Modulation of Nrf2-activation by Hepatitis B Virus

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Meiner Familie

Glücklich sind die,
die Träume haben und bereit sind,
den Preis zu zahlen,
damit sie wahr werden.
Phantasie ist wichtiger als Wissen,
denn Wissen ist begrenzt.

Albert Einstein

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1 Introduction

1.1 Hepatitis B Virus Infection

Hepatitis is an inflammatory liver disease which can be caused by contusion, drugs, radiation, toxins or pathogens like parasite, bacteria or viruses. Most cases of hepatitis are due to virus infections. The known viruses causing hepatitis are classified in Hepatitis A, B, C, D, E and G and are non-related, except C and G. In addition also some herpes viruses, Coxsackie virus, yellow fewer virus, adenovirus, paramyxovirus and rubella virus can cause a hepatitis phenotype (Gerok *et al.*, 2000).

1.1.1 Disease

The clinical symptoms of acute hepatitis are weariness, adynamia, headache, nausea, loss of appetite, elevated blood levels of transaminases, and symptoms of disturbed liver metabolism including icterus, cholestase, portal hypertension and dark urine (Gerok *et al.*, 2000). In some cases the infection leads to fulminant hepatitis with severe complications including liver failure. Chronic hepatitis B is defined as the infection persisting for more than 6 month. It can be asymptomatic although the viral surface antigen (HBsAg) is detectable in the blood of the patient. About 10 % of acutely HBV infected adults and 90 % of acutely infected children become chronically infected (de Franchis *et al.*, 2003). Chronic hepatitis B infection can lead to liver cirrhosis and hepatocellular carcinoma (Gerok *et al.*, 2000; Lupberger & Hildt, 2007).

1.1.2 Epidemiology

It is estimated that about 2 billion people worldwide have come into contact with HBV (tested positive for antibodies directed against viral core protein) and 400 million people are chronically infected with HBV (Buster & Janssen, 2006). In the year 2000 about 250.000 incidences of hepatocellular carcinoma (HCC) were diagnosed worldwide in HBV carriers (Lupberger & Hildt, 2007).

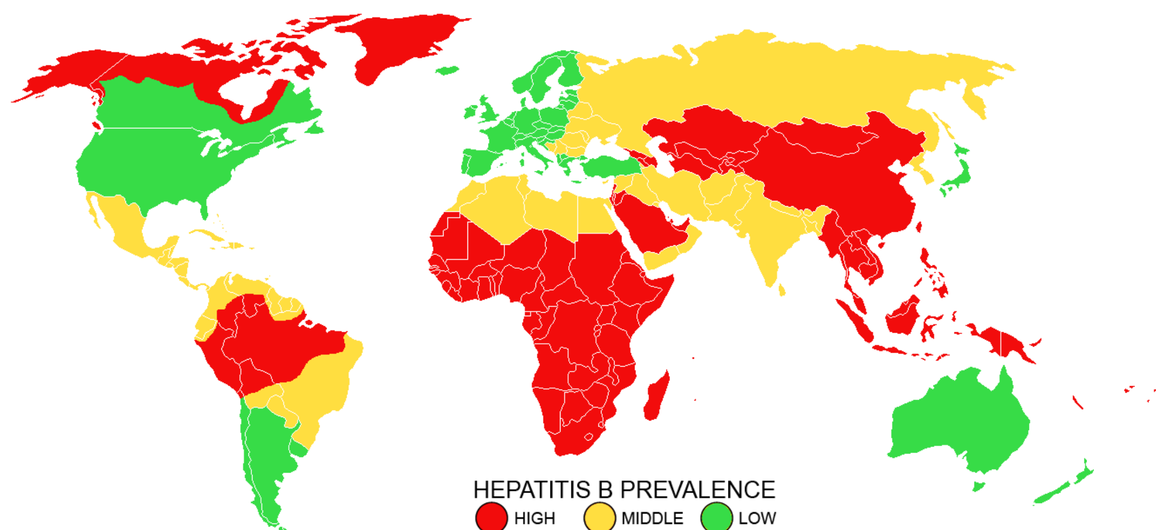


Figure 1: Worldwide prevalence of chronic hepatitis B. >8 % high prevalence (red), 2-8 % middle prevalence (yellow), <2 % low prevalence (green). Data based on WHO (Hollinger & Liang, 2001).

High endemic regions are defined as areas with a chronic hepatitis B prevalence of 8-20 % of the population (Figure 1, red) which include the population of Alaskan and Greenland Indians, the Amazon basin, sub-Saharan Africa, parts of the Middle East, Central Asian republics, Southeast Asia, and the Pacific basin (excluding Japan, Australia, and New Zealand) (Hollinger & Liang, 2001). In China, Thailand, and Senegal the infection rate in children exceeds 25 % whereas in Panama, Solomon Islands, New Guinea, Greenland, and in the population of Alaskan Indians the infection rates in infants are relatively low but increase rapidly during early childhood (Hollinger & Liang, 2001). In high endemic regions, about 70-90 % of the population becomes HBV-infected before the age of 40.
Areas with a chronic hepatitis B prevalence of less than 2 % of the population are defined as low endemic regions (Figure 1, green). They include North and parts of South America, Central and Western Europe, Turkey, Australia, New Zealand, and Japan. In these regions less than 20 % of the population becomes HBV-infected before the age of 40 (Hollinger & Liang, 2001).
The rest of the world falls under intermediate endemic regions (Figure 1, yellow) with a chronic hepatitis B prevalence of 2-8 % of the population (Hollinger & Liang, 2001).

1.1.3 Immunopathogenesis

The interaction between the hepatitis B virus and the innate and adaptive immune system ascertains the final outcome of the infection. The lack of available animal models (Dandri et al. 2001) and in vitro cell lines enabling the characterization of HBV infection (Zoulim 2006) has limited the study of HBV-associated pathogenesis. Analysis of acute HBV infection of chimpanzees and the transgenic mice model endorses the hypothesis that clearance of viral

DNA is encompassed mostly by antiviral cytokines produced during the innate and adaptive immune response where interferon-γ (IFN-γ), interferon-α/β (IFN-α/β) and tumor necrosis factor α (TNF-α) are supposed to play a major role (Bertoletti & Gehring, 2006). They are supposed to induce pathways leading to the inhibition of viral replication without cytolytic removal of infected cells (Guidotti *et al.*, 1999; Wieland *et al.*, 2000).
The development of chronic HBV infections is often associated with mild or even absent symptoms of an acute infection. In accordance to these clinical symptomatic observations, neonatally infected woodchucks with low IFN-γ and TNF-α production developed a chronic infection and were unable to establish an efficient antiviral immune response (Cote *et al.*, 2000; Menne *et al.*, 2002; Nakamura *et al.*, 2001). When the infection could not be cleared and HBV persistence leads to diffuse defects of helper and cytotoxic T-cell responses (Wherry *et al.*, 2003; Zhou *et al.*, 2004). This state of HBV-specific T-cell tolerance is likely to be maintained by the constant high levels of viral antigens and consequent T-cell exhaustion mediated by programmed death (PD-1) receptor and PD-1 ligand (PD-L1) interaction (Boni *et al.*, 2007; Sobao *et al.*, 2002; Webster *et al.*, 2004). Regulatory T-cells and defects in dendritic cells might also contribute (Alatrakchi & Koziel, 2009; Tan *et al.*, 2008).
The cause of increasing liver damage in chronic HBV patients is still not fully understood but it is already well established that the chronic, virus-specific immune response plays a major role which was demonstrated by Chisari and colleagues (Chisari, 2000; Nakamoto *et al.*, 1998; Rehermann, 2003; Rehermann & Nascimbeni, 2005). From experiments with transgenic mice they concluded that chronic liver inflammation mediated by the virus-specific immune system, continuous apoptosis and subsequent cell proliferation to substitute dead cells could increase the frequency of genetic alterations and the risk of cancer development (Chisari, 2000; Chisari *et al.*, 1985; Ferrari *et al.*, 2003; Visvanathan & Lewin, 2006).

1.1.4 Transmission, Prevention and Treatment

Globally, the major mode of HBV infection is perinatal transmission and exposure to blood secretions from chronic carriers. The introduction of HBsAg screening of plasma donors has greatly reduced the risk of HBV transmission. In most developed countries the spread is caused by drug use and particularly by sexual contact as the major risk factor (Doerr, 2002).

Immunization with recombinant HBV surface antigen has been available since 1986 and forms the most important disease prevention. Since chronic HBV infection is one of the most potent factors causing hepatocellular carcinoma (HCC) immunization also describes a vaccination against one of the most common type of cancer: HCC (Lupberger & Hildt, 2007). This could be imposingly demonstrated by a universal HBV vaccination program in Taiwan. About 20 years after the program was launched chronic HBV infection rates have been

reduced from 10 - 17 % to 0.7 - 1.7 % and HCC incidence rates as well have decreased from 0.52 - 0.54 to 0.13 - 0.20 per 100˙000 people in Taiwan (Chang, 2009).

An acute infection is usually not treated but monitored. The major target of the current treatment for chronic hepatitis B is the HBV polymerase. Its activity can be inhibited by nucleoside analogs (e.g. Lamivudine, Entecavir) and nucleotide analogues (e.g. Adefovir). But after long-term application of nucleos(t)ide-analogs the incidence of drug resistant escape mutants is high (Buster & Janssen, 2006). Nucleos(t)ide-analogs are usually applied in combination with pegylated interferon-alpha, which stimulates the antiviral response of the host immune system (Buster & Janssen, 2006). Nevertheless, there is a low chance for successful cure of a chronic HBV infection.

1.2 Hepatitis B Virus (HBV)

1.2.1 History

Already from antiquity there are reports about epidemic occurrence of jaundice, but the first well described documentation of a jaundice epidemic caused by smallpox vaccinations was written in 1883 and is considered as the first report of a Hepatitis B virus infection. About 80 years later, in 1963, B.S. Blumberg discovered an antigen in the serum of an Australian aborigine during population genetics studies of different ethnical groups. Misleadingly it was first associated with leukaemia until in 1968 researchers showed that this “Australian Antigen” could specifically be detected in sera of Hepatitis-B-patients (Blumberg *et al.*, 1966; Blumberg *et al.*, 1968; 1969; Levene & Blumberg, 1969; London *et al.*, 1969a; London *et al.*, 1969b; Millman *et al.*, 1969). After the discovery and characterization of this antigen, later called HBsAg (Hepatitis B surface antigen), an intensive research began about the infectious particle and the associated disease, the Hepatitis B infection. In 1970 Dane could first identify and show the infectious virus by electronmicroscopical studies (Dane D.S., 1970). Therefore this infectious virion was later called “Dane particle”. Only a few years later people recognized the significance of neutralizing antibodies against HBsAg for the development of a HBV vaccination and finally in 1975 the first vaccination studies with highly-purified, inactivated HBsAg-particles started (Millman *et al.*, 1970a; Millman *et al.*, 1970b).

1.2.2 Morphology, Structure and Genome Organisation

The Hepatitis B virus belongs to the family of *Hepadnaviridae* that can be further divided in two subfamilies: the *Avihepadnavirus* (birds) and *Orthohepadnavirus* (mammalian). HBV is

considered as the prototype member of *Hepadnaviridae* and belongs to the genus *Orthohepadnavirus* together with the ground squirrel HBV (GSHV), woolly monkey HBV (WMHBV), arctic ground squirrel HBV (ASHV) and woodchuck HBV (WHV), whereas duck HBV (DHBV), storck HBV (STHBV), snow goose HBV (SGHBV), maned duck HBV (MDHBV), grey teal HBV (GTHBV) and heron HBV (HHBV) form the *Avihepadnavirus*. The name *Hepadnavirus* derives from the terms *hepa* (*hepa* = liver) and *dna* revealing two main characteristics of HBV: it is an enveloped DNA-virus and is featured by a distinctive liver tropism. Besides the 42 nm infectious virion (Dane-particle), two other morphological forms can be found in the serum of HBV-infected patients. The non-infectious 22 nm spherical and filamentous subviral particles only consist of HBsAg (Hepatitis B surface antigen) spiked viral envelope. Interestingly the subviral particles (SVPs) exist in approximately 1˙000 - 100˙000 fold excess compared to Dane-particles (Ganem, 1991) (Figure 2).

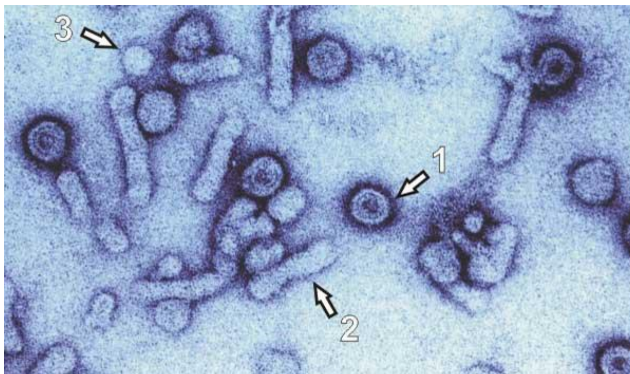


Figure 2: Electron microscopy of HBV particles 1 = 42 nm Dane particle, 2 = 22 nm filamentous subviral particle, 3 = 22 nm filamentous subviral particle (EM picture by of H.-W. Zentgraf, Heidelberg).

The function of the SVPs for the infection of HBV still remains unclear. Considering the fact that the viral surface protein HBs plays an important role in the immune response against HBV infection people hypothesize that the SVPs might function as some kind of deflection or decoy for the immune system facilitate the Dane-particle to escape and establish an infection. In contrast to soluble forms of Hepatitis B Virus envelope proteins, the SVPs do not inhibit infection of primary human hepatocytes (Chai *et al.*, 2008). Former studies suggested that SVPs enhance viral replication due to binding to the infected hepatocyte and subsequent activation of intracellular signaling (Bruns *et al.*, 1998). Furthermore SVPs are considered as an auspicious tool regarding the development of new vaccine strategies (Ludwig & Wagner, 2007; Patient *et al.*, 2009; Ramqvist *et al.*, 2007).

The virion (Dane-particle) consists of an outer lipid envelope and an icosahedral nucleocapsid composed of core protein. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity (Locarnini, 2004). The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells. The 3.2 kb partial double-stranded viral DNA genome has at least four

partially overlapping open reading frames (ORF) coding for seven viral proteins: the viral core protein (HBc) forming the nucleocapsid, the early antigen HBeAg (secretory protein), the viral polymerase (P), the HBx protein and the small (SHBs), middle (MHBs) and large (LHBs) surface proteins. Figure 3 shows a schematic view of the HBV structure.

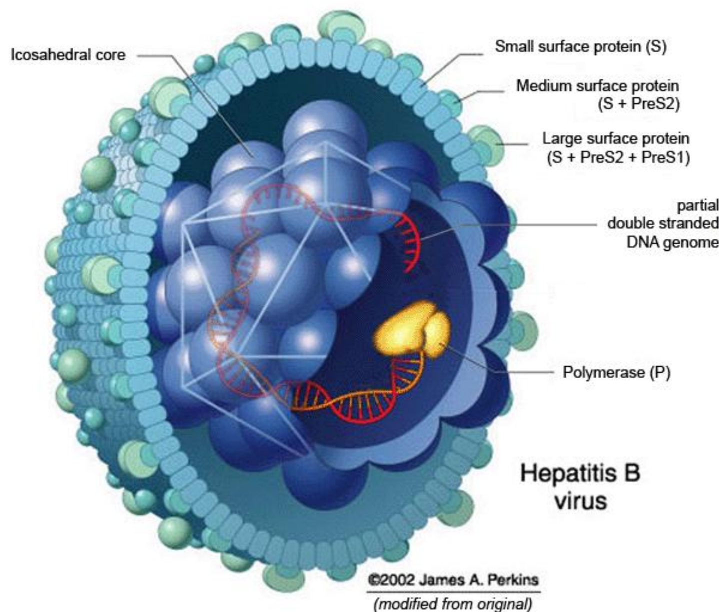


Figure 3: HBV structure. A partial double-stranded DNA genome is bound covalently to the viral polymerase (P). The genome complex is enclosed by an icosahedral capsid consisting of monomeric core proteins. The viral capsid is enveloped by the host membrane from the pre-Golgi compartment harboring the viral surface proteins LHBs, MHBs, and SHBs.

The 42 kDa large surface antigen (LHBs) consists of an S domain and a preS1 plus preS2 domain (PreS1-PreS2-S), the 31 kDa middle surface antigen is composed of preS2 and S domain (PreS2-S) and the 24 kDa small surface antigen consists only of the S domain (S) (Seeger & Mason, 2000). In the mature virus, the viral polymerase (P) is covalently attached to the 5'-end of the genome minus-strand. The P-genome complex is protected by an icosahedral capsid assembly consisting of the 21 kDa viral core protein (C). The HBV capsid is enveloped by host cell membrane, which harbours the small, middle, and large surface antigens (SHBsAg, MHBsAg, and LHBsAg, respectively) (Seeger & Mason, 2000) (Figure 3). Besides the viral core protein (C) an additional 25 kDa precursor protein is translated which is composed of the preC and C protein. The preC region of the precursor protein harbors a signaling sequence that directs the chain into the secretory pathway, where it is cleaved to a 17 kDa early antigen (HBeAg) and secreted to the bloodstream (see also Figure 4) (Seeger & Mason, 2000). The function of HBeAg still remains unknown. Former studies showed that HBeAg negative mutants replicate well *in vitro* and arise frequently during natural infections (Takahashi *et al.*, 1983).

The minus strand of the partial double-stranded DNA is complete whereas the positive strand is incomplete and has a variable length of 1000 - 2500 nucleotides. All coded proteins are translated from at least four RNA transcripts (see also Figure 4 and 5).

