scrambled siRNA and treated with 10 µg/ml CoPP.

***(A)** Southern blot analysis of Hind III-digested total cellular DNA using a ^{32}P labelled HBV DNA probe. HBV replicative intermediates were normalized to HBV integrates following phosphoimager quantification.*

***(B)** DNA isolated from cell culture media was subjected to LightCycler™ real-time PCR. HBV DNA copy number per cell is given, (*) $p<0.05$.*

Taken together, HO-1 induction in hepatocytes directly decreases intracellular HBV core protein levels and inhibits HBV replication at the posttranscriptional step.
Since HBV core particles are required to fulfill the pool of cccDNA, I next studied the effect of HO-1 induction on the accumulation of cccDNA.

4.2.2 HO-1 induction reduces HBV cccDNA

HBV cccDNA accumulates and persists as an episome in HBV infected cells, and serves as a viral transcription template in natural infection. I analyzed the effect of HO-1 on HBV cccDNA in HepG2-H1.3 cells, which replicate HBV from a single integrated 1.3-fold overlength HBV genome and establish an HBV cccDNA pool *(Webb & Protzer, unpublished)*. Treatment of HepG2-H1.3 cells with CoPP reduced the amounts of nuclear cccDNA by 90% (Fig. 27A&B). This effect was due to HO-1 induction, since it was reversed by knock-down of HO-1 by specific siRNA.
To elucidate the mechanism by which HBV cccDNA was affected, I treated the cells with either the nucleoside analogue, lamivudine (15 µM), completely blocking reverse transcription of HBV, or CoPP or a combination of both (Fig. 27C). Half-life times of cccDNA were 2.62, 2.59 and 2.53 days, respectively, and thus almost identical under all treatments.
Taken together, these results indicate that HO-1 induction represses HBV replication directly in hepatocytes at a posttranscriptional step, that the block of HBV replication is sufficient to reduce HBV cccDNA levels and that this effect depends on the expression level of HO-1.

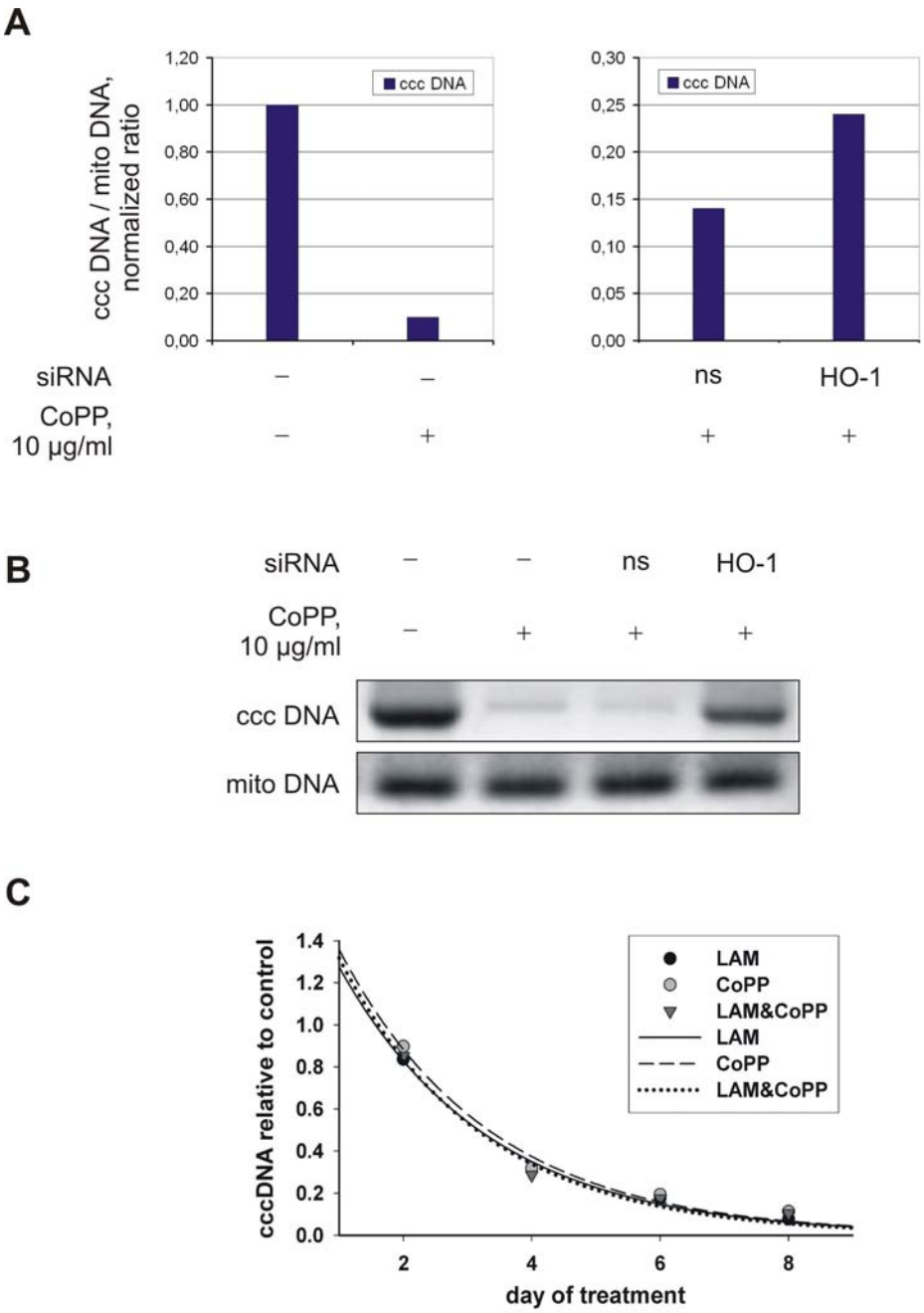


***Fig. 27:* HO-1 suppresses cccDNA amplification in HepG2-H1.3 cells**

cccDNA amounts were compared in HepG2-H1.3 cells transfected with 5 nM of either HO-1-specific or non-specific siRNA and treated with 10 µg/ml CoPP.

***(A)** total cellular DNA was subjected to LightCycler™ real-time RT-PCR. Normalized expression levels of cccDNA relative to mitochondrial DNA are given.*

***(B)** PCR products after PCR amplification of either cccDNA or mitochondrial DNA were visualized using 2% agarose gel.*

***(C)** HepG2-H1.3 cells were seeded on collagen-IV-coated dishes and cultivated as described in materials and methods. After cells reached confluence, they were treated with either 15 µM lamivudin or 10µg/ml CoPP or both and harvested every 2 days during the 10 day period of investigation. CccDNA was quantified as in (A). Regression curves set using SigmaPlot Software are shown.*

4.2.3 Induction of HO-1 during ongoing anti-HBV immune response in the mouse model of acute HBV infection, liver injury, and viral replication

Next, I studied the hepatoprotective and antiviral effects of HO-1 when HO-1 was induced during ongoing anti-HBV immune response in a mouse model of acute HBV infection. I first transduced mice with either AdHBV or an identical construct that contained premature stop codons introduced into all open reading frames of the HBV genome (AdHBVk/o). Second, I treated the animals intraperitoneally with CoPP on day 5 and day 10 after adenoviral transfer of HBV genome, when anti-HBV immune response was ongoing *(John von Freyend M. et. al., submitted)*. Finally, I analyzed mice on day 15 after transduction with AdHBV or AdHBVk/o. As an additional control, I used naïve C57Bl6 mice, which were similarly treated.

I first looked for the induction of HO-1. In C57Bl6, AdHBVk/o and AdHBV mice, CoPP efficiently induced HO-1 mRNA (13.4±0.4, 10.8±2.1 and 14.4±2.5-fold, $p<0.001$) and protein (9.7±0.4, 7.5±2.1 and 8.4±2.5-fold, and $p<0.001$) expression (Fig. 28). HBV-replicating mice (AdHBV) expressed 1.47±0.17-fold more HO-1 ($p<0.001$) mRNA than non-replicating mice (AdHBVk/o or non-infected) (Fig. 28A), which corresponded with 37% increase in protein level (Fig. 28B). Since induction of HO-1 by HBV itself was not observed in hepatoma cells [154], it could in first line be explained by the action of pro-inflammatory cytokines in response to HBV protein production.

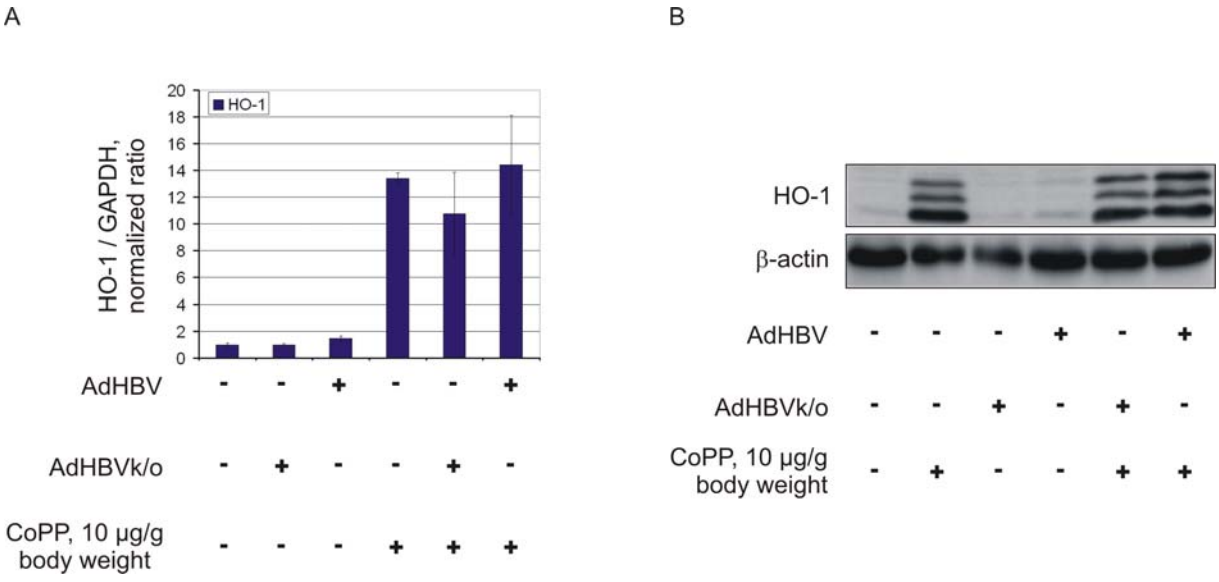


***Fig. 28:* Induction of HO-1 expression in the mouse liver during ongoing HBV-specific immune response**

(A) *1 µg total RNA from mouse livers was subjected to LightCycler™ real-time RT-PCR. Normalized expression levels of HO-1 relative to GAPDH are given.*
(B) *60 µg of total cellular proteins isolated from mouse livers were subjected to SDS-page and transferred to a nitrocellulose membrane. Membranes were stained for HO-1 and β-actin as a loading control.*

I further analysed the effect of HO-1 induction on liver injury.

Protzer et. al., who treated mice with CoPP prior to the transduction of animals with AdHBV, observed that induction of HO-1 significantly decreased ALT activity and improved liver histology. In contrast, in this study, induction of HO-1 during ongoing anti-HBV immune response in mouse livers was associated with a significant increase in liver inflammation as measured by serum ALT activity (Fig. 29). Induction of HO-1 by CoPP in naïve C57Bl6 mice did not cause any liver damage. Also, I did not find major differences in ALT activity between mice injected with AdHBVk/o and treated or not treated with CoPP. Liver injury was aggravated upon HO-1 induction only if mice produced HBV proteins and replicated HBV. When CoPP was not administered, mice transduced with AdHBV tended to have higher ALT activity than animals injected with the control AdHBVk/o vector. Interestingly, when HO-1 was induced, differences in the magnitude of liver injury between HBV-replicating and non-replicating mice became significant. Taken together, induction of HO-1 during ongoing anti-HBV immune response aggravates liver injury.

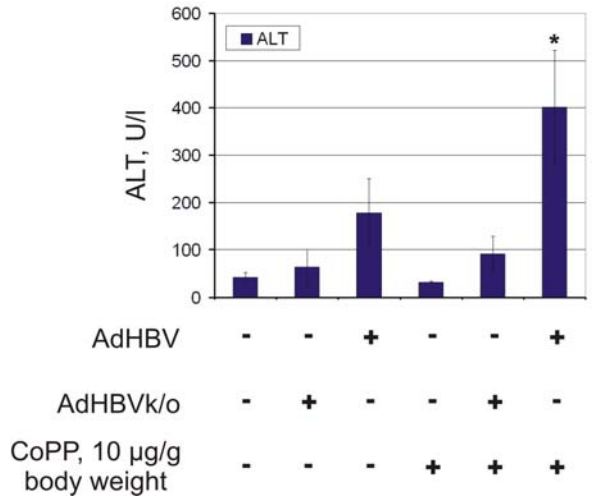


Fig. 29: ***Liver injury after induction of HO-1 expression during ongoing HBV-specific immune response***
Serum ALT activity was measured by Reflovet™ according to the manufacturer's instructions.

Afterwards, I studied whether the antiviral effect of HO-1 was retained in this experimental setting.

Consistent with previous results by *Protzer et. al.*, in mice that were transduced with AdHBV, HBV core protein, virus replication and viremia were markedly reduced (Fig. 30A-C).

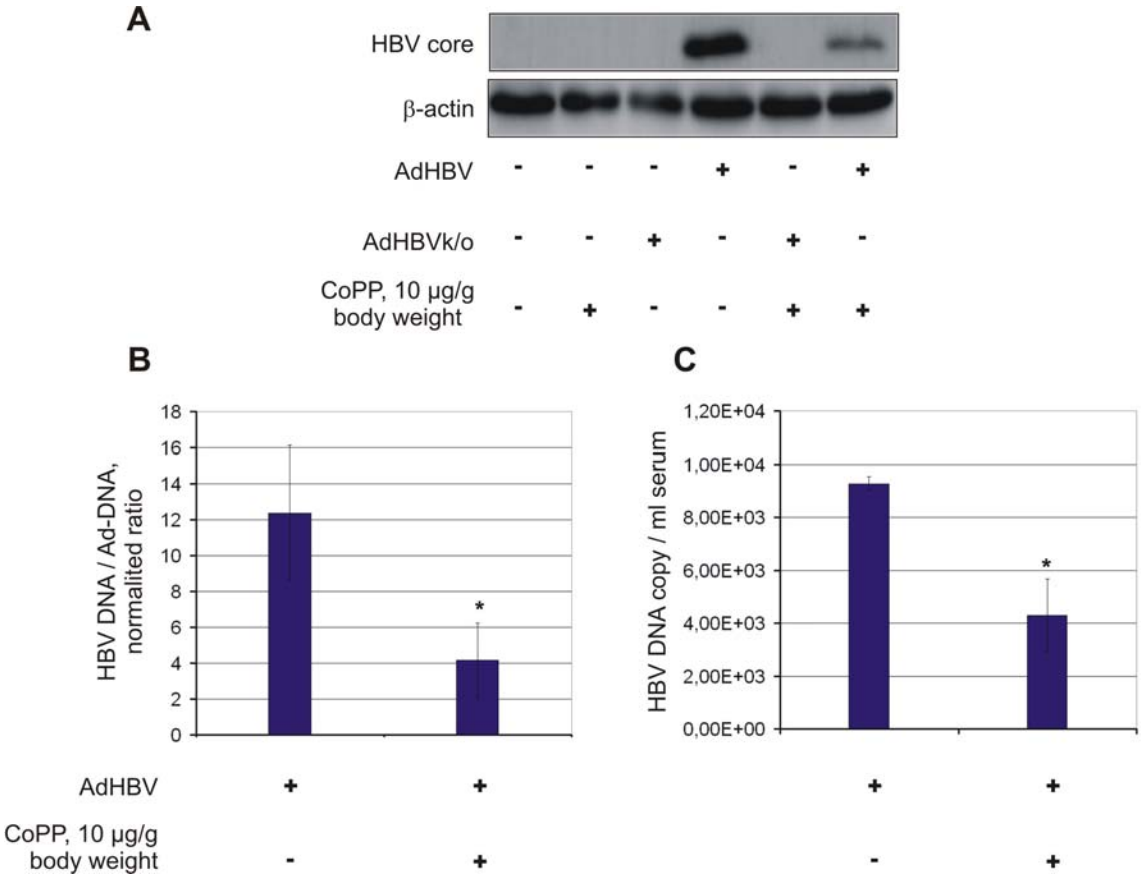


***Fig. 30:* HBV replication after induction of HO-1 during ongoing HBV- specific immune response in the mouse liver**

(A) *60 µg of total proteins from mouse livers were subjected to SDS-page and transferred to a nitrocellulose membrane. Membranes were stained for HBV core protein and β-actin as a loading control.*

(B) *DNA extracted from mouse livers was subjected to LightCycler™ real-time PCR. Normalized expression levels of HBV DNA relative to Ad-DNA are given, (*) p<0.05.*

(C) *DNA isolated from mice sera was subjected to LightCycler™ real-time PCR. HBV DNA copy number per 1 ml serum is given, (*) p<0.05.*

Thus, strong induction of HO-1 during ongoing anti-HBV immune response aggravates liver injury, but still has a pronounced antiviral effect by reducing levels of HBV core protein, decreasing HBV replication, and decreasing progeny HBV release.

Besides hepatocellular factors, extracellular mediators can also affect HBV replication and are therefore important for a detailed understanding of the virus-host interactions.

4.2.4 A decrease in HNF4α and HNF1α levels as well as HO-1 induction mediates the inhibition of HBV replication by IL-6 during the early steps of HBV infection

Interleukin-6 (IL-6) seems to be involved in the pathogenesis of HBV infection because its levels were higher in HBV-infected than HBV-non-infected persons and correlated with the clinical course of infection. Furthermore, the level of IL-6 in serum of HBV-infected patients correlated with a successful response to therapy with IFNα. Recently, *Dr. Hoesel et. al. submitted*, have shown that IL-6, induced upon HBV pattern recognition in mixed PHH cultures, inhibits HBV replication at the transcription level. However, it has not been studied so far, which cellular pathways are involved in the control of HBV replication by this cytokine.

4.2.4.1 IL-6 inhibits HBV transcription and replication

In order to examine the effect of endogenous IL-6 released after contact with HBV, 200 ng/ml of IL-6ab were added to PHH cultures prior to HBV infection. This resulted in an almost two-fold decrease in HBeAg and HBV progeny secretion, which indicates that endogenous IL-6 blocked HBV gene expression and replication (Fig. 31B).

Since the secretion of IL-6 was transient and abolished before virus replication began, recombinant IL-6 (rIL-6) was used to confirm the negative effect of IL-6 on HBV transcription and replication. 15 ng/ml of rIL-6 were added to HBV infected cells on day 1 and 3 post infection (p.i) after the peak amount of endogenous IL-6 in HBV-infected cells was removed by exchange of the culture medium. As a control, rIL-6 was pre-incubated with anti-IL-6 antibodies (1 µg/ml). Cells and supernatants were analyzed on day 5 p.i.

CRP expression indicated proper activation of the IL-6 signaling pathway by rIL-6 (Fig. 31A). In parallel, a 66% decline of HBV pgRNA (Fig. 31 A), a 77% drop in

HBeAg secretion and a greater than 50% reduction of HBV progeny release (Fig. 31 B) was detected.

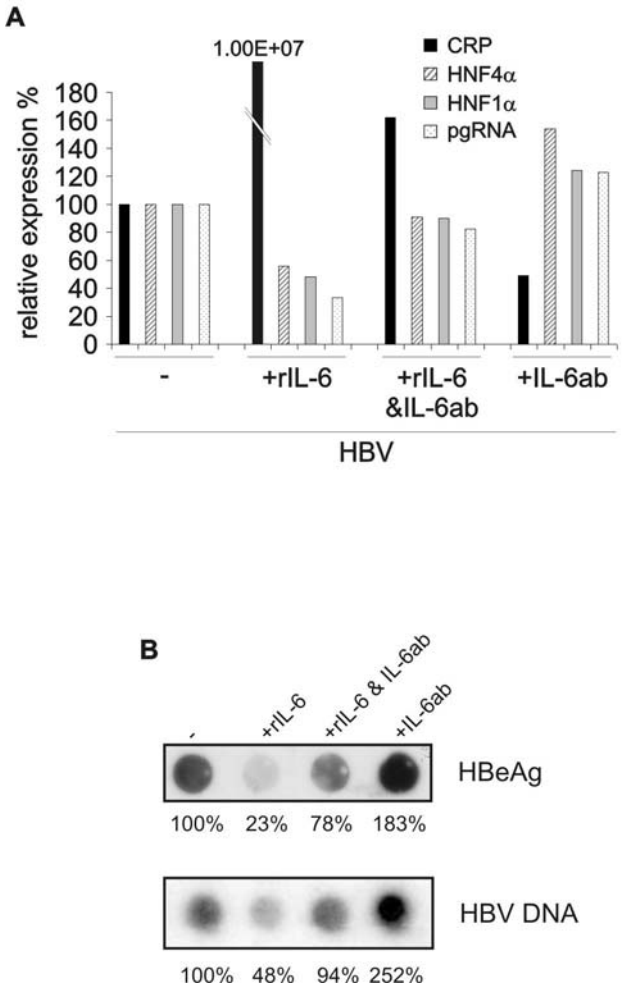


***Fig. 31:* Effect of IL-6 on HBV transcription and replication and on hepatocyte-enriched transcription factors**

(A) *Relative gene expression levels of CRP, HNF4α, HNF1α and HBV pg RNA were determined in HBV-infected hepatocytes by quantitative LightCycler™ real-time RT-PCR. Expression in untreated (-) HBV-infected cells was set to 100%. Recombinant IL-6 (rIL-6) was added to the cells with or and without neutralizing IL-6 antibodies (rIL-6ab). In addition, cells were incubated with rIL-6ab prior to HBV infection.*

(B) *Dot blot analysis of HBeAg using antibody detection (upper panel) and progeny HBV-DNA using a ^{32}P-labelled HBV-DNA probe (lower panel) secreted into cell culture supernatants. Amounts of HBeAg and HBV DNA in untreated HBV-infected PHH were set to 100%. One representative out of three experiments is shown.*
(This figure is kindly provided by Dr. M. Hoesel).

Western blot analyses of cytoplasmic protein fractions revealed that in HBV-infected cells treated with rIL-6, HBV core protein was reduced by 68% (Fig. 32A) and HBV

envelope proteins were diminished by 90% (L), 79% (M) and 60% (S) (Fig 32B). These effects could be reversed by neutralization with anti-IL-6 antibodies.
Thus, IL-6 suppressed HBV gene expression and replication.

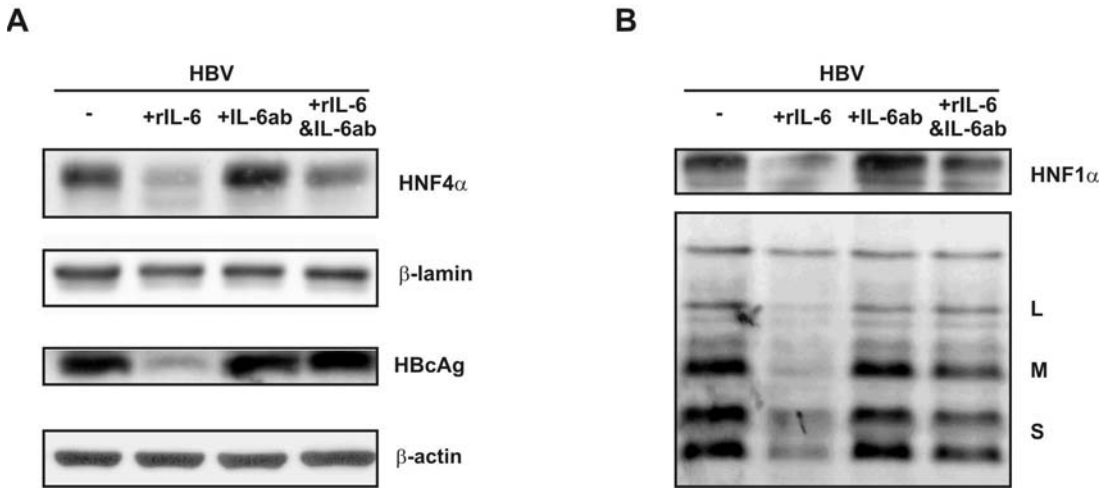


***Fig. 32:* Effect of IL-6 on expression of HBV proteins and hepatocyte-enriched transcription factors**
Western blot analyses of transcription factors HNF4α and HNF1α (30 µg of nuclear proteins) and HBV core and envelope proteins L, M and S (70 µg of cytoplasmic proteins). Expression of β-lamin and β-actin were used as loading controls for nuclear and cytoplasmic proteins, respectively.

4.2.4.2 MAP kinases activated by IL-6 down-regulate HNF 1α and HNF4α expression

Dr. Hoesel et. al. (submitted) has shown that contact with HBV inoculum containing virions as well as secretory HBsAg and HBeAg do not induce interferons or interferon-inducible genes.
I therefore searched for possible mechanisms to explain how IL-6, without interferon induction, inhibited HBV infection. Since the concerted action of the hepatocyte-specific transcription factors HNF1α and HNF4α is essential for HBV gene expression and replication, I examined whether IL-6 may control expression of these transcription factors.
Quantitative real-time RT-PCR analysis revealed a 48% and 56% decrease in HNF1α and HNF4α mRNA expression, respectively, upon rIL-6 stimulation in comparison to non-treated cells. The administration of the neutralizing IL-6ab prior to

HBV infection and treatment with rIL-6 resulted in a 24% and 54% increase of the HNF1α and HNF4α gene expression, respectively (Fig. 31 A). Accordingly, Western blot analyses of nuclear extracts prepared from HBV-infected PHH confirmed a 55%- and a 58% reduction in the amounts of HNF4α and HNF1α protein by treatment with rIL-6, respectively (Fig. 32 A&B). These results showed that both endogenously produced IL-6 and rIL-6 were able to control HNF1α and HNF4α expression in hepatocytes.

Since activated mitogen-activated protein kinases (MAPK) ERK1/2 and JNK have been reported to control HNF4α expression and IL-6-type cytokines may activate members of the MAPK family, I hypothesized that the activation of MAPK by IL-6 suppressed HBV replication.

I first examined whether MAPK family members ERK1/2, p38 and / or JNK were activated after HBV infection in PHH. As shown in Fig. 33A, levels of phosphorylated ERK and JNK, but not p38 were increased upon HBV infection. The activation of ERK and JNK was obviously mediated by IL-6 since pre-treatment of PHH with anti-IL-6 neutralizing antibodies (IL-6ab) abolished activation of ERK and JNK during HBV infection (Fig. 33 B). This was confirmed by treatment with rIL-6, which further activated ERK and JNK (Fig. 33 B), but not p38.

To determine the effect of IL-6-induced MAPK activation on HNF4α and HNF1α expression, I used specific inhibitors of upstream activators of the phosphorylation of MAPK before rIL-6 stimulation of PHH: PD98059 for pERK and SP600125 for pJNK, respectively. Treatment with PD98059 largely reduced the amount of activated ERK, while exposure to SP600125 before rIL-6 treatment reduced the amount of phosphorylated JNK to undetectable levels (Fig. 33 C). Inhibition of JNK activation completely overcame and ERK activation partially overcame rIL-6-mediated down-regulation of HNF4α and HNF1α (Fig. 33 D).

This results showed that in primary hepatocytes, IL-6 activated the MAPK ERK and JNK, which down-regulate expression of HNF4α and HNF1α, and consequently, HBV replication.

I concluded that ERK- and JNK- activation and subsequent down-regulation of the essential transcription factors HNF4α and HNF1α is responsible for the negative control of HBV replication by IL-6.

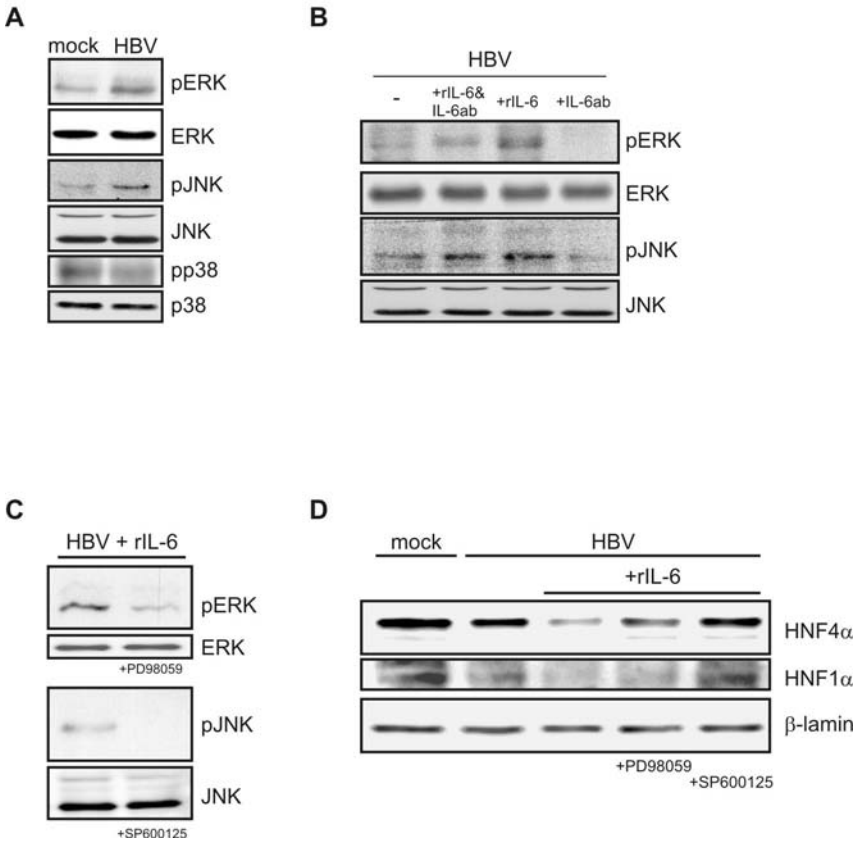


***Fig. 33:* Involvement of MAP kinase pathways in IL-6-mediated down-regulation of HNF1α and HNF4α**

(A) *70 µg of total proteins isolated from mock- or HBV-infected PHH cultures at 24 h p.i. were analyzed by Western blot with antibodies to activated (phosphorylated) ERK, JNK and p38. The respective unphosphorylated forms were used as a loading control.* ***(B)*** *Western blot analysis of pERK, ERK, pJNK and JNK using total proteins (40 µg) isolated from HBV-infected PHH without treatment (-), treated with rIL-6 or rIL6 preincubated with neutralizing anti-IL-6 antibodies (rIL-6&IL6ab) or from cells pre-incubated with IL-6ab prior to HBV infection.* ***(C)*** *Detection of pERK and pJNK in HBV-infected PHH untreated or treated with 50 µM of pERK upstream inhibitor PD98059 or pJNK upstream inhibitor SP600125 for 30 min prior to stimulation with rIL-6.* ***(D)*** *Nuclear proteins (20 µg per lane) were prepared from mock- or HBV-infected cells. Cells were either not treated (-) or stimulated with rIL-6 or preincubated with PD98059 and SP600125 before rIL-6 stimulation. Expression of HNF4α and HNF1α were analysed by Western blot. Expression of β-lamin served as a control.*

4.2.4.3 IL-6 may also inhibit HBV replication by induction of HO-1

IL-6 is also known to induce HO-1. Therefore, I next asked whether HO-1 was induced upon treatment of primary hepatocyte cultures with IL-6 and also played a role in the inhibition of HBV replication (Fig. 34). I found a 2.0-fold increase in protein

levels of HO-1 after HBV was given to the PHH cultures. However, when endogenous IL-6 was blocked by neutralizing antibodies, this effect was abolished. Protein levels of HO-1 were increased 2.4- and 3.5-fold upon addition of rIL-6 to mock cells or HBV-infected hepatocytes, respectively. Conversely, when rIL-6 was given together with neutralizing IL-6 antibodies, this effect was diminished. This implied the possible involvement of HO-1 in IL-6-induced inhibition of HBV replication.

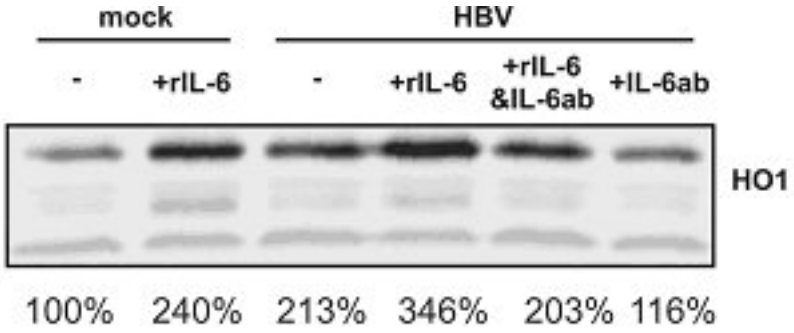


***Fig. 34:* Induction of HO-1 by IL-6**

Western blot analyses of HO-1 (30 µg of total proteins). Expression of β-actin controlled the loading of cytoplasmic proteins. Expression in untreated (-) HBV-infected cells was set to 100%. Recombinant IL-6 (rIL-6) was added to the cells with or without neutralizing IL-6 antibodies rIL-6ab. In addition, cells were incubated with rIL-6ab prior to HBV infection.

Thus, I concluded that IL-6 inhibited HBV replication at the transcriptional level through the activation of JNK and, to a lesser extent, ERK and consequent down-regulation of HNF4α and HNF1α expression. Additionally, inhibition of HBV replication by IL-6 may occur at the posttranscriptional level via induction of HO-1.

5 Discussion

5.1 Expression levels of HNF4α, HNF1α and PGC-1α determine replicative potential of HBV in the developing liver

5.1.1 Significance of the study models to investigate the dependence of HBV replication on hepatocyte differentiation

5.1.1.1 Cells of hepatocyte origin as a study model to investigate the dependence of HBV replication on hepatocyte differentiation

Hepatocyte differentiation is a multi-step process of morphological and functional changes in cells governed by a number of liver-enriched transcription factors [78].

In this study, in cells of hepatocyte origin, I demonstrated that HBV replication is highly dependent on the degree of hepatocyte differentiation.

I compared the efficiency of HBV replication in primary human hepatocytes (PHH) against conventional highly differentiated hepatoma cell lines (HepG2 and Huh7) and a low differentiated hepatocyte cell line (Pop10). Here, I isolated and cultivated PHH according to the protocol established by *Schulze-Bergkamen H et. al.*[155], which enables to maintain primary hepatocytes differentiated and suitable for studying all steps of the HBV replication cycle for up to two weeks in culture. In addition, I followed changes in the efficiency of HBV replication during differentiation of HepaRG cells. Upon differentiation, hepatoma HepaRG cells display hepatocyte-like morphology and functional profile and become infectable by wild-type HBV [156]. Using late (PEPCK, TDO, OATP-C and BSEP) [81, 84, 87, 89], but not early (albumin or ferritin) markers of hepatocyte differentiation, I was able to confirm differentiation of HepaRG cells and to prove that PHH are the most and Pop10 are the least differentiated cells in my study.

Many previous studies focused on the dependence of HBV replication on hepatocyte differentiation analyzed HBV replication in hepatoma cell lines with different genetic backgrounds and degrees of cell differentiation. In those studies, efficiency of HBV replication was not traced during differentiation of the respective hepatoma cell line. To date, no direct comparison of HBV replication efficiency between hepatoma cell lines and primary human hepatocytes has been performed. Using only hepatoma cells, it is not possible to precisely determine to what extent the efficiency of HBV replication depends on hepatocyte differentiation, because all hepatoma cells are by definition transformed and possess altered signaling

pathways. For the same reason, using only hepatoma cells, it is not possible to delineate which hepatocellular factors link HBV replication to hepatocyte differentiation. Furthermore, although cells of hepatocyte origin with different degrees of differentiation were used, none of the previous studies documented their differentiation status, for example, by controlling the expression of hepatocyte-specific differentiation markers. The exact evaluation and monitoring of cell differentiation status during experiments is necessary because it may vary even in primary hepatocytes depending upon the isolation procedure, time of cultivation and cultivation conditions [233]. Hepatocyte dedifferentiation may already be induced by ischemia-reperfusion stress during the isolation process, disruption of the normal tissue architecture as well as adaptation to the *in vitro* environment [233]. Maintenance of differentiated hepatocytes *in vitro* has been achieved by culturing cells in serum-free [234] DMSO-supplemented medium [235, 236, 237] using specific attachment surfaces, such as extracellular matrix and its components [238, 239]. In contrast, when hepatocytes are cultured in serum-supplemented standard tissue culture medium, hepatocyte morphology and production of liver-specific proteins are rapidly lost, the levels of liver-specific mRNA expression steadily drop within 5 days of culture and the transcription rates of liver-specific genes, 24h after plating, are between 1% and 10% of that seen in the liver [235].