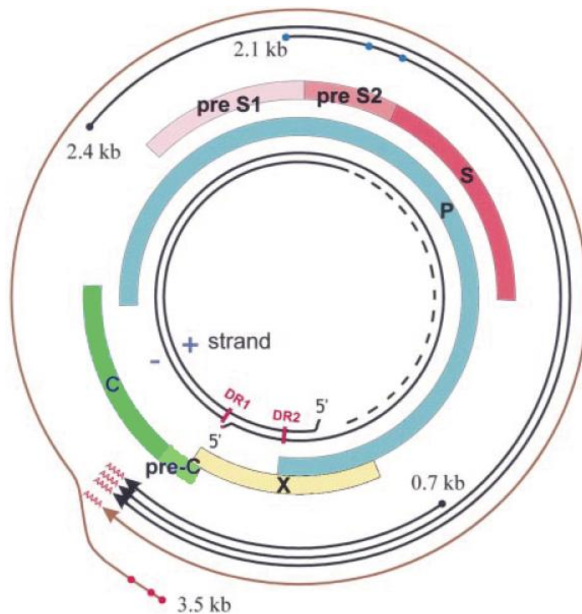


Figure 4: HBV genome organization. The 3.2 kb partial double-stranded DNA genome (black center lines) harbours 7 overlapping open reading frames (ORF): viral polymerase (P), HBx protein (X), HBc core protein (C), HBe protein (E; Pre-C+C), large surface protein LHBs (PreS1+PreS2+S), middle surface protein MHBs (preS2+S), and small surface protein SHBs (S). Figure modified from original (Kidd-Ljunggren *et al.*, 2000).

The 3.5 kb pregenomic RNA (pgRNA) is an overlength transcript, which is an intermediate for both viral replication and translation of the viral proteins. The 3.5 kb mRNA codes for the viral polymerase (P), the core protein (C), and the precursor early antigen (preC-C). The surface antigens (HBsAg) are translated from the 2.4 kb and the 2.1 kb mRNAs and are co-terminally with their carboxy end but differ in additional amino terminal domains (Block *et al.*, 2007; Seeger & Mason, 2000). The 0.7 kb mRNA is translated into the 17 kDa X protein, which is a regulatory protein (Twu & Schloemer, 1987; Wollersheim *et al.*, 1988). The existence of the 0.7 kb mRNA has been verified in cell culture but not *in vivo* (Nassal, 2008).

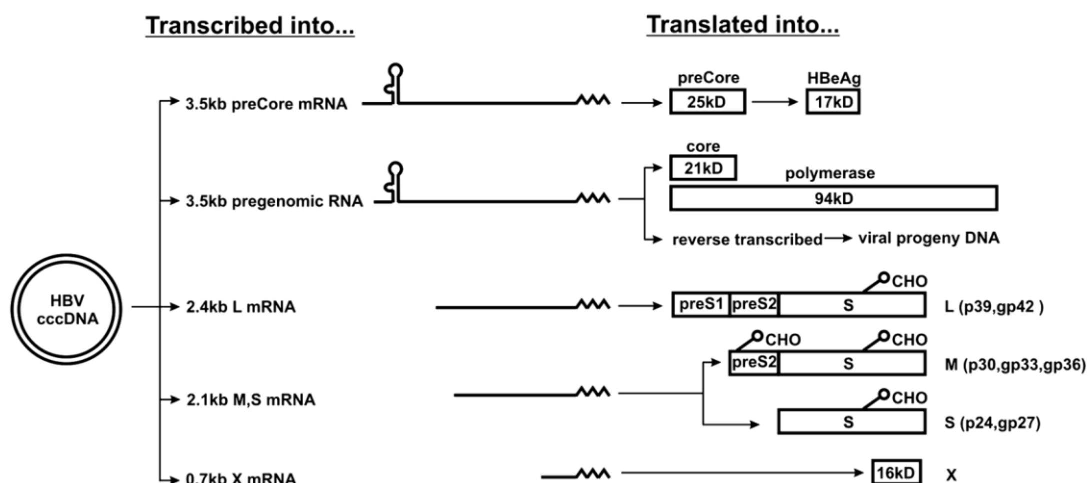


Figure 5: Viral mRNA transcripts and the corresponding translational products. N-linked glycosylation is indicated by CHO and is placed at the appropriate modification sites. Figure modified from (Block et al., 2007).

1.2.3 HBV species and subtypes

Replication by reverse transcription of a RNA intermediate and a lack of proofreading activity of the HBV polymerase makes the virus prone to mutation. The nucleotide substitution rate, per site and per year, is nearly as high as in retroviruses (10^{-5}) and is 10^{4} times higher than in DNA virus genomes (Orito *et al.*, 1989) which enables most viruses to adapt quickly to changing environments. The HBV genotypes are defined with regard to sequence divergence of more than 8 % of the whole genome and at least 4 % divergence within the HBsAg reading frame. First there were only four major genotypes described (A-D) but during the last 15 years four additional genotypes (E-H) were identified. The worldwide distribution of the different HBV genotypes and their phylogenetic relationship is shown in Figure 6.

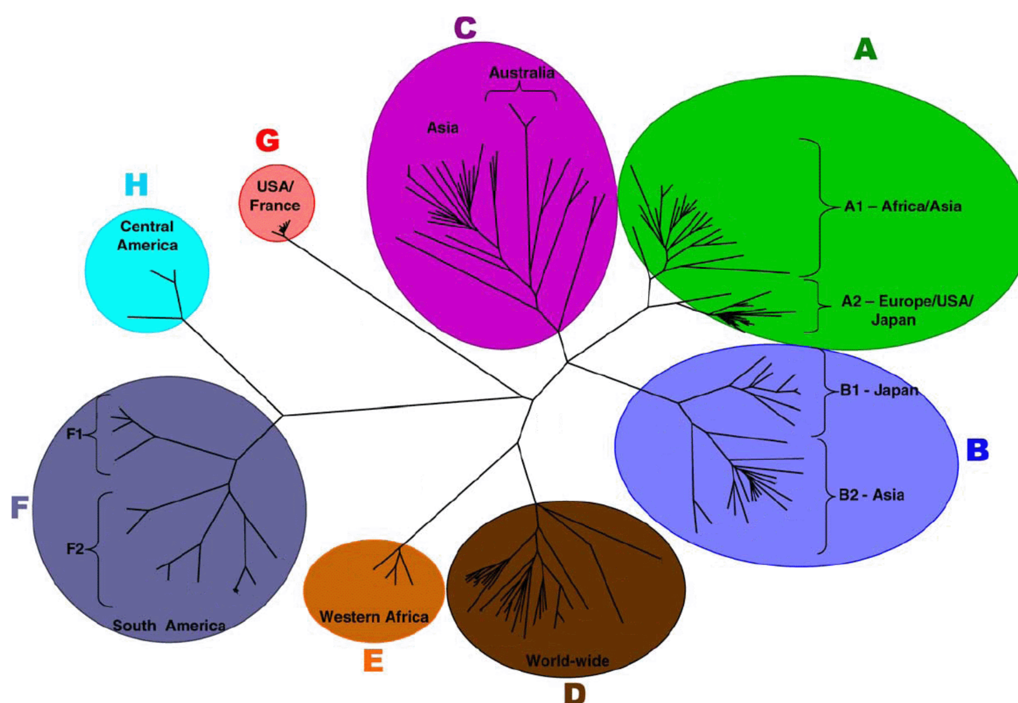


Figure 6: Worldwide distribution and phylogenetic relationship of HBV genotypes. 175 published HBV genomes and subgenomes are compared by neighbour-joining gene analysis and are displyed as a single phylogenetic branch. The different genotypes and their worldwide distribution are illustrated as follows: A (green) Asia, Japan, Africa, Europe, USA; B (light blue) Asia, Japan; C (purple) Asia, Australia; D (dark brown) worldwide; E (light brown) Western Africa; F (dark blue) South America; H (turquoise) Central America; G (pink) USA, France. Image modified from original (Kramvis *et al.*, 2005).

Furthermore, the treatment of chronic HBV infections with nucleos(t)ide-analogues can promote the development of new “quasispecies” (Kramvis *et al.*, 2005). Long term therapy with these nucleos(t)ide-analogues can cause drug resistant polymerase mutations that can also effect the overlapping HBsAg reading frame (Allen *et al.*, 1998).

The viral surface antigens are exposed on the viral envelope and display the major epitopes for the humoral immune response. Due to the high selective pressure, besides the viral polymerase, the coding region of HBsAg is the most variable part of the viral genome. Therefore, HBV can be further classified in serotypes based on the heterogeneity of the viral surface antigen. To date four serological subtypes (serotypes) were identified: ayw, adr, ayr and adw which have been further supplemented after identification of additional sub-determinants within the HBsAg (Kramvis *et al.*, 2005). Table 1 shows the geographic distribution and correlation of HBV genotypes and serotypes.

Genotypes	Serotypes	Distribution
A	adw2, ayw1	NW Europe, N America, Central Africa
B	adw2, ayw1	SE Asia, China Japan
C	ayr, adrq+, adrq-, adw2	SE Asia, China Japan
D	ayw2, ayw3	S Europe, Middle East, India
E	ayw4	Africa
F	adw4q-	American natives, Polynesia, Central and South America
G	adw2	United States, France

Table 1: Geographic distribution and correlation of HBV genotypes and phenotypes (Figure modified from (Chu & Lok, 2002)).

The heterogeneity in disease manifestations and response to anti-viral treatment among chronic HBV patients raises the question whether there is a correlation of HBV genotypes and viral latency, pathogenesis, treatment response and resistance to anti-viral drug therapy. Up to the present there are no data on clinical relevance of all HBV genotypes available. Several Asian studies revealed that genotype B is associated with slower progression to liver cirrhosis and less occurrence of hepatocellular carcinoma (HCC) compared to HBV carriers with genotype C (Ding *et al.*, 2001; Sakugawa *et al.*, 2002; Sumi *et al.*, 2003). Furthermore there are hints that HBV genotype influences the response to standard interferon therapy. Sánchez-Tapias and colleagues found a higher seroconversion rate for genotype A compared to D (Sanchez-Tapias *et al.*, 2002) and two studies from Taiwan and Hongkong suggested a higher rate of HBeAg seroconversion for genotype B as for genotype C (Kao *et al.*, 2000; Wai *et al.*, 2002). Consequently, there is growing evidence that HBV genotypes is more clinically important as it is already the case for hepatitis C virus (Zeuzem *et al.*, 2009).

1.2.4 The Viral Life Cycle

All members of the hepadnavirus are characterized by a high species and tissue specificity. HBV efficiently infects only hepatocytes although it is discussed that it can enter bile duct epithelium cells, some cells from the kidney, lymphoid system, and pancreas supposable to ensure viral persistence (Seeger & Mason, 2000). HBV has a non-cytolytic lifecycle and enters the hepatocyte by endocytosis which is mediated by the binding of the large HBsAg to a still unknown receptor complex (Figure 7).

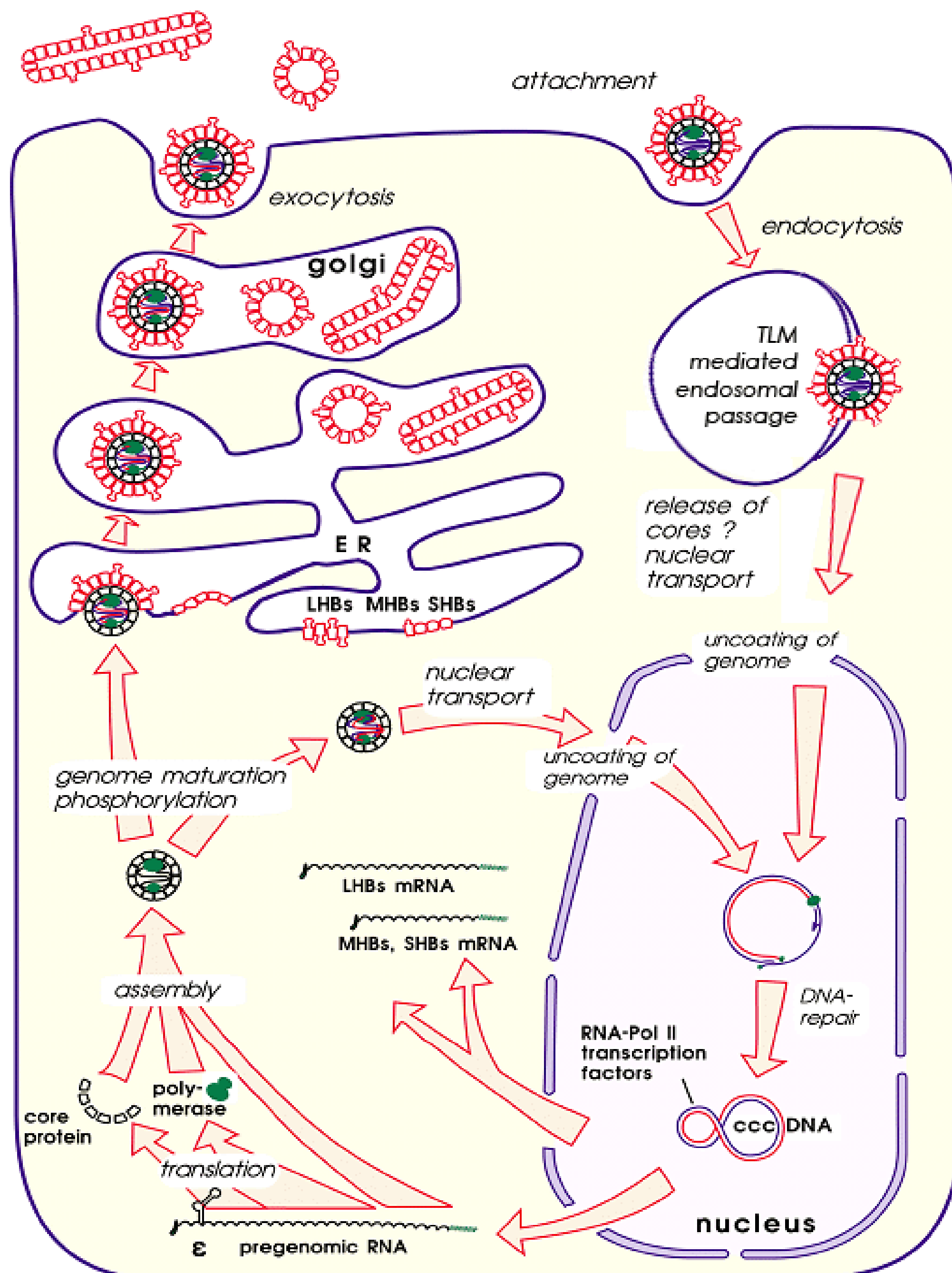


Figure 7: Scheme of the HBV lifecycle within a hepatocyte. Image modified from original (Kann *et al.*, 1995)

Inside the endosome the viral surface protein is presumably cleaved by an unknown protease leading to a large-scale exposure of a cell permeable translocation motif (TLM) within the PreS2 region of the LHBsAg that mediates the non-destructive passage of the whole virus through the endosomal membrane (Stoeckl *et al.*, 2006). The endosomal processing and the reducing conditions are probably responsible for the uncoating of the viral envelope in the cytoplasm. The entry of the viral relaxed-circular DNA genome (RC-DNA) into the nucleus is still not fully understood. On the one hand it is discussed whether the intact nucleocapsid mediates the delivery of the P-genome complex into the nucleus (Rabe *et al.*, 2003) or on the other hand if a partial disassembly of the capsid within the nuclear pore complex or in a perinuclear domain finally leads to the genome release and its import into the nucleus (Brandenburg *et al.*, 2005).

Within the nucleus the viral DNA polymerases and cellular repair mechanisms complete the shorter (+)-DNA strand. The two strands are covalently ligated and the terminal modifications has to be removed to form a very stable non-integrated HBV mini-chromosome, named covalently closed circular DNA (cccDNA) (Nassal, 2008; Zoulim, 2005). The cccDNA represents the central intracellular intermediate in viral replication and also serves as a template for the host RNA polymerase II processing the viral mRNAs (Figure 4 and 7) that are transported to the cytoplasm where the viral proteins are translated at the rER.

Upon steric activation by host chaperones Hsp40, Hsp70, Hsp90, Hop, and possible additional factors, the P protein binds to a secondary structure, the encapsidation signal ε, at the 5'-end of the pregenomic RNA of DHBV (Hu *et al.*, 2002). How the interaction of ε and polymerase take place is not known so far, but it is supposed to play a role in the recruitment of core protein homodimers that finally leads to capsid formation through self-assembly (Nassal, 2008). Besides initiation of pgRNA-polymerase encapsidation, the ε-polymerase interaction also induces reverse transcription where first the (-)-DNA strand is synthesized, followed by be (+)-DNA strand generation finally leading to RC-DNA (Nassal, 2008). A tyrosine residue of the polymerase serves as a protein primer that induces reverse transcription, using a bulge within ε to initiate synthesis of the first 3-4 nucleotides of the minus-strand DNA (Wang & Seeger, 1993; Zoulim & Seeger, 1994). The viral polymerase serves as a reverse transcriptase and stays covalently attached to the newly synthesized minus-strand of the HBV genome. The ε-structure recognition by P protein and the encapsidation by core protein oligomers are tightly coupled events (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990).

The viral core protein is required for maturation of the viral nucleocapsid phosphorylation (Melegari *et al.*, 2005) and dephosphorylation (Perlman *et al.*, 2005). The involvement of several cellular kinases are under consideration, for example cdc2 (Liao & Ou, 1995),

SPRK1 and SRPK2 (Daub *et al.*, 2002), and an unknown 46 kDa protein which has still to be characterized (Kau & Ting, 1998).
Further it is assumed that in the early stage of infection with low intracellular HBsAg levels the majority of mature nucleocapsids are redirected to the nucleus to increase the intranuclear cccDNA level to 10-50 molecules per cell (Newbold *et al.*, 1995). In a latter phase mature HBV capsids are enveloped at a pre-Golgi compartment which is mediated by membrane associated viral surface proteins (Bruss, 2004) and finally released by the Golgi secretory pathway (Figure 7). For recent review see (Schädler & Hildt, 2009).