Up to now, there is no consensus in defining the term: “differentiated hepatocyte” [80]. Many research groups use albumin, transferrin, α-1-antitrypsin and CK8/18 as markers of hepatocyte differentiation [240, 241, 242]. However, albumin, transferrin, α-1-antitrypsin and CK8/18 are already expressed at early stages of fetal liver development and indicate specification of hepatocyte lineage rather than hepatocyte differentiation. Enzymes involved in various hepatocyte-specific metabolic pathways (i.e. PEPCK or TDO) or markers of hepatocyte polarization (i.e. transporters OATP-C or BSEP), whose expression starts in almost mature hepatocytes either at the late stages of fetal liver development or even during postnatal liver development, can rather be considered as markers of hepatocyte differentiation [81, 84, 87, 89].

I compared efficiency of HBV replication in cells of hepatocyte origin after transduction with adenoviral vectors encoding 1.3-fold overlength HBV genome. I chose this system because adenoviral vectors proved to efficiently deliver target genes into a broad range of cells and provide efficient transcription of HBV genes

from the extrachromosomal template in the context of their own promoters [151, 231]. Using this approach, it is possible to transduce or deliver equal amounts of HBV genome into different cells of hepatocyte origin.

To date, to study the dependence of HBV replication on hepatocyte differentiation, transient transfection of hepatoma cells or stable HBV-producing hepatoma cell lines were used. However, transient transfection of hepatoma cells is unsatisfactory because transfection efficiency is notoriously low, variable and subject to the characteristics of the transfecting vector. Stably transfected cell lines rely on viral integration into the host genome, deviating further from the *in vivo* situation, where HBV genome integration is an exceedingly rare event [243].

5.1.1.2 HBV-transgenic mice as a model to study the replicative potential of HBV during liver development

In this work, I studied replicative potential of HBV during liver development in HBV-transgenic mice possessing one copy of 1.3-fold overlength HBV genome in every cell of their body. The HBV-transgenic mice replicate the virus to a level comparable with those in chronically infected patients [148]. Furthermore, transgenic mice allow for the study of HBV replication independent of early steps of the viral replication cycle (i.e. viral uptake and establishment of the viral genome in the nucleus). Moreover, all stages of liver development are well characterized in the mouse model [72, 78], and mice are easy to handle and maintain.

I showed that HBV replication starts after birth, increases along with hepatocyte maturation and achieves levels seen in adult animals between the second and fourth week of postnatal development. The late onset of HBV replication relied on low levels of viral pregenomes and delayed expression of the HBV core protein during prenatal liver development. While HBV pgRNA was already detected at ED 12.5, the expression of HBV core protein was not observed until ED 18.5 and that of L protein - 0.5 dpn.

The major concern of this study is whether the data about the replicative potential of HBV obtained during liver development in rodents can be translated to liver development in humans.

The comparable stages of liver development in humans and rodents are well described for the embryonic period. According to the data by *Godlewski et. al*, the

status of liver development ED 16.5 in rodents corresponds to the 8th week of gestation in humans [244].

A comparison of late fetal and postnatal stages relies on functional data. The fetal liver at 22nd week of gestation is a major site of fetal hematopoiesis in man, and is at the critical turning point between immigration and emigration of cells of the hematopoietic system. In rodents, these events are dated to the ED 18.5 [78]. The microscopically visible glycogen granules appear in hepatocyte precursors of rodents after ED 18.5 [78] and in hepatocyte precursors of humans after 21st week of pregnancy [245]. It is very likely that hepatocyte differentiation status at ED 18.5 in rodents corresponds to that at the 21st – 22nd week of pregnancy in humans.

So, during early fetal liver development, the maturation of rodent liver is delayed relative to the development of the human liver. However, during the late fetal stages, at birth and early postnatal development, the differentiation status of hepatocytes measured by expression levels of liver enzymes [246] and markers of hepatocyte polarization (i.e. basolateral transporters (NTCP)) [247, 248, 89] closely resembles that in rodents.

According to these data, the expression of HBV core protein could start as early as the 21st – 22nd week of pregnancy in humans. In addition, the starting point of HBV replication and the formation of infectious viral particles could be detected in the early postnatal period of human development.

In the HBV-transgenic mouse model, no conclusion can be made about the infectious potential of the virus in the developing liver. To understand at what stage of liver development HBV can infect hepatocytes, further experiments in an infectious model are required, for example using woodchucks and woodchuck hepatitis B virus (WHV).

There are two studies on infection of human fetal hepatocytes with HBV. *Lin et. al* found that human fetal liver cells isolated from a 6-week old fetus and cultivated for a week can be infected with HBV and support viral replication [249]. However, human fetal liver cells were cultivated in serum-free medium with epidermal growth factor, basic fibroblast growth factor and DMSO, which led to hepatocyte differentiation. Indeed, upon a week of cultivation, cells started to store glycogen, thus displaying a function of late stage hepatocyte maturation. T *Ochiya et. al.* isolated human fetal hepatocytes from fetal livers at 20-24 weeks of gestation and demonstrated infection of cells with HBV and viral replication after a week of

cultivation [250]. When permissiveness of cells for HBV was analyzed, the human fetal hepatocytes displayed functional (glycogen storage; expression of glucose-6-phosphatase and γ-glutamyl transpeptidase) and morphological (mononuclear, polyhedral, arranged in trabeculae) features attributed again to the late stages of liver development. Since human fetal liver cells in both studies underwent differentiation before infection with HBV, hardly any conclusion can be made about the replicative potential of the virus at the stage of cell isolation.

5.1.2 Level of pgRNA synthesis determines the efficiency of HBV replication in hepatocytes

I identified transcription of the HBV pgRNA to be the limiting step for the efficiency of HBV replication *in vitro*, in cells of hepatocyte origin, as well as *in vivo*, in HBV-transgenic mice.

HBV pgRNA serves as the template for reverse transcription and for translation of the viral core and polymerase proteins.

In my *in vitro* experiments, levels of HBV pgRNA and efficiency of HBV replication were the highest in the most differentiated PHH and undetectable in the least differentiated Pop10 cells, as well as strongly increased upon differentiation of HepaRG cells. Expression of HBV core protein displayed the same tendency.

In vivo, in HBV 1.3 transgenic mice, *Guidotti et al.* had previously shown that HBV 3.5 kb RNA containing viral pregenomes was first detectable 2 weeks postnatally by Northern blot analysis [148]. Using highly sensitive qPCR, I show here that HBV pregenomes are already transcribed at very low levels at ED 12.5, the earliest time point analyzed, and steadily increase thereafter. However, only at 1 wpn expression levels of HBV pgRNA reached those in adult livers, matching the starting point of significant HBV replication.

Since essential steps of HBV replication take place in the viral nucleocapsid, components of the nucleocapsid such as HBV core protein and the viral polymerase are crucial for HBV replication. Both proteins are translated from the HBV pgRNA. The expression of HBV core protein was observed first on ED 18.5, when levels of HBV pgRNA had risen further. Failure to detect HBV core protein at earlier time points, when HBV pgRNA, its translation template, is expressed, could result from the detection limit of the Western blot. I could not exclude false-negative results;

although, I used 60-80 μg of total cellular proteins per sample and used highly sensitive WestDura and WestFemto (see materials and methods) development substrates, which allow for the detection of as little as 1 fg of a given protein. Therefore, in addition to Western blotting, I stained liver sections and cytospins for HBV core protein. Neither method revealed expression of HBV core protein until ED 18.5. Thus, I consider production of HBV core protein to only start on ED 18.5. During further liver development, HBV core protein levels continuously increased. Though levels of HBV pgRNA equaled those in adult livers at 1 wpn, intracellular amounts of HBV core protein were lower than in adult liver even at 4 wpn because HBV core protein tends to aggregate in hepatocyte nuclei from where it can not reenter the replication cycle [148].

Consistent with the continuous increase in HBV pgRNA and HBV core protein levels, the amount of the liver HBV DNA significantly increased over baseline during the first postnatal week, indicating the onset of HBV replication. Up to two weeks postnatally, mice with lower HBV pgRNA levels still have amounts of liver HBV DNA below the baseline. Conversely, mice with higher HBV pgRNA levels start to replicate the virus earlier. These results are consistent with data of *Guidotti et. al.*, who proposed that the expression threshold for HBV pgRNA, HBV core protein and viral polymerase must be reached before HBV replication can occur [148].

There are three possible options for higher levels of HBV pgRNA in differentiated hepatocytes: (1) increased transcription of HBV pgRNA; (2) increased stability of HBVpgRNA; (3) both.

The transcription of pregenomic RNA is controlled by the HBV precore/core promoter, whose activity was reported to depend on hepatocyte differentiation [135]. Therefore, an increase in HBV pgRNA transcription can, to a large extent, explain higher levels of viral pregenomes and HBV core protein in differentiated hepatocytes. Also, the activity of preS1/S-promoter in the differentiated hepatoma cell lines was approximately 5 to 90 times greater than that observed in the undifferentiated or non-hepatocyte cell lines, indicating that the activity of this promoter is highly specific for differentiation state and cell type [135]. Consistent with these data, the expression of HBV L protein in my experiments was also highly dependent on hepatocyte differentiation *in vitro* as well as *in vivo*. Although HBV L protein is required for HBV particle formation, it is dispensable for intracellular HBV replication.

So, HBV- transgenic mice lacking HBV L protein replicate HBV at the same level or even more efficiently than mice expressing HBV L protein [251].

Heise T et. al. identified HBV RNA-binding liver nuclear proteins (p45, p39, and p26) that bind to a 91-bp element located at the 5' end of a previously defined posttranscriptional regulatory element (PRE) and affect the stability of HBV pgRNA [252]. Later, p45, p39, and p26 proteins were attributed to the full-length La-antigen and its proteolytic cleavage products [253]. I can not exclude that expression of La-antigen or its proteolytic cleavage products or any other cellular proteins binding to the PRE is affected by hepatocyte maturation and regulates the abundance of HBV pgRNA transcripts in hepatocyte.

A number of hepatocyte-enriched transcription factors: HNF4α, HNF3α/β/γ, C/EBPα/β in concert with their co-factors are known to bind HBV precore/core and preS1/S promoters [254] and could therefore link HBV replication to hepatocyte differentiation and be responsible for the onset and the dynamics of HBV replication during liver development.

5.1.3 A concerted action of HNF4α and HNF1α determines the transcription of HBV pregenome and efficiency of HBV replication in dependence on hepatocyte differentiation state

My experiments show that among all hepatocyte-enriched transcription factors studied, only HNF4α, the central regulator of hepatocyte differentiation, proved essential in controlling intracellular HBV replication. HNF4α regulated transcription of the HBV pregenome as a function of the hepatocellular differentiation state. *Tang et al.* had reported earlier that a concerted action of nuclear hormone receptors including HNF4α is needed to induce transcription of HBV pgRNA and replication of HBV in non-hepatocytes [138]. The authors have also shown that the presence of HNF4α alone is sufficient to enable efficient HBV replication. In this study, increasing amounts of plasmid encoding HNF4α were co-transfected into non-hepatocytes together with a vector containing an HBV genome. Therefore, the results may in part be explained by the limited transfection efficiency. Also, non-hepatocytes might lack a number of co-factors of HNF4α required for its transcriptional activity, e.g. SRC-1, GRIP1, p300 [255], PGC-1α [139, 256]. In cells of hepatocyte origin, where hepatocyte-enriched transcription factors are constitutively expressed, I found constant levels of

nuclear hormone receptor PGC1α, apparently sufficient to serve as a cofactor for HNF4α. In contrast, I found a strong positive correlation between intracellular amounts of HNF4α and efficiency of viral replication. COUP-TF competing with HNF4α for the same binding site [137, 140] was present in all cells at constant levels. This explains why a threshold of HNF4α expression had to be reached above which HBV replication depended on the level of HNF4α.

HBV replication rises when HBV-producing cells are kept under differentiating conditions [158]: plating onto extracellular matrix, culture medium containing dexamethasone and DMSO but low FCS ([162] and Fig. 3). Dexamethasone enhances HNF4α and HNF1α expression via an upstream steroid responsive element [257]. DMSO induces cellular differentiation by a so far unknown mechanism and enhances HBV replication [156, 161, 162]. Furthermore, it regulates histone acetylation and methylation [258], which may render target sites in the viral genome accessible to hepatocyte nuclear factors.

Although HNF4α was the key player in regulating transcription of the HBV pregenome in my experiments, knock-down of HNF1α also influenced HBV replication and progeny virus release. Since HNF1α is essential for expression of the viral envelope protein L [259], it was expected to control release of viral particles from infected cells. Its influence on viral pgRNA transcription and replication was unexpected since neither the overlapping HBV precore/core-promoter / enhancer II nor the upstream enhancer I, which control pgRNA transcription, contain HNF1α binding sites unless they are mutated [260].

Since HNF1α is essentially involved in the control of HNF4α expression [112, 257], I speculated that its knock-down diminished HBV replication by affecting the transcription of HNF4α. Indeed, I observed a substantial decrease in the amounts of HNF4α at day 3 and 5 after knock-down of HNF1α, coincidentally with down-regulation of HBV pgRNA transcription during HNF1α knock-down.

However, in HBV-transgenic mice transcription of the HBV pregenome was not measurably affected by knock-out of HNF1α and intracellular HBV replication was even slightly increased [251]. Since the effect of HNF1α on expression of HNF4α as well as on HBV replication ceased after five days in my experiments, I suppose that hepatocytes compensate for the lack of HNF1α.

Guidotti et al. reported that HBV replication per cell remained constant after partial hepatectomy in livers of HBV-transgenic mice [261]. This does not argue against a strong dependence of HBV replication on a highly differentiated hepatocyte, because liver regeneration after partial hepatectomy takes place by proliferation of highly differentiated hepatocytes without shortening of the G1-phase [262] or reduction of HNF4α expression levels [263].

I observed a significant positive correlation between levels of HNF4α and HBV core protein or pgRNA in tumor-peritumor tissues of patients with HCC and chronic hepatitis B. However, I did not see significant differences between tumor and peritumor tissue of the same patient. The first possible explanation may relate to the significant inter-individual variability in the expression of HNF4α, HBV core protein or pgRNA and the relatively low number of tumor and peritumor samples studied. The second possible explanation relies on the status of the peritumor, which was highly fibrotic or even cirrhotic in most samples. The progression of liver fibrosis has been shown to be accompanied by a drop in intracellular HNF4α levels [264].

5.1.4 HNF4α and HNF1α may additionally influence HBV replication by controlling cell metabolism and polarization

Although I clearly demonstrated that HNF4α in concert with HNF1α link transcription of HBV pgRNA and HBV replication to hepatocyte differentiation, I cannot exclude any additional influences of hepatocyte metabolic functions and cell polarization controlled by these transcription factors [112, 121, 265]. It has been only recently pinpointed that HNF4α is the key regulator of morphological and functional differentiation of hepatocytes, essential for metabolic function, formation of cell-cell contacts [120] and formation of a polarized hepatic epithelium [121]. HNF1α primarily regulates hepatocyte polarization [108]. Accordingly, I found that HNF4α and HNF1α knock-down affected expression of bile acid transporters OATP-C and BSEP, which are only expressed in highly differentiated polarized hepatocytes. *Funk et al.* demonstrated the importance of cell polarity for egress of DHBV particles into intercellular compartments, which restricts their diffusion and favors transmission of virus to adjacent cells [147]. *Bhat, P et. al.* indicated that functional polarity of hepatocytes is required for efficient export of infectious HBV particles [243].

I also showed that knock-down of HNF4α and HNF1α down-regulated expression of SREBP-2, PEPCK and TDO, which indicate high metabolic activity of hepatocytes. In this context, *Bhat, P et. al.* demonstrated that loss of metabolic function negatively affects virus export in both polarized and non-polarized hepatocytes [243].

Thus, I suggest that HNF4α in concert with HNF1α influence the efficiency of HBV replication directly, by regulating the expression of HBV pregenomes and indirectly, by controlling the metabolism and polarization of hepatocytes.

5.1.5 HNF3γ is not an absolute requirement for efficient HBV replication

In the initial experiments, I found a correlation between the efficiency of HBV replication and the expression levels of HNF3γ. Levels of HNF3γ were highest in the PHH, lowest in the least differentiated Pop10 cells and increased upon differentiation of HepaRG cells. Also, levels of HNF3γ increased in parallel to HBV pgRNA and core and L proteins during differentiation of stable HBV-producing cell lines. However, after knock-down of this transcription factor, no inhibition was found, but rather I observed an increase in transcription of HBV pgRNA and production of HBV progeny. Since all Foxa proteins share significant homology in their structure and binding sites [93], I supposed that HNF3α and HNF3β could have compensated for the lack of HNF3γ. In fact, after knock-down of HNF3γ, I observed an increase in the expression of HNF3α and HNF3β mRNA.

5.1.6 HNF4α, HNF1α and PGC-1α and replicative potential of HBV in the developing liver

During liver development in HBV-trangenic mice, I confirmed that expression levels of HNF4α and HNF1α correlated closely with those of HBV pgRNA, HBV core and HBV L proteins.

Besides HNF4α and HNF1α, the markers of HBV replication in HBV-transgenic mice also closely correlated with levels of PGC-1α, an important co-factor of HNF4α [139]. High expression levels, rather than the presence of PGC-1α alone, are necessary for efficient expression of genes regulated by HNF4α and HNF6α [98].

Up to ED 18.5, expression of COUP-TF dominated that of HNF4α, the competitor for its binding site. This might explain, why the amount of pregenomic RNA remained low before birth. I suggest that an increase in the expression level of HNF4α is responsible for high-level transcription of HBV pregenomes and onset of HBV replication, and in concert with those of HNF1α and PGC-1α, for morphological and metabolic differentiation of hepatocytes required for formation and secretion of HBV virions.

Using immunostaining of murine tissue samples, I observed cytoplasmic expression of HBV core protein predominantly in hepatocytes of the pericentral area of hepatic lobules, which was consistent with observations by *Guidotti et. al.* [148]. Using *in situ* hybridization analysis with antisense probes, *Guidotti et. al.* detected HBV RNA in virtually all hepatocytes, whereas expression of the HBV pgRNA containing HBV pregenomes and translational template for core and polymerase proteins was stronger in the centrilobular region [148].

Zonal gene expression in hepatic lobules is reported to be primarily regulated at the level of transcription. Transcription regulators involved in maintenance of hepatic zonal heterogeneity have not been studied in detail so far [54].To date, only the role of Wnt/beta-catenin and Apc for pericentral and periportal gene regulation in the adult liver has been demonstrated [266]. Besides Wnt/beta-catenin and Apc, HNF4α may also contribute to the hepatic zonal nature of gene expression. Using in-situ hybridization and Northern blotting, *Nagy et. al.* [267] and *Lindros et. al.* [268] found somewhat stronger staining of HNF4α and significantly higher levels of mRNA of this transcription factor in the perivenous region. The authors suggest that HNF4α could modulate but not govern the zonal expression of liver genes in the mature liver. Later, *Stanulovic et. al.* showed that HNF4α suppresses the expression of pericentral proteins in periportal hepatocytes, possibly via an HDAC1-mediated mechanism [269].

Higher expression levels of HNF4α in the pericentral compared to the periportal areas of the hepatic lobule may be responsible for high-level expression of HBV pgRNA and core protein perivenously and explain the fact that HBV replication predominantly occurs in the cytoplasm of pericentral hepatocytes. Further studies are necessary to confirm co-localization of HNF4α, HBV pgRNA and core protein in the hepatic lobule.

5.1.7 Hepatitis B X protein and replicative potential of HBV in the developing liver

HBV X (HBx), a small protein of 154 amino acids encoded by HBV [37], is usually considered to increase transcription of HBV genes and viral replication. In fact, it interacts with components of the transcriptional machinery [270], influences the activity of some DNA cis elements and binds directly to some transcriptional activators including the leucine zipper family, p53 and Smad4 [271].

In this work, I investigated the replicative potential of HBV in the developing liver using HBV-transgenic mice lacking HBx expression (HBV xfs) because they showed higher HBV DNA levels. To exclude false-negative results due to the absence of HBx expression in the experimental system, I repeated the relevant time points with HBV-transgenic mice expressing wild-type HBV including HBx (HBV 1.3). I did not observe any stimulatory effect of HBx on the replicative potential of the virus in the developing liver. Moreover, in my study, HBV-deficient mice expressed higher levels of HBV pgRNA than animals producing HBx.

The data on the role of HBx protein in the HBV replication cycle remain controversial and largely depend on the model of HBV infection used.

Tang et. al. [271] and *Keasler et. al.* [52] found a strong reduction in HBV replication when HepG2 cells were transfected with a X-deficient HBV replicon compared to a wild-type one. In addition, both groups showed that ectopically expressed HBx restores HBV replication in the X-deficient replicon to the level of the wild-type one. Furthermore, *Tang et. al.* identified a region of HBx critical for stimulation of HBV replication and at the same time responsible for co-activation of HBV pgRNA transcription [271]. These results indicate that stimulation of virus replication by HBx protein occurs at the transcriptional level.

However, *Blum et. al.* [272] did not show any major differences in the efficiency of HBV replication from wild-type or X-defective HBV DNA in Huh7 cells or rat hepatocytes. Since there were no major differences in HBV DNA constructs used by *Blum et. al.* [272] and others, the discrepancy may be due to the use of different cell culture systems. HBx protein does not possess any DNA-binding domains, therefore it functions as a co-activator. Hence, Huh7 cells might lack factors being recruited by HBx in HepG2 cells to activate transcription.

Other studies found no stimulatory effect of HBx on the transcription of viral genes and attribute the increase of HBV replication by HBx to the activation of intracellular signaling pathways via an increase of cytosolic calcium levels and Src kinases phosphorylation [273] or inhibition of cellular proteosomes [51].

In vivo data on the stimulatory role of HBx on HBV replication also seem to be contradictory. HBx has been shown to be necessary for establishment of viral infection in woodchucks, since productive WHV infection was observed only in woodchucks injected with the wild-type but not with the ORF-X mutated WHV DNA [274, 275]. HBx seemed to increase HBV replication efficiency after breeding X-deficient with X-expressing HBV-transgenic mice [276] and when an HBx-coding plasmid was co-introduced with an X-deficient HBV plasmid into mice by a hydrodynamic injection [52]. In contrast, *Reifenberg, K et. al.* did not report any decrease in HBV replication and virion export in X-deficient compared to wild-type HBV-transgenic mice [277].

To rule out the possibility that HBV xfs mice in my study have more integrates of the HBV genome as HBV 1.3 mice, I performed real-time PCR with total tail DNA and quantified HBV DNA relative to HPRT. Since HBV does not replicate in mouse tail tissues, I detected only integrates of HBV genome. When I quantified HBV DNA relative to HPRT, I excluded any influence of differences in the quality and amounts of starting material on the final results. I did not find any differences in the amounts of HBV genome integrates between the mouse strains and therefore concluded that both lines carry a single integrate of 1.3 overlength HBV genome [148].

On the other hand, my results could be explained by the difference in the integration sites of HBV genome between the mouse strains.

In adult mice, even within the same mouse strain, I observed pronounced differences in the efficiency of HBV replication. Other studies with HBV wild-type and X-deficient transgenic mice [276] also indicated a large variation of circulating HBV titers among different mouse lines carrying the same transgene, be it the wild-type or the mutated HBV genomes. This suggests that the effect of HBx on viral replication cannot be determined by simply comparing the serum viral titers between different transgenic mouse lines.

I also speculate that the mouse may be the wrong model to study the effect of HBx on HBV replication. HBV-transgenic mice are known not to establish cccDNA [148]. CccDNA, the transcriptional template of HBV during natural HBV

infection, accumulates as a stable episome in the nucleus and is organized into minichromosomes by histone and nonhistone proteins. Recently, *Levrero* and co-workers showed that HBV replication is regulated by the acetylation status of the cccDNA-bound H3/H4 histones [278]. They found that class I histone deacetylase inhibitors induced an evident increase of both cccDNA-bound acetylated H4 and HBV replication. Conversely, histone hypoacetylation and histone deacetylase 1 recruitment onto the cccDNA in liver tissue correlated with low HBV viremia in hepatitis B patients. HBx is reported to bind to DDB1, which is part of the STAGA histone acetylase complex [279]. The interactions between HBx and DDB1 might be involved in the increase of HBV transcription and replication by HBx, since the HBx mutant, defective in DDB1-binding, could not complement in trans. Moreover, the knock-down of DDB1 reduced HBV transcription and replication from the wild-type HBV replicon. Indeed, absence or minimal transcription of cccDNA in X-deficient woodchucks would explain why they cannot establish productive WHV infection. The fact that a stimulatory effect of HBx protein on HBV replication can be observed in mice after a hydrodynamic injection does not argue against this hypothesis, since injected plasmid encoding for HBV DNA can behave like cccDNA. However, the stimulatory effect of HBx protein on HBV replication was also observed in 18-week old transgenic mice [276]. Here, age-related changes in the chromatin activity might explain the requirement of HBx to stimulate transcription from integrated HBV genome.

In this study, I did not observe any stimulatory effect of HBx on replicative potential of the virus in the developing liver. Therefore, the late onset of HBV replication in HBx-deficient transgenic mice as well as the slow increase in the efficiency of HBV replication during liver development do not result from the absence of HBx, but can rather be explained by the strong dependence of HBV replication efficiency on expression levels of HNF4α, HNF1α and PGC-1α and therefore hepatocyte differentiation.

5.1.8 Possible clinical application of the obtained data

The results of this study provide new insights into virus-host cell interaction that will be helpful for the generation of new models of HBV infection, for the

development of therapeutics against HBV and prophylaxis against intrauterine infection with the virus.

Since HNF4α and HNF1α are key regulators of the functional and morphological phenotype of hepatocytes, down-regulation of these transcription factors as a therapeutic approach does not seem feasible. However, recently, helioxanthin analogue 8-1 has been described to suppress HBV replication by decreasing HNF4α levels in virus-harboring cells without any signs of toxicity [280].

Furthermore, the results of this study may be taken into consideration when prescribing certain drugs such as phenobarbital [281], which increase nuclear expression of HNF4α to patients with hepatitis B.

The information about the replicative potential of HBV in the developing liver could contribute to the development of prophylaxis against infection with HBV *in utero*. As it has already been discussed, the transfer of my data onto HBV replication during human liver development should be made with caution. While comparable stages of liver development are well described for the embryonic period [244], a comparison of later fetal and postnatal stages relies on functional data, showing that differential expression levels of liver enzymes during human liver development also closely resemble those in rodents [246].

While my studies do not allow for an analysis of the dependence of infection on the hepatocyte differentiation state or any potential immune response, my studies allow me to conclude, that if *in utero* infection occurs, it will be hard to detect since detection relies on expression of viral antigens and/or on virus replication.

Also, the late start of replication in the developing liver might explain why the active-passive immunoprophylaxis after birth protects infants at high-risk from chronic viral infection or viral hepatitis B [20], though it cannot prevent transplacental transmission and infections occurring prenatally.

Currently, two approaches for the prevention of HBV transmission *in utero* are being much discussed: passive vaccination by administration of anti-HBs IgG or treatment with nucleoside analogues. *Li et. al.* [282] and *Zonneveld et. al.* [283] strongly suggest that the administration of lamivudine to mothers with high serum HBV DNA concentrations in the third trimester of pregnancy was more effective than passive and active immunization alone in reducing the risk of mother to child transmission. The application of lamivudine is hampered by the fact that lamivudine treatment has not been adequately evaluated in humans in terms of birth defects. It is generally

considered that lamivudine could be used if the benefit of treating during pregnancy outweighs potential risks to the mother or fetus [11]. The most recent data suggests that the rate of birth defects when using lamivudine does not exceed that in the general population [284]. Currently, lamivudine and zidovudine are recommended for HIV-1 infected women during pregnancy. My data show that HBV starts to replicate only after birth. Therefore, for prophylaxis of intrauterine HBV infection, application of lamivudine to mothers with high viremia can be considered in the last treatment of pregnancy in order to decrease maternal viral replication and therefore the probability of perinatal infection of a child. The problem of antiviral resistance, however, makes the use of lamivudine monotherapy problematic, even for a short time. A potential complication for the mother is that flares of hepatitis B can occur if nucleoside analogue therapy is stopped after childbirth [11].

The purpose of anti-HBs IgG treatment is to prevent entry of HBV into hepatocytes. In a number of studies, application of anti-HBV IgG in the last trimester of pregnancy was efficient in the prophylaxis of intrauterine HBV transmission independent of the viral load and HBeAg status of mothers [285, 286]. The application of anti-HBV IgG significantly decreased rates of vertical HBV transmission from HBeAg-positive mothers to their children. Although anti-HBV IgG did not significantly reduce vertical transmission of HBV in HBeAg-negative mothers, it was effective in improving the neonatal immune response to hepatitis B vaccine.
However, lamivudine [33] as well as anti-HBV IgG [287] might not prevent perinatal transmission of HBV infection in every newborn .

The failure to prevent perinatal HBV infection in every high-risk newborn using active-passive vaccination or by additional administration of lamivudine or anti-HBs-IgG in the last trimester of pregnancy does not argue against my data that HBV replication starts after birth. These failures could rather be explained by the fact that HBV can cross placenta in the first trimester of pregnancy and remain infectious [243]. I speculate that transmitted HBV could be stored in hepatoblasts or in extrahepatic reservoirs until hepatocyte differentiation is completed and the virus can replicate. That suggests that a bi-phasic administration of anti-HBV IgG (in the first trimester and during last weeks of pregnancy) might be necessary to prevent transmission of HBV *in utero*.

5.2 HO-1 induction and HBV replication

5.2.1 HO-1 mediates antiviral effect of CoPP in HBV infection

An antiviral effect of CoPP in stable HBV-producing cell lines, HBV-transgenic mice and mice transduced with AdHBV has previously been shown in our laboratory. Since in the model of HIV infection, Vzorov et. al. found that porphyrins possess antiviral activity [214], I investigated whether inhibition of HBV replication by CoPP is mediated by HO-1 or is merely an effect of porphyrin.

In this study, I demonstrated that HO-1 mediates suppression of HBV replication by CoPP in hepatocytes at a posttranscriptional step that results in a reduction of HBV cccDNA, the viral persistence form in hepatocyte nuclei.

In order to study whether the antiviral effect of CoPP in HBV infection is specific to HO-1 induction, I knocked down HO-1 expression using an siRNA approach. HO-1 specific siRNA reduced HO-1 protein content by 74% and concomitantly reversed the inhibition of HBV replication by HO-1 as evidenced by measurement of HBV core protein expression, formation of HBV replicative intermediates, HBV progeny production and HBV cccDNA content. This strongly argues for an antiviral activity of HO-1 and not to a mere porphyrin effect. Porphyrins have been described to inhibit binding of HIV to its cellular receptor, CD4 [214]. In contrast, I observed an antiviral activity of HO-1 at a posttranscriptional step of HBV replication. Induction of HO-1 reduces the stability of HBV core protein and thereby hinders the formation of HBV capsids, in which reverse transcription and thus synthesis of new viral genomes takes place [154].

In my experiments, HO-1 induction led to a profound reduction of HBV cccDNA pool in HepG2-H1.3 cells, a cell line, which was recently established in our laboratory. HepG2-H1.3 cells are stably transfected with one copy of a 1.3-fold overlength HBV genome. This cell line establishes a pool of nuclear HBV cccDNA, the natural HBV transcription template, by reimporting viral genomes from newly synthetized viral capsids. Since *Webb et. al* (unpublished result) found that a continuous refill was necessary to maintain a constant cccDNA pool in these cells, my results suggest that inhibition of HBV replication by HO-1 eliminated HBV capsids and thus blocked the amplification of the cccDNA pool. A similar non-cytopathic mechanism of HBV cccDNA reduction has been described during clearance of acute hepatitis B in

chimpanzees [229] as well as during antiviral treatment of chronic hepatitis B with nucleoside analogues [288], which block reverse transcription.

To prove this hypothesis and to exclude a direct effect of HO-1 on nuclear HBV cccDNA, I treated HepG2-H1.3 cells with nucleoside analogue lamivudine with and without HO-1 induction. This led to identical regression kinetics of HBV cccDNA, and thus indicated that HO-1 induction inhibited the refill of nuclear cccDNA by newly formed viral DNA as effectively as inhibition of reverse transcription by lamivudine. Since the half-life of cccDNA was not reduced by combined treatment, I concluded that HO-1 had no additional direct effect on HBV cccDNA in the nucleus.

To characterize the molecular mechanisms of the anti-viral effect of HO-1, the first experiments were performed using the reaction products of HO-1 (i.e. CO, biliverdin, and Fe^{2+}). These ruled out that ferritin induced by Fe^{2+}, which has been shown to mediate several biological properties of HO-1, mediates the anti-viral effect since the CoPP concentrations used in the study induced ferritin light chain neither *in vivo* nor *in vitro* (unpublished observations). Initial results indicated that carbon monoxide as well as biliverdin can act as mediators of HO-1 antiviral activity.

5.2.2 Induction of HO-1 during ongoing anti-HBV immune response

In the current study, in strong contrast to data obtained in our laboratory, I found a significant increase in serum ALT activity after administration of CoPP, when an anti-HBV immune response was ongoing. This indicates that induction of HO-1 on ongoing anti-HBV immune response aggravates liver injury.

However, in various models of immune-mediated inflammation, HO-1 induction has been shown to prevent or at least significantly inhibit inflammation-related (TNFα-, CD95/CD95L-, lipopolysaccharide - induced) [289] or apoptotic liver injury [203]. HO-1 induction is reported to suppress functions of macrophages, maturation of dendritic cells [290], antigen presentation, secretion of IL-12 and IFNγ. In addition, HO-1 inhibits T cell proliferation via reduction of IL-2 production [192], directs the Th-cell response to Th2 rather than to Th1 direction and down-regulates IFN I pathway [199]. Furthermore, HO-1 induction significantly up-regulates Foxp3, TGF-beta, IL-10, CTLA-4 expression in CD4+ T-lymphocytes [291] and is even engaged in Foxa3+-mediated immune suppression [194].

I hypothesized that, either mechanisms underlying anti-inflammatory and hepatoprotective effects of HO-1 could not be realized if the enzyme is induced on ongoing anti-HBV immune response or, that anti-inflammatory and hepatoprotective activity of HO-1 might be restricted to a certain range of overexpression.

The major difference in the design of experiments between my and former experiments [154] was the timing of CoPP treatment. In an earlier experiment, HO-1 was induced before transduction of mice with HBV genome-coding adenoviral vector. Previous works focused on hepatoprotective and anti-apoptotic effects of HO-1 in the setting of immune-mediated liver injury also induced enzyme overexpression before liver damage was carried out [289], [203]. In my experiment, mice were treated with CoPP 5 and 10 days after HBV replication had been initiated.

The anti-inflammatory and antiviral activity of HO-1 could be attributed to the effects of biliverdin or CO (Protzer U., unpublished). *Bilban et. al.* reported that administration of exogenous CO leads to a transitory burst of ROS [292]. This rapid increase in ROS likely results in concomitant induction of antioxidant enzymes and protective genes such that upon the addition of pro-oxidant stimuli further production of ROS is attenuated. Thus, CO is not acting as an antioxidant molecule; rather, it forces the cell to undergo oxidative conditioning leading to cytoprotective gene expression, which indirectly modulates the ensuing cellular response to the stimulation. In the current work, HO-1 induction and therefore CO production were stimulated when HBV replication had started and anti-HBV immune response had developed, so that oxidative conditioning, important for protective effect of CO against oxidative injury, could not be realized.

The second hypothesis is supported by the results of *Suttner and Dennery,* who showed that high levels of HO-1 expression reverse its cytoprotective effect in a hamster fibroblast cell line due to accumulation of reactive iron [293]. In my case, HO-1 was induced 14.4±2.5-fold at the mRNA level and 8.4±2.5-fold-fold at the protein level, which according to the data of *Suttner et. al.* is similar to the HO-1 induction levels seen with toxic influence in a given cell. The accumulation of reactive iron as a result of excessive induction of HO-1 in my experiments could have augmented the oxidative stress caused by HBV replication [294, 295] and consequently led to the depletion of intracellular glutathione and sensitization of hepatocytes to TNF-induced apoptosis [296]. Moreover, excessive oxidative stress also results in necrosis of hepatocytes. HO-1 induction does not protect hepatocytes from caspase-3-

independent necrotic liver injury [203]. Thus, the cytoprotective action of HO-1 may be restricted to a narrow threshold of overexpression.

Additional experiments are required to prove both hypotheses and to evaluate the optimal timing and dosing of HO-1 induction before application moves to clinical practice.

5.2.3 Possible candidates for HO-1 induction in humans

In this study, I used CoPP to induce HO-1 expression *in vitro*, in hepatoma cells, as well *in vivo*, in mice. However, CoPP is reported to be toxic in higher concentrations [297, 298], which would be necessary to induce HO-1 in humans. Therefore, CoPP is very unlikely to be administered in clinical studies.
HO-1 can be induced by drugs already approved for treatment of human diseases, e.g. cyclooxygenase inhibitors, statins or rapamycin.

Aspirin, a well-known COX-2 inhibitor, induces HO-1 in a dose-dependent manner in cultured endothelial cells derived from human umbilical vein either by NO-dependent pathways [299, 300] or by lipoxin A(4) [301]. The induction of HO-1 by aspirin protects endothelial cells from H_2O_2-mediated toxicity that is in the first line mediated by increased ferritin synthesis [299]. Importantly, the dose of aspirin used in the studies described above is higher than that used clinically. The application of even low doses of the drug increases the risk of upper gastrointestinal bleeding [302]. In patients with severe acute hepatitis or flares of chronic hepatitis, blood coagulation and hemostasis are often severely impaired [303]. Therefore, administration of high dosages of aspirin required to induce HO-1 in such patients could result in a dramatic increase in the risk of any major bleeding. Thus, aspirin seems not to be a probable drug candidate for HO-1 induction in patients with acute or flares of chronic hepatitis B.

Statins are the widely used lipid-lowering agents. Simvastatin and lovastatin increase HO-1 mRNA levels in cultured endothelial cells derived from human umbilical vein [304, 305], in vessel smooth muscle cells [306] and mouse macrophages [307]. The increase in HO-1 mRNA levels was associated with antioxidative and antiproliferative effects. Moreover, simvastatin and lovastatin significantly induce HO-1 and confer antioxidant and anti-inflammatory actions in the liver when they are administered orally to mice [308]. The induction of HO-1 by statins involves p38 and

ERK MAPK [308] and p13K-Akt [306] signaling pathways. Upon HO-1 induction, effects of statins are mediated by production of carbon monoxide and bilirubin [309]. Like all drugs undergoing metabolism in the liver, statins may cause liver toxicity [310, 311]. However, in a cohort of patients with cardiovascular diseases and either elevated liver enzyme levels, nonalcoholic fatty liver disease, hepatitis C, cirrhosis, liver transplants or HCC, statins show benefits without increased risk of adverse effects [312]. Therefore, statins seem to be promising candidates to induce HO-1 in humans with HBV-mediated liver disease.

Rapamycin, a macrolide antibiotic, is a cytostatic agent that blocks cell cycle progression in the G1 phase [313]. *Visner et al.* reported that rapamycin induces HO-1 and suppresses platelet-derived growth factor-dependent vascular smooth muscle cell growth and proliferation [314]. Later, *Zhou et. al.* showed that HO-1 is critical for the antiproliferative and vascular protective effects of rapamycin *in vitro* and *in vivo* in monocrotaline-induced pulmonary hypertension [315]. However, rapamycin abolishes effects of hepatocyte growth factor and transforming growth factor β in hepatocytes [316]. In addition, rapamycin down-regulates major cytokines and growth factors important for liver regeneration: TNFα, hepatocyte growth factor, platelet-derived growth factor, platelet-derived growth factor receptor, insulin-like growth factor-1, TGFβ and significantly retards proliferation of hepatocytes after 2/3 hepatectomy. Furthermore, the drug significantly increases the apoptosis rate in the liver, especially between day 3 and 7 after the partial hepatectomy [317, 318]. Thus, rapamycin could substantially compromise liver regeneration, necessary to restore the function of the organ in HBV-caused liver disease, and thus does not seem to be a good candidate.

Resveratrol, an important component in certain varieties of red grapes, has recently been shown to induce HO-1 via phosphorylation of p38 MAPK and Akt [319]. Pretreatment with resveratrol markedly reduces infarct size 24 h after myocardial infarction and increases capillary density in the peri-infarct myocardium along with better left ventricular function compared with controls [320]. Resveratrol treatment prevents partial hepatectomy-induced lipid peroxidation, decreases in hepatic glutathione and NO levels as well as inhibition of glutathione-transferase activity. Compared to the control operated animals, resveratrol diminishes severe morphological changes: mitochondrial degeneration, vacuoles, lipid droplets and myelin-like figures [321]. However, resveratrol modestly stimulates apoptosis through

cell cycle arrest in G1 and G2/M phases [322]. The latter effects of the drug could compromise hepatocyte regeneration and therefore limit potential therapeutic application in patients with severe acute or flares of chronic hepatitis B.

Curcumin, a widely used spice and coloring agent in food, possesses antitumor, anti-oxidative and anti-inflammatory properties and has recently been reported to induce HO-1 protein levels in vascular endothelial cells [323]. The ability of curcumin to induce HO-1 is presumably due to the presence of phenol moieties and may also work through the activation of the HO-1 promoter at the transcriptional level [324]. The application of curcumin in the model of CCl4-induced hepatic damage and reduces hepatic enzyme activity and therefore significantly facilitates hepatocellular recovery [325].

Most pharmacological compounds mentioned above induce HO-1 at the transcriptional level. Here, the strength of enzyme induction in an individual person highly depends on the GT length of the HO-1 promoter. So, patients with long GT repeats are more resistant to HO-1 induction and may require higher doses of the pharmacological compounds, which ultimately lead to the increased rate of side effects. An increase in HO-1 expression via viral-mediated delivery of HO-1 gene circumvents this problem and provides for a more selective approach in targeting this gene to specific tissues. Changes in CO and bilirubin formation and in heme content as a result of increased HO-1 protein expression via genetic intervention are modest and less abrupt or volatile than those obtained after bolus administration of chemical inducers. The delivery of the human HO-1 gene has proved successful for achieving long-term overexpression [326].

5.3 Decrease in intracellular levels of HNF4α and HNF1α as well as induction of HO-1 mediates inhibition of HBV replication by IL-6

Together with Dr. M. Hoesel, I analysed mechanisms underlying the effects of IL-6 on HBV replication in primary human hepatocytes. The results by Dr. M. Hoesel clearly demonstrate the negative influence of recombinant and endogenous IL-6 on HBV replication. Since IL-6 suppressed rather than induced IFN response pathways in primary liver cells, I wondered about the mechanism by which IL-6 may inhibit HBV replication. All experiments indicated a primary negative influence on HBV transcription although the survival of the cells was improved and other transcripts

remained unaffected or were even upregulated. I showed that inhibition of HBV transcription was due to decreasing levels of HNF4α and HNF1α. Additionally, the induction of HO-1 may contribute to the inhibition of HBV replication by IL-6.

Up to date, the role of IL-6 in HBV-infection remains controversial. *Waris and Siddiqui* incubated HBV-replicating HepG2.2.15 hepatoma cells with human IL-6 and found that IL-6-induced STAT-3, interacted with HNF3, bound to the HBV enhancer I and stimulated HBV transcription [224]. However, in HBV-transgenic mice, administration of recombinant IL-6 led to the suppression of steady state mRNA expression 16 to 20 h after administration [327]. These *in vivo* results are in accordance with our findings in primary liver cell cultures, and the differences observed in hepatoma cells may be due to altered signaling pathways. Galun *et al.* observed that incubation of human liver tissue with HBV and human IL-6 before transplantation into SCID mice leads to a higher rate of HBV DNA positive animals than incubation of liver tissue with the virus only [223]. This may be explained by the improvement of hepatocyte survival by IL-6, since IL-6 up-regulates the expression of a number of survival genes, such as cIAP2, Mn-SOD and IGFBP1 (*Hoesel M, unpublished*).

As detailed above, I have shown that a concerted action of HNF4α and HNF1α, which also determines morphological and functional differentiation of hepatocytes, is required for efficient HBV transcription and thereby replication [328]. I, therefore, examined whether IL-6 treatment has a negative effect on the expression of HNF1α and HNF4α transcription factors. Indeed, I found that the addition of recombinant IL-6 markedly reduced expression of these transcription factors at both mRNA and protein levels. This reduction was IL-6-specific, since addition of recombinant IL-6 pre-incubated with IL-6 neutralizing antibodies diminished the inhibiting effect of IL-6. Thus, my data suggest that IL-6-mediated down-regulation of HNF1α and HNF4α is responsible for reduced HBV gene expression and pregenome transcription, thus controlling HBV replication at the level of transcription. In support of my data, IL-6 has been shown to be responsible for inhibition of the HNF1α expression and transcription from genes regulated by HNF1α in sepsis [329].

Finally, I attempted to identify a mechanism explaining how IL-6 mediates down-regulation of HNF1α and HNF4α transcription factors. It has been reported, that the family of IL-6-type cytokines besides STAT-3 activates the mitogen-activated protein kinases (MAPK) ERK1/2, p38 and JNK (reviewed in: *Heinrich PC et. al.* [330]). In this study, I showed that HBV infection as well as IL-6 treatment upregulates

MAPK JNK and ERK, but not p38. Activated ERK1/2 has been reported to reduce HNF4α expression in human hepatoma cells [331, 332]. Activated JNK has been shown to down-regulate HNF4α in hepatoma HepG2 cells [333] and in primary human hepatocytes [334]. By using specific upstream inhibitors of MAPK, I showed that inhibition of pJNK and to a lesser extent pERK prevented down-regulation of transcription factors HNF1α and HNF4α, which are essential for HBV transcription. Therefore, blocking activation of MAPKs JNK and ERK prevented control of HBV gene expression by IL-6. My results thus explain why activation of ERK1/2 suppressed HBV replication in HBV-transfected hepatoma cells at a transcriptional level as reported by *Zheng et al.* [335]. Since HNF4α also controls expression of HNF1α, it remains open whether there is a direct effect of MAPK on HNF1α, or whether this is secondary to the suppression of HNF4α.

Additionally, I cannot exclude any decrease of HNF4α or HNF1α binding to their target genes. The transcriptional activity of HNF4α has been shown to be impaired by the induction of JAK2 signal transduction pathways, which in turn are induced by binding of IL-6 to its receptor [336]. IL-6 also impairs the DNA-binding activity of HNF1α [329].

6 Summary

6.1 Summary (English)

The importance of host cellular factors for Hepatitis B Virus replication

Hepatitis B virus (HBV), a small enveloped DNA virus, transiently and persistently infects the liver, which may result in an inflammatory liver disease - acute or chronic hepatitis B. HBV is transmitted by perinatal, percutaneous, and sexual exposure. Perinatal transmission of HBV is the most frequent cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide despite available vaccination and is generally assumed to occur during or after birth. However, recently HBV has been shown to be able to cross the intact trophoblastic barrier in early pregnancy and remain infectious.

However, it is unknown, whether and at what stage of liver development HBV infects hepatocytes or hepatocyte precursors and when HBV replication in these cells is initialized. So far, it has not been thoroughly studied, to what extent the efficiency of HBV replication depends on the degree of hepatocyte differentiation and which hepatocellular factors link HBV replication to hepatocyte differentiation. The lack of knowledge in this regard has major clinical impact, since it may impede a more efficient prophylaxis of vertical transmission.

To identify the starting point of HBV replication in the developing liver and analyze changes in replication efficiency along with liver development, I took advantage of an HBV-transgenic mouse model, in which every hepatocyte contains a replication competent 1.3-fold overlength HBV genome and which allows for studies independent of the early steps of infection. I studied markers of HBV replication: HBV DNA, HBV pgRNA, HBV core and L proteins at critical time points of fetal and postnatal liver development and compared them with that seen in adult animals.

I found that even if all hepatocytes carry the HBV genome, HBV replication does not start before birth and determined the starting point of HBV replication to be within the first week after birth. Low levels of HBV pgRNA were detectable beginning at (embryonic day) ED 12.5, but HBV core protein was first seen at ED 18.5 and large envelope protein not before birth. All studied markers of viral replication increase continuously along with liver development. Here, a close correlation between levels of HBV replication, transcription of HBV pgRNA and HBV core protein was observed.

The results strongly indicate that a threshold of HBV pgRNA and HBV core protein levels must be achieved before HBV replication starts.

Using primary human hepatocytes, hepatoma (HepG2, Huh7, differentiated and undifferentiated HepaRG) and hepatocyte cell lines, I confirmed that HBV replication is highly dependent on hepatocyte differentiation state with HBV pgRNA being the limiting step.

I determined that high expression levels of HNF4α but not its potential cofactors or other hepatocyte-enriched transcription factors were essential for efficient HBV replication. The results of this study provide evidence that expression levels of HNF4α in concert with those of HNF1α link efficiency of viral replication to hepatocyte differentiation. In addition, I showed that increase in the expression level of HNF4α during liver development is responsible for high-level transcription of HBV pregenomes and onset of HBV replication. Also, expression levels of HNF4α in concert with those of HNF1α and PGC-1α account for morphological and metabolic differentiation of hepatocytes required for formation and secretion of HBV virions.

While my studies do not allow for an analysis of the dependence of infection on the hepatocyte differentiation state or any potential immune response, my studies do allow to state, that if *in utero* infection occurs, it will be hard to detect since detection relies on expression of viral antigens and/or on virus replication. Also, the late start of replication in the developing liver might explain why the active-passive immunoprophylaxis after birth protects infants at high-risk from chronic viral infection or viral hepatitis B, though it cannot prevent transplacental transmission and infections occurring pre- or perinatally.

Besides hepatocyte-enriched transcription factors, other hepatocellular factors can influence the efficiency of HBV replication.

Recently, administration of cobalt protoporphyrin IX (CoPP), inductor of heme oxygenase I, has been shown to inhibit HBV replication in stable HBV-producing cells, in HBV-transgenic mice as well as in mice transduced with adenoviral vector encoding HBV genome. Since porphyrins were reported to possess their own antiviral effect, it is important to investigate whether the antiviral effect of CoPP in HBV infection results from HO-1 induction or is merely caused by porphyrins.

Here, I showed that HO-1 induction in hepatocytes directly decreases intracellular HBV core protein levels and inhibits HBV replication at the posttranscriptional step.

The results also indicate that the block of HBV replication by HO-1 is sufficient to reduce HBV cccDNA levels.

Also, HO-1 induction has been recently demonstrated to ameliorate liver injury in the model of acute HBV infection, when the enzyme was induced by CoPP prior to the onset of HBV replication and development of anti-HBV immune response. Here, considering a therapeutic application of HO-1 induction in acute or flares of chronic hepatitis B, it is important to study the effect of enzyme overexpression during ongoing anti-HBV immune response. In the current study, the pronounced antiviral effect of HO-1 by reducing levels of HBV core protein, decreasing HBV replication, and decreasing progeny HBV release was retained when enzyme was induced during ongoing anti-HBV immune response. In contrast to previous studies, I found that strong induction of HO-1 during ongoing anti-HBV immune response aggravates liver injury. Further studies are required to carefully evaluate dosing and timing of HO-1 induction before application moves to clinical practice.

Besides intracellular factors, extracellular factors can also affect HBV replication. Interleukin-6 (IL-6) seems to be involved in the pathogenesis of HBV infection because its levels were higher in HBV-infected than HBV-non-infected persons and correlated with the clinical course of infection. Recently, IL-6, induced upon HBV pattern recognition in mixed PHH cultures, has been shown to inhibit HBV replication. However, it remained unclear, which cellular pathways are involved in the control of HBV replication by this cytokine.

The current work has shown that IL-6 inhibits HBV replication at the transcriptional level through the activation of JNK, and, to a lesser extent ERK, and consecutive inhibition of HNF4α and HNF1α expression. In addition, inhibition of HBV replication by IL-6 may also occur at the posttranscriptional level via induction of HO-1.

The results of this work provide new insights into virus-host cell interactions that will be helpful for the generation of new models of HBV infection, to improve prophylaxis of perinatal HBV infection and to develop therapeutics for treatment of chronic HBV infection.

6.2 Summary (German)

Die Bedeutung von zellulären Wirtsfaktoren für die Hepatitis B Virus Replikation

Das humane Hepatitis B Virus (HBV) ist ein kleines umhülltes DNA-Virus aus der Gruppe der Hepadnaviridae, welches Menschen und humanoide Primaten infiziert und eine akute oder chronische Lebererkrankung – Hepatitis B verursachen kann.

Mit mehr als 2 Mrd. Infizierten, darunter ca. 350 Mio. chronische Träger wird die HBV Infektion zu einer der welthäufigsten Infektionskrankheiten gezählt. Der Verlauf der Erkrankung hängt in großem Masse von Alter und Immunstatus des Infizierten ab. Während nur 5% der Erwachsenen eine chronische Hepatitis B Erkrankung entwickeln, beträgt die Chronisierungsrate bei Kindern und Personen mit einer Immunschwäche 30% und steigt bei Neugeborenen auf über 90% an. 15% bis 40% der chronisch Infizierten entwickeln eine Leberzirrhose und als Folge dessen eine hepatische Dekompensation und HCC. Die verfügbaren antiviralen Medikamente führen zu keiner vollständigen Beseitigung des Virus und beschränken sich auf eine langfristige Hemmung der HBV Replikation und Remission der Lebererkrankung. Daher bleibt die Gesamtprognose der Patienten mit einer chronischen Hepatitis B Erkrankung unbefriedigend. Im Fokus der Behandlung sollte daher die Prophylaxe der HBV Infektion insbesondere im Kindesalter stehen. Obwohl eine Aktiv-Passive-Immunisierung der Neugeborenen von HBV - infizierten Müttern gegen HBV als effizient betrachtet wird, ist eine perinatale u.a. eine intrauterine Transmission des HBV in hoch endemischen Gebieten häufig.

Eine hämatogene Übertragung des Virus durch eine Leckage der Plazenta oder ein Transfer des HBV durch die Zellen der Plazenta werden als mögliche Mechanismen der intrauterinen Infektion diskutiert. Vor kurzem wurde gezeigt, dass Zellen des Trophoblasts eine Transzytose des HBV ermöglichen, wobei das Virus infektiös bleibt.

Es war jedoch weitgehend unklar, ob und in welchem Stadium der Leberentwicklung das übertragene Virus die Vorläuferzellen der Hepatozyten infizieren und seine Replikation etablieren kann. Außerdem lagen bis jetzt keine detaillierten Daten vor, inwiefern die Effizienz der HBV Replikation vom Differenzierungsgrad der Hepatozyten abhängt und welche hepatozelluläre Faktoren dafür verantwortlich sein

könnten. Der Einblick in das infektiöse und replikative Potential des HBV würde ein wichtiger Fortschritt für die Entwicklung neuer Prophylaxeoptionen darstellen.

Im ersten Teil der vorliegenden Arbeit wurde die Expression der HBV Gene und Virusreplikation während der Leberentwicklung untersucht. Die Abhängigkeit der HBV Replikation von der Differenzierung der Hepatozyten und die dafür verantwortlichen Wirtsfaktoren wurden weiterhin detailliert in verschiedenen Zellen mit hepatozytärer Abstammung analysiert.

Um HBV Replikation in der sich entwickelnden Leber zu untersuchen, nutzte ich die Vorteile eines HBV transgenen Mausmodells aus, bei dem die Virusreplikation von einem in Chromosom-DNA integrierten 1.3-fachen Überlängegenom des HBV stattfindet. Somit konnte ich HBV Replikation unabhängig von den Frühphasen des Virusreplikationszyklus analysieren. Die Replikation des Virus wurde an kritischen Zeitpunkten der fötalen und postnatalen Leberentwicklung untersucht: embryonalem Tag (ED) 12.5, 15.5, 18.5 und postnatalen Tag (dpn) 0.5, 2 und Woche (wpn) 1, 2 and 4 und mit der in entsprechenden erwachsenen Mäusen verglichen. Als Marker der HBV Replikation analysierte ich virale Prägenome und intrazelluläre DNA mit einer Echtzeit Polymerasenkettenreaktion sowie Core und L-Proteine mittels Western Blot Analyse. Um Sensitivität der HBV Core Protein Detektion zu erhöhen, führte ich zusätzlich Immunfärbungen der Zytospins und der Leberschnitte durch.

Obwohl alle Hepatozyten der transgenen Tiere ein HBV Genom besitzen, konnte der Startpunkt der Virusreplikation erstmals in der ersten postnatalen Woche detektiert werden. In der weiteren postnatalen Leberentwicklung stieg die Menge der HBV DNA kontinuierlich an.

Um Ursache für diesen späten Start in der HBV Replikation während der Leberentwicklung zu erklären, analysierte ich als nächstes Transkription der HBV pgRNA, die als Matrize für die reverse Transkription sowie für die Translation von HBV Core und Polymerase Proteine dient. HBV pgRNA war am ED 12.5 kaum nachweisbar, stieg aber während der weiteren fötalen und frühen postnatalen Leberentwicklung an. In Übereinstimmung mit dem Start der HBV Replikation erreichte die Traskription der pgRNA das Niveau erwachsener Mäuse in der ersten postnatalen Woche. Ich beobachtete eine enge Korrelation zwischen Menge an Prägenomen und einer Neusynthese der HBV DNA.

Trotz Detektion der HBV pgRNA schon in Frühstadien der Leberentwicklung, zeigte sich die erste, geringe Expression des HBV Core Proteins erst am ED 18.5. In der

weiteren Leberentwicklung, parallel zu der HBV pgRNA nahm diese ebenfalls kontinuierlich zu, blieb allerdings unter dem Niveau entsprechender erwachsener Tiere.

Um zu analysieren, in welchem Stadium der Leberentwicklung infektiöse HBV-Partikel gebildet werden können, wurde die Expression des HBV L Proteins analysiert. Hier gelang die Detektion geringer Mengen von HBV L Protein erst am Tag 0.5 nach Geburt. Auch die Produktion des HBV L Proteins stieg in der postnatalen Phase der Leberentwicklung an.

Somit konnte ich zeigen, dass eine HBV Replikation erst in der ersten postnatalen Woche beginnt und stark von der Expression der HBV pgRNA und des Core Proteins abhängt. Die Ergebnisse weisen daraufhin, dass ein Schwellenwert von HBV pgRNA und Core Protein erreicht werden muss, bevor die HBV DNA Synthese initiiert werden kann.

Um eine Abhängigkeit der HBV Replikation vom Differenzierungsgrad der Hepatozyten zu bestätigen und um festzustellen, welche zelluläre Faktoren für einen späten Anfang der HBV Replikation verantwortlich sind, habe ich HBV Replikation sowie die Expression der Stoffwechselmarker und Marker hepatozytärer Polarisierung auf mRNA und Proteinebene in primären humanen Hepatozyten (PHH), in Hepatoma Zellinien (HepG2, Huh7 und undifferenzierten und differenzierten HepaRG) und in der Hepatozytenzellinie Pop10 verglichen. Die stärkste Expression der Stoffwechselmarker und Marker hepatozytärer Polarisierung zeigte sich in PHH und erhöhte sich mit steigendem Differenzierungsgrad der HepaRG Zellen. Um die HBV Replikation in den oben genannten Zellen zu untersuchen, transduzierte ich sie mit einem adenoviralen Vektor, der cDNA des grün-fluoreszierenden Proteins und 1,3 Überlängengenom von HBV kodiert. Die PHH, die am meisten differenzierten Zellen in dieser Studie, zeigten ein höheres Niveau in der HBV Replikation als Hepatoma Zellen. Hingegen wurde in Pop10 Zellen, mit geringer oder keiner Expression hepatozytärer Differenzierungsmarker, keine HBV Replikation nachgewiesen. Die Unterschiede in der Effizienz der HBV Replikation hängen in großem Maße von dem Expressionsniveau der HBV Prägenome ab.

Es ist mir somit gelungen sowohl *in vitro*, in Zellkultur, als auch *in vivo*, während der Leberentwicklung der HBV transgenen Mäuse zu beweisen, dass die HBV

Replikation stark vom Grad der hepatozytären Differenzierung abhängt. Hierbei stellt die Expression der HBV Prägenome den limitierenden Schritt dar.

Da die Effizienz der HBV Replikation im Kontext hepatozytärer Differenzierung durch die Expression von pgRNA limitiert wird und die Expression der Differenzierungsmarker ebenso auf dem Transkriptonsniveau reguliert wird, wurde im Folgenden die Expression der Hepatozyten-spezifischen Transkriptionsfaktoren (HNF1 α/β, HNF3$\alpha/\beta/\gamma$, HNF4α, C/EBPα/β, LRH-1, PGC-1α, COUP-TF) untersucht.

Hier war die Expression von HNF4α, HNF1α und HNF3γ unter allen anderen Hepatozyten-spezifischen Transkriptionsfaktoren am höchsten in PHH und stieg während der Differenzierung der HepaRG Zellen an. Pop10 Zellen hingegen exprimierten nur wenig HNF3γ, HNF4α und keinen HNF1α. Während der Differenzierung der HBV stabil produzierenden Hepatoma Zellinien HepG2-H1.3 und HepG2 2.15 beobachtete ich eine enge Korrelation zwischen den Expressionslevel der HNF4α, HNF1α und HNF3γ und der Transkription der HBV Gene, Produktion der HBV Proteine, HBV Replikation und Sekretion der HBV Virionen. Diese Ergebnisse deuteten daraufhin, dass HNF4α, HNF1α und HNF3γ eine Verbindung zwischen der Effizienz der HBV Replikation und Differenzierung der Hepatozyten darstellen könnten.

Um dies zu bestätigen und um festzustellen, ob das Vorliegen von HNF4α, HNF1α und HNF3γ alleine oder hohe Expressionslevel dieser Transkriptionsregulatoren die Effizienz der HBV Replikation bestimmen, führte ich ein Knock down von HNF4α, HNF1α und HNF3γ mit spezifischen siRNAs in HepG2-H1.3 Zellen durch.

Der knock down von HNF4α und HNF1α führte zur signifikanten Inhibition der Expression hepatozytärer Differenzierungsmarker und Transkription der HBV pgRNA, Expression der HBV Proteine, Replikation des Virus, Sekretion der HBV Virionen sowie der Akkumulation der covalently closed circular DNA (cccDNA) im Zellkern. Ich konnte ausschließen, dass unsere siRNAs gegen HNF4α und HNF1α eine Interferonantwort in den Zellen induzierten. Somit war die Inhibition der HBV Replikation spezifisch durch die Verminderung der Mengen von HNF4α und HNF1α bedingt. Mittels Ko-Immunfärbung auf HNF4α und HBV Core Protein konnte zudem gezeigt werden, dass die Inhibition der HBV Replikation auf einen direkten Effekt der Verminderung der HNF4α Level zurückzuführen und nicht durch sekundäre Mediatoren verursacht war. Die große Bedeutung hoher Expressionslevel der Hepatozyten-spezifischen Transkriptionsfaktoren für eine effiziente HBV Replikation

konnte außerdem *in vivo,* anhand von Tumor/Peritumorgewebe von Patienten mit HCC und chronischer Hepatitis B nachgewiesen werden. Hier korrelierten die Level der HBV pgRNA und des Core Proteins eng mit den Expressionslevel der HNF4α.
Darüber hinaus zeigte die Analyse der Expression der Hepatozyten-spezifischen Transkriptionsfaktoren während der prä- und postnatalen Leberentwicklung, dass die Expressionslevel von HNF4α, dessen Ko-Aktivator PGC-1α und HNF1α parallel zu der hepatozytären Reifung anstiegen und eng mit der Expression von HBV pgRNA und HBV Replikation korrelierten.
Somit konnte ich zeigen, dass die HBV Replikation erst nach der Geburt anfängt und parallel zu der Reifung der Hepatozyten in der postnatalen Phase der Leberentwicklung zunimmt. Ich habe demonstriert, dass HBV Replikation durch den Grad hepatozytärer Differenzierung bestimmt wird. Außerdem konnte gezeigt werden, dass die Hepatozyten-spezifischen Transkriptionsfaktoren HNF4α und HNF1α und während der Leberentwicklung auch PGC-1α, die Verbindung zwischen der hepatozytären Differenzierung und der Effizienz der HBV Replikation darstellen und für den späten Beginn der HBV Replikation während der Leberentwicklung verantwortlich sind.
Die Ergebnisse dieser Studie erlauben keine Aussage über das infektiöse Potential von HBV in der sich entwickelnden Leber oder über eine mögliche Immunantwort. Dazu sind weitere Studien an einem Tiermodell notwendig, welches mit HBV oder einem verwandten Virus z.B. Murmeltier Hepatitis Virus (WHV) infizierbar ist. Allerdings steht anhand meiner Daten fest, dass eine intrauterine Infektion vor der Geburt nur sehr schwer detektiert werden kann, da der Nachweis auf die Expression viraler Antigene und/oder auf die Virusreplikation angewiesen ist. Weiterhin, erklären meine Ergebnisse, warum Aktiv-Passive Immunisierung gegen HBV bei Neugeborenen so effizient ist, obwohl sie eine transplazentare Übertragung des Virus und Infektion nicht verhindern kann.
Zusätzlich implizieren die Ergebnisse dieser Studie, dass Patienten mit einer HBV Infektion keine Medikamente, wie z.B. Barbiturate, erhalten sollen, die Expressionslevel der HNF4α und HNF1α erhöhen und somit HBV Replikation verstärken.
Da die Effizienz der verfügbaren Behandlungsoptionen für chronische Hepatitis B Erkrankung limitiert ist, wird die Entwicklung neuer Therapieansätze dringend

erforderlich und setzt ein detailliertes Verständnis der Interaktionen zwischen dem HBV und dem Hepatozyten voraus.

Vor kurzem konnten wir einen antiviralen und hepatoprotektiven Effekt der Hämexogenase-I (HO-1) in HBV produzierenden Hepatoma Zellinien, HBV transgenen Mäuse sowie im Mausmodell einer akuten HBV Infektion beobachten. Für die Bestätigung einer antiviralen Aktivität der Enzyminduktion fehlte jedoch der Beweis, dass ein Knock down der HO-1 Expression den antiviralen Effekt rückgängig macht. Weiterhin war es nicht klar, ob eine HO-1 Induktion die Anreicherung der cccDNA beeinflussen kann.

Deswegen wurde im zweiten Teil dieser Arbeit untersucht, ob durch die Inhibition der Expression der HO-1 die antivirale Aktivität der Enzyminduktion rückgängig gemacht werden kann. Darüber hinaus wurde der Effekt der HO-1 auf die cccDNA Akkumulierung im Zellkern analysiert. Zusätzlich untersuchte ich die anti-entzündliche und hepatozytenschützende Aktivität von HO-1 in den Mäusen 14 Tage nach der Induktion der HBV Replikation durch einen HBV Genom kodierenden adenoviralen Vektor. Dieser Zeitpunkt entsprach dem Höchstpunkt der HBV-spezifischen Immunantwort.

Um eine antivirale Aktivität der HO-1 Induktion gegen HBV bestätigen zu können, habe ich die stabil HBV produzierende Zellinie HepG2-H1.3 mit der HO-1-spezifischen siRNA transfiziert und mit CoPP behandelt. Spezifische siRNA führte zu einer effizienten Inhibition der durch CoPP induzierten HO-1 Expression. Die weitere Untersuchung der Marker der HBV Replikation zeigte eine Verminderung der HBV Core Protein Expression, Synthese der replikativen Intermediate und Sekretion der Virionen nach der Behandlung der Zellen mit CoPP. Dieser konnte durch die Transfektion mit der spezifischen siRNA entgegen gesteuert werden. Mittels Echtzeit Polymerasenkettenreaktion konnte ich zeigen, dass HO-1 die Anreicherung von cccDNA im Zellkern der Hepatozyten inhibiert. Es handelte sich hierbei um einen indirekten Effekt, da eine Kombinationsbehandlung der Zellen mit CoPP und dem Inhibitor der HBV Replikation Lamivudin keine synergistische Wirkung auf die Halbwertszeit der cccDNA hatte.

Die Ergebnisse zeigten, dass HO-1 eine antivirale Aktivität besitzt und die HBV Replikation auf dem Posttranskriptionsniveau sowie die Akkumulierung der persistierenden Form der HBV cccDNA im Zellkern inhibiert.

Zusätzlich, habe ich im Mausmodell einer akuten HBV Infektion untersucht, ob der anti-virale, anti-entzündliche und hepatozytenschützende Effekte der HO-1 Induktion erhalten bleiben, wenn das Enzym auf dem Höchstpunkt der HBV-spezifischen Immunantwort in der Leber induziert wird. Ich konnte feststellen, dass die Induktion der HO-1 auf dem Höchstpunkt der HBV-spezifischen Immunantwort in der Leber die Expression des HBV Core Proteins, die Synthese replikativer Intermediate und die Sekretion der HBV Virionen hemmt. Im Gegensatz zu den vorausgegangenen Studien, fand ich allerdings eine Zunahme der Leberschädigung. Die Zunahme der Leberschädigung könnte durch eine zu starke Induktion der HO-1 Expression mit konsekutiver Umkehrung des anti-oxidativen Effektes und Verstärkung des oxidativen Stresses erklärt werden. Hieraus wird klar, dass weitere Studien benötigt werden, um festzustellen, welche Medikamente und in welchen Dosierungen das optimale Niveau der Induktion der HO-1 Expression hervorrufen. Viele zugelassene Medikamente wie z.B. Statine induzieren HO-1 Expression und besitzen minimale hepatotoxische Nebeneffekte und werden folglich passende Kandidaten zur Induktion der HO-1 Expression in einer Therapie.

Neben den bereits genannten hepatozellulären Faktoren, kann die HBV Replikation auch durch extrazelluläre Mediatoren beeinflusst werden. Bis heute wird der Effekt von Interleukin-6 (IL-6) auf die Infektion mit HBV und Virusreplikation sehr kontrovers diskutiert. Vorausgegangene Studien in unserer Arbeitsgruppe zeigten, dass sowohl das endogene als auch das rekombinante IL-6 die Transkription der HBV pgRNA und die HBV Replikation herunterreguliert. Es blieb jedoch unklar, welche zellulären Faktoren dafür verantwortlich sind.

Im dritten Teil dieser Arbeit wurde in Kulturen primärer humaner Hepatozyten untersucht, welche hepatozellulären Faktoren die Inhibition der HBV Replikation durch IL-6 vermitteln. Dafür wurden die Zellen mit dem Wildtyp HBV infiziert.

In dieser Arbeit wurde bereits gezeigt, dass hohe Expressionslevel der HNF1α and HNF4α für eine effiziente HBV Replikation essentiell sind.

Eine Untersuchung dieser Transkriptionsfaktoren nach Behandlung der PHH Kulturen mit rekombinantem IL-6 zeigte eine Verminderung der Expression von HNF1α and HNF4α auf mRNA- sowie Protein-Niveau. Eine Zugabe IL-6 spezifischer Antikörper hob diesen Effekt auf.

Da MAPK ERK1/2 und JNK von IL-6 aktiviert werden und diese die Expression von HNF4α beeinflussen können, vermutete ich, dass MAPK Aktivierung durch IL-6 zur Inhibition der HBV Replikation führt.

Ich habe festgestellt, dass Phosphorylierung von MAPK ERK1/2 und JNK aber nicht p38 nach der Infektion der PHH mit HBV anstieg. Unter Verwendung spezifischer Inhibitoren, konnte ich zeigen, dass Inhibierung der JNK – Aktivierung vollständig und ERK - Aktivierung partiell die IL-6 induzierte Inhibition der HNF4α and HNF1α Expression überwand.

Ich habe somit bewiesen, dass die Inhibition der HBV Replikation durch IL-6 über die Aktivierung von MAPK ERK and JNK und die konsekutive Hemmung der Expression von HNF4α and HNF1α vermittelt wird.

Neben der MAPK, induziert IL-6 auch die Expression der HO-1. Nach der Infektion der PHH Kulturen mit HBV verdoppelte sich die Expression der HO-1. Eine Inkubation naiver oder HBV infizierter PHH-Kulturen mit rekombinantem IL-6 führte zu einer deutlichen Induktion der HO-1 Expression. Die Zugabe der IL-6 Antikörper verhinderte diese Effekte.

Somit wird die HBV Replikation von IL-6 auf dem Transkriptionsniveau durch Aktivierung von JNK, weniger auch von ERK und konsekutive Verminderung der Expressionslevel von HNF4α and HNF1α inhibiert. Darüber hinaus kann IL-6 die Virusreplikation posttranskriptionell durch die Induktion von HO-1 hemmen.