1.2.5 The Viral Regulatory Proteins HBx and PreS2

1.2.5.1 HBx

Among all mammalian hepadnaviruses the HBx gene is conserved and expressed at very low levels during chronic and acute hepatitis. Various regulatory functions are discussed for the HBV X protein and two major mechanisms of stimulation are described: (i) by direct binding of HBx to various members of the transcriptional machinery and e.g. enhancing DNA binding activity of transcriptional factor CREB (cAMP responsive element-binding protein) or (ii) by stimulation of cytoplasmic signal transduction pathways (Bouchard & Schneider, 2004).

The integrity of X is essential for WHBV replication in woodchucks (Zoulim & Seeger, 1994) but its role for HBV life cycle is still enigmatic. On the one hand there are reports showing that viral genome expression occurs independently from HBx functionality (Blum *et al.*, 1992; Hafner *et al.*, 2003; Stockl *et al.*, 2003) and on the other hand a relevance of HBx was described for HBV replication (Melegari *et al.*, 2005). HBx is a transcriptional activator that stimulates gene expression by several transcriptional factors e.g. NF-κB, AP-1, AP-2, ATF/CREB or the calcium activated factor NF-AT (Bouchard & Schneider, 2004; Lupberger & Hildt, 2007). Regarding the interference of HBx with PKC signaling there are both reports describing an HBx-dependent activation of PKC which is mediated by an elevated DAG level in HBx-producing cells (Kekule *et al.*, 1993; Luber *et al.*, 1993) and reports showing that HBx neither affects activity of PKC nor that PKC is essential for HBx-dependent transcriptional activation (Hildt *et al.*, 2002; Lucito & Schneider, 1992; Murakami *et al.*, 1994).
Furthermore the Ras/GTP complex formation is increased by HBx, thus inducing the c-Raf signaling cascade (Benn & Schneider, 1994). It was found that HBx causes calcium release from the mitochondria (Bouchard *et al.*, 2001) leading to a subsequent activation of proline-rich tyrosine kinase (Pyk2), Src kinases, and focal adhesion kinase (FAK) which results in a Ras-dependent activation of the mitogen-activated protein (MAP) kinase pathways c-Raf/MEK/ERK and MEKK-1/JNK (Bouchard & Schneider, 2004; Bouchard *et al.*, 2006; Klein

et al., 1999). The stimulated MAP kinase pathways inhibit apoptosis and stimulate cell proliferation which results in enhanced HBV gene transcription in HBV infected cells (Peyssonnaux & Eychene, 2001).

Due to the fact that HBx affects the expression of multiple genes that are involved in the control of proliferation, cell cycle or apoptosis, it was considered to play a crucial role in HBV-associated carcinogenesis (Chan & Sung, 2006; Cougot *et al.*, 2005; Koike *et al.*, 2002; Staib *et al.*, 2003). Experiments with transgenic mice suggested that HBx indeed could exert a tumor promoter-like function since irradiation of HBx-transgenic mice or exposure of these mice to the mutagen diethynitrosamine caused a higher increase in the amount of pre-neoplastic lesions as compared to control animals (Madden *et al.*, 2001; Zhu *et al.*, 2004).

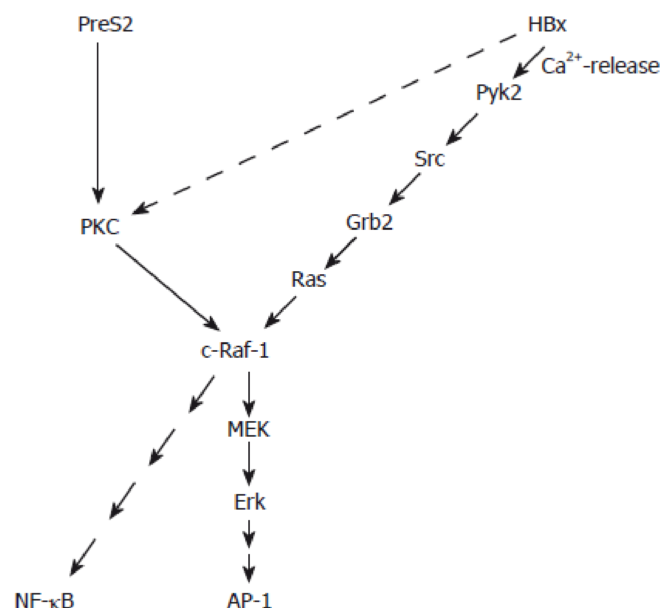


Figure 8: Signaling pathways activated by the viral regulatory proteins PreS2 and HBx. (Figure modified from (Lupberger & Hildt, 2007)).

1.2.5.2 PreS2

Besides the HBx-regulatory protein the PreS2 activators describe the second family of regulatory proteins and are composed of the PreS2 region of the LHBs and the C-terminally truncated MHBs (MHBs^{t}). Detailed analysis revealed a minimal PreS2 activator that lacks any membrane insertion domain (MHBs^{t55}) and includes the complete PreS2 domain and is localized within the cytoplasm (Hildt *et al.*, 1996b; Lauer *et al.*, 1992; Madden *et al.*, 2001). This class of regulatory proteins was termed PreS2 activators since the PreS2 domain is sufficient to exert the regulatory protein function.

The PreS2 region has a transcriptional activator function that requires the integrity of the PreS2 domain and its cytoplasmic orientation (Hildt *et al.*, 1996a; Kekule *et al.*, 1990; Schluter *et al.*, 1994). The structural MHBs and the regulatory MHBs^{t} differ in the PreS2 domain topology which faces the cytoplasm in case of the MHBs^{t} explaining the lack of N-glycosylation at Asn4 and additionally MHBs^{t} are not secreted as structural MHBs (Hildt *et al.*, 1995; Hildt *et al.*, 1993). Since the PreS2 domain is directed to the cytoplasm it is able to interact with cytostatic components and thereby triggers intracellular signaling cascades. It was shown that PreS2 activates the c-Raf/MEK/ERK pathway in a PKC-dependent Ras-independent manner that enhances gene transcription (Hildt *et al.*, 2002). Thereby PreS2 activators bind PKC-α in the cytoplasm resulting in a DAG (1,2-diacylglycerol)-independent activation of PKC and phosphorylation of the PreS2 domain (Hildt *et al.*, 2002). Furthermore the integrity of the c-Raf/MEK/ERK pathway is crucial for HBV replication and that HBx and PreS2 can replace each other in respect to c-Raf/MEK/ERK pathway activation but simultaneous knock out of PreS2 and HBx impedes HBV replication (Stockl *et al.*, 2003).

A distinctive feature of the LHBs is the dual membrane topology of the PreS1-PreS2 domain (Bruss *et al.*, 1994; Ostapchuk *et al.*, 1994; Prange & Streeck, 1995). The L protein can dispose its N-terminal preS domain both to the cytoplasmic and the luminal side of the membrane. As already mentioned above, the cytoplasmatic orientation of the PreS2 domain in case of MHBs^{t} proteins is causative for their regulatory function. Thus the PreS2 domain of LHBs initially remaining on the cytosolic side of the ER membrane after translation exhibits a regulatory protein function and therefore belongs to the family of PreS2 activator proteins. There is evidence for a PKC-dependent activation of AP-1 and NF-kappa B by LHBs. Downstream of the PKC the functionality of c-Raf is a requirement for LHBs-dependent activation of AP-1 and NF-kappa B since inhibition of c-Raf kinase abolishes LHBs-dependent transcriptional activation of AP-1 and NF-kappa B (Hildt *et al.*, 1996a).

Since the c-Raf/MEK/ERK signal transduction cascade can carry out a tumor promoter-like function according to the classical two-step model of carcinogenesis (Boutwell, 1974) an educated guess was made regarding the role of the Pres2 activators in tumor formation. Indeed, transgenic mice expressing a C-terminally truncated MHBs protein at aa 76 (MHBs^{t76}) showed an increased hepatocyte proliferation rate and in mice older than 15 months, an increased incidence of liver tumors occurs (Hildt *et al.*, 2002). The same results could be observed for LHBs-transgenic mice (Chisari *et al.*, 1989). These mice showed a strong overproduction of the large surface protein that result in an intracellular accumulation of the protein and subsequent permanent inflammation leading to the formation of critical mutations (Chisari, 2000; Chisari *et al.*, 1989; Dunsford *et al.*, 1990). See also figure 8 for a schematic view of the signaling pathways involved.

1.2.6 Infection Models

As mentioned earlier the receptors that mediate viral entry of HBV after binding are still not identified. For studying the mechanisms of HBV replication immortalized hepatoma cell lines are widely used. However, the virus uptake during the early phase of HBV infection is blocked due to unknown reasons and therefore only a few infection models are available to study HBV infection because HBV is very tissue and species specific (Dandri *et al.*, 2005). Infection of primary human hepatocytes would represent the most appropriate model but availability of human liver tissue is restricted and preparation of a sufficient amount of susceptible hepatocytes from a tissue sample is difficult. Other experimental models to study HBV infection are the closely related viruses as the duck hepatitis B virus (DHBV) (Mason *et al.*, 1980), Heron hepatitis B virus (HHBV) (Sprengel *et al.*, 1988) or woodchuck hepatitis B virus (WHBV) (Aldrich *et al.*, 1989). For example duckling liver tissue can be obtained easily and the preparation of hepatocytes by liver perfusion is more efficient as compared to human liver tissue samples.

Besides humans chimpanzees are as well susceptible for an infection with human HBV but this is controversial in both an ethical point of view and the expensive keeping of the animals. A few years ago it was demonstrated that HBV can infect and replicate in primary hepatocytes from the Asian tree shrew *Tupaia belangeri* which displays an important alternative infection model (Kock *et al.*, 2001; Walter *et al.*, 1996). Furthermore, a HBV susceptible hepatoblastoma cell line was reported that can be infected in the presence of corticoids and dimethyl sulfoxide (Gripon *et al.*, 2002). Nonetheless, all model systems require a large amount of human HBV with a defined genome for a successful infection and to perform reproducible experiments.

1.3 The Nrf2-ARE signaling pathway

1.3.1 Nrf2

The transcription factor Nuclear Factor-Erythroid 2-related Factor 2 (Nrf2) was first described during studies of β-globin gene expression and the characterization of the locus control region which have critical regulatory properties (Forrester *et al.*, 1986; Tuan *et al.*, 1985). This regulatory region contains a tandem AP1-NF-E2 (activating protein 1 and nuclear factor erythroid 2) motif, originally termed as the DNase hypersensitive site 2, which has a strong enhancer activity (Ikuta & Kan, 1991; Moi & Kan, 1990). After identification of this gene motif several groups identified several members of the cap 'n' collar (CNC) subfamily of the basic leucine zipper (bZIP) transcription factors which bind to this AP1-NFE2 site: p45-NFE2 and Nrf1 (Chan *et al.*, 1993), Nrf2 (Moi *et al.*, 1994) and Nrf3 (Kobayashi *et al.*, 1999). These proteins function as heterodimeric transcription factors by dimerizing with other bZIP proteins such as small Mafs (sMafs) (Igarashi *et al.*, 1994). In contrast to Nrf1-knock-out mice, Nrf2-disrupted mice were fertile, and did not show a phenotype of developmental deficits (Chan *et al.*, 1996), suggesting that Nrf2 is not essential for murine development and survival. But recently it was demonstrated that in Nrf2-/- mice liver regeneration is significantly impaired (Beyer *et al.*, 2008).

Despite the high sequence homology p45-NF-E2 is only expressed in megakaryocytes, erythroid cells, and mast cells while Nrf2 is not restricted to haematopoietic cells and is present in a broad number of tissues (Itoh *et al.*, 1997; Moi *et al.*, 1994). A high Nrf2 expression was especially found in organs involved in detoxification, such as liver and kidney, and organs exposed to the external environment, such as skin, lung, and digestive tract (Motohashi *et al.*, 2002). Besides them the family of Nrf2 transcription factors also contains the closely related protein Bach1 and Bach2 (Motohashi *et al.*, 2002). Nrf2 plays a crucial role in cellular defence against oxidative stress and activates expression of phase II detoxification enzymes and antioxidant proteins by binding to a *cis*-acting regulatory or enhancer element, called antioxidant response element ARE (Nguyen *et al.*, 2004).

1.3.2 ARE

The antioxidant response element (ARE) is a *cis*-acting enhancer sequence that is responsive to phenolic antioxidants (Kensler *et al.*, 2007; Rushmore *et al.*, 1990). It mediates transcriptional activation of genes in cells exposed to oxidative stress and was first identified to be a DNA consensus motif (gene enhancer) on the 5'-flanking region of the rat glutathione s-transferase A2 subunit gene (GSTA2) which was activated upon exposure to electrophilic

and planar aromatic compounds and phenolic antioxidants (Friling *et al.*, 1990; Rushmore *et al.*, 1991; Rushmore & Pickett, 1990).

This unique enhancer region can therefore mediate a transcriptional response to a broad spectrum of structurally diverse chemicals. Further sequence analysis by reporter gene assays revealed that the core ARE sequence is essentially represented by 5'-RGTGACnnnGC-3' or 3'-YCACTGnnnCG-5' (Rushmore *et al.*, 1991). In addition to being antioxidant-inducible, this ARE could as well contribute to the basal (constitutive) expression of this rat GST isoform. At that time, the transcription factor(s) responsible to interact with this consensus motif remained unknown. Nevertheless, the high similarity between this ARE sequence and the NF-E2-AP1 motif, which was found to bind Nrf2 and other members of the AP-1 family of transcription factors, as well as the TRE (phorbol-12-O-tetradecanoate-13-acetate (TPA)-responsive element) type-Maf recognition element (T-MARE) has been noted (Kataoka *et al.*, 1994b; Prestera *et al.*, 1993; Xie *et al.*, 1995).

Regarding differences in the ARE sequences among species people asked if there are similar or indispensable nucleotide sequence in the core ARE of all ARE-regulated genes. It was found that an active ARE located in the promoter of human glutamate cysteine synthetase modulatory subunit (GCSm) gene has a variant ARE sequence (Erickson *et al.*, 2002). These findings evoked a revision of the core ARE to 5'-RTKAYnnnGCR-3'.

The first report about Nrf2 as an activator of ARE comes from the work of Venugopal and Jaiswal in 1996. By means of EMSA and ARE-reporter gene activity assays they identified a cis-element resembling the documented ARE sequence in 5'-flanking regulatory region of the human NAD(P)H:quinone oxidoreductase (hNQO1) can physically bind Nrf1 and Nrf2 which resulted with an increased transactivation activity and NQO1 gene induction (Venugopal & Jaiswal, 1996). Further evidence was subsequently provided by Itoh and coworkers who observed an impaired constitutive and butylated hydroxyanisole (BHA)-induced expression of the phase II enzymes GSTs Ya and Yb in Nrf2-knock-out mice (Itoh *et al.*, 1997).

1.3.3 ARE-regulated Genes

As already mentioned above ARE-regulated genes are involved in a variety of cellular functions including antioxidant defence, glutathion homeostasis, drug metabolism, efflux transport pathways of chemicals or redox proteins and have been identified as downstream targets of Nrf2 (Nguyen *et al.*, 2003b). Examples are NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx), glutamate-cysteine synthestase catalytic subunit (GCSc) or glutathione-S-transferase (GST) ya or GSTp (Aleksunes & Manautou, 2007; Mates, 2000) (see also table 2).

Cellular Process	Nrf2-related genes	Function
Glutathione Homeostasis	Glutamate-cysteine synthetase (GCS)	Catalyze formation of γ-glutamylcysteine
	Glutathione synthetase (GS)	Catalyze additin of glycine to γ-glutamylcysteine
	Glutathione-S-transferase (GST)	Conjugate glutathione to chemicals
	Glutathione peroxidase (GPx)	Reduce hydrogen peroxide and alkyl hydroperoxides
Drug Metabolism	NAD(P)H quinone oxidoreductase (NQO1)	Reduce quinones and endogenous antioxidants, scavenge superoxide
	UDP-glucuronosyltransferase (UGT)	Catalyze addition of glucuronic acid to chemicals
	Microsomal epoxide hydrolase (mEH)	Hydrolyze epoxides
Stress Response / Iron Metabolism	Heme oxygenase-1 (HO-1)	Catabolize heme to carbon monoxide, biliverdin, and free iron
	Ferritin	Sequester free iron
Excretion / Transporters	Multidrug resistance-associated proteins (Mrp)	Efflux chemicals across cell membrane
	Multidrug resistance proteins (Mdr)	Efflux chemicals across cell membrane
Proteasomal protein degradation	proteasome subunit PSMB5	protein degradation

Table 2: Examples of Nrf2-regulated genes (Table modified from (Aleksunes & Manautou, 2007)).

These proteins play an important role against oxidative stress- or chemical-induced cellular damage which could be clearly demonstrated with numerous studies with Nrf2-knock-out mice. Chan and coworkers, as well as Cho et al showed the basic necessity for protection against pulmonary toxicity in mice (Chan & Kan, 1999; Cho *et al.*, 2002). Besides the lung, the liver is another organ where Nrf2 gene regulation plays an important cytoprotective role. It was shown that hepatocytes of Nrf2-deficient mice are more susceptible to ethanol-induced toxicity (Gong & Cederbaum, 2006), bile acid toxicity (Tan *et al.*, 2007) and acetaminophen-induced damage (Chan *et al.*, 2001). Furthermore, a crucial role of Nrf2 was found in liver regeneration (Beyer *et al.*, 2008) and in protection from fibrosis (Xu *et al.*, 2008). Since oxidative stress is thought to be one of the components contributing to the progression of chronic neurodegenerative diseases like Parkinson´s or Alzheimer´s disease the role of ARE-driven genes was as well analyzed in neuroprotection. It has been demonstrated that neural cells from Nrf2-deficient mice were more susceptible to oxidative stress compared to cells from wt mice (Lee *et al.*, 2003a; Lee *et al.*, 2003b) and Nrf2-overexpression resulted in a significant increase of neuronal resistance towards oxidative cell death (Shih *et al.*, 2003). Besides induction of genes coding for phase II detoxificating enzymes it was also shown

recently that Nrf2 regulates expression of proteasomal subunits PSMB5 (Kwak & Kensler, 2006; Kwak *et al.*, 2003).