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9 Attachments

9.1 Curriculum vitae / Lebenslauf

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09/84 – 06/91	8 Klassen des Gymnasiums № 45 mit erweitertem Unterricht der Mathematik, Sankt Petersburg. (Notendurchschnitt – 5,0 – (entspricht 1,0 in Deutschland))
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Beruflicher Werdegang

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05/99 – 04/01	Forschungsarbeit zum funktionalen Zustand des Komplement-Systems bei Patienten mit einer chronischen Hepatitis B+C Forschungsarbeit zum funktionalen Zustand des Komplement-Systems bei Patienten mit verschiedenen Formen der bakteriellen Dysenterie
10/99	Praktikum an der Medizinischen Fakultät von Carl Gustav Carus der TU Dresden
09/00 – 03/02	Spezialisierung im Bereich Infektionskrankheiten in der Abt. für Infektionskrankheiten der Universität (Ordinatur) Forschungsarbeit: Untersuchung des klinischen Verlaufs einer chronischen Hepatitis C bei Patienten mit einer terminalen, dialysepflichtigen Niereninsuffizienz

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07/04 – 03/08	Assoziiertes Mitglied der Nachwuchsforschergruppe der medizinischen Fakultät der Universität zu Köln, Leiterin: PD Dr. med. Ulrike Protzer Forschung im Bereich: „Der Differenzierungsgrad von Hepatozyten und Expressionslevels der leberspezifischen Transkriptionsfaktoren: Einfluss auf die Hepatitis B Virus Infektion“, gefördert durch das spezielle Forschungsförderungsprogramm für hochqualitative Forschungsprojekte des wissenschaflichen Nachwuches KölnFortune „Immuntherapie des hepatozellulären Karzinoms durch adoptiven Transfer HBsAg-spezifischer T-Lymphozyten“, gefördert durch das Lise-Meitner-PostDoc Programm des Landes NRW
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9.2 List of publications

9.2.1 Published Papers

9.2.1.1 „A concerted action of HNF4α and HNF1α links hepatitis B virus replication to hepatocyte differentiation“

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A concerted action of HNF4α and HNF1α links hepatitis B virus replication to hepatocyte differentiation

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Summary

Hepatitis B virus (HBV) is an important human pathogen, which targets the liver extremely efficient, gaining access to hepatocytes by a so far unknown receptor and replicating in a hepatocyte-specific fashion. Cell differentiation seems to determine HBV replication. We here show that the level of hepatocyte differentiation, as indicated by hepatocyte polarization and metabolic activity, is closely correlated to the transcription of the HBV RNA pregenome. Pregenome transcription determined the level of HBV replication in various cell lines of hepatocellular origin and in primary human hepatocytes. A variety of hepatocyte-enriched nuclear factors have been described to regulate transcription of the pregenome, but it remained unknown which factors link HBV replication to hepatocyte differentiation. We determined that high expression levels of HNF4α but not its potential cofactors or other hepatocyte-enriched transcription factors were essential for efficient HBV replication, and link it to hepatocyte differentiation. HNF1α contributed to the control of HBV replication because it regulated the expression of HNF4α. Thus, a concerted action of HNF4α and HNF1α, which also determines morphological and functional differentiation of hepatocytes, links HBV replication to hepatocyte differentiation.

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Introduction

Human hepatitis B virus (HBV) transiently and persistently infects the liver, which may result in an inflammatory liver disease – acute or chronic hepatitis B. Chronic hepatitis B often results in liver cirrhosis or hepatocellular carcinoma (HCC). HBV is an enveloped virus whose small (3.2 kb), relaxed circular (rc) DNA genome shows an extremely compact organization. The genomic viral DNA is encapsidated together with the viral polymerase within an icosahedral nucleocapsid consisting of the HBV core protein. The viral envelope is densely packed with large (L), middle (M) and – predominantly – small (S) viral envelope proteins. In addition, the virus encodes the regulatory protein X (for review see Seeger and Mason, 2000; Ganem and Schneider, 2001). Like all hepadnaviruses, HBV multiplies its genomes by reverse transcription of an RNA pregenome (Summers and Mason, 1982).

Following infection, the rcDNA genome is imported into the hepatocyte nucleus where it is completed to a covalently closed cirular DNA (cccDNA). cccDNA serves as the viral transcription template containing four unidirectional, overlapping open reading frames. Four promoters in concert with enhancers I and II (Shaul *et al*., 1985; Yee, 1989; Su and Yee, 1992) control transcription of independent mRNAs which all remain unspliced: *preC/C*, *preS1*, *preS2/S* and *X* (summarized in Ganem and Schneider, 2001). The 0.9 kb *X* (X protein), 2.1 kb *preS2/S* (M and S protein) and 2.4 kb *preS1* (L protein) are subgenomic RNAs and serve as mRNAs. *preC/C* RNA (3.5 kb) consists of a subset of more than genome-length RNAs, which are transcribed from two physically overlapping but functionally distinct promoters (Yaginuma and Koike, 1989; Yu and Mertz, 1996). *C* RNA can serve as mRNA for translation of HBV core and polymerase proteins or as pregenomic RNA (pgRNA), which is encapsidated and reverse transcribed in the viral capsid (Ganem and Schneider, 2001). *preC* mRNA is only translated into the hepatitis B e antigen (HBeAg) (Nassal *et al*., 1990).

Hepatitis B virus transcription by host RNA-polymerase II is regulated finely by hepatocyte-enriched transcription factors: CCAAT/enhancer-binding protein (C/EBP) (Lopez-Cabrera *et al.*, 1990; 1991) and hepatocyte nuclear factors (HNF), such as HNF1 (Raney *et al.*, 1990), HNF3 (Ori and Shaul, 1995) or HNF4 (Raney *et al.*, 1997; Tang and McLachlan, 2001) in concert with nuclear receptors PGC-1α (Shlomai *et al.*, 2006) and COUP-TF (Raney *et al.*, 1997; Yu and Mertz, 2003). These factors confer hepatocyte-specific activity of the viral *preS1* (Lopez-Cabrera *et al.*, 1991; Raney and McLachlan, 1995; Raney *et al.*, 1995) and *preC/C* promoters (Lopez-Cabrera *et al.*, 1990; Johnson *et al.*, 1995; Raney *et al.*, 1997; Tang and McLachlan, 2001; 2002; Zheng *et al.*, 2004; Shlomai *et al.*, 2006), and viral enhancer elements (Yee, 1989; Yuh and Ting, 1993), and determine the hepatocyte tropism of HBV in addition to a yet unknown receptor on the cell surface.

Hepatocyte-enriched transcription factors also play an important role in liver development and hepatocyte differentiation. It has recently been shown that HNF4α is essential for morphological and functional differentiation of hepatocytes, whereas expression of HNF1α is not an absolute requirement for mammalian liver development (Li *et al.*, 2000; Hayhurst *et al.*, 2001). HNF4α also is the dominant regulator of the epithelial phenotype of hepatocytes and thus is essential for a normal liver architecture (Parviz *et al.*, 2003). Forty-two per cent of the genes occupied by RNA polymerase II in hepatocytes are bound by HNF4α (Odom *et al.*, 2004). Primary human hepatocytes (PHH), which *per se* are highly differentiated, and hepatoma cells cultivated under differentiating conditions, have been reported to better support HBV infection and replication (Gripon *et al.*, 1989; 2002; Glebe *et al.*, 2001; Sprinzl *et al.*, 2001). In addition, the activity of preC/C – and especially preS1 – promoters seems to depend on the hepatocyte differentiation state and is enhanced in quiescent hepatocytes (Chang and Ting, 1989; Raney *et al.*, 1990; Ozer *et al.*, 1996).

However, none of these studies analysed in detail which hepatocellular factors link HBV transcription and replication to hepatocyte differentiation. Considering hepatocyte-enriched transcription factors as putative candidates, it is not known whether only one or a combination of these transcriptional regulators is responsible for differentiation-dependent activity of HBV promoters. Moreover, it is still unclear whether hepatocyte-enriched transcription factors act on HBV promoters in a dose-dependent manner. Thus, the essential link between the efficiency of HBV replication and the degree of hepatocyte differentiation remained to be elucidated.

In this study, we provide evidence that (i) HBV replication is highly dependent on hepatocyte differentiation state with transcription of pgRNA being the limiting step, and (ii) among all hepatocyte-enriched transcription factors, HNF4α is the essential link between the efficiency of HBV replication and degree of hepatocyte differentiation, regulating the former in a dose-dependent manner.

Results

Markers of hepatocyte differentiation

To analyse the dependence of HBV replication on hepatocyte differentiation, we chose PHH, hepatoma cell lines HepG2, HuH7 and HepaRG and hepatocyte cell line Pop10. HepaRG cells, which differentiate and become permissive for HBV if cultivated with corticosteroids and dimethyl sulfoxide (DMSO) (Gripon *et al.*, 2002; Engelke *et al.*, 2006), were compared in an undifferentiated and a differentiated state. To prove the differentiation status of our cells, we characterized the expression of hepatocyte-specific differentiation markers.

All cells stained positive for CK8/18 (data not shown), and expression levels of neither albumin nor ferritin (Fig. 1A) correlated with the expected level of hepatocyte differentiation. Therefore, we analysed key proteins of metabolic pathways in hepatocytes. Expression levels of cytochrome p450 family member CYP1A2, steroid regulatory element-binding protein 2 (SREBP-2), apolipoprotein B (ApoB 100) (Fig. 1A), 2′3′-tryptophandioxygenase (TDO) and cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 1B) were markedly higher in primary hepatocytes than in HepG2 or HuH7 cells and increased upon differentiation in HepaRG cells. All cells expressed comparable levels of hydroxymethylglutaryl-CoA-reductase and pterin-4 alpha-carbinolamine dehydratase (data not shown). In addition, we determined expression of mitochondrial cytochromes (liver-specific antigen, LSA). Only in primary hepatocytes and differentiated HepaRG cells expression of LSA was detected (Fig. 1A).

As polarization is an important feature of hepatocytes and other epithelial cells, we exemplarily analysed organic anion transporter polypeptide C (OATP-C) and bile salt exporting pump (BSEP) localized in basolateral and apical hepatocyte plasma membranes respectively. Western blotting detected OATP-C only in primary hepatocytes (Fig. 1A), in which basolateral distribution and thus polarization was confirmed by immunofluorescence staining (data not shown). Levels of OATP-C and BSEP mRNA were the highest in PHH, and increased upon differentiation in HepaRG cells indicating regulation mainly at the level of transcription (Fig. 1B). In Pop10 cells, no TDO mRNA and only minimal amounts of PEPCK, OATP-C and BSEP mRNA were detected.

Taken together, differentiated hepatocytes or hepatoma cells expressed a set of proteins indicating high metabolic activity as well as hepatocyte polarization. Expression of

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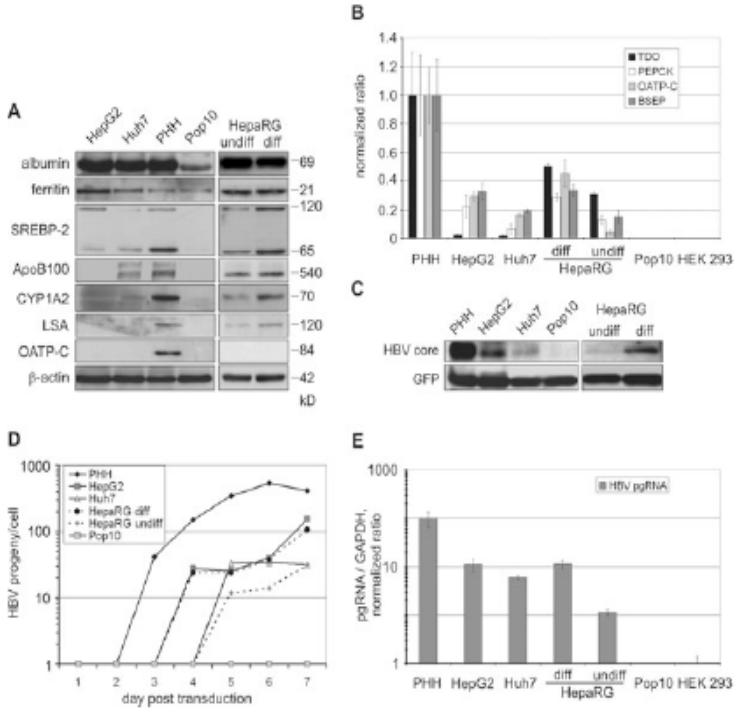

Fig. 1. HBV replication and hepatocyte differentiation markers in cells of hepatocyte origin.
A. Western blot analysis of OATP- C, CYP1A2, LSA, albumin, ferritin, SREBP-2, *ApoB100* and β-actin. Molecular weights of corresponding proteins are given in kDa.
B. Expression levels of TDO, PEPCK, OATP-C and BSEP were determined relative to GAPDH by LightCycler™ real-time reverse transcription polymerase chain reaction (RT-PCR). The normalized expression level in PHH was set to 1. Mean values ± SD from three different samples of each cell line are given (except HepaRG: two samples each). PHH from four different donors were included.
C. Western blot analysis of HBV core protein and GFP.
D. PHH, HepG2, Huh7, Pop10 and differentiated and undifferentiated HepaRG cells were transduced with Ad-HBV to induce HBV replication. Enveloped HBV progeny released from transduced cells was quantified by DNA dot blot analysis following CsCl gradient sedimentation. Numbers of enveloped HBV particles released per cell were calculated. In (A), (C) and (D) representative experiments are shown.
E. HBV pgRNA at day 7 after transduction with Ad-HBV was determined relative to GAPDH by LightCycler™ real-time RT-PCR. The normalized expression level in PHH was set to 1. Mean values ± SD from two independent experiments are given.

TDO, PEPCK, BSEP and OATP-C proved most appropriate as indicators of hepatocyte differentiation as their expression levels (i) were markedly higher in PHH than in hepatoma cells and strongly increased upon differentiation of HepaRG cells, and (ii) were undetectable in non-hepatocytes and very low or undetectable in hepatic cells not replicating HBV (Pop10). Notably, all differentiation markers were regulated primarily at the transcriptional level.

Efficiency of HBV replication in different hepatocytes

To study the efficiency of HBV replication independent of the early steps of virus uptake, we transduced PHH, HepG2, HuH7, differentiated and undifferentiated HepaRG and Pop10 cells with an HBV genome and a GFP cDNA using adenoviral vector Ad-HBV. Comparable (90–95%) transduction rates were confirmed by fluorescence microscopy and Western blot analysis of GFP expression (Fig. 1C).

As shown in Fig. 1D, PHH started to release enveloped progeny HBV particles at day 3, HepG2 and HepaRG cells at day 4 and HuH7 cells at day 5 after transduction with Ad-HBV. Pop10 cells did not release progeny HBV at all (detection limit: one HBV particle per cell). PHH secreted with > 500 virions per cell per day the highest amounts, markedly more than HepG2 cells (13.6-, 13.4-, 2.7-fold, at day 5, 6 and 7 post transduction respectively), differentiated HepaRG cells (14.4-, 14.3-, 3.9-fold) or HuH7 cells (10.5-, 15.7-, 13-fold). Differentiated HepaRG

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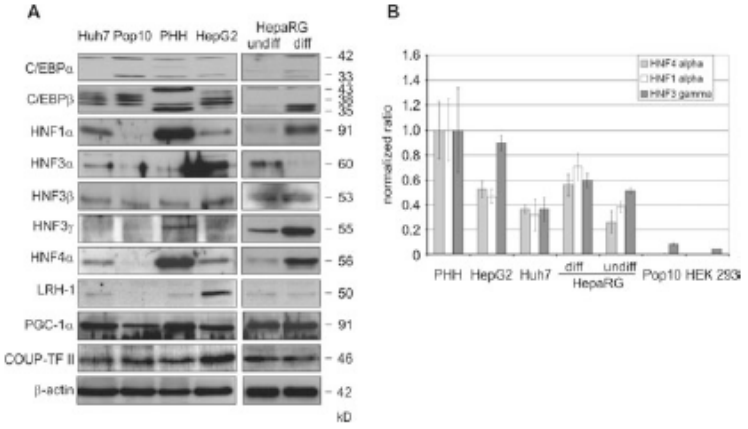

Fig. 2. Expression patterns of liver enriched transcription factors.
A. Western blot analysis of HNF1α, 3α, 3β, 3γ and 4α, C/EBPα and β, LRH-1, PGC-1α and COUP-TF II expression in primary human hepatocytes (PHH), HepG2, Huh7, Pop10, differentiated and undifferentiated HepaRG cells. Molecular weights of corresponding proteins are given in kDa.
B. LightCycler™ real-time RT-PCR analysis of HNF1α, 3γ and 4α expression. Normalized expression levels in PHH were set to 1. Mean values ± SD from three different samples of each cell line are given (except HepaRG: two samples each). PHH from four different donors were included.

cells released 2- to 3.3-fold more HBV progeny than respective undifferentiated cells.

By analysing the HBV replication cycle, we found expression levels of 3.5 kb HBV RNAs containing the HBV pregenome (pgRNA) to be the limiting step. Although transduced to equal levels, HepG2, differentiated HepaRG and HuH7 cells expressed only 9%, 8.5% and 6% of the amount of HBV pgRNA detected in PHH at day 7, respectively, and Pop10 cells no HBV pgRNA at all (Fig. 1E). Upon differentiation, HBV pgRNA increased 5.7-fold in HepaRG cells. Accordingly, expression of HBV core protein was the highest in primary hepatocytes and increased upon differentiation of HepaRG cells (Fig. 1C).

Taken together, primary hepatocytes, the highest differentiated cells in our study, replicated HBV better than hepatoma cells and released more progeny HBV. Pop10 cells, which express none or little of studied hepatocyte differentiation markers, did not replicate HBV at all. The differences in the efficiency of HBV replication depended largely on the expression level of the HBV pregenome. Thus, efficiency of HBV replication was regulated at the transcriptional level.

Cells efficiently replicating HBV express high levels of hepatocyte nuclear transcription factors HNF1α, HNF3γ and HNF4α

As HBV replication as well as hepatocyte differentiation was primarily regulated at the transcriptional level, we analysed hepatocyte-specific transcription factors HNF1α/β, HNF3α/β/γ, HNF4α, C/EBPα/β, LRH-1, PGC-1α and COUP-TF II as candidate common denominators.

Primary human hepatocytes expressed the highest levels of HNF1α, HNF3γ and HNF4α. Upon differentiation, HepaRG cells expressed increasing amounts of HNF1α, HNF3γ, HNF4α and C/EBPα and β, but decreasing amounts of HNF3α. In addition, PHH as well as HepaRG cells expressed different isoforms of C/EBPβ than the other cells analysed. Pop10 cells, in contrast, only contained trace amounts of HNF3γ and HNF4α and no detectable HNF1α (Fig. 2A and B).

To correlate transcription factor expression levels with HBV replication, we cultivated HBV-producing cell lines under conditions promoting differentiation: a collagen matrix and medium containing dexamethasone, DMSO and low FCS. Levels of OATP-C and BSEP mRNA rose 2.9- and 5.7-fold in HepG2.2.15 and 11.2- and 6.4-fold in HepG2-H1.3 cells, respectively, indicating successful differentiation during long-term culture (data not shown). In parallel, HNF4α, HNF1α and HNF3γ levels rose 3.2-, 3.3- and 4.6-fold in HepG2.2.15 cells and 7.0-, 4.5- and 2.6-fold in HepG2-H1.3 cells (Fig. 3C), respectively, whereas C/EBPα/β, HNF3α, HNF3β, LRH-1, PGC-1α and COUP-TF II expression did not show a clear tendency (data not shown). To exclude an influence of the cell culture conditions on the overall synthetic capacity of our hepatoma cells, we normalized expression levels of HNF4a to those of albumin, the major synthetic product of hepatocytes (Fig. 3D).

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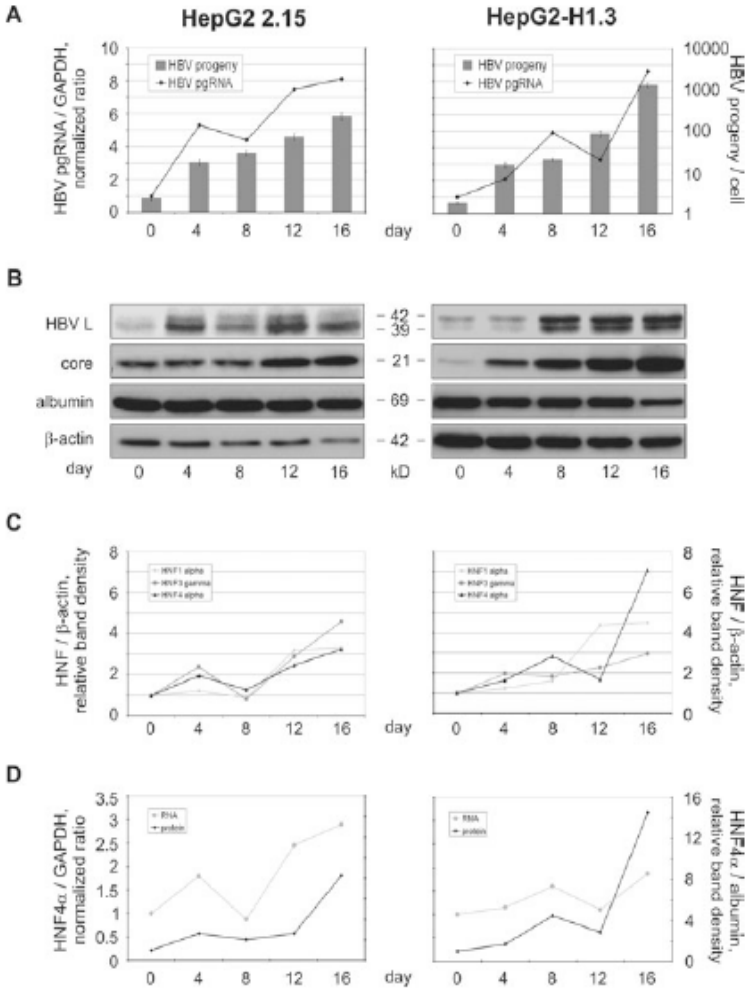

Fig. 3. HBV replication and hepatocyte differentiation in stably HBV-producing cell lines. Stably HBV-producing cell lines HepG2 2.15 (left) and HepG2-H1.3 (right) were cultured under differentiating conditions and lysed at indicated time points. A representative, parallel experiment is shown. Values obtained at day 0 were set to 1.
A. HBV-DNA was quantified by LightCycler™ real-time PCR in 200 µl of each of three parallel cell culture medium samples relative to an external standard. The amount of HBV progeny per cell was calculated (given as mean values ± SD) (grey bars). HBV pgRNA was determined in three parallel samples relative to GAPDH by LightCycler™ real-time RT-PCR (black line).
B. Western blot analysis of HBV core and L protein as well as albumin and β-actin. Molecular weights are given in kDa.
C. HNF1α, HNF3γ and HNF4α content was quantified relative to β-actin by chemiluminescence imaging of Western blots.
D. HNF4α mRNA was quantified by LightCycler™ real-time RT-PCR and is given in comparison with the protein quantified relative to albumin by chemiluminescence imaging of Western blots.

Accordingly, levels of HBV pgRNA increased 8.1-fold in HepG2.2.15 and 8.6-fold in HepG2-H1.3 cells. The amount of progeny HBV released into the cell culture medium increased from day 0 to 16 from 2.3 ± 0.14 to 217.4 ± 43.38 (HepG2.2.15 cells) and from 1.79 ± 0.1 to 1281.8 ± 149.4 HBV-DNA copies per cell (HepG2-H1.3 cells) (Fig. 3A). HBV core and L protein also increased over time: 10- and 7.1-fold in HepG2.2.15 cells and 14- and 8.8-fold in HepG2-H1.3 cells respectively (Fig. 3B).

Taken together, differentiation of the two HepG2-based cell lines resulted in increasing expression of HNF1α, HNF3γ and HNF4α and rising HBV replication. In both cell

lines, HBV replication depended on transcription of HBV pgRNA which correlated most closely with rising levels of HNF4α. This led us to the hypothesis that HNF1α, HNF3γ and/or HNF4α provide the essential link between hepatocyte differentiation and HBV replication.

High expression levels of HNF4α and HNF1α are required for efficient HBV replication

To test whether efficient HBV replication depends on high expression levels of HNF4α, HNF1α or HNF3γ, we performed knock-down experiments in HepG2-H1.3 cells using specific siRNAs for these transcription factors. As control, we used HepG2-H1.3 cells transfected with AlexaFluor488-labelled, non-silencing siRNA to which all effects of specific siRNAs were related.

We achieved a long-lasting 50%, 67% and 82% knock-down of HNF1α, HNF3γ and HNF4α respectively (Fig. 4A). At day 5 after the knock-down of HNF4α and HNF1α, mRNA levels of OATP-C decreased 2.55 ± 0.23- and 2.30 ± 0.13-fold, of BSEP 1.50 ± 0.16- and 1.30 ± 0.07-fold, of TDO 2.60 ± 0.01- and 1.75 ± 0.19-fold, and of PEPCK 2.80 ± 0.19 and 2.30 ± 0.13-fold (Fig. 4B).

Transcription of HBV pgRNA was significantly reduced in three independent experiments after knock-down of HNF4α (4.7 ± 0.3-fold at day 3; 2.2 ± 0.1-fold at day 5 post transfection) and HNF1α (4.05 ± 0.25-fold at day 5) (Fig. 4C). HBV core protein was diminished after the knock-down of HNF4α, HBV L protein after the knock-down of HNF1α (Fig. 4D and E), both leading to a significant decrease in HBV progeny release (Fig. 4F). Unlike HNF1α and HNF4α, HNF3γ knock-down did not have any inhibitory effect (Fig. 4C–F).

Southern blot analysis showed a 70% and 75% diminished HBV replication after the knock-down of HNF1α and HNF4α respectively (Fig. 4G). Concomitantly, the accumulation of HBV cccDNA in the nucleus of HepG2-H1.3 cells was inhibited (inhibition by HNF1α 88%, HNF4α 76%, Fig. 4H). As these siRNAs induced neither interferon-γ-inducible protein-10 (IP-10) nor 2′-5′-oligoadenylate-synthetase, we excluded that induction of interferon type I as a possible side-effect of siRNAs affected HBV replication (data not shown).

To show the direct effect of HNF4α on HBV replication in a given cell, we performed immunostaining for HNF4α and HBV core protein at day 7 after transfection with siRNA for HNF4α or with non-silencing siRNA (Fig. 5). In cells with reduced or undetectable HNF4α, HBV core protein was also strongly reduced. From these experiments, we concluded that high expression levels of HNF4α and HNF1α are needed for efficient HBV replication as well as expression of hepatocyte differentiation markers.

Levels of HNF4α determine HBV core protein expression and HBV replication in vivo *in human tumour and non-tumour liver tissue*

To test whether expression levels of hepatocyte-enriched transcription factors also determine the efficiency of HBV replication *in vivo* in livers of infected patients, we analysed expression of hepatocyte-enriched transcription factors, production of HBV core protein and pgRNA in tumour as well as corresponding peritumour tissues of HCC patients chronically infected with HBV. Tumours were graded as shown in Table 3. Minor differences of either hepatocyte-enriched transcription factors or HBV core protein (Fig. 6A) or HBV pgRNA (Fig. 6B) were detected between tumour and peritumour tissue samples of a given patient, whereas we found a high interindividual variation. In all samples analysed, amounts of HNF4α significantly correlated with amounts of HBV core protein (Pearson correlation 0.82, $P < 0.01$) (Fig. 6A). Also, levels of pgRNA correlated with expression levels of HNF4α (Pearson correlation 0.57, $P = 0.057$) (Fig. 6B). Thus, the data confirm *in vivo* in patient material that efficient HBV replication relies on high expression levels of HNF4α.

Discussion

In this study, we demonstrated that (i) HBV replication is highly dependent on the degree of hepatocyte differentiation, that (ii) pgRNA transcription determines the efficiency of HBV replication in relation to hepatocyte differentiation in cultivated cells as well as in liver tissue, and that (iii) HNF4α is the key regulator of HBV replication in context of hepatocyte differentiation. Not the presence of HNF4α alone, but its intracellular amounts determined the efficiency of HBV pregenome transcription and thus HBV replication in hepatoma cell lines as well as in primary human hepatocytes. This was confirmed when liver tissue samples from HBV-infected individuals were studied. As HNF4α also regulated morphological and functional differentiation of hepatocytes, it linked efficient HBV replication to hepatocyte differentiation.

We identified transcription of the HBV pgRNA to be the crucial step in the viral life cycle. Among all hepatocyte-enriched transcription factors studied, only HNF4α proved essential in controlling intracellular HBV replication. HNF4α regulated transcription of the HBV pregenome as a function of the hepatocellular differentiation state. Tang and McLachlan (2001) had reported earlier that a concerted action of nuclear hormone receptors including HNF4α is needed to induce transcription of HBV pgRNA and replication of HBV in non-hepatocytes. In cells of hepatocyte origin, where hepatocyte-enriched transcription factors are constitutively expressed, we found constant levels of nuclear hormone receptor PGC1α

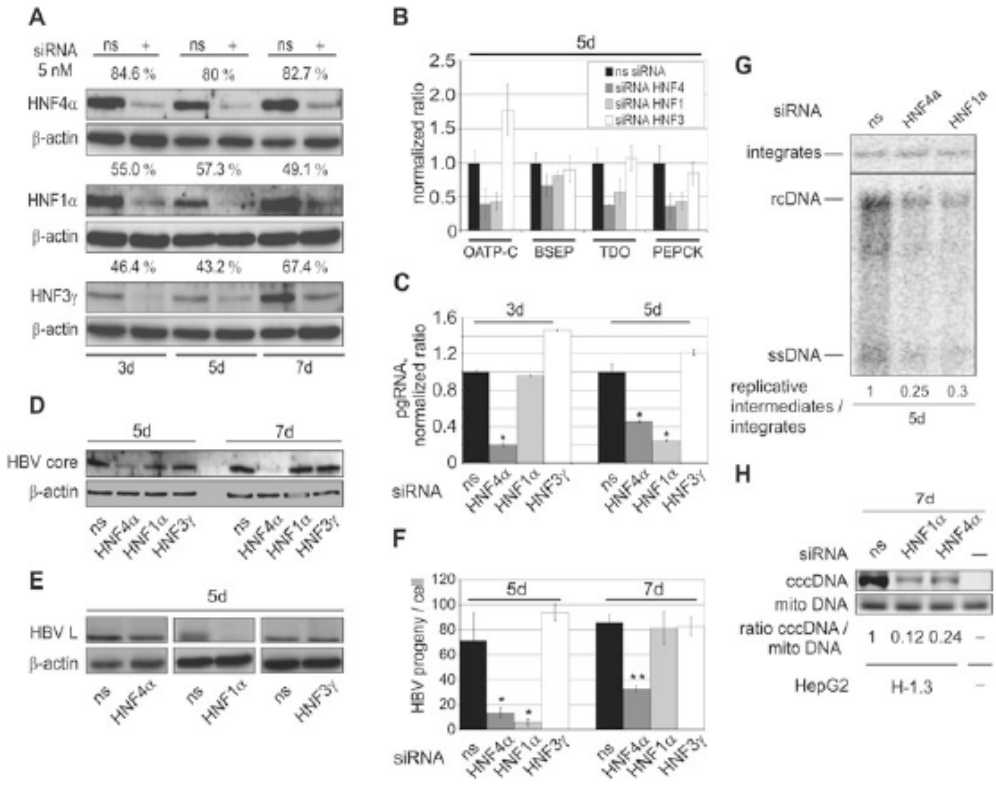

Fig. 4. Knock-down of HNF4α, HNF1α and HNF3γ and its effect on HBV replication and expression of hepatocyte-specific differentiation markers. HepG2-H1.3 cells were transfected with 5 nM siRNA (+) specific to either HNF4α, HNF1α or HNF3γ or non-silencing (ns). Cells were harvested on day (d) 3, 5 and 7 after transfection as indicated.
A. Western blot analysis of HNF4α, HNF1α and HNF3γ. Knock-down efficiency of specific siRNAs is given in percentage (relative to ns siRNA).
B. Normalized expression levels of OATP- C, BSEP, TDO and PEPCK were determined by LightCycler™ real-time RT-PCR relative to delta aminolevulinate synthase (ALAS).
C. Normalized expression levels of HBV pgRNA were determined by LightCycler™ real-time RT-PCR relative to delta aminolevulinate synthase (ALAS).
D and E. (D) Western blot analysis of HBV core protein at days 5 and 7 and (E) HBV L protein at day 5.
F. HBV-DNA was quantified by LightCycler™ real-time PCR relative to an external standard. The amount of HBV progeny per cell was calculated.
G. Southern blot analysis of HindIII-digested total cellular DNA using a ^{32}P-labelled HBV-DNA probe. HBV replicative intermediates were normalized to HBV integrates following phosphoimager quantification.
H. PCR amplification products of HBV cccDNA and mitochondrial DNA. Ratios were determined from real-time PCR analysis. Parental HepG2 cells were used as control.
In (B) and (C) ns siRNA-transfected cells were set to 1. In (A), (D), (E), (G) and (H) representative experiments are shown. In (B), mean values ± SD of two, in (C) and (F), mean values ± SD of three independent experiments are given; $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

apparently sufficient to serve as a cofactor for HNF4α. In contrast, we found a strong positive correlation between intracellular amounts of HNF4α and efficiency of viral replication. COUP-TF competing with HNF4α for the same binding site (Raney *et al.*, 1997; Yu and Mertz, 2003) was present in all cells at constant levels. This explained why a threshold of HNF4α expression had to be reached, above which HBV replication depended on the level of HNF4α.

Hepatitis B virus replication rises when HBV-producing cells are kept under differentiating conditions (Ozer *et al.*, 1996): plating onto extracellular matrix, culture medium containing dexamethasone and DMSO but low FCS (Glebe *et al.*, 2001 and Fig. 3). Dexamethasone

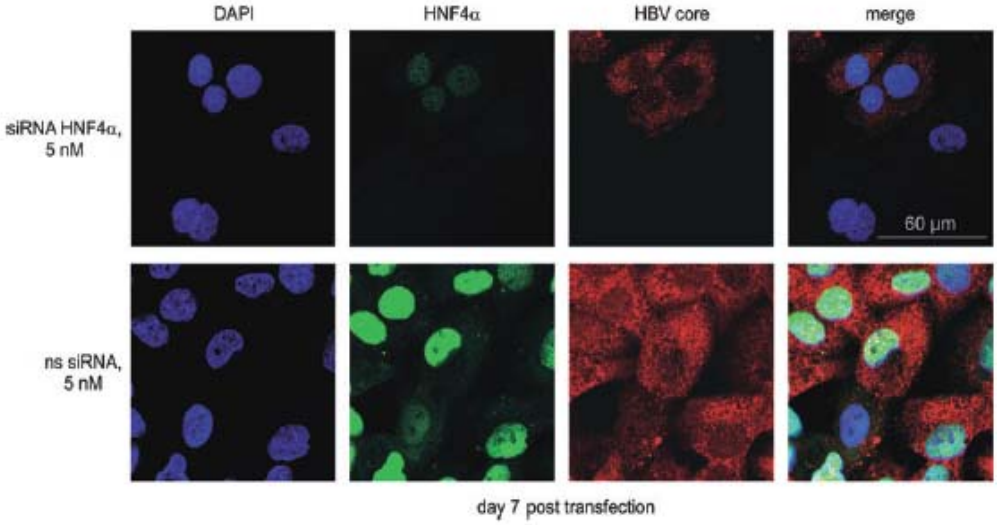

Fig. 5. Analysis of HBV core and HNF4α expression in single cells. Immunofluorescence staining of HNF4α (AlexaFluor488™, green), HBV core protein (AlexaFluor568™, red) or cell nuclei (DAPI, blue) in cells transfected with either HNF4α-specific or ns siRNA at day 7 post transfection. Laser scanning confocal microscopy, scale bar 60 µm.

enhances HNF4 and HNF1 expression via an upstream steroid responsive element (Bailly *et al.*, 2001). DMSO induces cellular differentiation by a so far unknown mechanism, and enhances HBV replication (Gripon *et al.*, 1989; 2002; Glebe *et al.*, 2001). Furthermore, it regulates histone acetylation and methylation (Sarg *et al.*, 2005), which may render target sites in the viral genome accessible to hepatocyte nuclear factors.

It was only recently that HNF4α was pinpointed to be the key regulator of morphological and functional differentiation of hepatocytes, essential for metabolic function and formation of a polarized hepatic epithelium (Parviz *et al.*, 2003) as well as for the formation of cell–cell contacts (Battle *et al.*, 2006). HNF1α primarily regulates hepatocyte polarization (Sakaguchi *et al.*, 2002). Accordingly, we found that HNF4α and HNF1α knock-down also affected expression of bile acid transporters OATP-C and BSEP, which are only expressed in highly differentiated, polarized hepatocytes, and metabolic enzymes such as SREBP-2, PEPCK and TDO, which indicated high metabolic activity.

Although HNF4α was the key player in regulating transcription of the HBV pregenome in our experiments, knock-down of HNF1α also influenced HBV replication and progeny virus release. As HNF1α is essential for expression of viral envelope protein L (Courtois et al., 1988), it controls release of viral particles from infected cells. Its influence on viral pgRNA transcription and viral replication, however, was unexpected as neither the overlapping HBV pre-core/core promoter/enhancer II nor the upstream enhancer I, which control pgRNA transcription, contain HNF1α binding sites unless they are mutated (Gunther et al., 1996).