1.3.4 Regulation of Nrf2-activity and -stability

Since also during cellular metabolism processes ROS can constantly emerge many antioxidant and phase II detoxification enzymes are constitutively expressed at a low level to protect cells against oxidative damage. In case of additional exposure to environmental toxicants, including electrophilic agents, antioxidants and phenolic antioxidants, expression of these enzymes is increased. As already mentioned before, Nrf2 plays a central role in the activation of gene transcription of antioxidative enzymes by binding to the ARE sequence (Nguyen *et al.*, 2003b) and on this account regulation of Nrf2 activation has been of particular interest.

1.3.4.1 Keap1

Keap1 (Kelch-like ECH-associated protein 1) is a zinc metalloprotein which is anchored to actin in the cytoplasm and was initially identified as potential interaction partner of Nrf2 by means of a yeast two-hybrid system experiment (Itoh *et al.*, 1999). Detailed analysis of Nrf2 activity and structure across various species has identified six evolutionarily conserved domains, named Neh1 - Neh6 (Neh = Nrf2-ECH (chicken Nrf2) homologous domain) (Itoh *et al.*, 1997) (Figure 9).

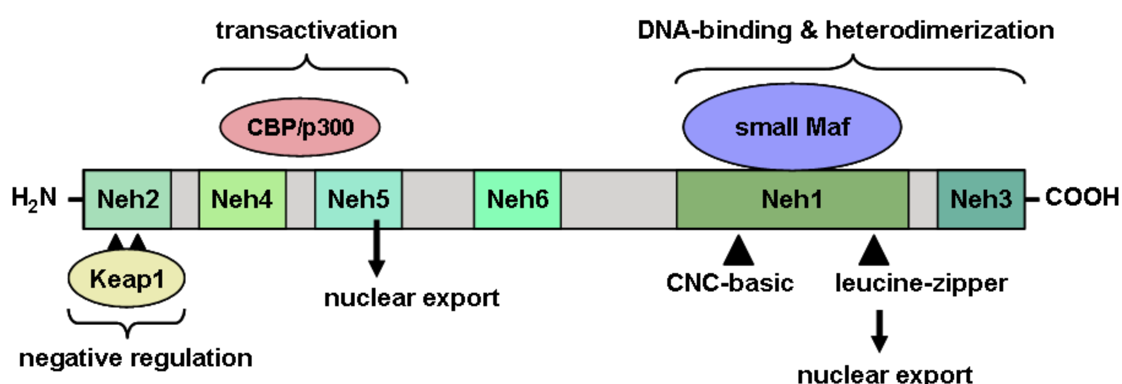


Figure 9: Structural topology of Nrf2 and function of the conserved domains. Nrf2 contains six highly conserved domains, named Neh1 to Neh6. The most highly conserved domain among species is Neh2 which is located at the N-terminus and which was demonstrated to interact with Keap1. Neh1, Neh3 and a basic leucine zipper structure is sited at the C-terminus that is involved in interaction with small Maf proteins and binding to ARE. Neh4 and Neh5 were shown to interact with the assumed coactivator CBP/p300 during transcriptional activation in the nucleus. Nuclear export signals located in the leucine zipper and Neh5 domain are supposed to desigante nuclear Nrf2 to the cytoplasmic proteasomal degradation.

The N-terminal domain Neh2 was discovered to have a negative regulatory role for the transactivating activity of Nrf2 since deletion of Neh2 was found to significantly increase Nrf2´s transactivation activity (Itoh *et al.*, 1999). The yeast two-hybrid analysis mentioned before displayed Keap1 as the major protein interacting with the Neh2 domain. Taken together, these findings hinted that Neh2 revealed a critical interaction site to which the negative regulator of Nrf2, Keap1, binds (Itoh *et al.*, 1999). Keap1 itself consists of five domains: the N-terminal region (NTR), the BTB/POZ (Bric-a-brac, tramtrack, broad-complex/poxvirus zinc finger), the intervening region (IVR), the double glycine repeat (DGR) or Kelch domain, and the C-terminal region (CTR) (Itoh *et al.*, 2004). The DGR or Kelch domain appears six times in Keap1 leading to the classification of Keap1 as a Kelch-repeat super family protein and was identified to bind to the Neh2 domain of Nrf2 and thus anchor it onto the actin cytoskeleton preventing nuclear accumulation (Itoh *et al.*, 1999; Kang *et al.*, 2004). Under normal physiological conditions this cytoplasmatic entrapment of Nrf2 and the formation of Nrf2-Keap1 complexes result in the low basal expressions of ARE-regulated genes. In response to oxidative stress, Nrf2 dissociate from Keap1 and can accumulate in the nucleus since the repressing activity of Keap1 is lost (Nguyen *et al.*, 2004). Since the C-terminal region of Keap1 was also demonstrated to bind to the Neh2 domain of Nrf2 (Tong *et al.*, 2006a), a "two-site molecular recognition model" has been suggested for the Nrf2-Keaps complex. According to this model, the two motifs DLG and ETGE located within the Neh2 domain independently associate with the DGR and CTR of Keap1 (Tong *et al.*, 2006b) which thereby might contribute to the overall stability of the Nrf2-Keap1 complex.

The regulation of Nrf2 release and nuclear translocation is still subject of ongoing discussion. It was observed that Keap1 contains a high density of cysteins which are assumed to provide target sites for direct attack by electrophilic compounds that can cause structural and functional changes leading to the release of the Nrf2 protein (Nguyen *et al.*, 2004). This leads to the supposition that the Nrf2/Keap1 complex might function as a cellular redox sensor by which the activation of ARE-dependent genes in response to oxidative stress is regulated (Itoh *et al.*, 1999). Studies with a purified Nrf2-Keap1 interacting system indeed showed that the most reactive cysteine residues of Keap1 (Cys257, Cys273, Cys288, Cys297) can be irreversibly modified by a thiol modifier, dexamethasone mesylate (Dinkova-Kostova *et al.*, 2002). Although further studies with electrophilic and antioxidant chemopreventive agents confirmed that multiple cysteine residues of Keap1 can be modified in similar but not entirely uniform manner (Hong *et al.*, 2005a; Hong *et al.*, 2005b) there are also studies showing that mutation of the Cys273 and Cys288 residues or electrophilic treatment did not affect the formation of the Keap1-Nrf2 complex (Eggler *et al.*, 2005; Kobayashi *et al.*, 2006). Thus,

release of Nrf2 appears to involve more complex mechanisms than only physical liberation from sequestration by Keap1 following modifications of the reactive cysteins.

Additionally, Keap1 exerts its negative regulatory role in regulating Nrf2 homeostasis through promoting Nrf2 degradation. The protein has a short half-life of approximately 13 - 20 min and is expressed at low levels although its mRNA is present during unstressed conditions which suggests a rapid turn-over mechanism (Itoh *et al.*, 2003; McMahon *et al.*, 2003; Nguyen *et al.*, 2003b). Furthermore inhibition of the proteasomal system with specific inhibitors increases levels of Nrf2 (Itoh *et al.*, 2003) and enables detection of ubiquitinated Nrf2 protein which is in accordance with a rapid turn-over mechanism. The fast Nrf2 decomposition arises from a facilitated ubiquitin-mediated proteasomal degradation in which Keap1 appears to play an important role (Nguyen *et al.*, 2003b; Sekhar *et al.*, 2002; Stewart *et al.*, 2003). The cysteine residues Cys273 and Cys288 were found to be essential for this function, since mutation of either residue to serine abolishes the ubiquitination activity of Keap1 (Levonen *et al.*, 2004; Zhang & Hannink, 2003). Recently it was demonstrated that the IVR domain of Keap1 can interact with Cullin 3 (Cul3), a subunit of the E3 ligase complex to promote ubiquitination of Nrf2 ubiquitination confirming that Keap1 is also responsible for the proteasomal targeting and degradation. Cul3-based E3 ligase complex is an ubiquitin-proteasome complex where E3 ligases catalyze the binding of ubiquitin to a target protein (Cullinan *et al.*, 2004; Furukawa & Xiong, 2005; Kobayashi *et al.*, 2004; Zhang *et al.*, 2004).

1.3.4.2 Small Maf Proteins

The family of Maf proteins is composed by the *v-maf* oncogene, originally isolated from avian retrovirus AS42 (Nishizawa *et al.*, 1989), its cellular counterpart *c-maf* and *v-maf*-related genes and can be divided into the large and small Maf subfamily. Together with the Cap ‘n’ collar (CNC) proteins they form a family of regulatory proteins which are important for transcriptional regulation (Motohashi *et al.*, 2002). The large Maf proteins play an important role in the regulation of embryonic development and cell differentiation (Blank, 2008) and encompass c-Maf, MafB (Kataoka *et al.*, 1994a), Nrl (Swaroop *et al.*, 1992) and L-Maf (Ogino & Yasuda, 1998). In contrast to the small Maf proteins, they possess an N-terminal acidic domain that serves as a transactivation domain. The small Mafs are composed of MafK, MafF (Fujiwara *et al.*, 1993), and MafG (Kataoka *et al.*, 1995) and function as obligatory heterodimeric partner molecules for members of the CNC family. Sequence analysis of Maf proteins revealed two highly conserved regions: a basic domain and an upstream 23 aa domain named extended homology region (EHR). They both transmit specific DNA binding of Maf proteins to palindromic consensus sequence (TGCTGAC(G)TCAGCA) known as the MARE (Maf recognition element) (Motohashi *et al.*, 2002).

Nrf2 as well as Nrf1 and Nrf3 contain the conserved CNC-like bZip motif and use small Maf proteins as obligatory heterodimeric partner molecules for binding to the MARE (Kobayashi *et al.*, 1999; Toki *et al.*, 1997). Similarly, binding of Nrf2 and Nrf1 to ARE requires another bZIP protein to heterodimerize, since they do not bind to ARE as homodimers or heterodimers (Venugopal & Jaiswal, 1996). Several studies could demonstrate heterodimerization of small Maf (MafF, MafG and MafK) with both Nrf1 and Nrf2 and subsequent ARE-mediated gene expression of NQO1 and GST-Ya in response to antioxidants and xenobiotics (Alam *et al.*, 1999; Itoh *et al.*, 1997; Venugopal & Jaiswal, 1996; Wild *et al.*, 1999). However, there are also reports showing that small Maf rather acts as transcriptional repressor rather than activator of ARE-mediated transcription and (Dhakshinamoorthy & Jaiswal, 2000; Nguyen *et al.*, 2000). Therefore the role of small Mafs still remains unsettled.

1.3.5 Nrf2 and Intracellular Signaling

Until now, three major signaling transduction cascades are suggested to be involved in regulation of the ARE: MAPK cascade, PKC, and PI3K. Since protein phosphorylation is one of the major posttranslational mechanisms in signaling processes, it is suggested that it could also play a role regulation of Nrf2 stability, and activity respectively. The following figure shows a potential scheme of the involved signaling pathways.

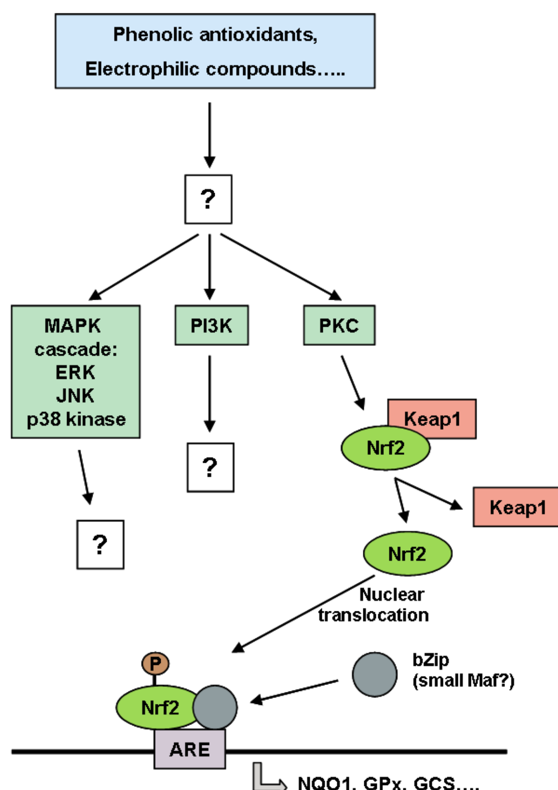


Figure 10: Signaling pathways involved in the transcriptional regulation of ARE-mediated gene expression. (Figure modified from (Nguyen *et al.*, 2003b)).

1.3.5.1 MAPK

The mitogen-activated protein kinase (MAPK) signalling pathways were the first cascade to be investigated regarding regulation of Nrf2-ARE-mediated gene induction. It was reported that the ARE-dependent gene expression is mediated by a MAPK pathway, which may involve direct activation of c-Raf by the inducers (Yu *et al.*, 1999). A follow-up study of Yu and colleagues revealed that MAP kinase pathways which are activated by MEKK1, TAK1, and ASK1 may link chemical signals to Nrf2, leading to the activation of ARE-dependent genes (Yu *et al.*, 2000a). However, in parallel it was observed that p38 kinase pathway functions as a negative regulator in the ARE-mediated induction of phase II detoxifying enzymes (Yu *et al.*, 2000b). Later, CBP/P300, a co-activator of the Nrf2 transcription complex, revealed its ability to directly be phosphorylated by the MAPK cascade which increases Nrf2-ARE transactivation activity (Shen *et al.*, 2004).
The extracellular signaling-related kinase 1/2 (ERK1/2) is a downstream, multi-kinase effector of MAPK that is able to phosphorylate a variety of transcription factors which are important for cellular antioxidant stress defence (Junttila *et al.*, 2008). By means of *in vitro* kinase assays purified recombinant Nrf2 was shown to be a target protein of ERK (Xu *et al.*, 2006; Zipper & Mulcahy, 2000). However, recent studies revealed that mutation at all conserved consensus phosphorylation sites of Nrf2 did not reduce its ability to respond to pyrrolidine dithiocarbamate (PDTC) treatment and to induce ARE-mediated gene expression, although it is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells (Zipper & Mulcahy, 2003). Therefore it is assumed that Nrf2 may be a direct target for ERK1/2 MAPK phosphorylation but the modulation might not be mandatory for Nrf2 activation and induction of ARE-mediated transactivation and the precise mechanism through which the MAPK pathway activates Nrf2-mediated gene expression has still to be elucidated as well as the differences observed for p38 pathway.

1.3.5.2 PKC

After stimulation with tert-butylhydroquinone (tBHQ) which is commonly used to induce ARE-mediated gene expression, human hepatoma cell line HepG2 showed an increased Nrf2 phosphorylation providing evidence for this hypothesis (Huang *et al.*, 2000). Furthermore, treating cells with the phosphatase inhibitor okadaic acid that induces cellular hyperphosphorylation (Cohen *et al.*, 1990) demonstrated a positive effect on nuclear Nrf2 accumulation and transcriptional activation of an ARE-driven reporter gene (Nguyen *et al.*, 2003a). Thus, these data suggests that stabilization of Nrf2 and subsequent activation of ARE-regulated genes is closely linked to protein phosphorylation. To date, direct phosphorylation of Nrf2 *in vivo* and *in vitro* was only shown for protein kinase C (PKC). This

phosphorylation, occurring at Ser40 which is phylogenetically conserved and located in the Neh2 domain of Nrf2 to which Keap1 binds, leads to interference with its interaction with Keap1 and promotes its nuclear accumulation (Huang *et al.*, 2002; Numazawa *et al.*, 2003). However, inhibition of PKC by staurosporine had no effect on tBHQ induced accumulation of Nrf2 in HepG2 cells suggesting that PKC phosphorylation is not directly responsible for Nrf2 stabilization (Bloom & Jaiswal, 2003; Nguyen *et al.*, 2003a). Taken together, these studies imply that PKC phosphorylation of Nrf2 might be an important mechanism to separate cytoplasmatic Nrf2-Keap1 interaction, but this process is not compellable for nuclear Nrf2 stabilization or target gene transactivation.

1.3.5.3 PI3K-AKT

Besides the MAPK cascade other studies have suggested that phosphatidylinositol 3-kinase (PI3K) which is an integral component of the insulin signaling pathway plays an essential role in the ARE-mediated rat GSTA2 induction by oxidative stress after sulphur amino acid deprivation (SAAD), which activates the p38 MAP kinase and leads to rGSTA2 induction (Kang *et al.*, 2000). Further evidence for the involvement of PI3k in the enhancement of ARE-mediated gene expression provided analysis of the human neuroblastoma cells line IMR-32. After treatment of the cells with LY 294002, a selective PI3-kinase inhibitor, ARE-driven gene transcription was diminished in response to tBHQ (Lee *et al.*, 2001). Later, a microarray approach showed an antioxidant responsive element-driven gene set involved in conferring protection from an oxidative stress-induced apoptosis in IMR-32 cells (Li *et al.*, 2002).

Since chronic oxidative stress can severely impair the regenerative potential of the liver (Parola & Robino, 2001; Weber *et al.*, 2003), the role of Nrf2 in liver regeneration was analyzed in detail recently. In Nrf2-deficient mice a delayed regeneration was observed which was attributed to a defect in insulin/IGF-1 signaling in the regenerating liver (Beyer *et al.*, 2008). PI3K-Akt and MAPK signaling cascade as well as several other pro-mitogenic and anti-apoptotic signaling pathways are activated by the insulin/IGF-1 signaling transduction (LeRoith & Roberts, 2003). For details about the involvement of Nrf2 in insulin/IGF-1 signaling see also figure 11.

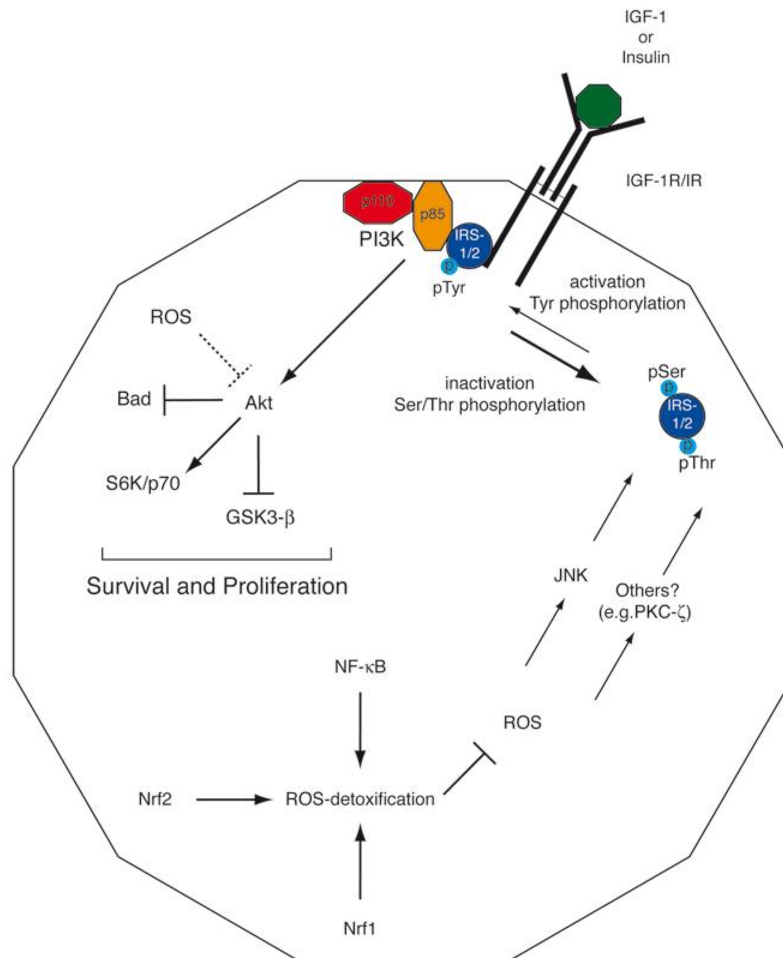


Figure 11: Involvement of Nrf2 in insulin/IGF-1 receptor signalling in the regenerating liver. (Figure modified from (Beyer & Werner, 2008)).

In contrast to wt-type mice, in Nrf2-knockout mice the activation of both p38 MAPK pathway and PI3K-Akt pathway was strongly reduced (Beyer *et al.*, 2008). In particular the significantly impaired PI3K signalling is suggested to be accountable for the delayed hepatocyte proliferation and increased apoptosis in Nrf2-knockout mice (Beyer & Werner, 2008).

2 Thesis Objectives

It was reported that hepatocytes of Nrf2-deficient mice showed a higher susceptibility to ethanol-induced toxicity (Gong & Cederbaum, 2006), bile acid toxicity (Tan *et al.*, 2007) and acetaminophen-induced damage (Chan *et al.*, 2001). Moreover, a recent study clearly demonstrated that Nrf2 plays a crucial role in liver regeneration (Beyer *et al.*, 2008) and in protection from fibrosis (Xu *et al.*, 2008). Additionally, both Nrf2 and HBV interact with the c-Raf/MAPK signaling cascade which finally leads to the assumption that their might also be an interaction between Nrf2 and HBV.

The aim of the present study was to characterize a potential modulation of the Nrf2/ARE-pathway by HBV, the molecular mechanisms involved and the physiological consequences for the host cell and the viral life cycle.

3 Materials

3.1 Cells, viruses and tissue samples

3.1.1 Prokaryotic Cells

Escherichia coli K12 strain DH5α

Genotype: F-φ80lacZΔM15 ΔfU169 recA1 endA1 hsdR17 phoA supE44 thi-1 gyrA96 relA1 λ-

3.1.2 Eukaryotic Cells

HuH7.5	Human hepatoblastoma cell line derived from HuH7 cells (Blight *et al.*, 2002)
HepG2	Human hepatoblastoma cell line (Knowles *et al.*, 1980)
HepG2.2.15	Human hepatoblastoma cell line derived from HepG2 harboring a 2.15 fold HBV genome (serotype ayw, genotype D) integrated into the chromosome (Sells *et al.*, 1987).
HepG2-4A5	Human hepatoblastoma cell line derived from HepG2 harboring a 1.2 fold HBV genome (subtype adr4) (Weiss *et al.*, 1996).
HepAD38	Inducible human hepatoblastoma cell line derived from HepG2 harboring an integrated tetracycline responsive 1.2 fold HBV genome (serotype ayw, genotype D) (Ladner *et al.*, 1997)
primary mouse hepatocytes	primary mouse hepatocytes were isolated from Nrf2-/- or wt C57BL/6 mice

3.1.3 Human liver samples

Consecutive sections of paraffin-embedded liver samples of chronic and acute HBV-infected patients were kindly provided by Karin Klingel, Tübingen University Hospital, Germany.

3.1.4 Viruses

Recombinant adenovirus containing 1.3 fold HBV-genome (adeno-HBV) was kindly provided by Michael Nassal, Freiburg, Germany.

3.2 Chemicals

3.2.1 Fine Chemicals

All fine chemicals were purchased by Carl Roth (Karlsruhe) or by Sigma-Aldrich (Seelze); relevant exceptions are named separately.

3.2.2 Drugs (Anaesthetics)

Ketavet (ketamine hydrochloride) Pharmacia & Upjohn, Erlangen
Rompun (xylazine hydrochloride) Bayer, Leverkusen

3.2.3 Inhibitors

3.2.3.1 Kinase Inhibitors

Inhibitor	Target and IC50 concentrations	Source
PD98059	MEK (IC50 = 2 µM)	Merck, Darmstadt
Sorafenib / Nexavar®	c-Raf-Kinase (IC50 = 6 nM, cell culture models)	Bayer, Leverkusen
SB203580	MAP2 (IC50 = 0.6 µM)	Promega, Mannheim
Calphostin-C	active PKC (IC50 = 50 nM)	Calbiochem, Darmstadt

Table 3: Kinase Inhibitors

3.2.3.2 Protease Inhibitors

Inhibitor	Target and concentration	Source
Aprotinin	serin proteases (1 µM)	Sigma-Aldrich, Sleeze
Leupeptin	serin-, cystein-proteases (4 µM)	Sigma-Aldrich, Sleeze
Pepstatin	acid-, aspartatic-proteases (1 µM)	Sigma-Aldrich, Sleeze
PMSF	serin proteases (1 mM)	Carl-Roth, Karlsruhe

Table 4: Protease Inhibitors

3.2.3.3 Proteasome Inhibitors

Inhibitor	Target and concentration	Source
MG132	Proteasome (4 mM)	Peptide Institute Inc, Japan
Lactacystin	Proteasome (25 µM)	Peptide Institute Inc, Japan

Table 5: Proteasome Inhibitors

3.2.4 Reagents for cell culture

Collagenase CLSII	Biochrom, Berlin
Collagen G	Biochrom, Berlin
Dexamethasone	Sigma-Aldrich, Sleeze
L-glutamine	PAN Biotech, Aidenbach
DMEM medium (high glucose, w/o L-glutamine)	PAN Biotech, Aidenbach
Fetal calf serum (FCS)	PAA, Austria
G418	Calbiochem, Darmstadt
Hydrocortisone-hemisuccinate	Sigma-Aldrich, Sleeze
Insulin	Sigma-Aldrich, Sleeze
Penicillin / Streptomycin	PAA, Austria
Trypsin / EDTA	PAA, Austria
Williams medium E (w/o L-glutamine)	Biochrom, Berlin

3.2.5 Synthetic Oligonucleotides for LightCylcer PCR

Nucleotide sequence starts at the 5'-end; bold indicated sequence is non-complementary to the template DNA. *6-FAM* = 6-carboxlfluorescein, *TAMRA* = carboxytetramethylrhodamine, *PH* = phosphate ester at the hydroxyl group of the 3'-end, Y= C/T. All synthetic oligonucleotides were synthesized by TibMolBiol, Berlin.

Primer	Sequence (5' – 3')
HBx_b	AGTCCAAGAGTYCTCTTATGYAAGACCTT
HBx_f	CCGTCTGTGCCTTCTCATCTG
HBx_sonde	6-**FAM**-CCGTGTGCACTTCGCTTCACCTCTGC-**TAMRA**-T-**PH**

Table 6: Synthetic Oligonucleotides

3.2.6 Plasmids

Plasmid	Description	Source
pcDNA3.1(-)	cloning vector used as control	Invitrogen, Karlsruhe
pUC18	cloning vector used as control	Invitrogen, Karlsruhe
pHBV1.2	1.2 fold HBV genome serotype ayw, genotype D	generated by J. Lupberger
pNQO1-Luc	Luciferase-reporter construct harboring the ARE-promoter region of NQO1 (Favreau & Pickett, 1995)	Sabine Werner, ETH Zürich
pGPx1-Luc	Luciferase-reporter construct harboring the ARE-promoter region of GPx1 (Banning *et al.*, 2005)	Sabine Werner, ETH Zürich
pGCS-Luc	Luciferase-reporter construct harboring the ARE-promoter region of γGCSc (Bea *et al.*, 2003)	Sabine Werner, ETH Zürich
phcaNrf2	constitutive active mutant of human Nrf2 (auf dem Keller *et al.*, 2006)	Sabine Werner, ETH Zürich
ptdnNrf2	transdominant negative mutant of human Nrf2 (auf dem Keller *et al.*, 2006)	Sabine Werner, ETH Zürich
pRaf-C4	dominant negative mutant of c-Raf	(Bruder *et al.*, 1992)
pYFP-γGCSc	γGCSc expression constructs tagged to yellow fluorescence protein (YFP)	ImaGenes GmbH, Berlin
pYFP-NQO1	NQO1 expression constructs tagged to yellow fluorescence protein (YFP)	ImaGenes GmbH, Berlin
p3.4kb-Luc	Luciferase-reporter construct harboring the complete ARE-promoter region of PSMB5	(Kwak & Kensler, 2006)
p1.1kb-Luc	Luciferase-reporter construct harboring a 5'-deleted promoter region (contains two ARE-regions)	(Kwak & Kensler, 2006)
p0.2kb-Luc	Luciferase-reporter construct harboring a 5'-5'-deleted promoter region (contains only one ARE-region)	(Kwak & Kensler, 2006)

Table 7: Plasmids

3.2.7 Antibodies

Antibody	Species and clonality	Dilution WB / IF	Manufacturer
anti-NQO1 (A180)	mouse monoclonal	1:300 / -	St. Cruz Biotech, USA
anti-γ-GCSc (H-300)	rabbit polyclonal	1:200 / 1:80 (IHC)	St. Cruz Biotech, USA
anti-sMaf F/G/K (H-100)	rabbit polyclonal	1:300 / 1:80	St. Cruz Biotech, USA
anti-Lamin A (H102)	rabbit polyclonal	1:200 / -	St. Cruz Biotech, USA
anti LMP2 (H200)	rabbit polyclonal	1:300 / 1:80	St. Cruz Biotech, USA
anti-β-actin	mouse monoclonal	1:10.000 / -	Sigma-Aldrich, USA
anti-GPx1 (C8C4)	rabbit monoclonal	1:1000 / -	Cell Signaling Technology, USA
anti-PSMB5	rabbit polyclonal	1:1000 / -	ABR Bioreagents, USA
anti-PSMB5i	rabbit polyclonal	1:5000 / -	Calbiochem, USA
anti-LMP7	rabbit polyclonal	1:1000 / -	Abcam plc, GB
anti-Proteasome subunit 2α (H-120)	rabbit polyclonal	1:300 / -	St. Cruz Biotech, USA
MA18/07	mouse monoclonal	1:300 / 1:100	Göttingen University, Germany (Heermann *et al.*, 1984)
anti-HBsAg	goat polyclonal	- / 1:100	DAKO, Denmark
anti-mouse-HRP	donkey polyclonal	1:2000 / -	GE Healthcare, Freiburg
anti-rabbit-HRP	donkey polyclonal	1:2000 / -	GE Healthcare, Freiburg
anti-mouse-IgG-Alexa488	donkey polyclonal	- / 1:400	Invitrogen, Karlsruhe
anti-goat-IgG-Alexa488	donkey polyclonal	- / 1:400	Invitrogen, Karlsruhe
anti-mouse-IgG-Cy3	donkey polyclonal	- / 1:400	Jackson ImmunoResearch Europe, Suffolk, UK
anti-rabbit-IgG-Cy3	donkey polyclonal	- / 1:400	Jackson ImmunoResearch Europe, Suffolk, UK
anti-mouse-IgG-Cy5	donkey polyclonal	- / 1:400	Dianova, Hamburg
anti-goat-IgG-Cy5	donkey polyclonal	- / 1:400	Dianova, Hamburg
anti-DNPH	rabbit monoclonal	1:200 / -	Millipore, Germany

Table 8: Antibodies

3.2.8 Molecular Weight Calibrators

Low molecular weight (LMW) marker	GE Healthcare, Freiburg
peqGOLD Protein-Marker I	PeqLab, Erlangen
peqGold 100 bp DNA ladder	PeqLab, Erlangen

3.3 Kits

QIAGEN Plasmid Maxi Kit	QIAGEN, Hilden
OxyBlot™ protein oxidation detection Kit	Millipore, Germany
Enzygnost HBeAg Monoclonal ELISA	Dade Behring, Marburg
Enzygnost HBsAg 5.0 ELISA	Dade Behring, Marburg
20S Proteasome Activity Assay Kit	Millipore, Germany
Vectastain Kit	Vector, USA

3.4 Devices

3.4.1 Electrophoresis

Horizontal electrophoresis systems GNA 100 and 200	BioRad, München
Vertical electrophoresis systems SE 260 and 600	GE Healthcare, Freiburg
Semi-dry blotting chambers Semiphor and Multiphor II	GE Healthcare, Freiburg
Hoefer Electrophoresis power supply 301	GE, Healthcare, Freiburg

3.4.2 Microscopy

Confocal laser scanning microscope LSM 510	Zeiss, Jena
Leica, Leitz DM RBE	Leica, Wetzlar

3.4.3 Imaging

Agarose gel documentation	Intas, Göttingen
AGFA CP-1000 film developer	AGFA, Köln
Biomax MR scientific imaging film	Kodak, Stuttgart
Intelligent Dark Box LAS-3000	Fujifilm, Düsseldorf

3.4.4 Centrifugation

Centrifuge 5415 R (refrigerated)	Eppendorf, Hamburg
Sorvall Evolution RC with rotors SLA-1500 and SS-34	Sorvall Instruments, Bad Homburg
Heraeus Fresco 21 Centrifuge	Thermo scientific,

	Osterode
Hettich centrifuge Micro 200R	Hettich, Tuttlingen
Beckman Optima^{TM} TLX Ultracentrifuge	Beckman Coulter, Germany

3.4.5 Others

Hereaus Bacteria incubator Typ B6760	Kendro, Langenselbold
Incubator for cell culture	Heraeus, Osterode
INFORS HT Multitron	INFORS AG, Bottmingen
pH meter 766 Calimatic	Knick, Berlin
Photometer Ultraspec 3000	GE Healthcare, Freiburg
Stuart roller mixer SRT9	Bibby Scientific, UK
Satorius balance LP 6000 200S	Satorius, Göttingen
Satorius universal analytical balance	Satorius, Göttingen
Sonicator W-220F	Heat System-Ultrasonics, USA
Sterile bench for cell culture	Heraeus, Osterrode
Thermomixer HLC MHR10	Eppendorf, Hamburg
UV Stratalinker 1800	Stratagene, USA
Vacuum dryer (Slap Dryer Model 443)	BioRad, USA

3.5 Image Processing Software

LSM Image browser	Zeiss, Jena
Photoshop CS2	Adobe, San Jose, USA

3.6 Relevant Materials

Abbocath 24G catheter	Abbott, Ireland
Hybond-P, PVDF membrane	Millipore, Germany
Hyperfilm ECL Chemiluminescence 18x24 cm	GE Healthcare, Freiburg
Hyperfilm ECL Chemiluminescence 5x7 inch	GE Healthcare, Freiburg
Hyperfilm MP autoradiography 18x24 cm	GE Healthcare, Freiburg
SuperSignal West Pico Chemiluminescent substrate	Thermo scientific, Germany

3.7 Buffers and Solutions

All Buffers and solutions were prepared with ddH_2O (double deionised water) obtained from a Milli-Q Utrapure water system (Millipore, Schwalbach). Exceptions are named separately.

20x Acetate buffer

1097 µl Acetic acid 100 %
14.825 g Sodium acetate (anhydrous)
1000 ml ddH_2O
Store at 4 °C

Agarose Loading Buffer 6x

6x TAE
30 % (w/v) Glycerol
0.1 mg/ml Bromphenolblue

Anode-buffer I

20 % Ethanol (v/v)
0.3 M Tris

Anode-buffer II

20 % Ethanol (v/v)
25 mM Tris

Buffer A (Nuclear Fractionation)

50 mM Tris/HCl
10 mM Sodium chloride
5 mM Magnesium chloride
0.5 % NP-40
pH 8.0

Buffer B (Nuclear Fractionation)

20 mM HEPES
0.5 M Sodium chloride
1 mM EDTA
1 mM DTT
pH 7.9

Cathode buffer

20 % Ethanol (v/v)
40 mM 6-aminohexanoic acid

Citrate buffer

10 mM Citric acid (anhydrous)
pH 6; store at 4 °C

DAB (Diaminobenzidin-tetrahydrochlorid)-solution

Part 1:
2.5 ml 20x Acetatepuffer
47.5 ml ddH_2O
Part 2:
Solve 10 mg 3-Amino-9-Ethylcarbazol in 1 ml DMSO/Triton (9:1). Prior to usage mix the two solutions and add 10 µl 30 % H_2O_2 (solution stable for 15 min).