As HNF1α is essentially involved in the control of HNF4α expression (Bailly et al., 2001; Odom *et al.*, 2004), we speculated that its knock-down diminished HBV replication by affecting the transcription of HNF4α. Indeed, we observed a substantial decrease in the amounts of HNF4α at days 3 and 5 after knock-down of HNF1α (data not shown), coincident with downregulation of HBV pgRNA transcription during HNF1α knock-down.

However, in HBV transgenic mice, transcription of the HBV pregenome was not measurably affected by a knock-out of HNF1α, and intracellular HBV replication was even slightly increased (Raney *et al.*, 2001). As the effect of HNF1α on expression of HNF4α as well as on HBV replication ceased after 5 days in our experiments, we suppose that hepatocytes compensate for the lack of HNF1α.

Guidotti *et al.* (1997) reported that HBV replication per cell remained constant after partial hepatectomy in livers of HBV transgenic mice. This does not argue against a strong dependence of HBV replication on a highly differentiated hepatocyte, because liver regeneration after partial hepatectomy takes place by proliferation of highly differentiated hepatocytes without shortening of the G1-phase (Fausto and Campbell, 2003) or reduction of HNF4α expression levels (Flodby *et al.*, 1993).

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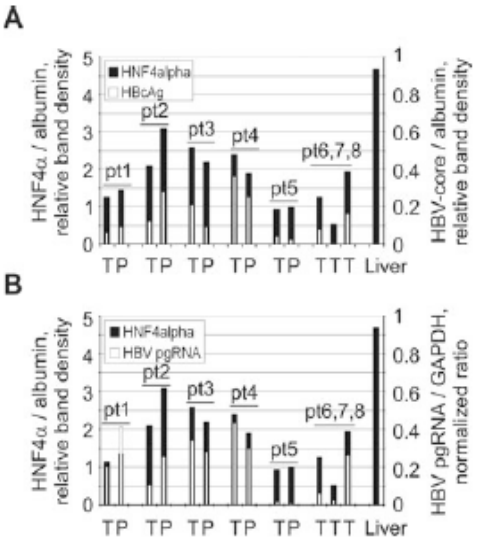

Fig. 6. Correlation of the amounts of HNF4α and HBV core protein in tumour (T)–peritumour (P) tissues of HCC patients chronically infected with HBV.
A. Western blot analysis for HNF4α and HBV core protein in tumour–peritumour tissue samples from five patients (pt 1–5), three unpaired HCC tissue samples (pt 6, 7, 8) and three normal liver tissue samples (pooled). HNF4α and HBV core protein, respectively, were quantified relative to albumin using chemiluminescence imaging. Relative band intensities are given.
B. Normalized expression levels of HBV pgRNA were determined by LightCycler™ real-time RT-PCR relative to GAPDH and are shown in comparison with HNF4α expression.

Although we clearly demonstrated that HNF4α and to a lesser extent HNF1α link HBV replication to hepatocyte differentiation by controlling transcription of its RNA pregenome, we cannot exclude an additional influence of hepatocyte metabolic functions controlled by these transcription factors (Naiki et al., 2002; Parviz et al., 2003; Odom *et al.*, 2004).

Taken together, we have shown that HBV replication strongly depends on the degree of hepatocyte differentiation, and that high HNF4α expression levels required for development of a highly differentiated hepatocyte link efficient HBV replication to hepatocyte differentiation.

Our results provide new insights into virus–host cell interaction that will be helpful for the generation of new models of HBV infection and for the development of therapeutics against HBV. Furthermore, the results of this study should be taken into consideration when prescribing drugs, e.g. phenobarbital (Bell and Michalopoulos, 2006) increasing nuclear expression of HNF4α to patients with hepatitis B.

Experimental procedures

Cell culture

Primary human hepatocytes were isolated from surgical liver resections after receiving informed consent of the donor. After a two-step collagenase perfusion of tissue remnants and subsequent differential centrifugation, PHH were plated onto collagen-coated dishes and cultivated as described (Schulze-Bergkamen *et al.*, 2003). Kidney cell line HEK293, HCC cell lines HepaRG, HepG2 and HuH7 as well as hepatocyte cell line Pop10 were cultivated as described (Sprinzl *et al.*, 2001; Gripon *et al.*, 2002; Nguyen *et al.*, 2005). HepG2.2.15 cells, an established cell line replicating HBV from four intergrated dimeric HBV genomes (Sells *et al.*, 1987), and HepG2-H1.3 cells, a cell line containing one copy of a 1.3-fold overlength HBV genome, which establishes HBV cccDNA as additional transcription template (Jost *et al.*, 2007; Protzer *et al.*, 2007), were plated onto collagen-coated dishes, and after achieving 80–90% confluence maintained in PHH medium/Dulbecco's modified Eagle's medium (1:1), containing 1% bovine serum.

Induction and analysis of HBV replication

To induce HBV replication, AdHBV, a first generation adenoviral vector (Ad5ΔE1/E3) containing a 1.3-fold HBV genome and a GFP expression cassette, was used (Sprinzl *et al.*, 2001). Cells were transduced with AdHBV to achieve 90–95% green fluorescent cells. For PHH, we used a multiplicity of infection of 5, for HuH7 and HepaRG of 30 and for HepG2 and Pop10 cells of 10 infectious units per cell.

Hepatitis B virus particles contained in 2.5 ml of cell culture medium were sedimented through a caesium chloride step gradient (density 1.15–1.4 g ml^{-1}). Enveloped, DNA-containing HBV particles were identified by dot blot hybridization of respective fractions using a ^{32}P-labelled HBV-DNA probe (Jost *et al.*, 2007; Protzer *et al.*, 2007), and quantified using a PhosphoImager (Bio-Rad Laboratories, Munich, Germany) relative to a dilution series of HBV-DNA.

Total cellular DNA (15 µg) was digested with HindIII, which excises the HBV integrate, separated through a 1.5% agarose gel, and analysed by Southern Blotting using a ^{32}P-labelled HBV-DNA probe as described (Sprinzl *et al.*, 2001). HBV replicative intermediates were quantified relative to the HBV integrate.

Real-time PCR

Total DNA was extracted from cell culture medium using QIAamp MiniElute Virus Spin Kit and from cells using DNeasy kit (Qiagen), and HBV-DNA including cccDNA was quantified as described (Untergasser *et al.*, 2006).

Total RNA was extracted using RNeasy® total RNA extraction kit (Qiagen, Hilden, Germany). One microgram of total RNA was transcribed into cDNA after DNase digestion using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA) for PCR. HBV pgRNA was detected as described previously (Untergasser *et al.*, 2006). For gene expression analysis, appropriate exon–exon spanning primer pairs were selected whenever possible. Primer positions are listed in Table 1. Real-time PCRs were performed using the LightCycler™ system and normalized to a

Table 1. Primers used for LightCycler™ real-time PCR.

Target gene	GenBank accession No.	Primer forward	Primer reverse
Organic anion transporter polypeptide C (OATP-C)	NM_006446	1939–1954	2038–2021
Bile salt-exporting pump (BSEP)	NM_003742	3577–3592	3901–3886
2′3′-Tryptophan dioxygenase (TDO)	NM_005651	921–936	1130–1114
Pterin-4 alpha-carbinolamine dehydratase (PDG)	NM_000281	316–332	519–503
Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	NM_002046	607–623	973–958
Delta-aminolevulinic acid synthetase (ALAS)	NM_000688	2007–2026	2192–2177
Cytosolic phosphoenolpyruvate carboxykinase (PEPCK)	NM_002591	1626–1643	1840–1824
Hepatocyte nuclear factor (HNF)4α	NM_000457	687–702	962–945
HNF1α	NM_000545	980–995	1309–1292
HNF3γ	NM_004497	327–344	571–550
Interferon-gamma inducible protein – 10 (IP-10)	NM_001565	145–160	310–294
2′5′-Oligoadenylate synthetase (2′5′ OAS)	NM_016816	377–392	601–585

dilution series of calibrator cDNA using the Relative Quantification Software (both Roche Diagnostics, Mannheim, Germany) as described in detail (Untergasser *et al.*, 2006).

Protein expression analysis

For extraction of total cellular proteins CHAPS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Chaps), for extraction of proteins from tissue samples SDS buffer (15 mM Tris HCl, pH 6.8, 2.5% glycerol, 0.5% SDS, 1 mM EDTA) after addition of protease inhibitors was used. For detection of transporters, membrane proteins were extracted with TED buffer (50 mM Tris, 1 mM DTT, 1 mM EDTA). For Western blot analysis, cellular lysates containing 15 µg of tissue lysates containing 30 µg of protein per lane were separated by SDS-PAGE (6.5–12.5% according to the expected protein size), transferred onto nitrocellulose membranes, stained with appropriate primary (see Table 2) and secondary antibodies (Sigma, Munich, Germany) and detected and quantified by enhanced chemiluminescence (WestDura, Pierce, Rockford, USA) using the Gel Doc 2000 System (Bio-Rad Laboratories, Munich, Germany). For reprobing, membranes were treated with 0.2 N NaOH for 10 min at room temperature before staining with the new antibody.

For immunostaining, cells were fixed with 3.7% formaldehyde and stained using monoclonal mouse anti-human HNF4α (Abcam, Cambridge, UK) and polyclonal rabbit anti-HBV core antibodies. Nuclei were stained with Diammino-2-phenylindol (DAPI). Fluorescence images were acquired using confocal microscope FluoView1000 (Olympus, Hamburg, Germany).

Knock-down using siRNAs

Synthetic siRNA against HNF4α (aacctagagattgttacagaa), HNF1α (caggacaagcatggtcccaca), HNF3γ (ttgatggatgttattggctaa) or non-silencing control (aattctccgaacgtgtcacgt) labelled with AlexaFluor488™ and transfection reagent HiPerFect was purchased from Qiagen, Hilden, Germany. A total of 3.5×10^5 HepG2-H1.3 cells per well were seeded onto collagen-coated six-well plates and transfected with siRNAs (5 nM) using the fast-forward protocol provided by the manufacturer as described

Table 2. Primary antibodies used for Western blot analysis.

Target	Source	Working dilution	Manufacturer
Liver-specific antigen (LSA)	Mouse	1:1000	Clone OCH1E5, DAKO
Organic anion transporter polypeptide C (OATP-C)	Mouse	1:1000	Clone mMDQ (provided by D. Keppler)
β-Actin	Mouse	1:4000	Clone AC-15, Sigma
Hydroxyl-methylglutaryl-CoA-reductase (HMG-CR)	Goat	1:400	Polyclonal antiserum, Santa Cruz
Steroid regulatory element binding protein – 2 (SREBP-2)	Mouse	1:5000	Clone IgG-1C6, BD Biosciences
Cytochrome p450 family member (CYP1A2)	Rabbit	1:1000	Polyclonal antiserum, BD Biosciences
Apolipoprotein B (ApoB)	Mouse	1:1000	Clone 13, BD Biosciences
Albumin	Rabbit	1:2000	Polyclonal antiserum, DAKO
Ferritin	Rabbit	1:1000	Polyclonal antiserum, Santa Cruz
Hepatocyte nuclear factor (HNF)4α	Rabbit	All 1:400	Polyclonal antisera, Santa Cruz
HNF1	Rabbit		
HNF3α	Rabbit		
HNF3β	Rabbit		
HNF3γ	Goat		
CCAAT/enhancer binding protein (C/EBP)α	Rabbit		
C/EBPβ	Rabbit		
PGC-1α	Rabbit		
ARP-1	Goat		
Liver receptor homologue – 1 (LRH-1)	Mouse	1:400	Clone H2325, R&D
HBV core and GFP	Rabbit	1:2000	Polyclonal antiserum (Kratz *et al.*, 1999)
HBV core protein (H800)	Rabbit	1:10000	Polyclonal antiserum (Nassal *et al.*, 1990)
HBV L and M (H863)	Rabbit	1:1000	Polyclonal antiserum (Engelke *et al.*, 2006)

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Table 3. Staging and grading of hepatocellular carcinoma samples.

Patient number	Serum HBsAg/anti-HBc	Tumour staging and grading	Peritumour, fibrosis stage	Peritumour, inflammation grade
1	+	pT3 N0 Mx G2 R0	3	3
2	+	pT1 N0 Mx G2-4 R0 areals of mixed (high and no) differentiation	2–3	2
3	+	pT1 N0 Mx G2 R0	2–3	2
4	+	pT1 N0 Mx G2 R0	nd	nd
5	+	pT2 N0 Mx G2 R0	2–3	2
6	+	pT1 N0 Mx G1 R0	4	2
7	+	pT3 N0 Mx G3 R0	4	1
8	+	pT1 N0 Mx G1 R0	4	1

nd, not determined.

(Protzer *et al*., 2007). Transfection efficiency was controlled by fluorescent microscopy of AlexaFlour488™-labelled siRNA. Knock-down efficiency was determined by quantitative Western blot analysis (see above).

Patient samples

Human HCC or surrounding, non-tumorous (peritumour) HBV-infected liver tissue samples were selected from the tissue bank of the Institute of Pathology, University Hospital Cologne, established after informed consent of patients. Selection criteria were: active HBV infection (HBsAg, anti-HBc and/or HBV-DNA positive in patient's serum), absence of any other obvious cause for HCC (e.g. HCV infection, haematochromatosis) and availability of snap frozen tumour and peritumour tissue. Tumours were graded according to the American Joint Commission on Cancer. For detailed information see Table 3. Healthy liver tissue was obtained from human liver grafts (HBV, HCV, HIV negative) not suited for transplantation.

Statistical analysis

The results were analysed using Student's *t*-test, ANOVA and Pearson correlation. All data are expressed as a mean ± standard deviation. A *P*-value of 0.05 or less was considered significant.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Differentiation markers in stably HBV-producing cell lines. Stably HBV-producing cell lines HepG2 2.15 (left) and HepG2-H1.3 (right) were cultured under differentiating conditions and lysed at indicated time points. OATP-C and BSEP mRNA was determined relative to GAPDH by LightCycler™ real-time RT-PCR. The normalized expression level at day 0 was set to 1 (in HepG2 2.15 cells, OATP-C mRNA at day 0 was undetectable). Representative experiment is shown.

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9.2.1.2 „Antiviral activity and Hepatoprotection by Heme Oxygenase-1 in Hepatitis B Virus Infection“

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Antiviral Activity and Hepatoprotection by Heme Oxygenase-1 in Hepatitis B Virus Infection

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CLINICAL-LIVER, PANCREAS, AND

Background & Aims: **Induction of heme oxygenase-1 (HO-1) has been shown to be beneficial in immune-mediated liver damage. We now investigate the effects of HO-1 induction in models of human hepatitis B virus (HBV) infection.** ***Methods:*** **Adenoviral transfer of an HBV 1.3 genome into wild-type mice was used as a model for acute hepatitis B. HBV transgenic animals were used as a model for chronic HBV infection. HBV replication was assessed by HBV viremia, antigenemia, and Southern blotting, liver damage was assessed by serum alanine aminotransferase activities and histopathology of liver sections. To investigate HO-1 effects on HBV replication at a molecular level, stably HBV-transfected hepatoma cells were used. HBV gene expression, protein stability, transcription, and replication were determined. HO-1 was induced by either cobalt-protoporphyrin-IX or over expressed by adenoviral gene transfer.** ***Results:*** **In the acute hepatitis B model, liver injury was reduced significantly after HO-1 induction. In addition, HO-1 showed a pronounced antiviral effect, which was confirmed in stably HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice. We showed that HO-1 induction repressed HBV replication directly in hepatocytes at a posttranscriptional step by reducing stability of HBV core protein and thus blocking refill of nuclear HBV covalently closed circular (ccc)DNA. Small interfering RNA directed against HO-1 proved that this effect depended on the expression level of HO-1.** ***Conclusions:*** **Besides its hepatoprotective effect, HO-1 showed a pronounced antiviral activity in HBV infection. Therefore, induction of HO-1 might be a novel therapeutic option for inflammatory flares of hepatitis B.**

Although an effective vaccine is available, more than 350 million people are persistently infected with hepatitis B virus (HBV) and at high risk to develop cirrhosis or hepatocellular carcinoma. Present treatment regimens are costly and limited with respect either to side effects or to drug-resistant HBV variants. In particular, treatment options for inflammatory flares are limited.

Because HBV infection is restricted to hepatocytes of human beings and primates, only very few small animal models are available that allow the study of HBV infection. As a model for the chronic carrier state of HBV infection, HBV transgenic mice have been established, replicating the virus to high levels in hepatocytes without any evidence of liver inflammation and damage, which allow the study of antiviral treatment strategies.[1,2] As a model for acute, self-limited hepatitis B, we recently developed an adenovirus-mediated transfer of HBV genomes into mouse livers, efficiently initiating HBV replication from an extrachromosomal template in adult immunocompetent mice.[3,4] In contrast to HBV transgenic mice, in which HBV replicates from an integrated genome, adenovirus encoding HBV (AdHBV)-infected mice develop necroinflammatory liver disease and a B- and T-cell immune response against HBV, and clear HBV replication after 4–6 weeks.[5]

During viral hepatitis, an antiviral T-cell response is responsible for both liver disease and viral clearance.[6,7] During clearance of HBV infection, T cells seem to act in a biphasic fashion: first, they secrete antiviral cytokines such as interferon-γ and tumor necrosis factor-α, which control viral replication and recruit other immune effector cells, and in a second phase, they eliminate remaining infected cells.[8] Liver disease seems to result from secondary recruitment and activation of non–T cells.[6]

We recently reported that heme oxygenase-1 (HO-1) prevents apoptotic liver damage in models of nonviral immune-mediated hepatitis in mice without affecting T-cell–dependent cytokine production.[9,10] We therefore hypothesized that HO-1 induction might be a novel

Abbreviations used in this paper: AdGFP, adenovirus encoding green fluorescent protein; AdHBV, adenovirus encoding hepatitis B virus; AdHBVk/o, adenovirus encoding hepatitis B virus with open reading frames knocked out; AdHO-1, adenovirus encoding heme oxygenase-1; cccDNA, covalently closed circular DNA; CoPP, cobalt-protoporphyrin-IX; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HO-1, heme oxygenase-1; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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strategy for treatment of inflammatory flares of hepatitis B and studied HO-1 effects in models of HBV infection.

Heme oxygenases catalyze the initial and rate-limiting step in the oxidative degradation of heme. The reaction products are carbon monoxide, biliverdin (which is converted rapidly to bilirubin), and free iron (which leads to induction of ferritin, an iron-binding protein). Among the 3 known heme oxygenases, HO-1, also known as heat shock protein 32, is the only inducible form of these enzymes.[11] HO-1 is induced by oxidative stress or during the resolution phase of inflammation.[12] HO-1 deficiency resulted in an enhanced proinflammatory cytokine response toward mitogenic stimuli and chronic inflammation, as shown in HO-1 knockout mice[13] as well as in a human patient with HO-1 deficiency.[14]

Over expression of HO-1 protects organs/tissues from immune-mediated organ injury, either through prevention of oxidative damage or via local immunomodulatory influence on inflammatory cells.[15] In the liver, induction of HO-1 by cobalt protoporphyrin-IX (CoPP) or over expression by adenoviral gene transfer (AdHO-1) have been reported to confer protection from ischemia/reperfusion injury secondary to transplantation,[16] as well as from adenovirus-induced liver inflammation.[17] Moreover, the reaction products of HO-1 protect from liver inflammation.[9,10,18-20] In our own studies, we found that both CoPP as well as AdHO-1 protected mice from immune-mediated hepatitis associated with apoptotic liver damage.[9,10]

Here we show that HO-1 induction and its over expression suppressed HBV replication in vivo in a mouse model of acute, self-limited hepatitis B and in HBV transgenic mice representing the chronic HBV carrier state, as well as in stably HBV-transfected cell lines.[21] Moreover, we observed that inflammation and increased liver cell injury in the model of acute hepatitis B were ameliorated by HO-1 induction.

Materials and Methods

Animals

Ten- to 12-week-old male C57BL/6 wild-type mice or HBV transgenic mice (kindly provided by H. Schaller, Heidelberg, Germany) selected for comparable levels of HBV replication were used.[1] Legal requirements for biological safety and animal care were met. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the US Academy of Sciences and published by the National Institutes of Health.

Dosage and Application Routes

CoPP (10 mg/kg body weight; Alexis Deutschland GmbH, Grunberg, Germany) was dissolved in 0.2 mol/L NaOH, adjusted to neutral pH level, and administered intraperitoneally in a final volume of 200 μL/20 g mouse. Adenoviruses encoding either rat HO-1 (AdHO-1, kindly provided by Thomas Ritter, Berlin, Germany[9]), or a 1.3-fold overlength HBV genome genotype D and a green fluorescence protein (GFP) cassette (AdHBV),[3,4] or as a control GFP alone (AdGFP) or an HBV genome with all open reading frames knocked out (AdHBVk/o) were injected intravenously

Analysis of Liver Injury

Serum alanine aminotransferase (ALT) activity was determined using specific bioreaction strips on a Reflovet Plus reader (Roche Diagnostics, Mannheim, Germany). Liver tissues were formalin fixed for 24 hours, embedded in paraffin, and sectioned. Three-micrometer sections were stained with H&E using a standard protocol and liver histology was analyzed by light microscopy. Immunohistochemical detection of HBV core antigen was performed using a polyclonal rabbit anti-HBV core antigen antibody (DAKO, Glostrup, Denmark).

Cell Culture

HepG2.2.15 cells[21] or HepG2-H1.3 cells, human hepatoma HepG2 cells stably transfected with an HBV 1.3-fold overlength genome (Webb and Protzer, unpublished) were grown on collagen type IV–coated dishes in Williams E medium or Dulbecco's modified Eagle medium (high glucose), respectively, supplemented with 5%/1% fetal calf serum, 100 IU/mL of penicillin and 100 μg/mL of streptomycin, 1% nonessential amino acids, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate. Cells were allowed to replicate HBV for several days before medium was changed to medium containing 10 μg/mL CoPP or tin-protoporphyrin-IX (Biotrend GmbH, Cologne, Germany) or AdHO-1 (5 IU/cell) if indicated. CoPP or tin-protoporphyrin-IX–containing medium was changed every 24 hours (HepG2 2.15) or 48 hours (HepG2-H1.3). After 5 days of incubation, cells and supernatants were harvested for further analysis.

Gene Knock-Down by Small Interfering RNA

Small interfering RNA (siRNA) was purchased from Eurogentec Deutschland (Cologne, Germany). Target sequences for siRNA design were as follows: GAG CCT GGA AGA CAC CCT AAT for human HO-1 and AAT TCT CCG AAC GTG TCA CGT for scrambled siRNA. HepG2-H1.3 cells were transfected with 25 nmol/L siRNA using HiPerFect (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA from liver tissue, HepG2.2.15 cells, or HepG2-H1.3 cells was extracted using Trizol reagent and after DNase digestion reverse transcribed into complementary DNA using SuperScript II or III Reverse Transcription kit (both Invitrogen, Paisley, UK) according to

the manufacturer's instruction. Total DNA was extracted from mouse sera using the DNeasy Kit and from cell supernatants using the QIAamp MinElute Virus Spin Kit (both Qiagen). Real-time polymerase chain reactions (PCRs) were performed using a LightCycler rapid thermal cycler system and the LightCycler-FastStart DNA Master Plus SYBR Green mix (Roche Diagnostics) as described recently.[22] The following oligonucleotide pairs were used: β-actin mouse (729-752 and 1076-1053, GenBank X03765); β-actin human (553-574 and 1706-1683, GenBank BC009275); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) human (607-623 and 973-958, GenBank NM002046); GAPDH mouse (512-527 and 782-767, GenBank NM001001303); HO-1 mouse (318-338 and 716-696, GenBank NM010442); HO-1 rat (679-696 and 825-808, GenBank NM012580); and HO-1 human (700-718 and 929-911, GenBank NM002133). To detect total HBV DNA, primers HBV1745-1767 and HBV1844-1823, to detect HBV cccDNA, primers HBV 2251-2267 and HBV 92-71 (HBV genotype D, subtype ayw, numbering from core core start codon = 1) were used.

Western Blot Analysis

Western blot analysis of livers or cell pellets was performed as described previously.[3] The following antibodies were used: rabbit anti-HO-1 SPA-896 (Stressgen Biotechnologies Inc, San Diego, CA); rabbit anti-HBV core (aa1-149) (kindly provided by Michael Nassal, University of Freiburg, Germany); anti-β-actin sc-1615 (Santa Cruz Biotechnology, Santa Cruz, CA), or AC-15 (Sigma-Aldrich, Munich, Germany).

Analysis of Secreted HBV Antigens

In mouse serum (1:20 diluted), hepatitis B e antigen (HBeAg) and hepatitis B surface antigen, as well as antibodies against hepatitis B surface antigen were determined by enzyme-linked immunosorbent assay (Axsym; Abbott Laboratories, Abbott Park, IL). In cell culture media blotted onto a nitrocellulose membrane, HBeAg was detected by rabbit anti-core antibody and quantified relative to an external standard using the Gel Doc 2000 System (BioRad Laboratories, Munich, Germany).

Southern blot analysis of intracellular HBV DNA, Northern blot analysis of intracellular HBV RNA, and DNA dot-blot analysis of progeny HBV DNA in cell supernatant were performed as described previously.[1,3]

Metabolic Labeling

HepG2 cells were transfected with plasmid pCH-core, expressing HBV core protein under control of a CMV I/E promoter, and pulse labeled with Dulbecco's modified Eagle medium pulse medium (containing 5% fetal calf serum, 100 μCi ^{35}S methionine). After 12 hours, maximally labeled cells were harvested as a control for HBV core protein degradation kinetics, and medium was exchanged to Dulbecco's modified Eagle medium chase medium (containing 10% fetal calf serum, 1 mmol/L unlabeled methionine) with or without CoPP. Cells were harvested after a 2-, 4-, 6-, 12-, 24-, and 48-hour chase.

Immunoprecipitation

A total of 200 μg of cell lysate (for preparation see Western Blot Analysis section) were suspended in 500 μL of RIPA buffer and incubated with rabbit-anti-HBV core antigen antibody (Diagnostic BioSystem, Pheasanton, CA) at 4°C for 12 hours, followed by addition of 100 μL protein A-sepharose CL-4B beads (Pharmacia Biotech AB, Uppsala, Sweden) for 4 hours. Supernatants of the immunoprecipitation reaction were collected to determine GAPDH as a loading control. Beads were washed in RIPA buffer, protein was resolved in 50 μL of 3× sodium dodecyl sulfate loading buffer at 95°C for 5 minutes, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by autoradiography.

Statistical Analysis

The results were analyzed using the Student *t* test if 2 groups were compared or by analysis of variance followed by the Dunnett's test if more groups were tested against a control group. If variances were inhomogeneous in the Student *t* test, results were analyzed using the Welsh test. All data are expressed as a mean ± standard error of the mean. A *P* value of .05 or less was considered significant.

Results

Induction of HO-1 Protects Mice From AdHBV-Induced Liver Injury and Prevents HBV Replication

To study the effect of CoPP in a model of acute HBV infection in mice, we first infected mice with either AdHBV or as a control with an identical construct in which premature stop codons were introduced into all HBV open reading frames (AdHBVk/o), and analyzed the effect of CoPP in these animals in comparison with naive Bl6 mice after 7 days. In all mice, CoPP efficiently induced HO-1 expression (11.8 ± 2.1-, 15.8 ± 2.5-, and 13.4 ± 0.4-fold; $P < .001$). HBV replicating mice (AdHBV) expressed on RNA level 1.47 ± 0.17-fold more HO-1 ($P < .001$) than nonreplicating mice (AdHBVk/o or noninfected) (Figure 1*A*), corresponding to a 37% increase on protein level (Figure 1*B*). Interestingly, HBV core protein was reduced in AdHBV-infected animals, in which HO-1 was induced (Figure 1*B*).

For a more detailed analysis, we pretreated the animals with CoPP 24 hours before AdHBV infection (day −1) and detected HO-1 messenger RNA (mRNA) and protein as well as HBV core protein at days 1, 5, and 26 postinfection. Figure 2*A* and *B* show induced HO-1 gene expression and protein synthesis at days 1 and 5 after AdHBV administration, and reduced levels of HBV core protein at days 5 and 26 (Figure 2*B*).

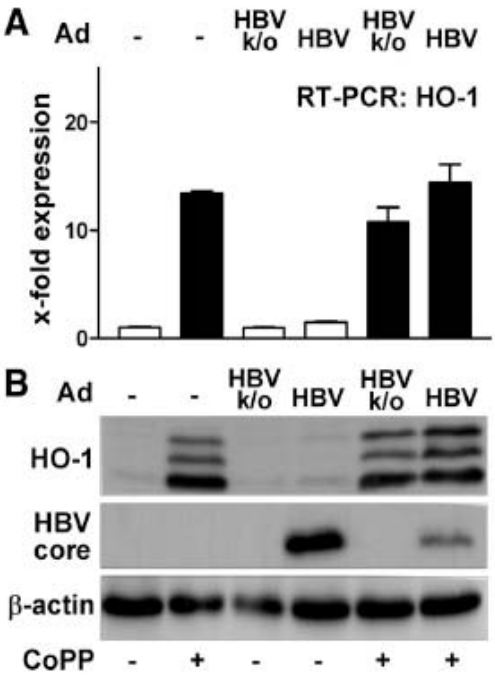

Figure 1. HO-1 induction by CoPP in mouse livers. CH57Bl/6 mice were infected intravenously with 10^9 IU of AdHBV or control vector AdHBVk/o or injected with saline, and after 7 days injected intraperitoneally with 10 mg/kg CoPP. (*A*) Expression levels of HO-1 mRNA were quantified by real-time reverse-transcription PCR (n = 3; data are expressed as mean ± standard error of the mean). (*B*) HO-1, HBV core, and β-actin were analyzed by Western blot in pooled liver lysates.

AdHBV-infected mice had increased serum activities of ALT within 1–26 days after infection, showing the highest levels of 116 ± 27 U/L at day 5 (untreated C57BL/6 mice: 45 ± 10 U/L). HO-1 induction prevented an increase of serum ALT activity throughout the experiment (Figure 2*C*). Accordingly, histopathology of liver sections revealed mild to moderate liver inflammation in the AdHBV-treated groups (Figure 3*C* and *G*), whereas HO-1 induction markedly reduced leukocyte infiltration (Figure 3*E* and *I*). Although HO-1 levels already had declined at day 26 after AdHBV infection (Figure 2*A* and *B*), ALT activities still were reduced significantly (Figure 2*C*), and liver inflammation was attenuated (Figure 3*I*). Thus, it seems that HO-1 induction at early time points during AdHBV infection was sufficient to reduce acute liver injury.

Immunohistochemical analysis of HBV core protein revealed that up to 20% of cells stained positive for cytoplasmic core protein at day 5, which slowly declined to about 10% at day 26 (Figure 3*D* and *H*). Again, HO-1 induction markedly reduced HBV core expression (Figure 3*F* and *J*).

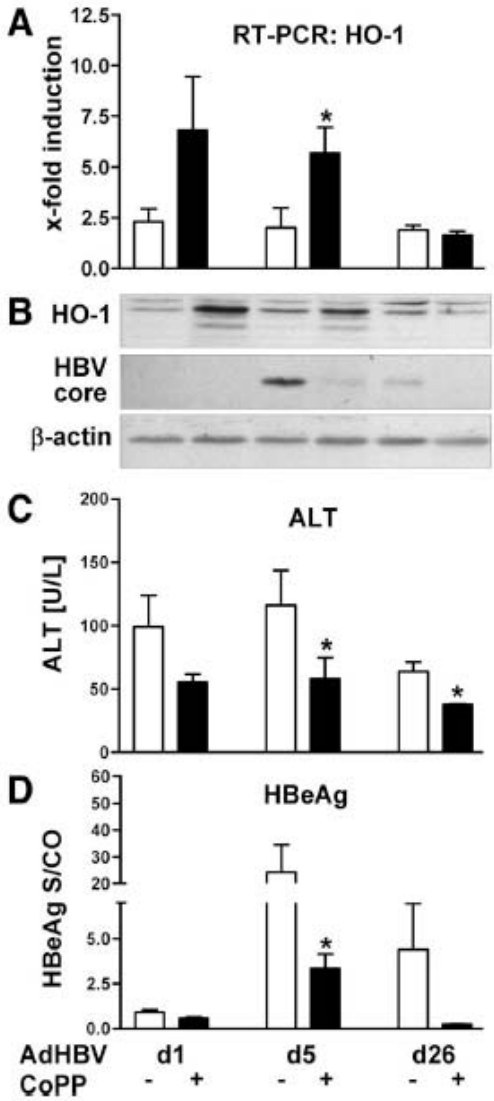

Figure 2. CoPP pretreatment inhibits HBV gene expression and the activity of alanine aminotransferase into sera of AdHBV-infected mice. Either 10 mg/kg CoPP or solvent was injected intraperitoneally 24 hours before infection with AdHBV in mice (n = 5; data are expressed as mean ± standard error of the mean). (*A*) At days 1, 5, and 26 postinfection, HO-1 mRNA expression was measured by real-time reverse-transcription PCR. Western blot analysis was performed of (*B*) pooled liver protein lysates, and (*C*) serum ALT activities were determined in mouse sera. (*D*) HBeAg serum levels were measured by an enzyme-linked immunosorbent–based assay. *$P \leq .05$ CoPP-treated vs mock-treated AdHBV-infected mice.

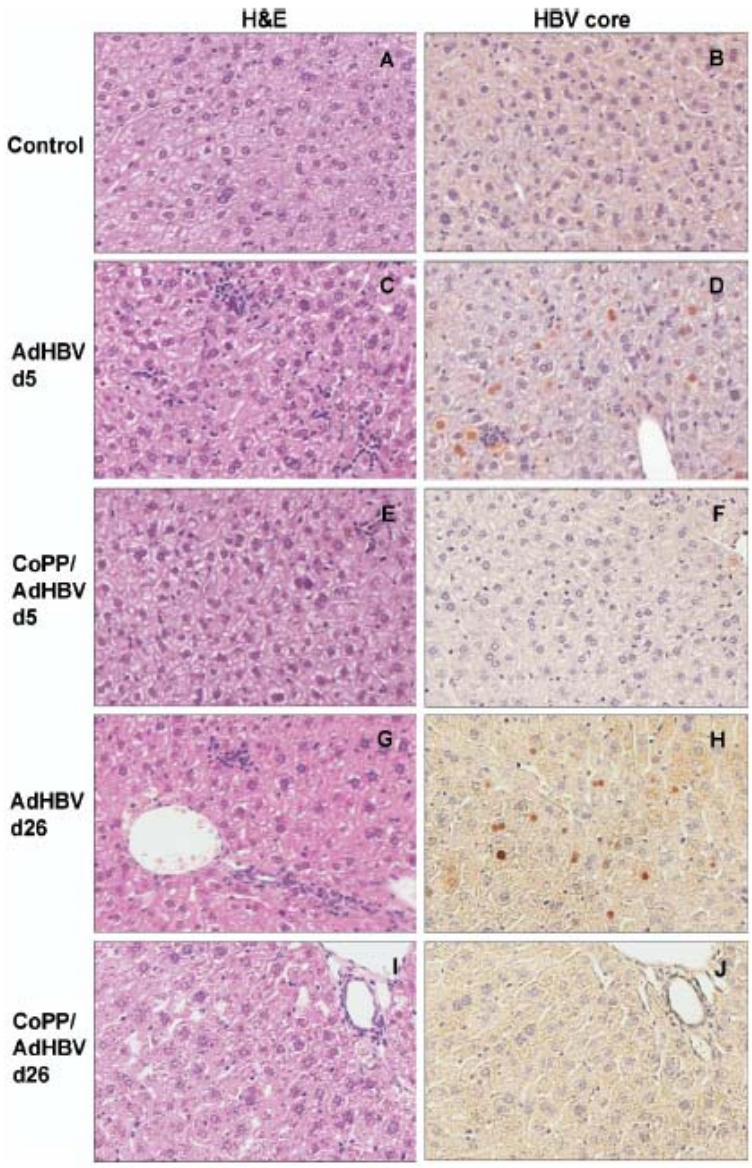

Figure 3. HO-1 induction by CoPP reduces AdHBV-induced hepatic necroinflammation and HBV core protein expression. Mice were treated with either 10 mg/kg CoPP or solvent intraperitoneally 24 hours before AdHBV infection. Histologic analysis (*A–I*, H&E staining) and HBV core antigen immunostaining (*B–J*) of liver sections of representative animals show persistent reduction of AdHBV-induced necroinflammatory lesions (*E* vs *C* and *I* vs *G* at day 5 and 26, respectively) and HBV core expression (*F* vs *D* and *J* vs *H* at day 5 and 26, respectively) in the CoPP-treated animals (*A* and *B*) solvent treated control without infection.