10x FA Gel Buffer

200 mM MOPS
50 mM Sodium acetate
10 mM EDTA
Prepare with DEPC water
pH 7.0 with NaOH

1x FA Gel Running Buffer

100 ml 10x FA gel buffer
20 ml 37 % (12.3 M) FA
880 ml DEPC water

5x FA RNA Loading Buffer

16 µl Saturated aqueous Bromphenolblue solution
80 µl 500 mM EDTA, pH 8.0
720 µl 37 % (12.3 M) FA
2 ml 100 % Glycerol
3084 µl Formamide
4 ml 10x FA Gel Buffer
Ad 10 ml with DEPC water

Stability: approximately 3 months at 4 °C

HANKS solution I

8.0 g	Sodium chloride
0.4 g	Potassium chloride
3.57 g	HEPES
0.06 g	Na_2HPO_4 x $2H_2O$
0.06	KH_2PO_4

Ad 1l ddH_2O, pH 7.4, sterilised.

Add prior to usage

2.5 mM	EGTA
0.1 %	Glucose

HANKS solution II

8.0 g	Sodium chloride
0.4 g	Potassium chloride
3.57 g	HEPES
0.06 g	Na_2HPO_4 x $2H_2O$
0.06	KH_2PO_4

Ad 1l ddH_2O, pH 7.4, sterilised.

Add prior to usage

0.3 mg/ml	Collagenase CLSII
5 mM	Calcium chloride

Luciferase Lysis buffer

25 mM	Tris
2 mM	DTT
2 mM	EGTA
10 %	Glycerol (v/v)
1 %	TritonX-100 (v/v)

pH 7.5

Luciferase Substrate

20 mM	Tris-HCl pH 7.8
5 mM	Magnesium chloride
0.1 mM	EDTA
33.3 mM	DTT
470 µM	Luciferin
530 µM	ATP

Luria-Bertani Medium (LB)

1 %	Trypton (w/v)
0.5 %	Yeastextract (w/v)
1 %	Sodium chloride (w/v)

Mowiol

10 %	Mowiol (w/v)
25 %	Glycerol (w/v)
2.5 %	DABCO
100 mM	Tris/HCl pH 8.5

Phosphate buffered saline (PBS) 10x

80.0 g	Sodium chloride
2.0 g	Potassium chloride
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4

Ad 1l H_2O, pH 7.4

Proteasome Lysis Buffer

50 mM	HEPES pH 7.5
5 mM	EDTA
150 mM	Sodium chloride
1 %	Triton X-100

Prior to usage add 2 mM ATP

Proteasome Buffer A

50 mM	Tris/HCl pH 7.5
5 mM	Magnesium chloride
2 mM	ATP
250 mM	Sucrose

Proteasome Buffer B

50 mM	Tris/HCl pH 7.5
5 mM	Magnesium chloride
2 mM	ATP
20 %	Glycerol

RFB1 buffer

100 mM	Rubidium chloride
30 mM	Potassium acetate
10 mM	Calcium chloride
50 mM	Magnesium chloride
15 %	Glycerol (w/v)

pH 5.8

RFB2 buffer

10 mM	MOPS
10 mM	Rubidium chloride
75 mM	Calcium chloride
15 %	Glycerol (w/v)

pH 5.8 (HCl)

RIPA buffer

50 mM	Tris/HCl pH 7.2
150 mM	Sodium chloride
0.1 %	SDS (w/v)
1 %	Sodium desoxycholate (w/v)
1 %	TritonX

Supplemented with protease inhibitors

SDS-running buffer (10x)

0.25 M	Tris
2 M	Glycin
1 %	SDS (w/v)

pH 8.3

4x SDS-loading buffer

4 %	SDS (w/v)
125 mM	Tris pH 6.8
10 %	Glycerol (v/v)
10 %	β-Mercaptoethanol (v/v)
0.02 %	Bromphenolblue (w/v)

Separation gel buffer

1.5 M	Tris
0.4 %	SDS (w/v)

pH 8.8

Stacking gel buffer

0.5 M	Tris
0.4 %	SDS (w/v)

pH 6.7

TAE-buffer (50x)

30 mM	Tris
20 mM	Acetic acid
1 mM	EDTA

TBST (10x)

100 mM	Tris pH 8.8
150 mM	Sodium chloride
2 %	Tween

pH 7.5

4 Methods

4.1 Cell Culture

4.1.1 Prokaryotic Cell Culture

E. coli K12 strain DH5α was cultivated in Luria Bertani (LB) medium at 37 °C with agitation in baffled Erlenmeyer flasks. For selection of transformed bacteria 100 µg/ml ampicillin or 25 µg/ml kanamycin was added to the medium. Frozen stocks were stored in 30 % (v/v) glycerol at - 80 °C.

4.1.2 Eukaryotic Cell Culture

Human hepatoma-derived HepG2, HepG2.2.15 and HuH7.5 cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml streptomycin. In addition, HepG2.2.15 cells were cultivated in presence of 500 µg/ml G418. HepAD38 cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 U/ml penicillin, and 100 µg/ml streptomycin, 500 µg/ml G418, 5 µg/ml insulin and 25 µg/ml hydrocortisone-hemisuccinate.

Primary mouse hepatocytes were plated on collagen-coated tissue culture dishes in William's medium E (WME) supplemented with 10% fetal calf serum, 100 nM dexamethasone, 2 mM L-glutamine and 1%-penicillin/streptomycin solution. After 4 h of incubation, cells were attached to collagen-coated tissue culture dishes and plating medium was removed and replaced by serum-free cell culture medium (WME supplemented with 100 nM dexamethasone, 2 mM L-glutamine and 0.1 U/ml penicillin, and 100 µg/ml streptomycin.

All cells were cultured at 37 °C in a water saturated atmosphere of 5 % (v/v) CO_2.

4.1.3 Isolation of Primary Mouse Hepatocytes

Primary mouse hepatocytes were isolated from Nrf2-/- (Chan & Kan, 1999) and wt C57BL/6 mice with a two-step collagenase perfusion. Anesthesia was administered by intramuscular injection of 5/100 mg body weight ketamin hydrochloride (Ketavet) 10 % and 1/100 mg body weight xylazine hydrochloride (Rompun) 2 %. After cleaning the abdomen under sterile conditions, the abdominal cavity was opened and the portal vein was cannulated with a 24G

catheter. After starting the liver perfusion with prewarmed HANKS solution I using a peristaltic pump at flow rate 8 ml/min the vena cava and the right heart ventricle were incised to permit sufficient outflow. The liver was perfused with HANKS solution I for 2 min, followed by prewarmed HANKS solution II containing collagenase CLSII for 5 - 7 min. The organ was perfused until the tissue lost flexibility and the color changed from dark red-brown to a light yellow-brown. The liver was excised, the gall bladder was removed and the liver capsule was opened to release the cells into Williams medium E supplemented with 100 nM dexamethasone, 10 % fetal calf serum, 1 µM insulin, 2 mM L-glutamine, 0.1 U/ml penicillin, and 100 µg/ml streptomycin. Hepatocytes were sieved by a 100 µm cell strainer and enriched by centrifugation for 2 min at 37.5x g. $1x10^6$ cells per well were seeded in 3 ml medium to a collagen coated 6-well plate. After 4 h the culture supernatant was removed and maintained as described in 4.1.2.

4.1.4 Infection of Primary Mouse Hepatocytes

Primary mouse hepatocytes from Nrf2-/- and wt C57BL/6 mice were infected with recombinant adeno-HBV, kindly provided by Michael Nassal, Freiburg, Germany, as described previously (Ren & Nassal, 2001). 2 days p.i. the the supernatant was collected for subsequent analysis of HBsAg and HBeAg level by ELISA.

4.1.5 Transfection of Hepatoma Cells

Human hepatoma-derived HepG2, HepG2.2.15, HepAD38 and HuH7.5 cells were transfected with linear polyethylenimine (1 mg/ml) according to the manual of ExGene500 transfection agent (Fermentas, Germany).

4.1.6 Cell Lysis

4.1.6.1 Protein Lysates

Confluent liver cells were washed twice in PBS and lysed on ice in 200 - 300 µl high detergent lysis buffer (RIPA; 20 mM Tris, 1 % (w/v) sodium deoxycholate, 1 % (v/v) Triton X-100, 0.1 % (w/v) sodium dodecyl sulfate, 150 mM NaCl, pH 7.2) supplemented with protease inhibitors (1 mM PMSF, 4 µM leupeptin, 1 µM aprotinin, 1 µM pepstatin). After incubation on ice for 10 min the lysed cells were collected by a rubber policeman. Prior to measurement of protein concentration with Bradford reagent lysates were sonicated.

4.1.6.2 Luciferase Lysates

Cells designed for luciferase assay were washed twice with PBS, incubated in luciferase lysis buffer (25 mM Tris, 2 mM DTT, 2 mM EGTA, 10 % glycerol, 1 % Triton X-100 pH 7,5) on ice for 10 min. After collection of cells and centrifugation, the amount of proteins was quantified by Bradford reagent.

4.1.6.3 Nuclear Fractionation

After washing cells twice with cold PBS 300 µl lysis Buffer A was added, the cell lysate was transferred into a reaction tube and incubated on ice for 10 min. After centrifugation (4 °C, 12.000 g, 15 min) the supernatant (cytosolic extract) was transferred into a new reaction tube The pellet was washed twice with Buffer A, resuspended in 70 µl Buffer B and centrifuged at 14.000 rpm, 4 °C for 20 min. The supernatant (nuclear extract) was collected in a new tube and protein concentration was measured with Bradford reagent for subsequent analysis by Western Blot.

4.1.6.4 Proteasome Isolation

Proteasomes were isolated by differential centrifugation based on the protocol of Robek et al. (Robek et al. 2007) In brief cells were disrupted in buffer A (50 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 250 mM Sucrose) by a potter-Elvehjem tissue homogenizer. Unbroken cells and nuclei were removed by centrifugation at 20.000 rpm for 10 min. The supernatant was centrifuged at 100.000 rpm for 18 min, followed by 80 min centrifugation at 100.000 rpm. All centrifugation steps were done with the rotor Beckmann TLA100.3. Centrifugation pellet 1 and 2 were resuspended in buffer B (50 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 20 % glycerol). Analysis of constitutive proteasome function was performed using a commercial assay system (20S Proteasome Activity Assay Kit, Millipore, Germany) measuring the fluorescence of 7-Amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC, and Bz-SPSV-AMC respectively to measure immune proteasome activity.

4.2 Molecular Biology

4.2.1 Production of Chemically Competent *E. coli K12* strain DH5α

Pre-warmed LB medium (200 ml) was inoculated with *E. coli K12* DH5α bacterial strains. After the bacteria were grown to an optical density of OD_{600} = 0.3 - 0.4, cells were pelleted by centrifugation using sterile centrifugal tubes and resuspended in 0.4 volume of buffer RFB1. The bacteria suspension was incubated on ice for 15 min and after subsequent centrifugation the cell pellet was resuspended in 0.04 volume of RFB2. After a final incubation step of 15 min on ice the suspension was aliquoted and immediately frozen in liquid nitrogen and stored at -80 °C.

4.2.2 Transformation of Chemically Competent bacteria

DNA was introduced into chemically competent *E. coli K12* strains DH5α by transformation as described by Sambrook et al. (Sambrook & Pollack, 1974). Briefly, 10 - 50 ng plasmid DNA was added to 100 µl competent bacteria, carefully mixed and incubated on ice for 30 min. After a subsequent heat shock for 30 sec at 42 °C, cells were again incubated on ice for 5 min. To allow the formation of antibiotic resistance 500 µl LB medium was added to the cell suspension and incubated for 1 h shaking at 37 °C. Finally, cells were plated on LB agar plates containing the suitable antibiotic for selection purposes of the transformed bacteria and incubated over night at 37 °C.

4.2.3 Isolation of Plasmid DNA

Plasmid DNA was extracted from 200 - 300 ml bacterial overnight culture using QIAGEN Plasmid Maxi Kit. The extraction procedure was performed as described by Qiagen. The plasmid DNA was eluted in sterile ddH_2O and stored at -20 °C. The DNA concentration was determined by measuring the absorbance at λ = 260 nm. The purity of DNA preparation was analyzed by measuring the absorbance at λ = 280 nm at the same time.

4.2.4 Isolation of RNA with TRIZOL

Total RNA was prepared using TRIZOL reagent (Invitrogen) as described by the manufacturer. The quality of the isolated RNA was controlled by running an agarose gel. RNA concentration and purity was measured by measuring the absorbance at λ = 260 nm and λ = 280 nm, respectively.

4.2.5 Agarose Gel Electrophoresis

Plasmid DNA was analyzed by agarose gel electrophoresis. Broad range agarose (Genaxxon, Biberach) was dissolved in 1x TAE buffer by heating. 0.1 µg/ml ethidium bromide was added to the liquid agarose before it was casted into an electrophoresis tray. The samples were loaded with an appropriate amount of 6x agarose gel sample buffer and separated by electrophoresis at 90 V. For imaging the DNA was visualized by UV light at 254 nm and documented with an INTAS-imaging system. The fragment sizes were compared to molecular weight calibrators.

4.2.6 Quantitative LightCycler PCR

For the quantification of target DNA molecules the Roche LightCycler 1.5 was used. The principle of quantification is the detection of the cleavage associated change in fluorescence of the probe, which binds in between the two binding sites of the primer set. In this study quantitative PCR systems were instrumental to quantify a 124 basepair (bp) DNA sequence within the HBV genome. This HBV assay was performed as described by Stoeckl and co-workers (Stockl *et al.*, 2003).

The PCR reaction mixture for the LightCycler formats is summarized in table 9. The Lightcycler temperature profile was 95 °C 5 min, 30x (95 °C 15 sec, 60 °C 30 sec, 72 °C 30 sec).

Reagents	**Amount in [µl] per sample**
10x PCR buffer w/o MgCl2	2
$MgCl_2$ (50 mM)	1.8
TE buffer, pH 8.0	0.24
BSA (600 µg/mL)	0.24
dNTP mix (25 mM each)	0.32
sense primer (10 µM)	0.5
antisense primer (10 µM)	0.5
probe (10 µM)	0.25
Immolase hotstart polymerase	0.1
sample	1

Table 9: LightCycler PCR reaction mixture

4.2.7 Northern Blot

4.2.7.1 Formaldehyde Agarose Gel Electrophoresis

One volume RNA (12 µg/lane) was mixed with 4 volumes of 5x RNA-Loading buffer and was size-fractionated in a 1.2 % agarose gel containing 0.67 % formaldehyde (FA). Prior to loading the samples, the gel was equilibrated in 1x FA gel running buffer containing 0.74 % FA for at least 30 min. After running, the gel was incubated for 20 min in 0.05 M NaOH, 1.5 M NaCl, followed by 20 min incubation in 0.5 M Tris pH 7.5, 1.5 M NaCl. Afterwards RNA was transferred onto Hybond-N membranes (Amersham) over night by capillary blotting using a Schleicher and Schuell's Turboblotter according to the manufacturer's instructions. After blotting RNA was crosslinked using an UV Stratalinker 1800.

4.2.7.2 Hybridization

DNA probes were radio-labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using Klenow Fragment (5U/µl) (Bioline). 50-80 ng DNA were heat denaturated for 5 min at 95°C and afterwards chilled on ice. DNA was combined with 5 µl Klenow Fragment Buffer (10x), 2 µl dATP/ dTTP/ dGTP (0,5mM), 1 µl random primer (500 ng/µl) (Stratagene), 1 µl Klenow Fragment, 5 µl radiolabeled [α-^{32}P]dCTP, filled up to a final volume of 50 µl with ddH$_2$O and incubated for 1 h at 37 °C. Radiolabeled probes were purified using the QIAquick PCR Purification Kit (QIAGEN) as described in the protocol.

Hybridization was performed over night using Roti-Hybri-Quick (Roth) as described by the manufacturer. Membranes were washed the next day once for 10 min with pre-heated Washing Buffer I and twice for 10 min with pre-heated Washing Buffer II at 65 °C. Membranes were wrapped in cling film and exposed to a roentgen film (Amersham) at -80°C.

4.3 Protein Biochemistry

4.3.1 Protein Quantification with Bradford Reagent

The binding of proteins to the dye Coomassie Brilliant Blue G-250 in the Bradford reagent stabilizes the anionic form of the dye. This shifts the light absorption maximum from 465 nm to 595 nm. Protein quantification was performed according to the manufacturer's instructions.

4.3.2 SDS-PAGE Electrophoresis

This method separates a protein mixture dependent on its molecular weight (MW) composition as described by Laemmli (1970). It was performed as described in standard protocols (Ausubel, 2004). The polymer density of the separation gel depends on the expected MW of the target protein (MW < 30 kDa = 15 % (w/v) PAGE gel, MW > 30 kDa = 10 % (w/v) PAGE gel), while for the stacking gel a polymer density of 4 % was used. Proteins are denaturated in the appropriate amount of 4x SDS-PAGE sample buffer by 5 min heat denaturation at 95 °C prior to electrophoresis in 1x SDS-PAGE buffer at 80 -120 V.

4.3.3 Western Blot

Separated proteins in a SDS-PAGE gel were transferred on a methanol pre-treated PVDF membrane (Immobilon-P, Millipore) using a semi-dry blotting chamber as described by Towbin et al (Towbin *et al.*, 1979; 1992). The proteins migrate from the gel onto the membrane in an electric field of 1.3 mA/cm² for 1 h. Figure 12 shows the scheme of a semi-dry blotting stack.

Unspecific binding sites were coated using blocking solution 10 % (w/v) skim milk powder in buffer TBS-T. Primary antibodies were diluted in the appropriate blocking solution as suggested by the manufacturer and incubated for 1h at room temperature or over night at 4 °C. Unbound antibodies were removed by washing with buffer TBS-T. Subsequent the membrane was incubated with a horseradish peroxidise-coupled secondary antibody diluted in 10 % skim milk/TBS-T for 1 h at room temperature, followed by final washing steps with TBS-T. For detection the membrane was incubated with SuperSignal West Pico Chemiluminescent substrate (Thermo scientific) as described by the manufacturer and exposured to a scientific imaging film (GE Healthcare).