Hence, we wondered whether the hepatoprotective effect of HO-1 was associated with an antiviral activity. We therefore determined HBeAg and hepatitis B surface antigen in sera of mice during the time course. Induction of HO-1 suppressed HBeAg secretion (Figure 2*D*) most prominently on day 5 post-AdHBV infection, when expression levels of viral proteins peaked. Hepatitis B surface antigen also was reduced at

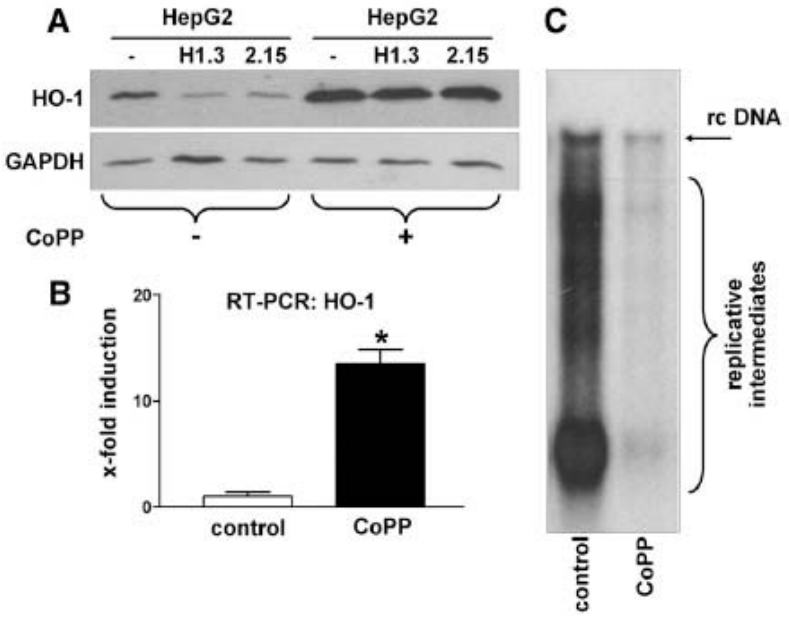

Figure 4. HO-1 induction inhibits HBV replication in stably transfected hepatoma cells. HO-1 was induced by daily incubation with 10 μg/mL CoPP in parental HepG2 hepatoma cells and in HBV-replicating HepG2-H1.3 and HepG2.2.15 cells. Cells were lysed after 5 days of treatment. (*A*) Western blot analysis of HO-1 protein in CoPP-treated and untreated cells. (*B*) Real-time reverse-transcription PCR analysis of HO-1 mRNA. *$P \leq .05$ CoPP-treated vs untreated cells ($n = 3$). Data are expressed as mean ± standard error of the mean. (*C*) Southern blot analysis of intracellular HBV DNA in HepG2.2.15 cells using a ^{32}P-labeled HBV-DNA probe.

day 5 postinfection (3.8 vs 12.9 signal/noise), but was below detection limit in all mice at day 26 owing to beginning antibodies against hepatitis B surface antigen seroconversion (data not shown).

These results show that HO-1 induction not only prevented liver damage, but also reduced expression levels of HBV proteins in a model of acute, self-limited HBV infection.

HO-1 Blocks HBV Replication in HepG2.2.15 and HepG2-H1.3 Cells at a Posttranscriptional Step

To study whether HO-1 directly acted on hepatocytes and blocked HBV replication, we induced the enzyme in stably transfected human hepatoma HepG2.2.15 or HepG2-H1.3 cells replicating HBV. Incubation with 10 μg/mL CoPP resulted in high expression of HO-1 after 5 days as measured by Western blot analysis and quantitative real-time reverse-transcription PCR (Figure 4*A* and *B*). Notably, HBV replication per se did not induce but rather reduced HO-1 in HepG2 cells (Figure 4*A*), indicating that HO-1 induction by AdHBV (Figure 1) resulted from inflammation rather than from HBV replication.

To analyze a potential antiviral effect of HO-1, we quantified HBV replicative intermediates relative to HBV integrates after Southern blot analysis. This revealed that HO-1 induction by CoPP reduced HBV replication in HepG2.2.15 cells by 84% (Figure 4*C*) and in HepG2-H1.3 cells by 91% (data not shown), providing evidence that CoPP has a direct antiviral effect in hepatocytes.

To confirm the finding that HO-1 mediates the antiviral effect of CoPP, we performed knock-down experiments using HO-1–specific siRNA and scrambled siRNA as control. HepG2-H1.3 cells were transfected directly after plating, treated with CoPP on days 1 and 3, and harvested at day 5 posttransfection. Western blot analysis proved that HO-1–specific siRNA inhibited CoPP-induced HO-1 expression by 74.1% (Figure 5*A*).

CoPP reduced HBV core protein (Figure 5*A*), HBV progeny ($4.96 \pm 0.84 \times 10^7$ vs $4.45 \pm 0.69 \times 10^6$ HBV DNA copies/mL), and HBeAg (6.0 vs 1.3 ng/mL) in HepG2-H1.3 cells. HO-1–specific siRNA reversed the reduction of HBV core protein by 27.3% (HO-1 siRNA vs nonsilencing siRNA, Figure 5*A*) as well as HBV replicative intermediates (52%) and progeny HBV (59%) (data not shown). Because levels of intracellular HBV RNA remained unchanged in treated and untreated cells (Figure 5*B*), we concluded that HO-1 affected HBV replication at a posttranscriptional step.

HO-1 Induction Reduces HBV cccDNA

HBV cccDNA accumulates and persists as an episome in HBV-infected cells, and serves as viral transcription template in natural infection. We analyzed the effect of HO-1 on HBV cccDNA in HepG2-H1.3 cells, which replicate HBV from a single integrated HBV overlength genome and establish an HBV cccDNA pool (Webb and Protzer, unpublished data). Treatment of HepG2-H1.3 cells with CoPP reduced the amounts of nuclear cccDNA

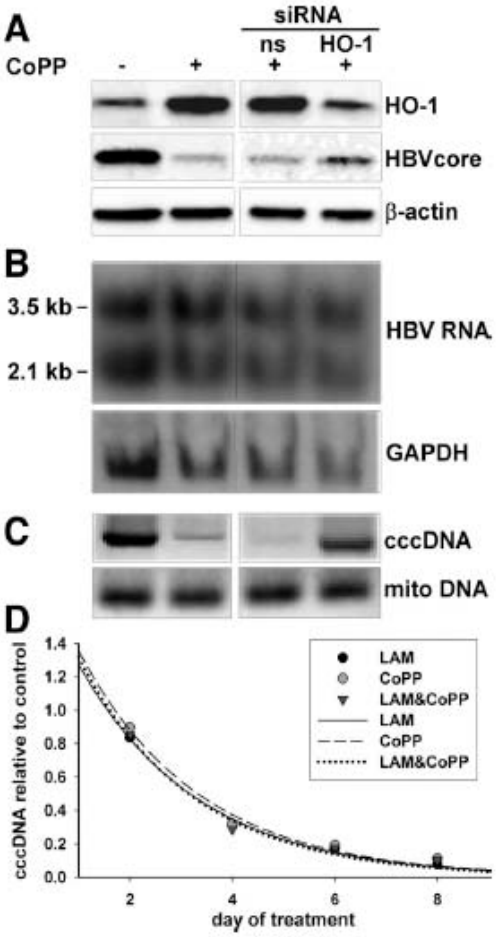

Figure 5. Analysis of the HBV replication cycle after induction and silencing of HO-1 in HepG2-H1.3 cells. HepG2-H1.3 hepatoma cells were transfected with HO-1–specific and nonsilencing (ns) scrambled siRNA, and treated with CoPP. (*A*) Western blot analysis of HO-1 and HBV core protein at day 5 posttransfection. (*B*) Northern blot analysis of intracellular HBV RNAs. (*C*) Nuclear HBV cccDNA and mitochondrial DNA analyzed by specific real-time PCRs. Agarose gel electrophoresis of PCR products is shown. (*D*) HepG2-H1.3 cells were grown to confluency and cultivated for 10 days to allow accumulation of HBV cccDNA before treatment with lamivudine (LAM), CoPP, or both was started. HBV cccDNA was quantified relative to mitochondrial DNA in cellular lysates collected every second day. Regression curve analysis of mean values determined from 3 independent vials per time point and treatment is shown. ●, LAM; ○, CoPP; ▼, LAM and CoPP; —, LAM; – – –, CoPP; - - -, LAM and CoPP.

by 90% (Figure 5C). This effect was owing to HO-1 induction because it was reversed by knock-down of HO-1 by specific siRNA.

To elucidate the mechanism by which HBV cccDNA was affected, we treated the cells with either nucleoside analogue lamivudine (15 μmol/L), which completely blocked reverse transcription of HBV, or CoPP, or a combination of both (Figure 5*D*). Half-life times of cccDNA were 2.62, 2.59, and 2.53 days, respectively, and thus almost identical under all treatments.

Taken together, these results indicate the following: (1) HO-1 induction represses HBV replication directly in hepatocytes at a posttranscriptional step, (2) the block of HBV replication is sufficient to reduce HBV cccDNA levels, and (3) this effect depends on the expression level of HO-1.

HO-1 Induction Reduces HBV Core Protein Stability

Because HO-1 induction resulted in markedly reduced levels of HBV core protein, we hypothesized that

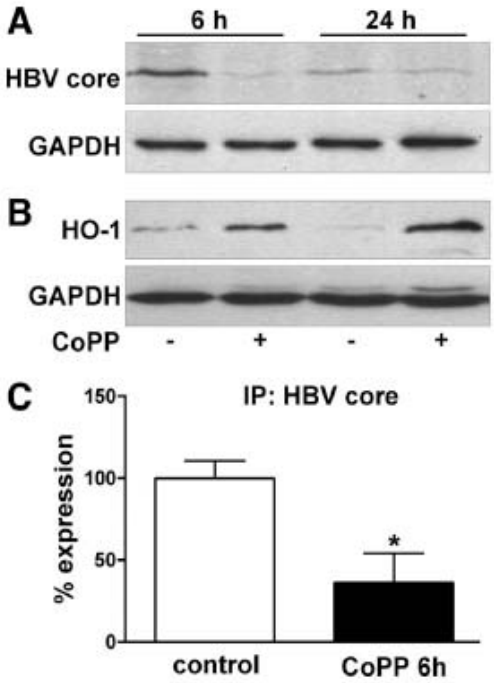

Figure 6. Effect of HO-1 induction on stability of HBV core protein. (*A*) Autoradiography of HBV core protein in HepG2 cells, which were transfected with plasmid pCHcore (encoding the HBV core protein) and pulse-labeled for 24 hours, after a 6-hour or 24-hour chase reaction in the presence or absence of CoPP (*upper panel*). Immunoprecipitation was performed using rabbit antiserum against HBV core protein in cell lysates containing 200 μg of protein each. GAPDH expression was analyzed by Western blot in immunoprecipitation supernatants (*lower panel*). (*B*) HO-1 induction by CoPP as well as GAPDH expression as a loading control was analyzed by Western blot in cellular lysates. (*C*) Quantification of remaining HBV core protein after HO-1 induction for 6 hours from 3 independent experiments detected by autoradiography using the Gel Doc 2000 System. *$P \leq .05$ for CoPP-treated vs untreated cells.

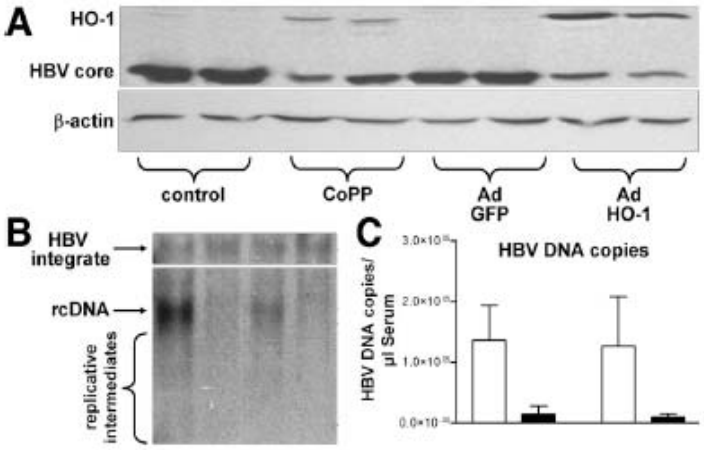

Figure 7. HO-1 induction or over expression decreases HBV replication in HBV transgenic mice. HBV transgenic mice were injected with either 10 mg/kg CoPP 3 times intraperitoneally or once with 10^9 IU AdHO-1 or AdGFP intravenously. HO-1 and HBV core protein expression was determined after 5 days. (*A*) Western blot of protein liver lysates from 2 animals per group. (*B*) Southern blot analysis of hepatic HBV replication using a ^{32}P-labeled HBV-DNA probe (40 µg pooled liver DNA/lane). (*C*) HBV viremia was measured by quantitative real-time PCR of HBV DNA in mouse sera (n = 4). Data are given as mean ± standard error of the mean.

HO-1 induction affects stability of HBV core protein. To test this independent of HBV gene expression and replication, we used a construct expressing HBV core protein under control of a ubiquitous promoter to perform pulse-chase metabolic labeling experiments in HepG2 hepatoma cells. Following the fate of ^{35}S methionine pulse-labeled HBV core protein, levels remained stable for 6 hours, started to decline at 12 hours, and decreased to 10% of the initial counts at 48 hours (data not shown). HBV core protein was degraded in response to HO-1 induction by CoPP within 6 hours (Figure 6). This indicated that HO-1 induction destabilizes HBV core protein, and thus controls HBV replication by interfering with capsid formation.

HO-1 Induction and Over Expression Control Viral Replication in HBV Transgenic Mice

To prove the antiviral effect of HO-1 in vivo, we took advantage of the HBV transgenic mouse model. These mice replicate the virus from an integrated genome permanently without signs of cytopathology and liver disease, and therefore represent the carrier state of a chronic HBV infection.[1] We investigated the effect of HO-1 induction by CoPP and over expression by AdHO-1 in these transgenic animals. As shown in Figure 7*A*, CoPP as well as AdHO-1, but not the control adenovirus AdGFP, had induced HO-1 protein production after 5 days in HBV transgenic mice. CoPP (109.8 ± 49.8 ng/mL, n = 5, vs untreated control 235.9 ± 10.1 ng/mL, n = 3, $*P \leq .05$), but not AdHO-1 (182.1 ± 54.0 ng/mL, n = 5), significantly suppressed serum concentrations of HBeAg. However, Southern blot analysis of HBV replicative intermediates showed that HO-1-induction by CoPP as well as over expression by AdHO-1 reduced hepatic HBV replication (Figure 7*B*) and HBV viremia (Figure 7*C*).

In summary, a pronounced antiviral effect of induction as well as over expression of HO-1 affecting HBV replication was confirmed in vivo in HBV transgenic mice representing the persistent carrier state of HBV infection.

Discussion

In this study, we showed that HO-1 induction ameliorates liver damage in a mouse model of acute hepatitis B, but also suppresses viral replication in stable cell lines as well as in vivo in HBV transgenic mice representing the carrier state of HBV infection without inflammation or obvious liver damage. Therefore, HO-1 elicits its antiviral effect directly in hepatocytes, and inhibits HBV replication efficiently at a posttranscriptional step by reducing the stability of HBV core protein. This resulted in a reduction of HBV cccDNA, the viral persistence form in hepatocyte nuclei, because the refill of cccDNA from mature viral capsids was blocked.

On HO-1 induction, we found a significant inhibition of transaminase release and a reduction of necroinflammatory liver damage in the AdHBV mouse model of acute, self-limited hepatitis B. With respect to production of proinflammatory cytokines, AdHBV increased intrahepatic expression as well as serum levels of tumor necrosis factor-α and interferon-γ, which were merely affected by HO-1 induction (data not shown). This observation is in line with our previous findings that HO-1 failed to block a T-celldependent cytokine response.[10] However, HO-1

induction showed a pronounced inhibitory effect on viral gene expression in this model. Hence, the protective effect observed in the acute hepatitis B model has to be attributed to the antiviral activity of HO-1 induction rather than to its anti-inflammatory effects. HO-1 was described to be up-regulated in livers of patients with chronic hepatitis B.[23] Accordingly, we found an up-regulation of HO-1 in HBV replicating mice with liver inflammation. HBV replication per se in the absence of inflammation, however, seemed to reduce HO-1 expression (Figure 4). Hence, up-regulation of HO-1 seen in the livers of hepatitis B patients may be a result of inflammation, not a direct effect of the virus.

To prove that the antiviral effect of HO-1 was indeed caused by inhibition of viral replication in hepatocytes, we used HepG2.2.15 and HepG2-H1.3 hepatoma cells stably transfected with HBV. As hypothesized, CoPP-induced HO-1 inhibited viral replication and virion release from these cells, thereby affecting a posttranscriptional step of the viral replication cycle. According to our results, it has been described that heme, which physiologically induces HO-1, inhibits human immunodeficiency virus-1 replication in T cells and macrophages by a so far unknown mechanism.[24] Here, we describe that induction of HO-1 reduces the stability of HBV core protein, which hinders formation of HBV capsids, in which reverse transcription and thus synthesis of new viral genomes takes place.

To study whether the antiviral effect is specific to HO-1 induction, we knocked-down HO-1 expression by a siRNA approach. HO-1–specific siRNA reduced HO-1 protein content by 74% and concomitantly reversed the inhibition of HBV replication by HO-1 as evidenced by measurement of HBV core protein expression, formation of HBV replicative intermediates, HBV progeny production, and HBV cccDNA content. This strongly argues for an antiviral activity of HO-1 and not to a mere porphyrin effect, which has been described to inhibit binding of human immunodeficiency virus to its cellular receptor, CD4.[25] In contrast to this effect on virus entry, we observed antiviral activity of HO-1 itself or its reaction products on HBV capsid stability and thus intracellular replication.

To characterize the molecular mechanisms of the antiviral effect of HO-1, we have initiated experiments using the reaction products of HO-1 (ie, CO, biliverdin, and Fe^{2+}). We can rule out that ferritin induced by Fe^{2+}, which has been shown to mediate several biological properties of HO-1, mediates the antiviral effect because the CoPP concentrations used in our study induced ferritin light chain neither in vivo nor in vitro (data not shown). Initial results indicate a role of carbon monoxide as well as biliverdin.

In our experiments, HO-1 induction led to a profound reduction of the HBV cccDNA pool in the HepG2-H1.3 cell line, which we recently established in our laboratory. HepG2-H1.3 cells are stably transfected with 1 copy of a 1.3-fold overlength HBV genome, and establish a pool of nuclear HBV cccDNA, the natural HBV transcription template, by re-importing viral genomes from newly synthesized viral capsids. Because we found that a continuous refill is necessary to maintain a constant cccDNA pool in these cells (Webb and Protzer, unpublished result), we suggested that inhibition of HBV replication by HO-1 eliminated HBV capsids and thus blocked filling up the cccDNA pool. A similar noncytopathic mechanism of HBV cccDNA reduction has been described during clearance of acute hepatitis B in chimpanzees,[26] as well as during antiviral treatment of chronic hepatitis B with nucleoside analogues, which block reverse transcription.[27]

To prove this hypothesis and to exclude a direct effect of HO-1 on nuclear HBV cccDNA, we treated HepG2-H1.3 cells with nucleoside analogue lamivudine with and without HO-1 induction. This led to identical regression kinetics of HBV cccDNA, and thus indicated that HO-1 induction inhibited the refill of nuclear cccDNA by newly formed viral DNA as effectively as inhibition of reverse transcription by lamivudine. Because the half-life of cccDNA was not reduced by combined treatment, we concluded that HO-1 had no additional direct effect on HBV cccDNA in the nucleus.

To study the antiviral effect of HO-1 in a model without inflammation and liver damage representing the chronic carrier state of HBV infection, we used HBV transgenic mice.[1] In addition to HO-1 induction by CoPP, we over expressed HO-1 in hepatocytes by an AdHO-1. Both CoPP as well as AdHO-1, but not the control adenovirus AdGFP, significantly suppressed production of HBV core protein, replication of the viral genome, and viremia in HBV transgenic mice. This strongly indicated that HO-1 is antivirally active in vivo in the setting of an established and ongoing HBV replication, even in the absence of inflammation.

This justifies speculation that HO-1 might prevent liver damage and help to control the virus during flares of hepatitis B. However, dosing and timing of HO-1 induction will have to be evaluated carefully because high levels of HO-1 expression have been described to reverse its cytoprotective effect in a hamster fibroblast cell line as a result of accumulation of reactive iron.[28] These observations may suggest that the cytoprotective action of HO-1 is restricted to a narrow threshold of over expression.

Our findings that HO-1 induction show a hepatoprotective as well as an antiviral effect in hepatitis B, in combination with the observation that HO-1 can be induced by drugs approved for treatment of human diseases (eg, cyclooxygenase inhibitors,[29] statins,[30] or rapamycin[31]), should encourage further studies. These may involve treatment of acute or even fulminant hepatitis B, flares of chronic hepatitis B, or treatment of hepatitis B in the transplant setting.

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9.2.2 Submitted Papers

9.2.2.1 „Active replication of Hepatitis B Virus in mice starts after birth"

Title: Active replication of Hepatitis B Virus in mice starts after birth

Short title: HBV replication during liver development

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Abbreviations

HBV, hepatitis B virus; ED, embryonic day; HNF, hepatocyte nuclear factor; wpn, weeks postnatally; dpn, days postnatally; qPCR, quantitative real-time polymerase chain reaction; GAPDH, glycerinaldehyd-3-phosphat-dehydrogenase; HPRT, hypoxanthine guanine phosphoribosyl transferase; SDS, sodium dodecyl sulfate; PGC, peroxisome-proliferator-activated receptor-gamma co-activator; COUP-TF, chicken ovalbumin upstream promoter-transcription factor

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Abstract

Background and Aims: Vertical transmission of Hepatitis B Virus (HBV) is the most frequent cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide despite available vaccination and is generally assumed to occur during or after birth. However, recently HBV has been shown to be able to cross the intact trophoblastic barrier in early pregnancy and to remain infectious. Since HBV replication depends on hepatocyte differentiation, the purpose of this study was to define the starting point and analyze changes in HBV replication along with hepatocyte maturation during fetal and postnatal liver development.

Methods: Markers of HBV replication and expression levels of hepatocyte-enriched transcription factors were analyzed in HBV-transgenic mouse models (HBV 1.3, HBV 1.3 xfs) by molecular biological, biochemical and immunohistological methods starting from embryonic day (ED) 12.5 through early liver development until 4 weeks postnatally.

Results: Synthesis of new viral DNA corresponding with viral replication was only detected postnatally. Low levels of 3.5 kb RNA were detectable beginning at ED 12.5, but HBV core protein was first seen at ED 18.5 and large envelope protein not before birth. All markers of viral replication increase continuously along with liver development, correlating closely with expression levels of hepatocyte nuclear factor (HNF) 1α and HNF4α.

Conclusions: In HBV-transgenic mice, HBV replication starts after birth, although transcription of HBV pregenomes is detectable at early stages of fetal liver development. Efficiency of HBV replication paralleles liver maturation, suggesting that differentiation of hepatocytes determines the starting point of virus replication.

HBV replication during liver development

Infection with hepatitis B virus (HBV) is the main cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide [1]. In high endemic areas, the vast majority of HBV infections is acquired perinatally or during early childhood despite available HBV prophylaxis and is followed by chronic HBV infection in up to 90 % of cases [2]. Importantly, 25% of patients infected as neonates die prematurely from cirrhosis or liver cancer [3]. Vertical transmission is the major cause of HBV infection in these cases and is generally assumed to occur during or after birth. However, intrauterine infection has been reported in up to 40 % of pregnancies at risk [4].

The risk of intrauterine infection has been related to the presence of HBV DNA in the placenta [5-7] and to maternal viremia [8], whereby even very low levels of viremia were sufficient to cause vertical transmission [9, 10]. Epidemiological studies in China indicate that intrauterine HBV infection occurs with higher frequency in pregnancies of mothers testing positive for HBsAg and HBeAg than of those testing positive for HBsAg alone [11]. These studies suggest that in addition to placental leakage active transplacental transfer may occur [11]. Supporting the latter, HBsAg and HBcAg levels in placentas from HBsAg positive mothers decreased from the maternal to the fetal side [4]. Recently, HBV has been shown to be able to cross the intact trophoblastic barrier and to remain infectious [4, 12]. Trophoblast-derived cells are able to transcytose HBV in endosomes, a process relying on an intact cytoskeleton and being reduced with formation of the syncytiotrophoblast as well as by the presence of specific antibodies [12]. Thus, infectious HBV may be able to cross the maternal-fetal barrier especially in the first trimester of pregnancy.

Since HBV infection can only be detected when gene expression has started and / or the virus has begun to replicate, it is often hard to determine the exact time point of transmission. Studies with transfected hepatoma cells as well as primary hepatocytes have demonstrated that HBV replication relies on differentiated, i.e. polarized and metabolically active hepatocytes [13-15]. However, it is unknown, whether and at what stage of liver development HBV can infect hepatocytes or hepatocyte precursor cells and when HBV replication in these cells is initialized.

Hepatocytes only gradually differentiate during liver development. At embryonic day (ED) 8.5 in the mouse, undifferentiated endodermal cells migrate from the ventral foregut into the septum transversum [16]. The morphology of the determined endodermal cells then changes to that of bipotent hepatoblasts that become committed to progress along the hepatocytic or the cholangiocytic lineage at ED 14 [17]. After ED 16, with transition from embryonic to fetal period, differentiating hepatocytes begin to form hepatic cords, and at ED 18 depositions of

glycogen and peroxisomes can be identified. Just prior to birth, hepatocytes become polygonal and begin to establish the polarized epithelium [18]. During the perinatal period, the zonal heterogeneity of the liver develops, and a large number of metabolic enzymes are induced within the hepatocytes [19]. However, final maturation of the liver structure is not completed before 2 weeks after birth [20].

Hepatocyte differentiation is known to be governed by a number of hepatocyte-enriched transcription factors [16, 18]. Among them, hepatocyte nuclear factor (HNF) 4α is a crucial factor for morphological and functional differentiation of hepatocytes [21, 22]. Transcriptional regulation of target genes by HNF4α is closely associated with PGC-1α acting as a positive co-factor [23] and COUP-TF often working as an antagonist of HNF4α [24].

HNF4α is also responsible for activation of the HNF1α promoter [25] and together, these transcription factors are key regulators of a polarized epithelial phenotype and essential for a normal liver architecture [26-29]. These and other hepatocyte-enriched transcription factors have also been shown to regulate transcription of HBV genes [30-34], whereby a concerted action of HNF1α and HNF4α links hepatocyte differentiation to efficiency of viral replication with transcription of pregenomic RNA being the limiting step [14].

Up to date, it has been unclear when HBV becomes able to replicate in the setting of differentiating hepatocytes and when HBV gene expression starts in the developing liver. Since intrauterine transmission of HBV probably occurs frequently, the question remains when the virus can multiply, spread in the liver and establish itself. The lack of knowledge in this regard has major clinical impact, since it may impede a more efficient prophylaxis of vertical transmission.

To identify the starting point of HBV replication in the developing liver and analyze changes in replication efficiency along with liver development, we took advantage of an HBV transgenic mouse model, in which every hepatocyte contains a replication competent HBV genome and which allowed studies independent of the early steps of infection i.e. viral uptake and establishment of the viral genome in the nucleus. Markers of HBV replication were studied from embryonic (ED 12.5 and ED 15.5) through fetal (ED 18.5) stages of murine liver development until 4 weeks postnatally (wpn). Finally, expression levels of transcription factors expected to determine changes in replication efficiency of HBV were quantified at all stages of liver development.

Material and methods

Animals

HBV 1.3 mice (strain 1.3.32 [35]) are transgenic for a replication competent, terminally redundant HBV DNA construct (1.3 genome lengths). HBV 1.3 xfs mice carry an analogous HBV construct with frame-shift mutations running into premature stop codons in the 3' and 5' X open reading frame and therefore lack expression of the HBV X-protein [36, 37]. Legal requirements for biosafety and animal care were met. All animals received human care according to the "Guide for the Care and Use of Laboratory Animals" prepared by the US Academy of Sciences and published by the National Institutes of Health.

Homozygous breeding pairs were selected for HBe antigen levels > 15 ng / ml indicating efficient HBV replication. Pregnancy was determined by plug check. Noon of the day a plug was detected was considered ED 0.5. Liver specimens were analyzed at ED 12.5, ED 15.5, ED 18.5, as well as 0.5 and 2 days postnatally (dpn) and 1, 2 and 4 weeks postnatally (wpn). Corresponding maternal livers were used as positive controls. For each time point, at least 3 different liver specimens per mouse strain with livers from 2 different breedings were analyzed. Fetal livers were microdissected and cells were isolated as described previously [38].

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from liver specimens from ED 12.5 onwards using RNeasy® total RNA extraction kit (Qiagen, Hilden, Germany) and was reverse transcribed into cDNA after DNAse digestion using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, USA). Total DNA was extracted from liver specimens from ED 15.5 onwards using DNeasy® kit (Qiagen). For gene expression analysis, exon-exon spanning primer pairs were selected whenever possible. Primers were selected to bind intronic sequences to recognize genomic DNA (Table 1). Differential expression was quantified by LightCycler™ quantitative real-time polymerase chain reaction (qPCR) (Roche Diagnostics, Mannheim, Germany) relative to glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) and β-actin RNA or relative to hypoxanthine guanine phosphoribosyl transferase (HPRT) 1 DNA, respectively, and normalized to a dilution series of calibrator cDNA / DNA using the Relative Quantification Software (Roche Diagnostics).

Protein expression analysis

For protein analysis, samples were lysed in buffer containing 0.5 % sodium dodecyl sulfate (SDS), 15mM Tris-HCl (pH 6.8), 2.5 % glycerol, 1 mM EDTA and proteinase inhibitors (Roche Diagnostics). 60 - 80 µg of total protein per lane were separated by 8.5 - 12.5 % SDS-

polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, stained with appropriate primary and peroxidase-conjugated secondary antibodies (Sigma, Munich, Germany) and detected and quantified by enhanced chemiluminescence (WestDura and WestFemto, Pierce, Rockford, USA) using the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, USA). For reprobing, membranes were stripped with 0.2 N NaOH for 10 minutes at room temperature. In all samples, levels of HBV core protein (antiserum H800 [39]), HBV large envelope (L) protein (antiserum H863 [40]) as well as HNF1α, HNF4α, peroxisome-proliferator-activated receptor-gamma co-activator (PGC) -1α and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA) were quantified by Western Blot analysis using β-actin (antibody from Sigma-Aldrich, St. Louis, MO) as internal control.

Immunochemistry

Immunochemical detection of epithelial-specific pancytokeratin and HBV core protein was performed on cytospins with fetal liver cells (ED 12.5, ED 15.5) or adult hepatocytes after acetone fixation. Monoclonal mouse anti-human pancytokeratin (Sigma-Aldrich) and polyclonal rabbit anti-HBV core protein (H800 [39]) primary antibodies were detected through Cy™3-conjugated donkey anti-mouse or Cy™2-conjugated donkey anti-rabbit IgG, respectively (Jackson Research Laboratories, West Grove, PA). Fluorescence images were acquired using fluorescent microscope IX81 (Olympus, Hamburg, Germany) and Cell P Analysis Software (AnalySIS, Soft Imaging System GmbH, Münster, Germany).

Formalin-fixed liver samples from different stages of liver development were paraffin-embedded. Sections of 4 µm were deparaffinized and treated with proteinase K for 15 minutes. After biotin blocking, endogenous peroxidase was quenched for 20 minutes with 0.3 % hydrogen peroxide and non-specific binding sites were blocked by normal rabbit serum before staining with antiserum H800 [39] (dilution 1:500) for HBV core protein detection over night at 4 °C and visualized using a biotinylated secondary goat anti-rabbit antibody (dilution 1:300) (Dako, Carpinteria, CA), horseradish peroxidase-conjugated avidin-biotin-complex and diaminobenzidine (Dako). Nuclei were counterstained with hematoxylin.

Statistical analysis

The results were analyzed using Kruskal-Wallis testing and Spearman correlation. A p-value of 0.05 or less was considered significant.

Results

Detection of HBV DNA during liver development

Starting point and dynamics of HBV replication, defined by new synthesis of viral DNA, during liver development were analyzed by qPCR. In order to distinguish newly synthesized HBV DNA from integrates of HBV genome present in all cells of the transgenic mice, levels of HBV DNA in tails of homozygous adult mice were taken as baseline. HBV DNA levels in liver samples of homozygous animals at ED 15.5 and ED 18.5 were equal to baseline and double that of heterozygous animals at ED 15.5, clearly indicating that only integrated HBV genomes were detected (Figure 1). While intracellular HBV DNA levels remained at baseline at 0.5 dpn, levels of intracellular HBV DNA slightly increased in 2 / 5 animals at 2 dpn and significantly increased at 1 wpn in 3 / 5 animals (Kruskal-Wallis, $p \leq 0.05$) (Figure 1). Thereafter, HBV DNA levels increased rapidly, achieving levels of adult mice between 2 and 4 wpn (Figure 1). However, at all stages of postnatal liver development, HBV DNA levels showed high variations among individual animals with some transgenic mice having very low, other rather high HBV DNA levels.

Kinetics of HBV replication in HBV 1.3 xfs mice, which carry an X-deficient HBV genome, were comparable to those in HBV 1.3 mice, but generally higher at corresponding time points (Figure 1). To achieve maximal sensitivity, HBV 1.3 xfs mice were used for most of our studies, while HBV 1.3 mice were used to control for an influence of the lacking expression of HBV X-protein. From these analyses we concluded that even if all hepatocytes carry the HBV genome, HBV replication does not start before birth and determined the starting point of HBV replication within the first week after birth.

Transcription of HBV 3.5 kb RNA during liver development

To analyze why HBV replication does not start before birth, expression levels of HBV 3.5 kb RNA including pregenomic RNA as a template for reverse transcription and the mRNAs for translation of HBV core, e and polymerase proteins were determined by qPCR (Figure 2 and Supplementary Figure 1). HBV 3.5 kb RNA was barely detectable at ED 12.5 with levels representing only 1.4 % of those detected in corresponding maternal adult livers (Figure 2). Along with fetal and early postnatal liver development, amounts of HBV 3.5 kb RNA increased continuously, but remained significantly lower than in adult liver until 1 wpn: 5.8 %, 19.4 %, 23 % and 45.1 % at ED 15.5, ED 18.5 0.5 dpn ($p \leq 0.01$) and 2 dpn ($p \leq 0.05$), respectively (Figure 2).

Interestingly, in HBV 1.3 mice, dynamics of HBV 3.5 kb RNA was comparable, but levels of HBV 3.5 kb RNA were generally lower than in HBV 1.3 xfs mice at corresponding time points (Figure 2). Irrespective of the mouse strain, levels of HBV DNA correlated closely with those of HBV 3.5 kb RNA in individual animals (Spearman correlation: 0.78, $p \leq 0.05$).

HBV protein expression during liver development

HBV core protein builds up the HBV nucleocapsid and is a prerequisite for HBV DNA replication. While 3.5 kb RNA was detected at very low level from ED 12.5 onwards, expression of HBV core protein was first detected by Western blot analysis at ED 18.5 (Figure 3A). Levels of HBV core protein increased continuously during postnatal liver development (Figures 3A and 3B) irrespective of the presence of HBV X-protein (Figure 3C), but did not reach levels detected in adult livers even at 4 wpn.

To study the expression of HBV core protein on the single cell level and with high sensitivity, fetal liver cells were isolated at ED 12.5 and ED 15.5 and subjected to immunostaining on cytospins. At neither time point, fetal liver epithelial cells as identified by co-staining with an epithelial-specific pancytokeratin marker expressed HBV core protein, whereas hepatocytes isolated from adult mouse livers stained clearly positive (Figure 4A).

To analyze the distribution of HBV core protein within the hepatic lobule, we performed immunocyto-/-histochemical stainings of liver samples at all stages of liver development. In accordance with the results obtained using Western blot analysis, single hepatocytes first stained positive for HBV core protein at ED 18.5 (Figure 4B). During postnatal liver development, the number of HBV core positive cells and in particular the number of cells with cytoplasmic HBV core staining increased continuously (Figure 4B). Until 1 wpn, HBV core positive cells were distributed throughout the whole liver lobule (Figure 4B). The characteristic pericentral zonation of HBV core protein expression as seen in adult liver became obvious at 2 wpn and was readily formed at 4 wpn (Figure 4B).

To determine the time point when infectious HBV particles may start to be released, the expression of HBV L protein as the essential component of the viral envelope was quantified by Western blot analysis. Low amounts of HBV L protein were first detected at 0.5 dpn (Figures 5A and B). Postnatally, production of HBV L protein increased continuously, achieving levels of adult mice at 4 wpn (Figure 5A). In HBV 1.3 mice, levels of HBV L protein were lower than in HBV 1.3 xfs mice at corresponding time points (Figure 5B).