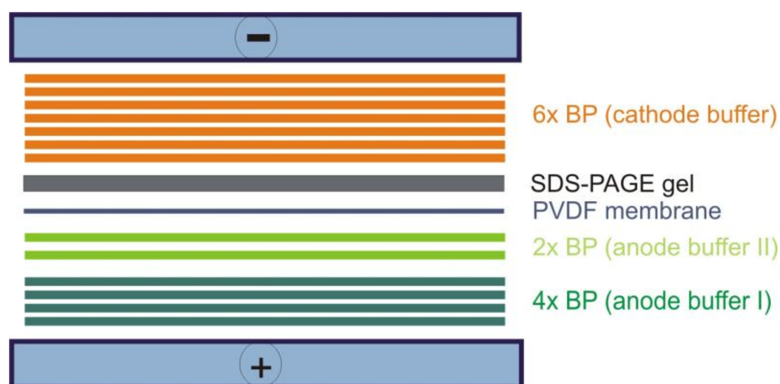


Figure 12: Scheme of a semi-dry blot stack. Blotting paper (BP) in the size of the SDS-PAGE mini gel were soaked with anode I (dark green) or II (light green) or cathode buffer (orange) and piled together with a PVDF membrane (marine blue) and the gel (grey) to a semi-dry blotting sandwich in the order as indicated. The stack is held by the anode (bottom) and the cathode (top).

4.3.4 Measurement of Protein Oxidation

Protein carbonylation was detected using OxyBlot™ protein oxidation detection Kit (Millipore, Germany). This kit provides the chemical and immunological reagents necessary for sensitive immunodetection of carbonyl groups introduced into proteins by oxidative reactions. The procedure is based on derivatization of carbonyl groups on the protein side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). To increase protein oxidation cells were treated with 2 mM H_2O_2 for 30 min or 20 mU glucoseoxidase for 4 h. After cell lysis with RIPA buffer, lysates were sonicated and protein concentration was measured with Bradford Reagent. The DNP-derivatized protein samples (40 µg of protein / lane) were separated by 12 % SDS-PAGE electrophoresis, followed by Western Blot analysis. The membrane was incubated with an anti-DNPH antibody, followed by incubation with a horseradish-peroxidase-linked secondary antibody, as provided by the kit. Proteins were visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo scientific).

4.3.5 Indirect Immunofluorescence

Cells grown on glass slides were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7 % formaldehyde in PBS for 15 min. After permeabilization with 0.05 % Triton X-100 in PBS for 5 min at room temperature, unspecific binding sites were blocked with 10 % Bovine serum albumin (BSA) in PBS (blocking solution) for 30 min. Cells were incubated for 1 h with the first and the appropriate secondary antibodies both diluted in blocking solution.

Nuclei were labeled with 4´,6-diamidino-2-phenylin-dole (DAPI). Immunofluorescence staining was analyzed by confocal laser scanning microscopy (LSM 510, Zeiss, Germany).

4.3.6 Immunohistochemistry

Consecutive sections of paraffin-embedded liver samples derived from HBV-patients with chronic or acute hepatitis kindly provided by Karin Klingel, Tübingen University Hospital, were immunostained with anti-γGCSc and anti-HBsAg using Vectastain Kit, Vector, USA. Prior to immunostaining liver samples were deparaffinized by incubation for 10 min in Xylol, followed by 10 min in EtOH 99 %, 10 min EtOH 70 %, 5 min ddH_2O and 5 min boiling in citrate-buffer. Endogenous peroxidase activity was inhibited with 0.3 % hydrogen peroxide followed by blocking with 10 % bovine serum albumin in TBST. Primary antibodies used were rabbit polyclonal anti-γGCSc and mouse monoclonal anti-HBs. Antigen visualization was performed using a biotinylated secondary antibody and streptavidin/peroxidase complex as provided by the kit.

4.3.7 ELISA for HBsAg- and HBeAg-detection

Enzyme immunoassay for the detection of HBsAg and HBeAg was performed with the commercially available assays from Enzygnost, Dade-Behring, Marburg, Germany and was performed in accordance with the manufacturer's recommendations.

4.3.8 Luciferase Reporter Gene Assay

Prior to lysis with luciferase lysis buffer cells were transfected using linear polyethylenimine (PEI) 1 mg/ml as described recently (Ehrhardt, 2006). For each well of a 6 well plate 0.5 µg pLucNQO1, pLucGCS or pLucGPx1 luciferase reporter plasmids were co-transfected with desired plasmids to analyze the HBV-dependent effect on Nrf2 activation (ptdnNrf2, phcaNrf2, pRaf-C4, pHBV1.2, or pcDNA3.1). Protein concentration was measured using Bradford Reagent. Luciferase activity with firefly luciferin as substrate was analyzed using a Luminometer (Berthold Detection Systems) according to standard protocols.

5 Results

5.1 HBV induces ARE-dependent gene expression *in vivo* and *in vitro*

The liver plays a crucial role in detoxification processes and ARE-dependent genes control the expression of a variety of cytoprotective genes. To investigate whether HBV has an effect on ARE-dependent gene expression reporter gene experiments were performed. Various reporter constructs containing a luciferase reporter gene under the control of the ARE promoter sequence derived from NQO1 (NAD(P)H quinone oxidoreductase 1; pNQO1-Luc), GPx1 (glutathione peroxidase 1; pGPx1-Luc) or γGCSc (γ-glutamylcysteine synthetase catalytic subunit; pGCS-Luc) (Banning *et al.*, 2005; Bea *et al.*, 2003; Favreau & Pickett, 1995) were cotransfected with a plasmid containing a 1.2 fold HBV genome (pHBV1.2) or an empty cloning vector (pcDNA, pUC18) as control. Stimulation with tBHQ (*tert*-butylhydroquinon) to induce ARE-regulated genes served as positive control. The luciferase reporter assay clearly showed a HBV-dependent increase of reporter gene expression indicating an activation of ARE-regulated genes by HBV (Figure 13A).

Following the reporter gene assays it was investigated whether the HBV-dependent activation of ARE-regulated genes is as well reflected by increased protein amounts. Western Blot analysis derived of total cell lysates of the HBV-producing cell lines HepG2.2.15 and HepAD38 revealed a significantly increased protein amount of NQO1, GCSc and GPx1 as compared to HBV-negative HepG2 cells (Figure 13B).

Since the prior results were all based on cell culture experiments the effect of HBV on ARE-mediated gene expression was as well analyzed *in vivo* using liver samples derived from patients with an acute or chronic hepatitis B infection. Consecutive sections were stained by immune histochemistry with a GCSc- or HBsAg-specific antibody. The staining pattern of these tissue sections shown in figure 13C displayed a close correlation between HBsAg positive cells and increased protein amount of GCSc in both chronic and acute infection.
Thus, HBV activates expression of ARE-regulated genes both *in vitro* and *in vivo*.

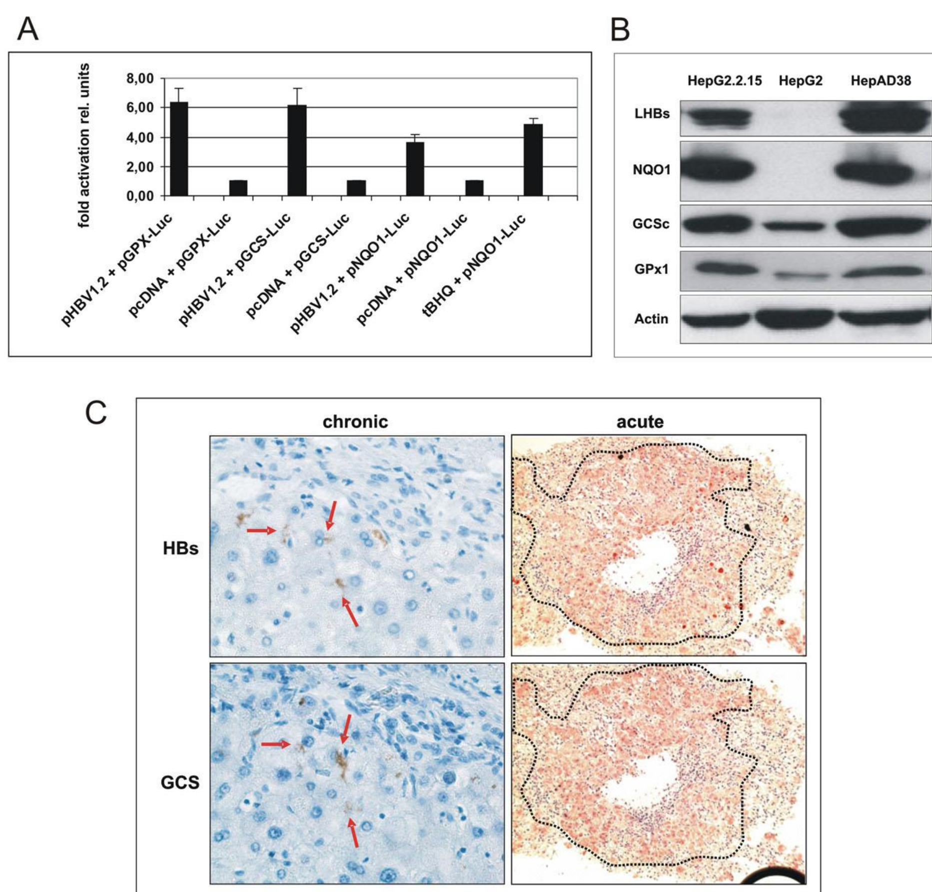


Figure 13: HBV induces expression of ARE-regulated genes *in vitro* and *in vivo*.
(A) HepG2 cells were cotransfected with 1.2 fold HBV genome (pHBV1.2) or pcDNA serving as control plasmid and a pGPx1-Luc, pGCS-Luc or pNQO1-Luc luciferase reporter construct. Cell lysates were analyzed by luciferase assay 48 h after transfection. Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation. (B) Western Blots of whole cell lysates derived from HepAD38 and HepG2 were stained with anti-NQO1, anti-GPx1 or anti-GCSc to compare the protein level in HBV-positive and HBV-negative cells. HBs-expression was analyzed by a LHBs-specific antiserum (MA18/7). β-Actin was used as loading control. (C) Immune histochemistry staining of consecutive sections of tissue samples from chronic or acute HBV-infected patients were analysed using HBsAg- and GCSc-specific antisera.

5.2 HBV-induced activation of ARE-regulated genes depends on the functionality of Nrf2

Since Nrf2 plays a crucial role in the regulation of ARE-regulated genes it is likely that Nrf2 is as well relevant for the HBV-dependent activation of ARE-regulated genes. To answer this question cells were cotransfected with the ARE-driven luciferase reporter constructs, a plasmid coding for HBV (pHBV1.2) or a control plasmid (pcDNA) and a trans-dominant negative Nrf2 mutant (ptdnNrf2). The luciferase reporter gene assay demonstrated that coexpression of the tdn Nrf2 mutant and subsequent inhibition of the transcription factor

abolished the HBV-dependent induction of the target genes (Figure 14A and B). Comparable results were obtained by Northern Blot hybridization of RNA isolated from HepG2 cells which have been cotransfected with pHBV1.2 or pcDNA and increasing amounts of ptdnNrf2 using NQO1-, GPx1- and HBV-specific probes (Figure 14C).

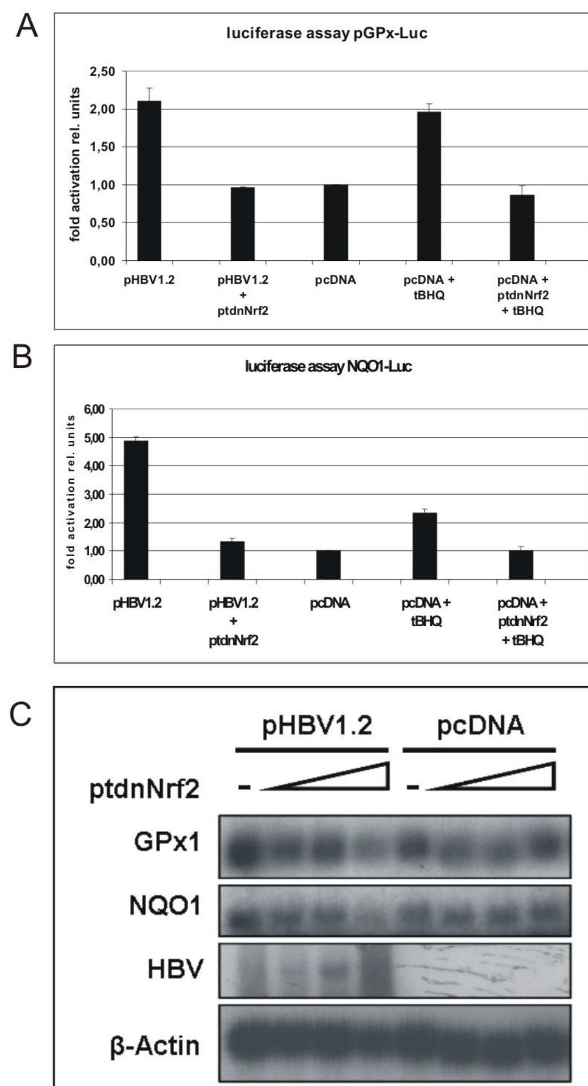
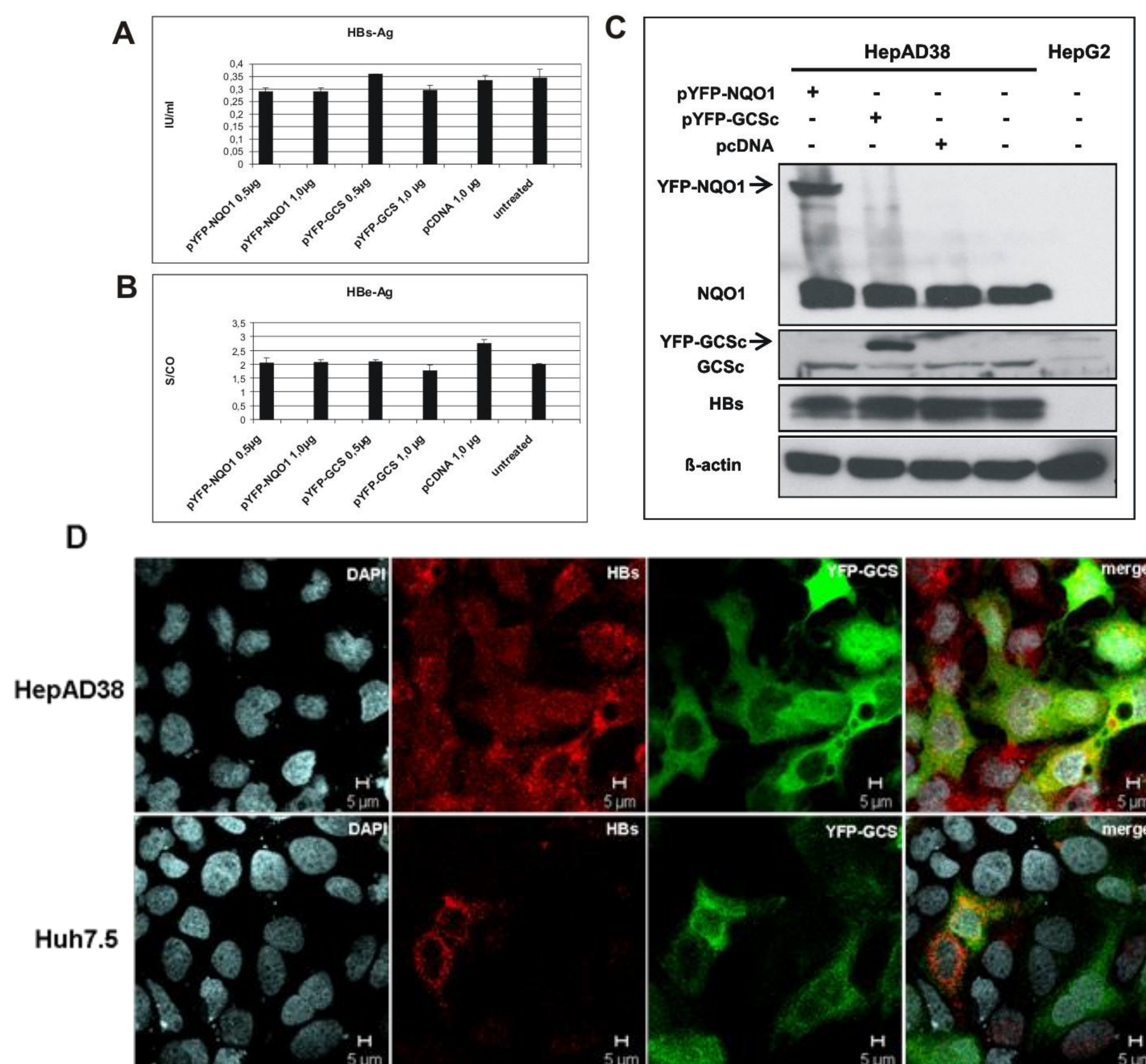


Figure 14: HBV-dependent activation of ARE-regulated genes requires functionality of Nrf2. Coexpression of a tdn Nrf2 mutant abolishes the HBV-dependent activation of ARE-regulated genes. Luciferase assays of HepG2 cells cotransfected with ptdnNrf2 and pHBV1.2 or control plasmid and a pGPx1-Luc (A) or pNQO1-Luc (B) luciferase reporter constructs. Cell lysates were analyzed by luciferase assay 48 h after transfection. Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation. (C) HepG2 cells were transfected with pHBV1.2 or control plasmid pcDNA. Nrf2 was knocked out by coexpression of a tdn mutant. Isolated RNA was analyzed by Northern Blot hybridization using NQO1-, GPx1- and HBV-specific probes. Actin served as loading control.