Hepatocyte-enriched transcription factors during liver development

To explore which hepatocellular factors were responsible for the late onset of HBV replication during liver development, hepatocyte-enriched transcription factors were quantified along with liver development. The expression of HNF1α, HNF4α, PGC-1α and COUP-TF during mouse liver development was studied at mRNA and/or protein level. As expected, expression of HNF1α, HNF4α and PGC-1α was detected at the mRNA level already at ED 12.5 and steadily increased thereafter (Supplementary Figure 2). In Western blot analysis, we first detected HNF1α, HNF4α and COUP-TF at ED 15.5 and PGC-1α at ED 18.5 (Figure 6). The protein levels of HNF1α, HNF4α and PGC-1α, which are required for transcription of HBV RNAs, increased from ED 15.5 onwards, achieving those in adult liver at 4 wpn (Figure 6). Expression of COUP-TF, which negatively regulates HBV pregenome transcription, readily increased until ED 18.5 and remained constant during further liver development (Figure 6). Among the studied transcription regulators, amounts of HNF1α and HNF4α correlated most closely with expression levels of HBV 3.5 kb RNA (Spearman correlation 0.949, $p < 0.01$ and 0.889, $p < 0.01$, respectively).

Discussion

HBV is a highly hepatocyte-specific virus whose replication depends on differentiation and functional activity of a hepatocyte. Up to date, it has been unclear when HBV gene expression starts in the developing liver and when HBV becomes able to replicate in the setting of differentiating hepatocytes. Since intrauterine transmission of HBV probably occurs frequently, the question remains when the virus can multiply, spread in the liver and establish itself. The lack of knowledge in this regard has major clinical impact, since it may impede a more efficient prophylaxis of vertical transmission of the virus from infected mothers to their offspring. We therefore used transgenic mouse models to define the starting point of HBV replication and changes in replication efficiency along with liver development.

We here show that HBV replication as defined by new synthesis of HBV DNA starts postnatally in hepatocytes of HBV 1.3 transgenic mice, although a replication competent HBV genome is present as a transgene in all cells of the organism and HBV is continuously replicating in the maternal livers. A significant increase of HBV DNA over baseline level was only registered at 1 wpn and HBV DNA levels equalled those in adult livers not before 4 wpn. Recently, we demonstrated that pregenomic RNA represents the limiting step of the viral replication cycle in dependence of hepatocyte differentiation in cultured cells [14]. In an earlier study, Guidotti et al. had shown by Northern blot analysis that HBV 3.5 kb RNA was first detectable 2 weeks postnatally in HBV 1.3 transgenic mice [35]. Using highly sensitive qPCR, we here show that HBV 3.5 kb RNA is already transcribed at very low levels at ED 12.5, the earliest time point analyzed, and steadily increases thereafter. However, only at 1 wpn expression levels of 3.5 kb RNA reached those in adult livers, matching the starting point of significant HBV replication.

Since essential steps of HBV replication take place in the viral nucleocapsid, components of the nucleocapsid such as HBV core protein and the viral polymerase are crucial for HBV replication and both proteins are translated from the 3.5 kb RNA. HBV core protein became first detectable at ED 18.5 and increased continuously during pre- und postnatal liver development. Though levels of HBV 3.5 kb RNA equalled those in adult livers at 4 wpn, intracellular amounts of HBV core protein were lower than in adult livers even at 4 wpn because HBV core protein tends to aggregate and accumulate in hepatocyte nuclei [35] from where it cannot enter the replication cycle again. Interestingly, typical pericentral zonation of HBV core protein started to form at 1 wpn and became obvious at 4 wpn, reflecting the

ongoing maturation of the liver parenchym after birth [20] with distinct metabolic activities in the perivenous zone favoring HBV replication/transcription [35].

Viral envelope proteins are crucial prerequisites for synthesis of new viral particles. In our study, HBV L protein as an essential component of the virion envelope, was first detected at 0.5 dpn. Among all viral envelope proteins, the production L protein strictly depends on the hepatocyte differentiation state since it is translated from 2.4 kb RNA which has been reported to be highly differentiation-dependent [14].

In adult animals, levels of viremia and antigenemia show interindividual variation. Additionally, male mice show generally higher virus titers than female mice [35, 36, 37]. Although we generally selected breeding pairs with highly replicating HBV, we observed that HBV 1.3 xfs mice missing an intact X-protein replicate HBV at higher levels than HBV 1.3 mice carrying a wild type HBV genome (Figure 1). This is in contrast to observations made in transient expression experiments [41]. Since both mouse lines carry only one HBV integrate as determined by PCR analysis (Figure 1), this is most probably due to the integration site in the respective mouse line. Notably, neither the start of HBV gene expression nor of HBV replication depended on the presence of the HBV X-protein.

The transgenic mouse models also allowed analyzing essential factors linking replication efficiency and hepatocyte differentiation state. Several *in vitro* studies had described that replication efficiency in primary hepatocytes and hepatoma cell lines depends on hepatocyte differentiation [15]. Previously, we described the transcription of pregenomic RNA to be the bottle neck and a concerted action of the transcription factors HNF1α and HNF4α to be crucial [14]. Other publications have indicated that PGC-1α is an important co-activator and COUP-TF a co-repressor of HNF4α-regulated transcription [24, 42]. Our *in vivo* data now confirm these *in vitro* studies and they even allow a more detailed analysis based on a stepwise and physiological differentiation process during liver development. Expression levels of HNF1α, HNF4α and PGC-1α during liver development correlated closely with those of HBV 3.5 kb RNA, HBV core and HBV L proteins. Up to ED 18.5, however, expression of COUP-TF dominated that of HNF4α, the competitor for its binding site. This might explain, why the amount of pregenomic RNA in the pool of 3.5 kb RNAs remained low before birth. We suggest that an increase in the expression level of HNF4α is responsible for high-level transcription of HBV pregenomes and onset of HBV replication and – in concert with those of HNF1α and PGC-1α - for morphological and metabolic differentiation of hepatocytes required for formation and secretion of HBV virions.

Using a transgenic mouse model, we show that HBV replication starts postnatally even though a transcription template is present before. However, the transfer of our data onto HBV replication during human liver development should be made with caution. While comparable stages of liver development are well described for the embryonic period [43], a comparison of later fetal and postnatal stages relies on functional data, showing that differential expression levels of liver enzymes during human liver development also closely resemble those in rodents [44]. While our studies do not allow for an analysis of the dependence of infection on the hepatocyte differentiation state or any potential immune response, our studies do allow to state, that if *in utero* infection occurs, it will be hard to detect since detection relies on expression of viral antigens and/or on virus replication. Also, the late start of replication in the developing liver might explain, why the active-passive immunoprophylaxis after birth protects infants at high-risk from chronic viral infection [45], though it cannot prevent transplacental transmission and infections occurring pre- or perinatally. Successful prevention of *in utero* HBV transmission has been reported for lamivudine [46] or administration of anti-HBs IgG [47, 48] in the third trimester of pregnancy. However, both treatment options might not prevent perinatal transmission of HBV infection in every newborn [9, 49].

In conclusion, HBV replication in HBV transgenic mice starts after birth, although transcription of HBV pregenomes is detectable at earlier stages of liver development. Efficiency of HBV replication paralleles liver maturation, suggesting that differentiation of hepatocytes with increasing levels of HNF1α, HNF4α and PGC-1α determines the starting point of HBV replication through an increase of pregenomic RNA. The late start of HBV replication might explain the high efficacy of active-passive immunoprophylaxis after birth in the clinical context.

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Table 1

Target gene	GenBank Acc	Forward primer	Reverse primer
HNF1α	NM_009327	3577 - 3592	3901 - 3886
HNF4α	NM_008261	1939 - 1954	2038 - 2021
PGC-1α	NM_008904	921 - 936	1130 - 1114
β-ACTIN	NM_007393.3	1440 - 1456	1651 - 1435
GAPDH	NM_001001303	607 - 623	973 - 958
HBV DNA	NM_000457	687 - 702	962 - 945
HPRT 1 (DNA)	NM_013556	2007 - 2026	2192 - 2177
HBV 3.5 kb RNA	HBV genotype D, subtype ayw, numbering from core AUG = 1	1626 - 1643	1840 - 1824

HBV replication during liver development

Figure legends

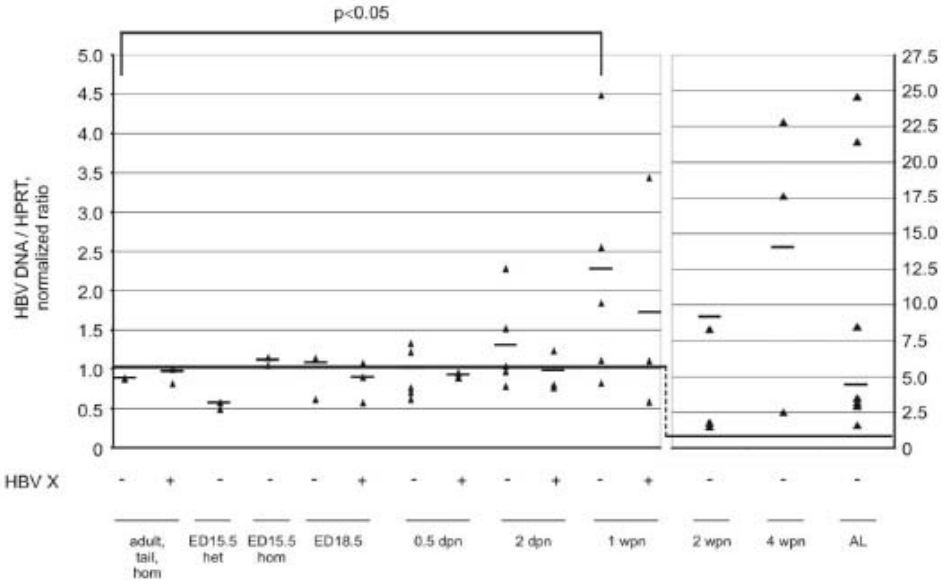

Figure 1: Detection of HBV DNA during liver development

Total DNA from livers and tails of adult HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-), was isolated and compared to fetal liver DNA obtained at ED 15.5 and ED 18.5 and to newborn liver DNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. HBV DNA was quantified by LightCycler™ real time PCR relative to house keeping gene HPRT and normalized to a dilution series of a calibrator DNA. Maximal levels of HBV DNA determined in tails of adult transgenic animals were set to 1 and used as baseline. Levels of HBV DNA from individual samples and mean values are given. At ED 15.5, heterozygous (het) and homozygous (hom) fetuses were analyzed.

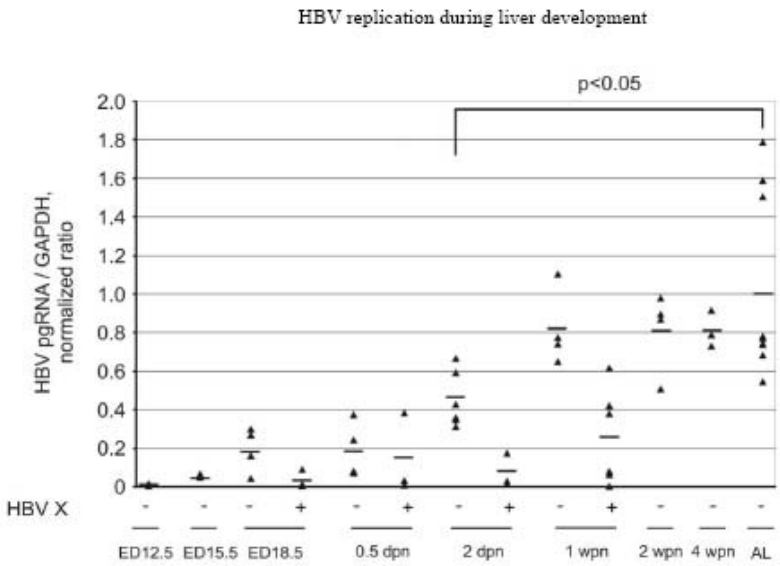

Figure 2: Expression of HBV 3.5 kb RNA during liver development

Total RNA from livers of adult HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-), which carry an X-deficient replication competent HBV genome, was isolated and compared to fetal liver RNA obtained at ED 12.5, ED 15.5 and ED 18.5 and to newborn liver RNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. Expression of HBV 3.5 kb RNA was quantified relative to GAPDH by LightCycler™ real-time PCR and normalized to a dilution series of calibrator cDNA using the Relative Quantification Software.

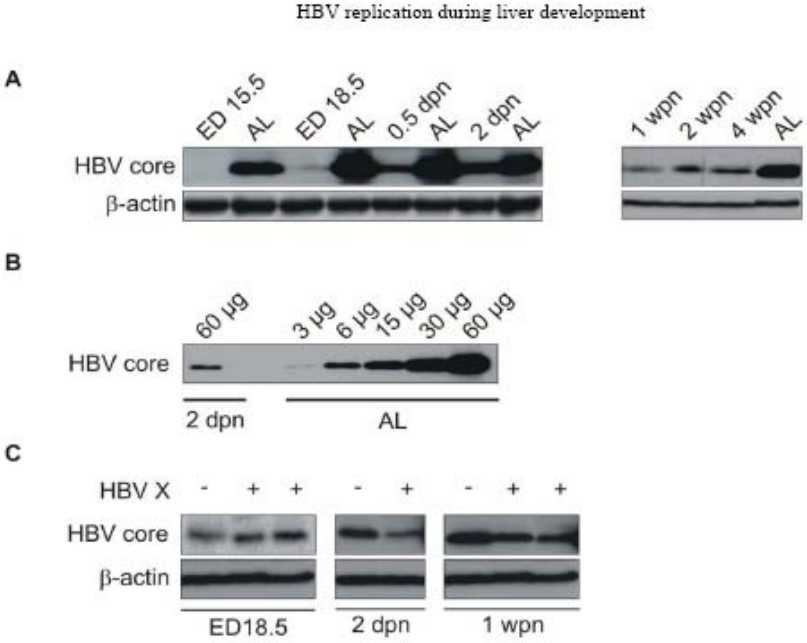

Figure 3: Expression of HBV core protein during liver development

(A) Total cellular proteins from livers of transgenic mice carrying replication competent, x-deficient HBV genomes were isolated at ED 12.5, ED 15.5 and ED 18.5, 0.5 and 2 days (dpn) as well as at 1, 2 and 4 weeks (wpn) after birth and from maternal adult livers (AL). 80 µg of proteins per sample were separated by SDS-PAGE, blotted and stained for HBV core protein and β-actin. Representative Western blots are shown.

(B) HBV core protein expression in 60 µg of total protein extracted from livers at 2 days postnatally (2 dpn) was compared to that in 3 to 60 µg of total protein in maternal livers.

(C) Expression levels of HBV core protein in HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-) were compared at different stages of liver development.

HBV replication during liver development

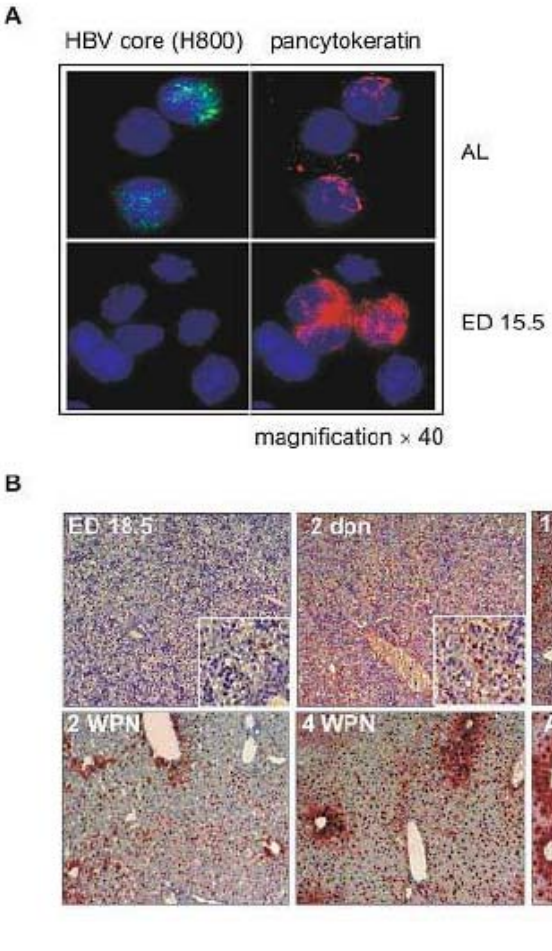

Figure 4: Immunocyto-/-histochemistry for HBV core protein during liver development

(A) Single cell suspensions obtained from ED 15.5 fetal or corresponding adult livers of HBV 1.3 xfs transgenic mice were co-stained on cytospins for HBV core protein (green) and pancytokeratin (red) expressed only in epithelial cells. Nuclei were stained with DAPI (blue).

(B) Livers of HBV 1.3 xfs transgenic mice were isolated at different stages of liver development and sections of paraffin-embedded liver tissues were stained for HBV core protein and developed using APC-system and horseradish peroxidase (brown).

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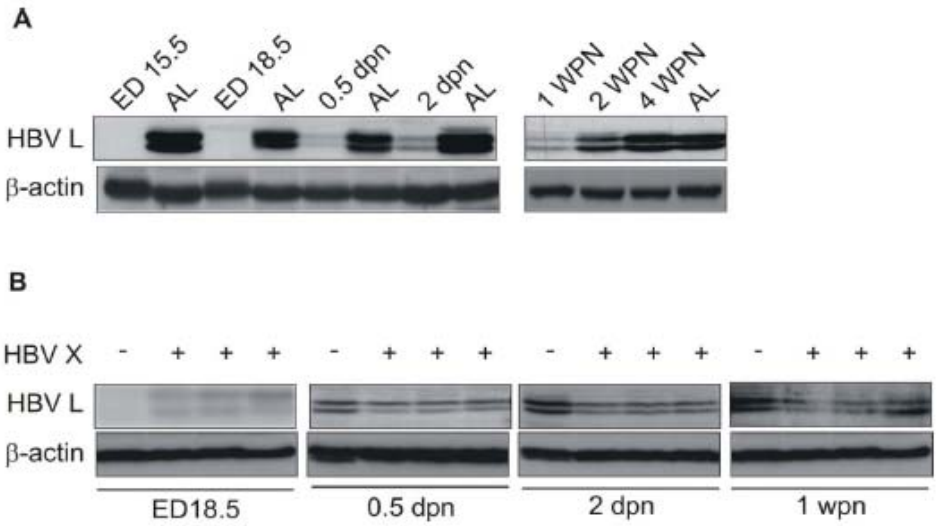

Figure 5: Expression of HBV L protein during liver development

(A) Total cellular proteins from livers of transgenic mice carrying replication competent, x-deficient HBV genomes were isolated at ED 12.5, ED 15.5 and ED 18.5, 0.5 and 2 days (dpn) as well as at 1, 2 and 4 weeks (wpn) after birth and from maternal adult livers (AL). 80 µg of proteins per sample were separated by SDS-PAGE, blotted and stained for HBV L protein and β-actin. Representative Western blots are shown.

(B) Expression levels of HBV L protein were compared in HBV 1.3 transgenic mice (+) and HBV 1.3 xfs mice (-) at different stages of liver development.

HBV replication during liver development

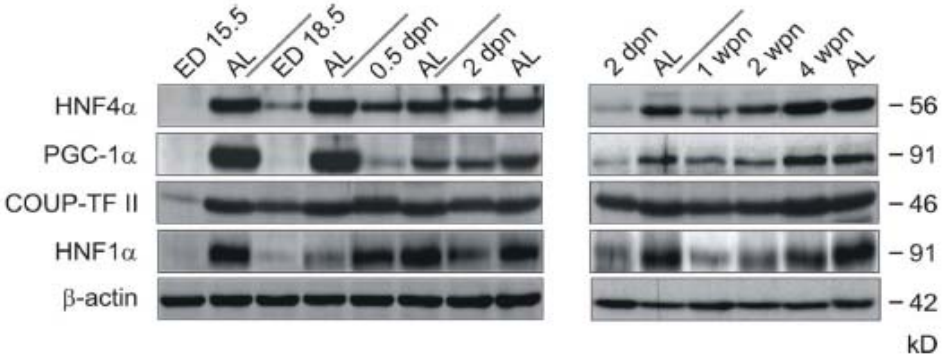

Figure 6: Hepatocyte-enriched transcription factors during liver development

Total cellular proteins from livers of transgenic mice carrying replication competent X-deficient HBV genomes (HBV 1.3 xfs) were isolated from maternal adult (AL) as well as fetal livers at ED 12.5, ED 15.5 and ED 18.5 and newborn livers obtained 0.5 and 2 days (dpn) as well as livers at 1, 2 and 4 weeks (wpn) after birth. 80 µg of proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were stained for HNF1α, HNF4α, PGC-1α, COUP-TF and β-actin as loading control. A representative Western blot is shown.

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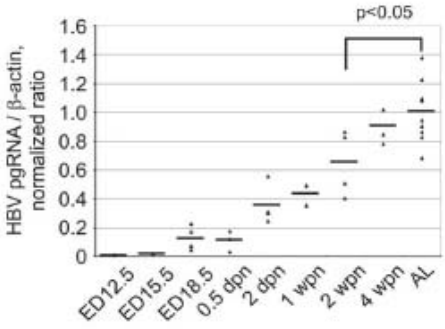
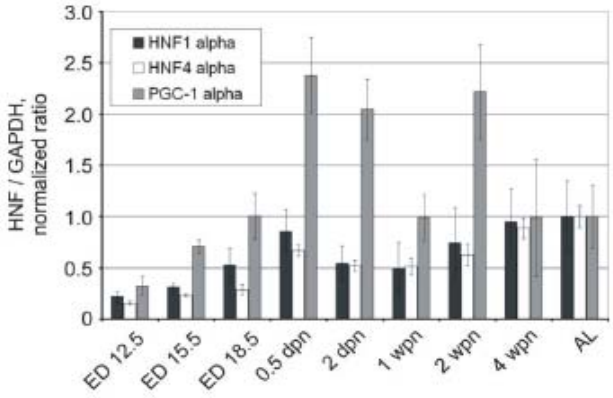
Supplementary Figures



Supplementary Figure 1: Expression of HBV 3.5 kb RNA during liver development

Total RNA was isolated from livers of HBV 1.3 xfs mouse mothers (AL) and compared to fetal liver RNA obtained at ED 12.5, ED 15.5 and ED 18.5 and to newborn liver RNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. Expression of HBV 3.5 kb RNA was quantified relative to β-actin by LightCycler™ real-time PCR and normalized to a dilution series of calibrator cDNA using the Relative Quantification Software.

HBV replication during liver development



Supplementary Figure 2: Hepatocyte-enriched transcription factors during liver development by real-time PCR

Total RNA was isolated from livers of HBV 1.3 xfs mouse mothers (AL) and compared to fetal liver RNA obtained at ED 12.5, ED 15.5 and ED 18.5 and to newborn liver RNA obtained 0.5 and 2 days (dpn) as well as 1, 2 and 4 weeks (wpn) after birth. Total cellular RNA was reverse transcribed into cDNA. Expression of HNF1α, HNF4α and PGC-1α was quantified relative to GAPDH by LightCycler™ real-time PCR and normalized to a dilution series of calibrator cDNA using the Relative Quantification Software. Medium ± SD of at least three animals per time point are given.

9.2.2.2 „Not interferon, but IL-6 controls early gene expression in Hepatitis B Virus (HBV) replication"

Not interferon, but IL-6 controls early gene expression in Hepatitis B virus (HBV) infection

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Running title: IL-6 controls HBV early gene expression

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Word count: summary 152 words; text (including spaces, figure legends and references): 52680 characters;

Summary

Hepatitis B virus (HBV) infection is non-cytopathic and hardly induces innate immunity. We here describe that HBV triggers pattern-recognition receptors on primary human non-hepatocytes in the liver. Although they are not infected, NF-kB is activated mainly in liver macrophages, Kupffer cells. This induces the release of IL-6 and other cytokines but no interferon. The response is transient, and inhibits responsiveness towards a subsequent challenge. IL-6 controls HBV gene expression and replication in hepatocytes at the level of transcription shortly after infection by activating the mitogen-activated protein kinases ERK and JNK. These inhibit expression of hepatocyte nuclear factors 1α and 4α essential for HBV transcription. Thus, an IL-6 response triggered by HBV pattern recognition is responsible for early control of the virus besides its known function in preventing hepatocyte damage. Thus, neutralization of IL-6 during treatment of certain diseases may represent a risk if the patient is HBV infected.

Introduction

Activation of the innate immune system plays a central role in virus-host interactions, because it triggers the adaptive immune response and influences the clinical outcome of an infection. The innate immune system relies on the recognition of molecular patterns associated with pathogens like bacteria or yeast but also viruses.

The liver plays an important role in the host defense against pathogens. Various liver cells including parenchymal hepatocytes and non-parenchymal Kupffer cells, liver sinusoidal endothelial cells and myofiboblasts (Ito cells), are involved in antigen processing and the induction of immune responses (for review see: (Knolle and Limmer, 2001; Racanelli and Rehermann, 2006; Winau et al., 2007)). Kupffer cells, the liver-resident macrophages, account for approximately one-third of non-parenchymal cells in the liver and constitute more than 70% of the total macrophage population of the body. They are able to effectively remove lipopolysaccharides (Lichtman et al., 1994) as well as Gram-negative bacteria (Katz et al., 1991) from the portal vein blood and the systemic circulation and are part of the innate immune system. Liver sinusoidal endothelial cells rapidly clear antigen from the blood through receptor-mediated endocytosis by pattern recognition receptors such as mannose-binding proteins and scavenger receptors (Limmer et al., 2000; Smedsrod, 2004). Isolated Kupffer cells and hepatic endothelial cells have been shown to produce inflammatory cytokines including IL-6, IL-1β and tumor necrosis factor (TNF) α, but also interferons to appropriate stimuli (Wu et al., 2007).

IL-6 is a pleiotropic cytokine with well known beneficial effects for the liver. IL-6 activates intracellular pathways via a heterodimeric IL-6 / gp130 receptor and activating intracellular STAT3. IL-6 promotes liver regeneration and protects against a multitude of liver-damaging influences such as alcohol and CCl4 intoxication. Recently, the IL-6-gp130-STAT3 pathway has been demonstrated to protect hepatocytes from T-cell mediated damage (Klein et al., 2005).

In HBV infection, the role of the adaptive immune response is well established, but our knowledge about the innate immune response is limited (reviewed in: (Rehermann and Nascimbeni, 2005)). In HBV transgenic mice, natural killer (NK) cells and NKT-cells challenged with alpha-galactosylceramide inhibit HBV replication (Kakimi et al., 2001) by secreting interferon (IFN) γ. Activation of toll-like receptors or non-parenchymal liver cells inhibits hepatitis B virus replication *in vivo* in an IFN α/β dependent manner (Isogawa et al., 2005; Wu et al., 2007). All these effects are mediated by antiviral cytokines, mainly IFNs but also TNF α.

Notably, most of the HBV DNA is cleared from the serum and the livers of experimentally infected chimpanzees prior to a detectable adaptive immune response in the liver impying an important role of the innate immune system in mounting the host response (Guidotti et al., 1999). Microarray analyses of serial liver biopsies of experimentally infected chimpanzees, however, revealed no detectable changes in the expression profile of intrahepatic genes in the first weeks of infection (Wieland et al., 2004). Despite this, a role for the innate immune response in the control of early HBV replication should not be dismissed, because expression of immune-response genes might occur below the level of detection of the microarray analysis that has been carried out (Rehermann and Nascimbeni, 2005).

Whereas the role of IFNs and TNFα in HBV control has been intensively studied, little is known about the inflammatory cytokines such as IL-6 and IL-1β.

The purpose of this study was to examine whether host recognition of HBV activates an innate immune response in primary human liver cells, and which consequences the release of cytokines upon pattern recognition of HBV may have for for both, the host cell and the virus.

Results

HBV infection stimulates secretion of IL-6 by non-parenchymal liver cells prior to HBV replication in hepatocytes.

To determine if HBV or its secretory antigens activate pattern recognition receptors on human liver cells, we prepared two different primary human liver cell cultures, and examined IL-6 secretion upon incubation with HBV inocula.

Primary human hepatocyte (PHH) cultures contained ≥ 85% hepatocytes and 3-15% of liver sinusoidal endothelial cells or liver macrophages, Kupffer cells (Schulze-Bergkamen et al., 2003). Non-parenchymal liver cell (NPC) cultures contained ≥ 85% NPC, mainly Kupffer cells (Fig. 1 A and B). Enrichment of Kupffer cells was controlled by quantitative real-time RT-PCR analysis, which revealed a five-fold higher expression level of the macrophage-specific CD68 mRNA in NPC than in PHH cultures. Additionally, NPC cultures were inoculated with fluorescent-labelled *E.coli*, which were phagocytosed and induced a phenotypic change from stellate to rounded cells within 2 h (Fig. 1 C and D).

To examine IL-6 secretion, PHH and NPC cultures were incubated with HBV inocula at a multiplicity of infection (moi) of 200 virions per cell on day 3 after seeding. Mock inocula were prepared from HBV-negative HepG2 cells and used at equal amounts. NPC cultures inoculated with fluorescent-labeled *E.coli* triggering toll like receptor (TLR)-4 were used as a positive control. Cell culture media were collected at 5h, 24h (day 1), 48h (day 2), 72h (day 3) and 96 h (day 4) post infection (p.i). Levels of secreted hepatitis B e antigen (HBeAg) and IL-6 were measured by immunosorbent assays.

Both, PHH cultures and NPC, rapidly started to secrete IL-6 within the first 5 h after inoculation with HBV, with maximal levels at 24 h p.i. IL-6 induction was transient equaling background levels again on day 3 p.i. *E.coli*-treated NPC secreted IL-6 with kinetics comparable to that in HBV-infected cells (Fig. 1 E). Mock inocula did not induce IL-6. Maximum levels of IL-6 were markedly higher in HBV-inoculated NPC (4464.6 ± 294.5 pg/ml) than in HBV-infected PHH cultures (2310.7 ± 128.6 pg/ml), although cell density in

NPC cultures was lower. On day 4 p.i., we detected newly synthesized HBeAg (21.4 ng/ml) in HBV-infected PHH but not in NPC cultures (detection limit 0.2 ng/ml; data not shown). This indicated HBV gene expression and beginning virus replication in PHH, and the absence of productively infected hepatocytes in NPC cultures. Thus, our data suggest that NPC but not hepatocytes produce IL-6 after their exposure to HBV, but prior to HBV replication in hepatocytes. Confirming the selective enrichment of hepatocytes or NPC, the acute phase response C-reactive protein (CRP) gene was up-regulated only in PHH cultures (Tab. 2).

To exclude LPS contamination of our preparations, we incubated TLR4-MD-2-transfected 293 cells with our HBV preparations, which did not lead to cell activation as compared to LPS used as positive control (data not shown). In order to analyze potential LPS-like tolerance effects of HBV infection, mock- or HBV-infected PHH cultures on day 6 p.i. were inoculated again with HBV or challenged with fluorescent-labeled *E.coli* particles. As shown in Fig. 1F, mock-infected PHH cultures secreted IL-6 at 24 h after addition of HBV or *E.coli* at levels comparable to those in 3-days old PHH cultures stimulated with HBV or *E.coli* (Fig. 1E). In contrast, PHH cultures, which had been previously infected with HBV, secreted barely detectable amounts of IL-6 (Fig. 1 F). Thus, once activated after HBV infection, NPC seem not to be capable of pathogen recognition and IL-6 production any more.

To test dose dependency, we infected PHH with HBV at a multiplicity of infection (moi) of 200, 50, 10 and 5 virions per cell. After 24 h, IL-6 concentrations of 3457, 3500, 1374 and 679 pg/ml were determined, respectively, indicating receptor saturation at the two highest doses. To analyze for dependency on virus replication, we pretreated the HBV inoculum with 100 mJ of UV-light. When UV-treated HBV was used for infection, transcription of HBV pregenomic (pg) RNA was reduced by 86% at day 4 p.i.. However, the secretion of IL-6 was not affected (1069.5 ± 15.5 and 1153 ± 6 pg/ml for UV-treated and untreated HBV, respectively), indicating that neither an intact viral genome nor its transcription and/or replication were required to induce IL-6 secretion (data not shown).

To determine whether other cytokines besides IL-6 were induced upon cellular HBV recognition, we analyzed cell culture supernatants from HBV-infected PHH or NPC cultures for the presence of IL1β, TNFα and IFNα by ELISA, and determined their mRNA expression levels by quantitative real time RT-PCR. IL-1β (PHH: 157.4 ± 22.2 pg/ml, NPC: 1862.7 ± 122.4 pg/ml) and TNFα secretion (PHH: 262.5 ± 23.6 pg/ml, NPC: 1189.5 ± 171.5 pg/ml) were detected with a kinetic very similar to that of IL-6 peaking at 24 h p.i confirming the involvement of NPC in virus recognition (data not shown). IL-6, IL-1β and TNFα gene expression was up-regulated in NPC, and to a lesser extent, in PHH cultures (Tab. 2). In contrast, IFNα was not detected in cell culture media by ELISA. In NPC, the IFNβ gene was ≤ 2-fold regulated. In HBV-infected PHH, the IFNβ, the IFN-inducible 2'5'-oligoadenylatsynthetase (2'5'OAS) and the IFN-inducible protein 10 (IP10) genes were 5.9- and 6.3-fold downregulated (Tab. 2), whereas they were 3- and 47-fold upregulated, respectively, upon exposure to E. coli (data not shown).

Taken together, release of IL-6, IL-1β and TNFα was induced rapidly after contact of hepatic cells with an HBV inoculum containing virions as well as secretory hepatitis B surface antigen (HBsAg) and HBeAg, whereas it was neither induced by nor depended on HBV replication. In contrast, HBV induced no interferon, and interferon-regulated genes were even down-regulated.

Activation of NFκB in primary liver cells early after HBV infection.

Since we proposed that non-parenchymal liver cells such as Kupffer cells or sinusoidal endothelial cells might be capable of recognizing HBV particles by pattern recognition receptors, we examined whether NFκB was activated upon contact with HBV.

Nuclear extracts from HBV-infected, but not from mock-infected PHH cultures exhibited a clear NFκB binding activity on day 1 p.i., not detected by EMSA any more on day 4 p.i. (Fig. 2 A). In HBV-infected PHH cultures (moi 20 virions / cell), DNA binding of NFκB was 5-

fold increased as early as 3 h p.i. whereas in mock-infected samples no activated NF-κB was detected by a sensitive, chemiluminiscent ELISA-based NFκB-DNA binding assay. At 24 h p.i., infected cells exhibited a slightly reduced but still substantial NF-κB activity. The addition of NF-κB competitor oligonucleotides confirmed the specificity of the assay (Fig. 2 B). From 48 h p.i. to day 6 p.i., we detected no NF-κB binding activity, neither in mock- nor in HBV-infected cells (day 4 p.i is shown in Fig. 2 B). Addition of an NF-κB inhibitory peptide (16.6 µg/ml) to PHH cultures 2.5 h before HBV infection resulted in 81% reduced activation of NF-κB and 50% reduced secretion of IL-6 (data not shown).

Next, we examined the activation of NF-κB in NPC cultures inoculated with HBV at moi 20 virions / cell. As a positive control, we used 100 fluorescent-labeled *E.coli* particles per cell, which we expected to activate NF-κB in a TLR4-dependent fashion. NFκB-DNA binding assay revealed 10.5-fold and 6.6-fold increase of NFκB binding 3 h after inoculation with HBV or fluorescent-labelled *E. coli*, respectively. After 24 h, NF-κB activity declined in both HBV- and *E.coli*-treated NPC (Fig. 2 C). Activation of NF-κB was more pronounced in HBV-inoculated NPC than in PHH cultures (Fig. 2 B and C). Taken together, our data demonstrate an activation of NF-κB in primary human liver cell cultures within the first 3 h p.i. with HBV, which subsequently leads to induction of inflammatory cytokines.

HBV envelope proteins induce induction of NF-kB and secretion of cytokines by NPC.

To show specific activation of pattern recognition receptors by HBV, we added 0.5 or 2.5 I.U. of neutralizing human anti-HBs antibodies (Hepatect®, Biotest Pharma GmbH, Dreieich, Germany) to the HBV inoculum (containing $2x10^7$ virions) prior to infection of PHH cultures (moi 20 virions / cell). 24 h p.i., we determined the levels of HBsAg, HBeAg and IL-6 in PHH culture media (Tab. 3). While equal levels of HBeAg indicated equal infectious doses, the addition of 0.5 I.U. of neutralizing human anti-HBs antibodies reduced the release of HBsAg by 63% and of IL-6 by 41%. The addition of 2.5 I.U. of anti-HBs antibodies reduced

HBsAg to undetectable levels and IL-6 release by 66%, whereas addition of equal amounts of unspecific human IgG did not reduce either of the two (data not shown). Accordingly, HBV inocula pretreated with 0.5 or 2.5 I.U. of anti-HBs neutralizing antibodies activated NF-κB at 26% and 78% lower levels than an untreated inoculum, respectively (Tab. 3).

Taken together, HBV envelope proteins seemed to largely contribute to the induction of NF-κB-regulated cytokines such as IL-6 in human non-parenchymal liver cells. However, an incomplete block of IL-6 secretion by anti-HBs neutralizing antibodies (Tab. 2) indicated an additional immune stimulatory activity of HBV-patterns besides the envelope proteins.

IL-6 inhibits HBV transcription and replication.

Since NF-κB activation was transient and was abolished before virus replication began, we wondered which cytokines - without interferon induction – were able to control HBV gene expression and replication early after infection, and to which extent IL-6 contributed to this. We therefore used recombinant IL-6 (rIL-6) and neutralizing anti-IL-6 antibodies (IL-6ab) to study the influence of IL-6 on HBV replication.

Addition of IL-6ab (200 ng/ml) to PHH cultures prior to HBV infection resulted in an almost two-fold increase of HBeAg and HBV progeny secretion, indicating that endogenous IL-6 blocked HBV gene expression and replication (Fig. 3 B). Next, we added 15 ng/ml rIL-6 to HBV infected cells every 48 hours, after the peak amount of endogenous IL-6 in HBV-infected cells had been removed by exchanging the culture medium 24 hours post infection. As a control, rIL-6 was pre-incubated with anti-IL-6 antibodies (1 µg/ml). Cells and supernatants were analyzed on day 5 p.i..

CRP expression indicated proper activation of the IL-6 signaling pathway by rIL-6 (Fig. 3 A). In parallel, a 66% decline of HBV pregenomic RNA (pgRNA) (Fig. 3 A), a 77% drop in HBeAg secretion and a >50% reduction of HBV progeny release (Fig. 3 B) was detected. Western blot analyses of cytoplasmic protein fractions revealed that in HBV-infected cells

treated with rIL-6, HBV core protein was reduced by 68% and HBV envelope proteins were diminished by 90% (L), 79% (M) and 60% (S) (Fig 3 C). These effects were antagonized by neutralization with anti-IL-6 antibodies.

To examine the effects of IL-6 on the state of the HBV-infected hepatocytes, we determined expression levels of the anti-apoptosis genes cIAP2, Mn-SOD and IGFBP1, which we recently found to be regulated by HBV infection (Hösel & Protzer, unpublished results). Treatment with rIL-6 resulted in a 3.8 -, 3.9 - and 24.6 up-regulation, respectively. In contrast, INFβ- or IFN-inducible genes were either not affected or even down-regulated (Tab. 4).

Thus, IL-6 suppressed transcription of the HBV pgRNA as well as HBV gene expression and as a result HBV replication and -in addition- supported cell survival early after HBV infection.

Mitogen-activated protein (MAP) kinases activated by IL-6 down-regulate hepatocyte nuclear factor (HNF) 1α and HNF4α.

We next searched for possible mechanisms explaining how IL-6, without interferon induction, inhibited HBV infection. Since hepatocyte specific transcription factors HNF1α and HNF4α control transcription of HBV pgRNA and HBV gene expression in a concerted action in hepatocytes (Quasdorff et al., 2008), we examined whether IL-6 may control expression of these transcription factors.

rIL-6 stimulation resulted in an 48% and 56% decrease in HNF1α and HNF4α mRNA expression, respectively, in comparison to non-treated, HBV-infected cells (Fig. 3 A), whereas expression levels of HNF3β or γ remained constant (data not shown). Neutralization of IL-6 released after HBV infection resulted in an increase of HNF1α and HNF4α gene expression by 24% and 54%, respectively (Fig. 3 A). Accordingly, Western blot analyses of nuclear extracts prepared from HBV-infected PHH confirmed a 55%- and a 58% reduction in the amounts of HNF4α and HNF1α protein by treatment with rIL-6, respectively (Fig. 3 C).

This showed that both, endogenously produced IL-6 as well as added rIL-6 controlled expression of HNF1α and HNF4α, two essential transcription factors driving HBV gene expression and replication.

Since the activated MAP kinases exogenous signal regulated kinase (ERK) 1/2 and jun N-terminal kinase (JNK) have been reported to control HNF4α expression, and IL-6-type cytokines may activate members of the MAPK family, we hypothesized that activation of MAPK by IL-6 suppressed HBV replication. Thus, we first examined whether MAPK family members ERK1/2, p38 and / or JNK were activated after HBV infection in PHH. As shown in Fig. 4 A, levels of phosphorylated ERK and JNK, but not p38 were increased upon HBV infection. This activation was obviously mediated by IL-6 since pre-treatment of PHH with neutralizing IL-6ab abolished activation of ERK and JNK during HBV infection (Fig. 4 B). This was confirmed by treatment with rIL-6, which also activated ERK and JNK (Fig. 4 B), but not p38 (data not shown).

To determine the effect of IL-6-induced MAPK activation on HNF4α and HNF1α expression, we used specific inhibitors of upstream activators of MAPK phosphorylation prior to rIL-6 stimulation of PHH: PD98059 for ERK and SP600125 for JNK, respectively. Treatment with PD98059 inhibited ERK phosphorylation to a large part, while SP600125 blocked JNK phosphorylation (Fig. 4 C). Accordingly, inhibition of JNK-activation completely and that of ERK-activation partially overcame rIL-6-mediated down-regulation of HNF4α and HNF1α (Fig. 5 D). HBeAg levels were restored when cells were treated with JNK inhibitor SP600125 and increased by 90% when treated with ERK inhibitor PD98059, whereas p38 inhibitor SB203580 showed no effect (data not shown).

This showed that in primary hepatocytes, IL-6 activated the MAPK ERK and JNK, and thus down-regulated expression of HNF4α and HNF1α, which in a concerted action drive HBV gene expression and consequently HBV replication. We concluded, that ERK- and JNK-

activation and subsequent downregulation of essential transcription factors HNF4α and HNF1α was responsible for the negative control of HBV replication by IL-6.

Discussion

It has previously been described that patients infected with HBV have elevated plasma levels of IL-1, TNFα and IL-6 (Anastassakos et al., 1988; Devergne et al., 1991; Sheron et al., 1991; Torre et al., 1994). We found that the contact of primary human liver cells with HBV in cell culture induced the expression and secretion of IL1β, TNFα and IL-6 within 5 h after inoculation, whereas we detected no induction of type I IFN. Using two different primary human liver cell cultures, we found that PHH cultures containing mainly (but not exclusively) parenchymal hepatocytes secreted less cytokines than NPC cultures containing mainly Kupffer cells. Within 3 h, HBV activated NF-κB in NPC cultures comparable to fluorescent-labeled *E.coli,* recognized through TLR4 by Kupffer cells (Su et al., 2000). Importantly, NF-κB activation followed by cytokine induction occurred early after infection, prior to virus replication in hepatocytes.

Careful controls excluded that this recognition was due to contamination of our virus stocks with e.g. co-purified lipoproteins. Treatment of the HBV inoculum with anti-HBs neutralizing antibodies resulted in a dose-dependent reduction of NF-κB activation and, accordingly, IL-6 secretion. Thus, our data strongly suggest that HBV envelope proteins are recognized by pattern recognition receptors on non-parenchymal liver cells, and that this recognition does not depend on HBV replication in hepatocytes.

Notably, the activation of NF-κB and proinflammatory cytokines was transient in our experiments and was not induced by newly synthesized virus, which was released from infected hepatocytes in our cultures. When we tried to stimulate PHH cultures, which had

been infected with HBV before, neither *E. coli* nor a secondary HBV infection were able to trigger the release of IL-6 (Fig. 1F). Therefore, we propose that HBV or its secretory antigens tolerize hepatic NPC by a mechanism which might resemble LPS tolerance and thus prevent continuous stimulation by newly synthesized virus. This is in accordance with the observation that recombinant HBsAg interferes with NF-κB signaling pathways (Cheng et al., 2005) and may explain why the virus hardly induces any detectable innate immune responses in chimpanzees (Wieland et al., 2004) although huge amounts of viral antigens circulate.

Although the induction of NF-κB and proinflammatory cytokines was transient, it was sufficient to cause changes in hepatocellular gene expression. For instance, expression of the acute phase response CRP was up-regulated in PHH as well as transcription of the NF-κB and STAT3-regulated anti-apoptotic gene cIAP-2, Mn-SOD and IGFBP1. NF-κB and its target genes are known to play an important role in protection against apoptosis (reviewed in: (Kucharczak et al., 2003)). The IL-6 / STAT-3 pathway is also essential for liver regeneration and inhibition of apoptosis (Kovalovich et al., 2001) (reviewed in: (Taub, 2003)). IL-6-type cytokines via activation of STAT3 induce the synthesis of acute phase proteins in hepatocytes, which also play a protective role for the cell during the acute phase response (Taub, 2003). Our own results showed activation of STAT3 in HBV-infected hepatocytes (Hösel & Protzer, unpublished). Thus, we suggest that HBV recognition and subsequent NF-κB activation ensure cellular homeostasis and prevent apoptosis of the infected hepatocyte since HBV obviously needs an intact cell for its multiplication.

IFNs are well known to control HBV replication. Although we detected an activation of NF-κB in NPC cultures, we did not observe an induction of interferon response in HBV-infected cells. IFN and IFN-inducible genes were even down-regulated in HBV-infected hepatocytes. In agreement with our data, induction of IFN-α/β and 2´5´-OAS genes has not been observed in livers of chimpanzees acutely infected with HBV (Wieland et al., 2004). It remains to be investigated whether HBV inhibits induction of an IFN response.

IFNγ and TNFα play an important role in controlling HBV infection in a non-cytopathic fashion in HBV-transgenic mice (Dumortier et al., 2005; Guidotti et al., 1996) and in HBV-infected chimpanzees (Guidotti et al., 1999). Both cytokines directly induce the elimination of HBV-RNA containing capsids from the cytoplasm in infected hepatocytes (Biermer et al., 2003; Klöcker et al., 2000; Pasquetto et al., 2002; Wieland et al., 2005). However, little is known about the effects of IL-6 on HBV replication.

We therefore analysed the effects of IL-6 on HBV infection and replication in primary human hepatocytes. Our results clearly demonstrate a negative influence of IL-6 on HBV gene expression and replication in primary human hepatocyte cultures. Levels of HBV pg RNA, HBeAg, core- and envelope proteins as well as viral progeny release were markedly reduced in HBV-infected PHH after administration of human recombinant IL-6 (Fig. 3A). Moreover, we showed that induction of endogenous IL-6 by HBV pattern recognition also controls HBV transcription and replication, since treatment of PHH with IL-6 neutralizing antibodies prior to infection increased HBV gene expression and its progeny release (Fig. 3B).

The role of IL-6 for HBV-infection has been discussed controversially. Waris and Siddiqui incubated HBV-replicating HepG2.2.15 hepatoma cells with human IL-6 and found that IL-6 induced STAT-3 interacted with HNF3, bound to the HBV enhancer I and stimulated HBV transcription (Waris and Siddiqui, 2002). However, *in vivo* in HBV-transgenic mice, administration of recombinant IL-6 led to the suppression of steady state mRNA expression 16 to 20 h after administration (Gilles et al., 1992). The latter results are in accordance with our findings in primary liver cell cultures, and the differences observed in hepatoma cells may be due to dysregulation of signalling pathways. Galun *et al.* observed that human liver tissue incubated with HBV and human IL-6 before transplantation into SCID mice lead to a higher rate of HBV DNA positive animals than liver tissue incubated only with HBV (Galun et al., 2000). This may be explained by the improvement of survival of hepatocytes by IL-6, since IL-6 does not directly interact with HBV (Heinz et al., 2001).

Since IL-6 rather suppressed than induced IFN response pathways in primary liver cells (Tab. 2), we asked how IL-6 might inhibit HBV replication. All experiments indicated a negative influence on HBV transcription although STAT-3 dependent genes were upregulated. HBV transcription by host RNA-polymerase II is regulated finely by a number of hepatocyte enriched transcription factors. These include HNF1 (Raney et al., 1990), HNF3 (Ori and Shaul, 1995) or HNF4 (Raney and McLachlan, 1997; Tang and McLachlan, 2001) which act in concert with nuclear receptors. Recently, we have shown that a concerted action of HNF4α and HNF1α, which also determines morphological and functional differentiation of hepatocytes, mediates efficient HBV transcription as a prerequisite of its replication (Quasdorff et al., 2008). HNF1α has been shown to be required for the efficient transcription from HBV pre-S1 promoter (Raney et al., 1991; Raney et al., 1990) and HNF4α is necessary for the stimulation of the HBV preC/C promoter (Raney et al., 1997) and binds to the viral enhancer I (Yu and Mertz, 2003).

We therefore examined whether IL-6 treatment would have a negative effect on the expression of HNF1α and HNF4α transcription factors. Indeed, we found that the addition of recombinant IL-6 markedly reduced expression of these transcription factors at both mRNA and protein levels, whereas e.g. HNF3 remained unchanged. This reduction was IL-6-specific, since addition of neutralizing IL-6ab antibodies reverted the effect (Fig. 3). Thus, our data indicate that IL-6-mediated down-regulation of essential transcription factors HNF1α and HNF4α are responsible for reduced HBV gene expression and pregenome transcription, and thus controls HBV replication at the level of transcription. Recently, the helioxanthin analogue 8-1 has been described to suppress HBV replication by down-regulating HNF4α in virus harboring cells (Ying et al., 2007), whereas other cytokines control HBV replication usually at a posttranscriptional step ((Biermer et al., 2003; Dumortier et al., 2005; Guidotti et al., 1996; Klöcker et al., 2000; Pasquetto et al., 2002; Wieland et al., 2005).

Finally, we tried to identify a mechanism underlying IL-6 mediated down-regulation of HNF1α and HNF4α transcription factor expression. It has been reported, that the family of IL-6-type cytokines besides STAT-3 activates the mitogen-activated protein kinases (MAPK) ERK1/2, p38 and JNK (reviewed in: (Heinrich et al., 2003)). In this study, we show that HBV infection as well as IL-6 treatment activate MAPK JNK and ERK, but not p38 (Fig. 4 A, B). Activated ERK1/2 has been reported to reduce HNF4α expression in human hepatoma cells (Hatzis et al., 2006; Reddy et al., 1999). Activated JNK down-regulated HNF4α in hepatoma HepG2 cells (Jahan and Chiang, 2005) and in primary human hepatocytes (Li et al., 2006). By inhibition of MAPK, we showed that upon IL-6 stimulation activated JNK and, to a lesser extend, ERK down-regulated transcription factors HNF1α and HNF4α, which are essential for HBV transcription, in primary hepatocytes. Our results thus explain, why activation of ERK1/2 suppressed HBV replication in HBV-transfected hepatoma cells at a transcriptional level as reported by *Zheng et al.* (Zheng et al., 2003). Since HNF4α also controls expression of HNF1α, it remains open whether there is a direct effect of MAPK on HNF1α, or whether this is secondary to the suppression of HNF4α.

Given the clinical benefit shown by the humanized neutralizing IL-6R monoclonal antibody Tocilizumab as a treatment for Crohn's disease, rheumatoid arthritis and Castleman's disease (Nishimoto and Kishimoto, 2006) it will be important to keep in mind that IL-6 seems to have beneficial effects during HBV infection and neutralization of this pathway could represent a risk for infected patients.

Taken together, we provide strong evidence for recognition of HBV envelope proteins by a yet unspecified pattern recognition receptor or receptors. This recognition occurs in or on non-parenchymal liver cells and results in control of HBV gene expression and transcription of the HBV pregenome in infected hepatocytes. For this control, IL-6 but not IFN plays a major role. By activating MAPK JNK and ERK, IL-6 controls expression of HNF1α and HNF4α, two transcription factors essential for HBV promoter activity. This represents a novel

mechanism by which a cytokine controls HBV at the transcriptional level. Thus, IL-6, well known to stabilize the hepatocyte, helps to minimize early induction of immune responses and thus is advantageous for the virus. If IL-6 is blocked for therapeutic purposes, the clinical course of hepatitis B should be carefully monitored.

Experimental Procedures

Reagents

The NFκB inhibitor peptide, AAVALLPAVLLALLAPVQRKRQKLMP, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), MAPK inhibitors PD98059 and SP600125 from Calbiochem (La Jolla, CA), Alexa Fluor 488 conjugated *Escherichia coli* from Molecular Probes, Invitrogen. Human recombinant IL-6 (van Dam et al., 1993) and mouse monoclonal anti-IL-6 antibody (IL-6ab) have been described previously (Fischer et al., 1997).

Isolation and culture of primary human liver cells.

PHH and NPC cultures were prepared from surgical human liver biopsies after informed consent of patients after a standard two-step collagenase perfusion by serial differential centrifugation at 50 x g (PHH) and 250 x g (NPC), respectively (Schulze-Bergkamen et al., 2003). PHH were seeded onto plastic dishes coated with collagen type IV, NPC onto collagen-free plates. Cell culture medium was Williams medium (Invitrogen, Pailsley UK) supplemented with L-glutamine (5 mM), glucose (0.06% (w/v)), HEPES (23 mM, pH7.4), gentamycin (50 µg/ml), penicillin (50 IU/ml), streptomycin (50 µg/ml), inosine (37µM), DMSO (1.75%), hydrocortisone (4.8 µg/ml) and insulin (1 µg/ml)). Cells were cultivated overnight with 10% (w/v) FCS, after 16 h with 5% FCS and after 72 h without FCS.

Hepatitis B virus infection.

HBV virus inoculum was concentrated from the medium of HepG2.2.15 cells (Williams E medium containing 5% FCS, 2.4 µg/ml hydrocortisone and 0.87% DMSO) as described before (Untergasser et al., 2006) using centrifugal filter devices (Centricon Plus-80, Biomax 100.000, Millipore Corp., Bedford, MA, USA), and stored in 10% glycerol at -80°C until further use. Mock inocula were concentrated from HepG2 cells accordingly. PCR analysis for mycoplasma was negative in both cell lines. HBV titers were determined as enveloped, DNA-containing viral particles after sedimenting viral particles into a CsCl density-gradient (density 1.15 - 1.4 g/ml) by dot blot analysis relative to an HBV DNA standard following hybridization with a ^{32}P labeled probe using a PhosphoImager (BioRad Laboratories, Munich, Germany) (Protzer et al., 1999). On day three after seeding, PHH or NPC cultures were inoculated with HBV at indicated moi in the presence of 5% PEG 6000. At 24 h p.i., the inoculum was removed, cells were washed three times with PBS and further cultivated in PHH medium.

Quantification of gene expression by real-time RT PCR.

Total RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, USA). 0.75 µg of total RNA was transcribed into cDNA using First-Strand Synthesis Supermix for qRT-PCR (Invitrogen) after DNAse digestion (Qiagen, Hilden, Germany). HBV pgRNA was detected as described previously (Protzer et al., 2007). Real-time PCRs were performed with the LightCycler FastStart DNA Master^{PLUS} SYBR Green Kit using the LightCycler™ system and normalized to a dilution series of calibrator cDNA using the Relative Quantification Software (all Roche Diagnostics, Mannheim, Germany) as described (Protzer et al., 2007). For detection, exon-exon-spanning oligonuclotide primers were used (Tab. 1).

Detection of cytokine release.

Cytokine concentrations in cell culture supernatants were determined by specific enzyme-linked immunosorbent assays (ELISA). Matched antibody pairs were used for detection of human IL-6 (BD Biosciences, San Diego, CA, USA), IL-1β (R&D Systems, Minneapolis, MN, USA), TNFα (Biosource, Camarillo, USA) and IFNα (PBL Biomedical laboratories, Piscataway, NJ, USA). The sensitivity of the assays was 15.6 pg/ml for IL-6, 7.8 pg/ml for IL1β, 62.5 pg/ml for TNFα, and 46 pg/ml for IFNα.

Western blot analysis.

Nuclear and cytoplasmic protein extracts were prepared with NE-PER nuclear and cytoplasmic protein extraction reagent (Pierce, Rockford, USA). Total cellular proteins were extracted with CHAPS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1% Chaps). Indicated amounts of proteins were separated by SDS-PAGE (10 to 12.5% according to the expected protein size), and electrophoretically transferred onto nitrocellulose membranes. The membranes were stained with appropriate primary and peroxidase-coupled secondary antibodies (Sigma, Munich, Germany). Signals were detected by WestDura chemiluminescence reagents (Pierce, Rockford, USA) and quantified using the Gel Doc 2000 System (BioRad Laboratories). For reprobing, membranes were treated with 0.2 N NaOH for 10 min at RT before staining with the new antibodies.

Primary antibodies were: rabbit anti-HBVcore antiserum H 800 (Protzer et al., 1999), goat polyclonal anti-S (Murex, Abott), rabbit polyclonal anti-ERK2, mouse monoclonal anti-phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-p38 and rabbit polyclonal anti-phospho-p38 MAP kinase, rabbit polyclonal anti-SAPK/JNK and mouse monoclonal anti-phospho-SAPK-JNK (Cell Signalling, Danvers, MA, USA), rabbit polyclonal anti-HNF1α, anti-HNF4α and anti-β lamin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-β actin (Sigma, St. Louis, MO,USA).

Detection of NF-kB activation.

For the electrophoretic mobility shift assay (EMSA), nuclear extracts from mock- or HBV-infected PHH were prepared from $2x10^6$ cells (Schreiber et al., 1989). EMSA was performed by incubating 5 µg of nuclear extract with ^{32}P-labeled double-stranded oligonucleotides containing the kB site of the HIV-1 LTR enhancer as described (Kruppa et al., 1992). Resolution of protein-DNA complexes was performed by 6%-nondenaturing PAGE and detected by autoradiography.

In chemiluminiscent ELISA-based NF-κB assay, the activation of NF-κB was measured using the EZ-Detect™ Transcription Factor kit for NF-κB p50 (Pierce). 2 µg of nuclear proteins were subjected to a microtiter plate coated with an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3'). Nuclear NF-κB p50 binding was quantified by chemiluminescence after staining with appropriate antibodies. Wild type NF-κB Competitor Duplex (40 pmoles) or mutant type NF-κB Competitor Duplex (40 pmoles) were used to ensure signal specificity.

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Figures

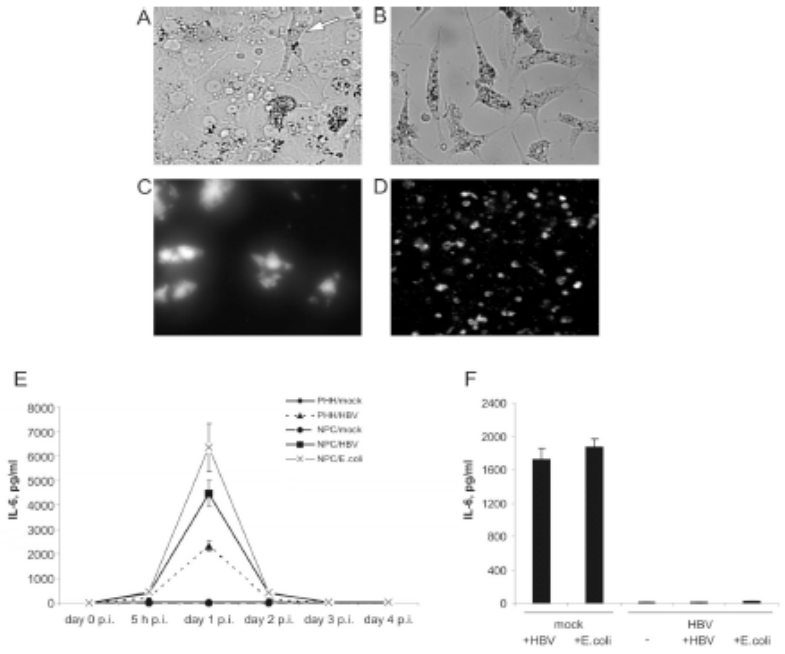

Fig. 1. Kinetics of IL-6 secretion in mock- and HBV-infected PHH or NPC cultures.

Phase contrast microscopy of PHH (A) and NPC (B) cultures isolated by differential sedimentation on day 3 after seeding. The arrow indicates a Kupffer cell in the PHH culture. (C, D) Fluorescent microscopy of NPC at 2 h after addition of green-fluorescent *E.coli* (A-C: 400-fold magnification; D: 100-fold magnification). (E) Cells were mock- or HBV-infected or inoculated with fluorescent bacteria on day 3 after seeding. IL-6 was measured by ELISA in cell culture supernatants at indicated time points. Medium values ± SD of three measurements each of two independent experiments are given. (F). PHH cultures were either mock- or HBV-infected on day 3 after seeding. On day 6 p.i., cells were left untreated (-) or inoculated

again with HBV or with fluorescent *E.coli*. IL-6 was measured by ELISA in cell culture supernatants at 24 h after treatment. Values represent medium ± SD of three measurements.

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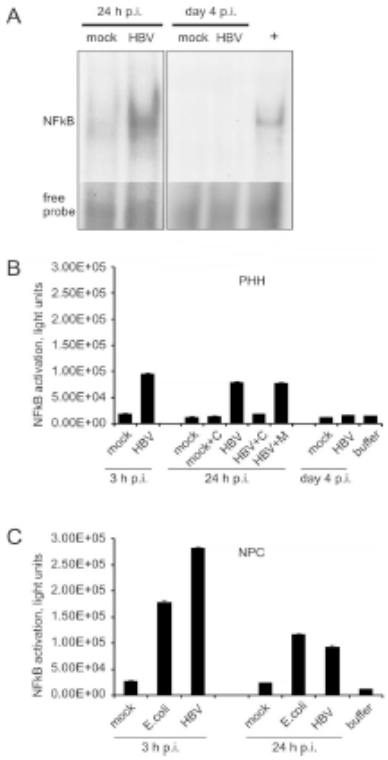

Fig. 2. Activation of NF-κB upon HBV infection of primary liver cells.

(A) Electro-mobility shift assay of nuclear extracts (5 µg protein per lane) prepared from mock- or HBV-infected primary human hepatocyte (PHH) cultures at day 1 and 4 p.i. using ^{32}P-labeled oligonucleotides containing the NF-κB binding site of the HIV-1 LTR. Nuclear extracts from HeLa cells stimulated with 10 ng/ml TNFα were used as a positive control (+). (B,C) Chemiluminiscent ELISA-based NF-κB assay of 2 µg of nuclear proteins isolated at time points indicated from mock- or HBV-infected PHH or NPC cultures set up in parallel. 40 pmoles of a competitor oligonucleotide duplex (+C) or a mutant competitor (+M) were added to ensure signal specificity. 10 µl of NER-protein extraction buffer were used to measure background luminescence (buffer). Nuclear extracts from *E.coli*-treated NPC were used as a positive control. Values are shown as medium ± SD of three measurements.

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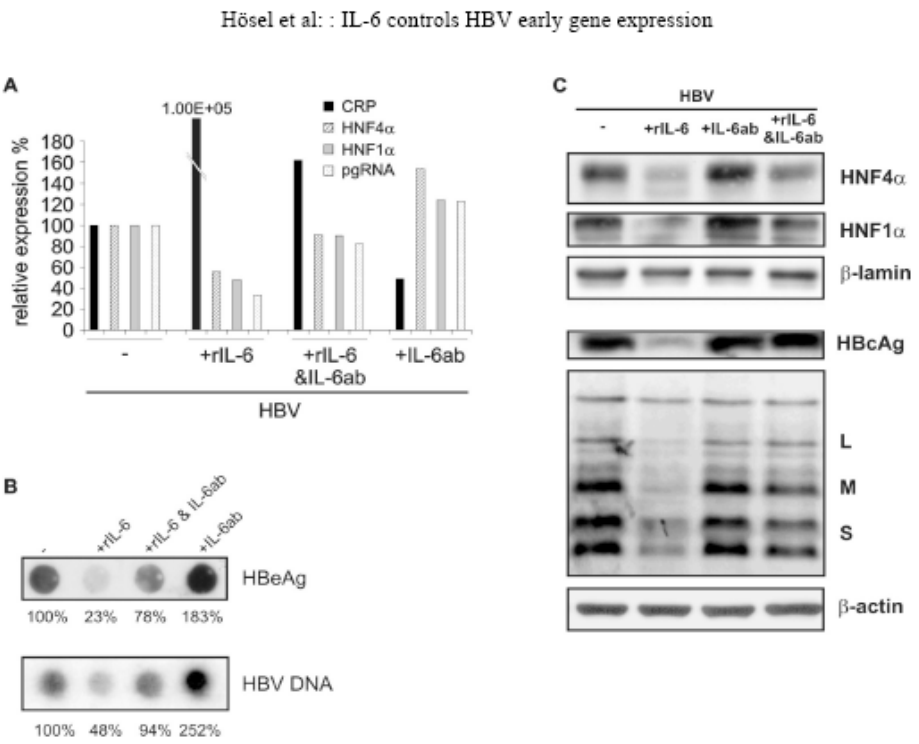

Fig. 3. Effect of IL-6 on HBV transcription and replication and on hepatocyte-enriched transcription factors.

(A) Relative gene expression levels of CRP, HNF4α, HNF1α and HBV pg RNA were determined in HBV-infected hepatocytes by quantitative LightCycler™ real-time RT-PCR. Expression in untreated (-), HBV-infected cells was set to 100%. Recombinant IL-6 (rIL-6) was added to the cells with or and without neutralizing IL-6 antibodies rIL-6ab. In addition, cells were incubated with rIL-6ab prior to HBV infection.

(B) Dot blot analysis of HBeAg using antibody detection (upper panel) and progeny HBV-DNA using a ^{32}P-labelled HBV-DNA probe (lower panel) secreted into cell culture supernatants. Amounts of HBeAg and HBV DNA in untreated HBV-infected PHH were set to 100%. One representative out of three experiments is shown.

(C) Western blot analyses of transcription factors HNF4α and HNF1α (30 µg of nuclear proteins) and HBV core and envelope proteins L, M and S (70 µg of cytoplasmic proteins). Expression of β-lamin and β-actin was controlled for the loading of nuclear and cytoplasmic proteins, respectively.

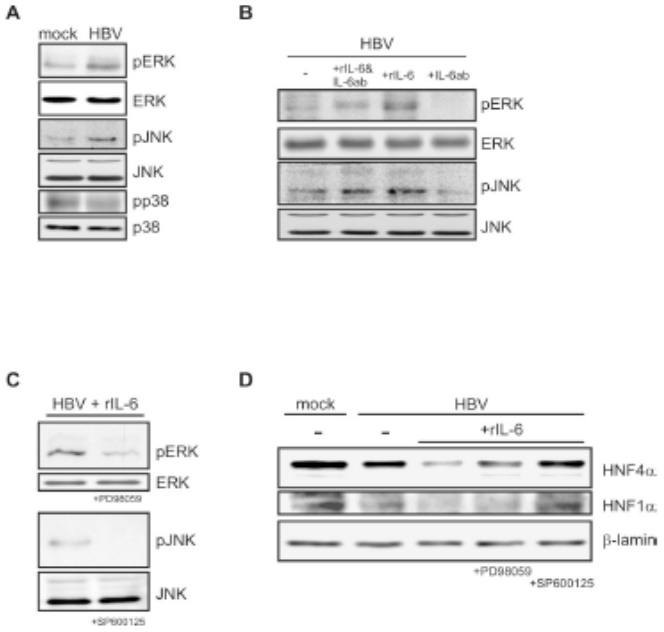

Fig. 4. Involvement of MAP kinase pathways in IL-6-mediated down-regulation of HNF1α and HNF4α.

(A) 70 µg of total proteins isolated from mock- or HBV-infected PHH cultures at 24 h p.i. were analyzed by Western blot with antibodies to activated (phosphorylated) ERK, JNK and p38. The respective unphosphorylated forms were used as a loading control. (B) Western blot analysis of pERK, ERK, pJNK and JNK using total proteins (40 µg) isolated from HBV-

infected PHH without treatment (-), treated with rIL-6 or rIL6 preincubated with neutralizing anti-IL-6 antibodies (rIL-6&IL6ab) or from cells pre-incubated with IL-6ab prior to HBV infection. (C) Detection of pERK and pJNK in HBV-infected PHH untreated or treated with 50 µM of pERK upstream inhibitor PD98059 or pJNK upstream inhibitor SP600125 for 30 min prior to stimulation with rIL-6. (D) Nuclear proteins (20 µg per lane) were prepared from mock- or HBV-infected cells. Cells were either not treated (-) or stimulated with rIL-6 or preincubated with PD98059 and SP600125 before rIL-6 stimulation. Expression of HNF4α and HNF1α were analysed by Western blot. Expression of β-lamin served as control.

Tables

Table 1. Primer sets used for RT-PCR.

Target gene	forward primer (5'-3')	reverse primer (5'-3')
IL-6	AAACAACCTGAACCTTCC	CAGGGGTGGTTATTGC
TNFα	AGCACTGAAAGCATGA	GGGTTTGCTACAACATGG
IL1-β	GTACGATCACTGAACTGC	GAGTGGGCTTATCATCTTT
IFNβ	GCCGCATTGACCATCT	AGTTTCGGAGGTAACCTG
IP10	ACTGTACGCTGTACCT	TGGCCTTCGATTCTGGA
2´5´OAS	CAGTTAAATCGCCGGG	AGGTTATAGCCGCCAG
CRP	ACATTCACAGGGCTCT	ACAAGGTTCGTGTGGA
IGFBP1	AGAGCACGGAGATAACT	TCCAAGGGTAGACGCA
Mn-SOD	AGCCCAGATAGCTCTTC	AGGTAGTAAGCGTGCTC
cIAP2	TGAAGCTGTGTTATATGAGCA	ACGAACTGTACCCTTGAT
HNF1α	TGTGCGCTATGGACAG	GTGTTGGTGAACGTAGGA
HNF4α	GAGTGGGCCAAGTACA	GGCTTTGAGGTAGGCATA
HBV pgRNA	CTCCTCCAGCTTATAGACC	GTGAGTGGGCCTACAAA
ALAS	CAATCAATTACCCTACGGTG	CAAAATGCAGTGGCCT

Table 2. Alteration of cellular gene expression in HBV-infected primary liver cells.

Cell culture	Fold change in gene expression, HBV vs. mock						
	IL1β	TNFα	IL-6	CRP	IFNβ	2'5'OAS	IP10

PHH	74 ↑	nc	11.5 ↑	24 ↑	6 ↓	5.9 ↓	6.3 ↓
NPC	137 ↑	7 ↑	36 ↑	nc	≤2 (↑	nc	≤2 (↑

↑- up-regulated gene, ↓ - down-regulated gene, nc – no change in gene expression detected.

Table 3. Inhibition of NFκB activation and IL-6 secretion after treatment of HBV inoculate with anti-HBs neutralizing antibodies.

Sample	HBeAg S/CO	HBsAg S/N	IL-6 [pg/ml]	NF-κB activity, x10^4 RLU
PHH/HBV	161.5 ± 4.8	39.8 ± 3.1	2119.7 ± 15.6	10.6 ± 4.51
PHH/HBV + anti-S 0.5 I.U.	158.9 ± 6.3	14.6 ± 0.4	1245.7 ± 67.5	7.82 ± 3.51
PHH/HBV + anti-S 2.5 I.U.	159.4± 9.9	nd	724.7 ± 25.3	2.29 ± 1.55

Results are shown as mean of triplicates ± SEM; production of HBeAg and HBsAg is shown as S/CO – signal-to-control and S/N – signal-to-noise ratio, respectively; nd – not detected, RLU – relative light units.

Table 4. Alteration of gene expression in HBV-infected PHH cultures after treatment with recombinant IL-6.

Fold change in gene expression: PHH/HBV vs. PHH/HBV+rIL-6

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CRP	cIAP2	Mn-SOD	IGFBP1	IFNβ	2'5'OAS	IP10
1017 ↑	3.8 ↑	3.9 ↑	24.6 ↑	nc	nc	7.4 ↓

↑- up-regulated gene, ↓- down-regulated gene, nc – no change in gene expression detected.

Eigenständigkeitserklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Fr. Prof. Dr. Protzer betreut worden.

Veröffentlichte Papers:

- „A concerted action of HNF4α and HNF1α links hepatitis B virus replication to hepatocyte differentiation“ (siehe Anlage 9.2.1.1)
- „Antiviral activity and Hepatoprotection by Heme Oxygenase-1 in Hepatitis B Virus Infection“ (siehe Anlage 9.2.1.2)

Eingereichte Papers:

- „Active replication of Hepatitis B Virus in mice starts after birth” (siehe Anlage 9.2.2.1)
- „Not interferon, but IL-6 controls early gene expression in Hepatitis B Virus (HBV) replication” (siehe Anlage 9.2.2.2)