5.3 HBV replication does not depend on Nrf2 and is not affected by overexpression of ARE-regulated genes

Since HBV induces ARE-regulated genes via Nrf2 it was investigated whether the amount of an ARE-regulated gene affects HBV replication. Therefore the stable HBV-transfected cell line HepAD38 was transfected with expression plasmids coding for NQO1 (pYFP-NQO1) or GCSc (pYFP-GCSc) or with pcDNA as control. 48 h after transfection the cellular supernatant was collected and analyzed by HBeAg- or HBsAg-ELISA (Figure 15A and B). For quantification of secreted virions the supernatant was additionally analyzed by real time PCR (Figure 16C). Both, ELISA and real time PCR showed that overexpression of neither NQO1 nor GCSc affects HBV-expression. Comparable results were obtained for Western Blot analysis of total cell lysates. Overproduction of NQO1 and GCSc was shown using NQO1- and GCSc-specific antisera (Figure 15C). Moreover, both HepAD38 and HuH7.5 cells grown on cover slips were cotransfected with NQO1- and GCSc-expression plasmids whereas HuH7.5 cells were additionally transfected with pHBV1.2. Cells were stained for immunofluorescence microscopy using HBsAg-specific antisera (Figure 15D - F).



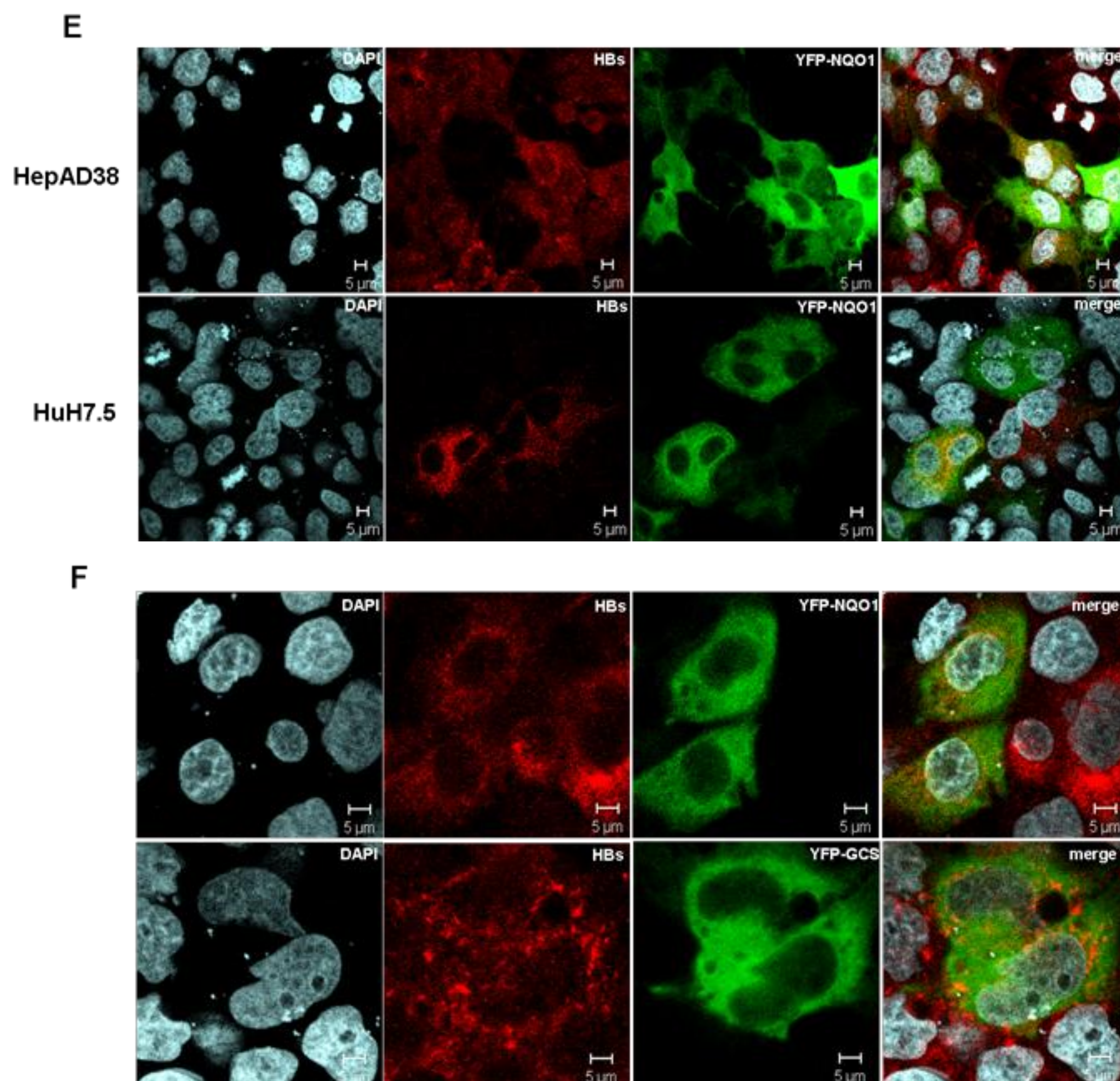


Figure 15: HBV-replication is not affected by overexpression of ARE-regulated genes.
HBV-positive cell line HepAD38 was transfected with an YFP-NQO1- or YFP-GCS-expression vector and analyzed 48 h after transfection. Secretion of HBsAg and HBeAg was quantified by an HBsAg- or HBeAg-specific ELISA (A and B). (C) Western Blot analysis of whole cell lysates of transfected cells using NQO1-, GCSc-, and HBsAg-specific antisera. For immunofluorescence microscopy YFP-GCS- (D) or YFP-GCSc-transfected (E) HepAD38 and HuH7.5 cells grown on cover slips were immunostained with a goat-anti HBsAg specific antibody 48 h after transfection. (F) More detailed picture of pYFP-NQO1- or pYFP-GCSc-transfected HepAD38 cells. Viral HBs protein was visualized with a goat-anti HBs antibody.

With the next set of experiments were done to analyzed whether general activation or inhibition of ARE-regulated genes have an effect on HBV-expression. Therefore the stable HBV-transfected cell line HepAD38 was cotransfected with a constitutive active Nrf2 mutant (phcaNrf2) or a tdn Nrf2 mutant (ptdnNrf2) to achieve a general overexpression or inhibition,

respectively. 48 h after transfection the supernatant was analyzed by HBeAg- or HBsAg-ELISA (Figure 16A and B) and by real time PCR (Figure 16C). Both ELISA and real time PCR demonstrated that neither general activation by caNrf2 nor general inhibition by tdnNrf2 of ARE-regulated gene expression did affect HBV replication.

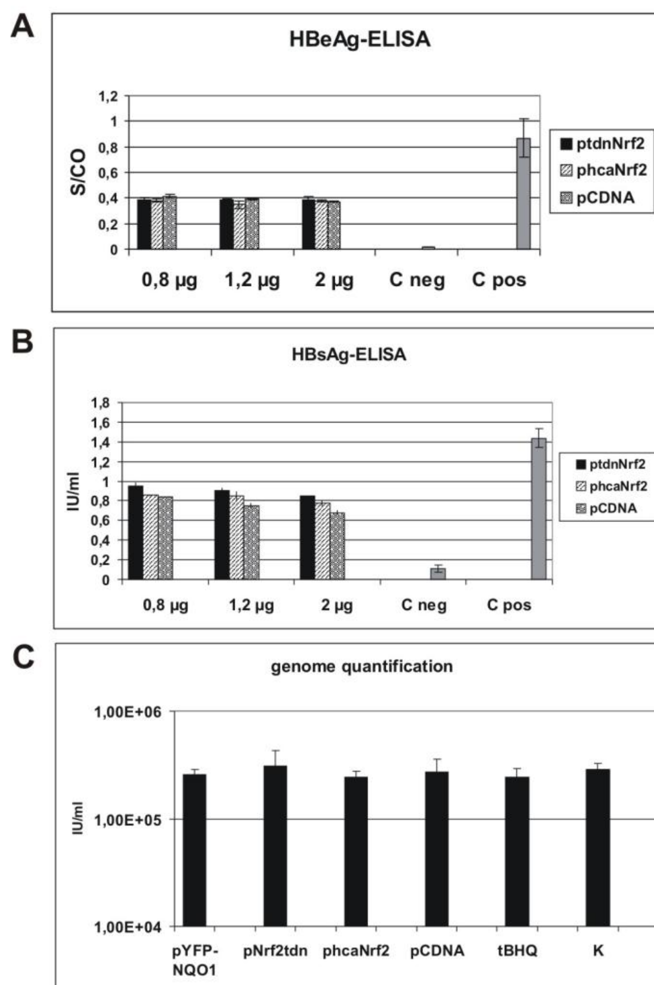


Figure 16: HBV-expression does not depend on Nrf2.

The HBV-positive cell line HepAD38 was transfected with a constitutive active Nrf2 mutant (phcaNrf2) or a tdn Nrf2 mutant (ptdnNrf2) and analyzed by different approaches. Transfection with pcDNA served as control. Secretion of HBsAg and HBeAg was quantified by an HBsAg- or HBeAg-specific ELISA (A and B). (C) Cells were additionally transfected with an YFP-NQO1- or YFP-GCS-expression vector Quantification of secreted viral particles by real time PCR. Transfection with pcDNA served as control as well as stimulation with tBHQ.

In addition to the cell culture based transfection experiments, primary mouse hepatocytes were isolated from Nrf2-/- (Chan & Kan, 1999) or from the appropriate wt C57BL/6 mice and infected with recombinant adenovirus containing 1.3 fold HBV genome (adeno-HBV) (Ren & Nassal, 2001). 48 h after infection supernatant was collected and analyzed by ELISA. Quantification of HBeAg- and HBsAg-secretion revealed no difference between Nrf2-/- and

wt C57BL/6 mice (Figure 17) confirming the previous results obtained from transfection experiments.

Taken together these data show that neither general inhibition nor constitutive activation of Nrf2 or overexpression of specific ARE-regulated genes, respectively, does affect HBV replication.

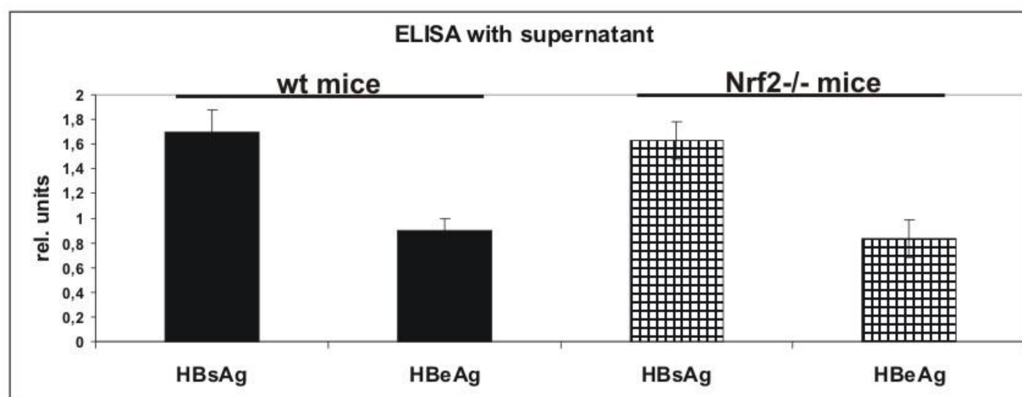


Figure 17: Nrf2 knock-out in primary mouse hepatocytes has no effect on HBV replication. Primary mouse hepatocytes isolated from Nrf2-/- and wt C57BL/6 mice were infected with adeno-HBV. 48 h p.i. supernatant was collected for subsequent quantification of HBeAg- and HBsAg-secretion by ELSIA.

5.4 HBV-dependent activation of Nrf2 requires the integrity of the c-Raf/MEK signaling pathway

Induction of ARE-regulated genes can be achieved on the one hand by increased radical levels (Mates, 2000). There are reports describing the capacity of HBV to induce radical formation (Chemin & Zoulim, 2009). To study whether increased radical levels are relevant for activation of ARE-regulated genes by HBV HepG2 cells were cotransfected with pHBV1.2 and pGCSc-Luc or pGPx1-Luc. 24 h after transfection cells were incubated with increasing concentrations of N-acetylcysteine (NAC) for 16 h which serves as a radical scavenger. Analysis of cellular lysates by luciferase reporter gene assay showed that even high concentrations of NAC resulted only in a small decrease of HBV-dependent activation of ARE-regulated genes (Figure 18A).

On the other hand it could be triggered by a c-Raf-dependent signaling pathway (Kensler *et al.*, 2007; Shen *et al.*, 2004). Since the viral proteins PreS2 and HBx are known to modulate the cellular c-Raf signaling cascades (Benn & Schneider, 1995; Klein *et al.*, 1999) the potential relevance of c-Raf signaling pathways was determined by luciferase reporter assays of pHBV1.2- and pNQO1-Luc-cotransfected HepG2 cells. These cotransfection experiments displayed that inhibition of c-Raf either by the small molecule inhibitor Nexavar®

(Sorafenib, BAY43-9006) (Himmelsbach *et al.*, 2009) or by coexpression of the tdn mutant Raf-C4 (Bruder *et al.*, 1992) abolished the HBV-induced activation of the reporter gene (Figure 18B). Comparable results were obtained after inhibition of MEK with PD98059 (Hildt *et al.*, 2002) which significantly reduced ARE-induction whereas inhibition of p38 MAPK with SB203580 (Beyer *et al.*, 2008) and inhibition of PKC activity with Calphostin-C (Hartzell & Rinderknecht, 1996) had no effect on HBV-dependent induction of the ARE-reporter gene construct (Figure 18B).

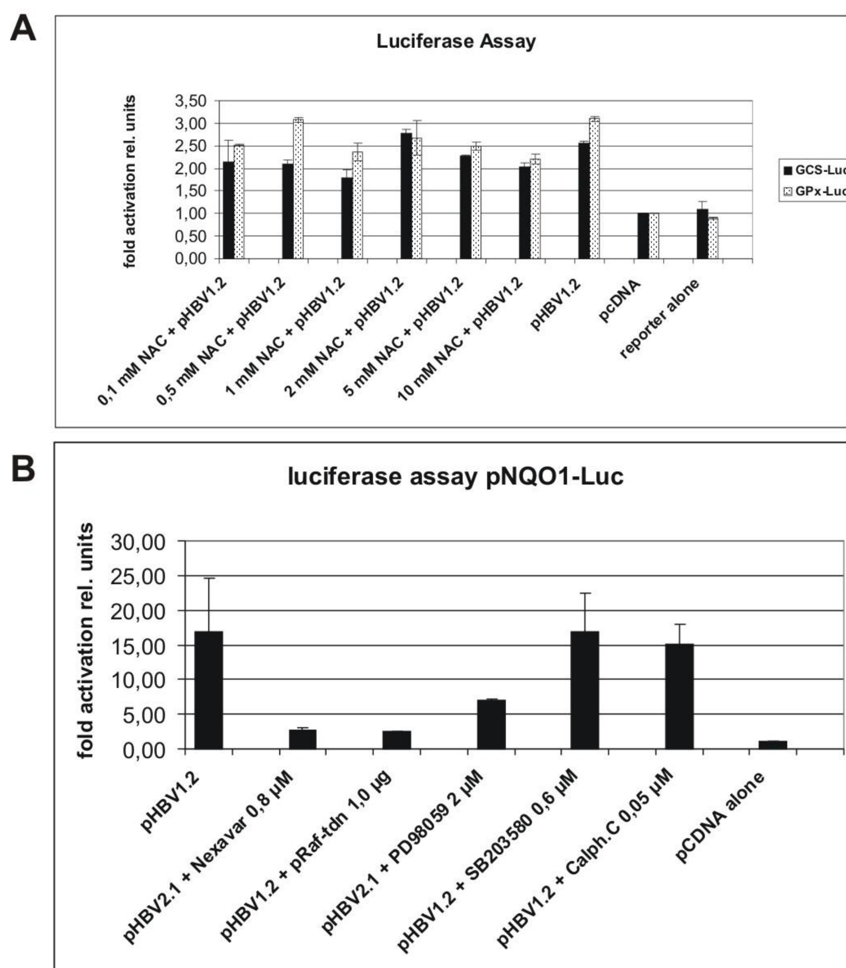


Figure 18: HBV-dependent activation of Nrf2 is mediated by the c-Raf/MEK signaling cascade.
(A) HepG2 cells were cotransfected with pHBV1.2 and pGCSc-Luc or pGPx1-Luc. 24 h after transfection cells were treated with increasing concentrations of the radical scavenger N-acetyl-cysteine (NAC) for 16 h and analyzed by luciferase reporter assay. (B) 24 h after transfection with pHBV1.2 and pNQO1-Luc HepG2 cells were treated with different kinase inhibitors for 24 h. Inhibition of c-Raf was achieved by 0.8 µM Nexavar® or coexpression of the tdn c-Raf construct pRaf-C4 (1 µg). MEK was inhibited by 2 µM PD98059, p38 MAPK by 0.6 µM SB203580 and 0.05 µM Calphostin-C was used to inhibit PKC. Cotransfection with pcDNA served as control. Cells were lysed for subsequent analysis by luciferase reporter gene assay. Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation.

Regarding the regulation of Nrf2 activity it is known that small Maf proteins play a dual role. On the one hand Nrf2 heterodimerizes in the nucleus with Maf but on the other hand increased amounts of Maf inhibit Nrf2-dependent activation of ARE-regulated genes (Dhakshinamoorthy & Jaiswal, 2000). Therefore the effect of HBV on the amount of small Maf proteins was investigated. Confocal immunofluorescence microscopy of pHBV1.2-transfected HuH7.5 cells and the HBV-producing cell line HepAD38 showed a decreased amount of small Maf proteins in HBV-positive cells as compared to control cells (Figure 19A-C). In addition, Western Blot analysis of nuclear and cytosolic fractions derived from HepAD38 and HepG2 cells using a small Maf-specific antibody confirmed these results and showed a reduced Maf level in the nuclear fraction of the HBV-positive cells.

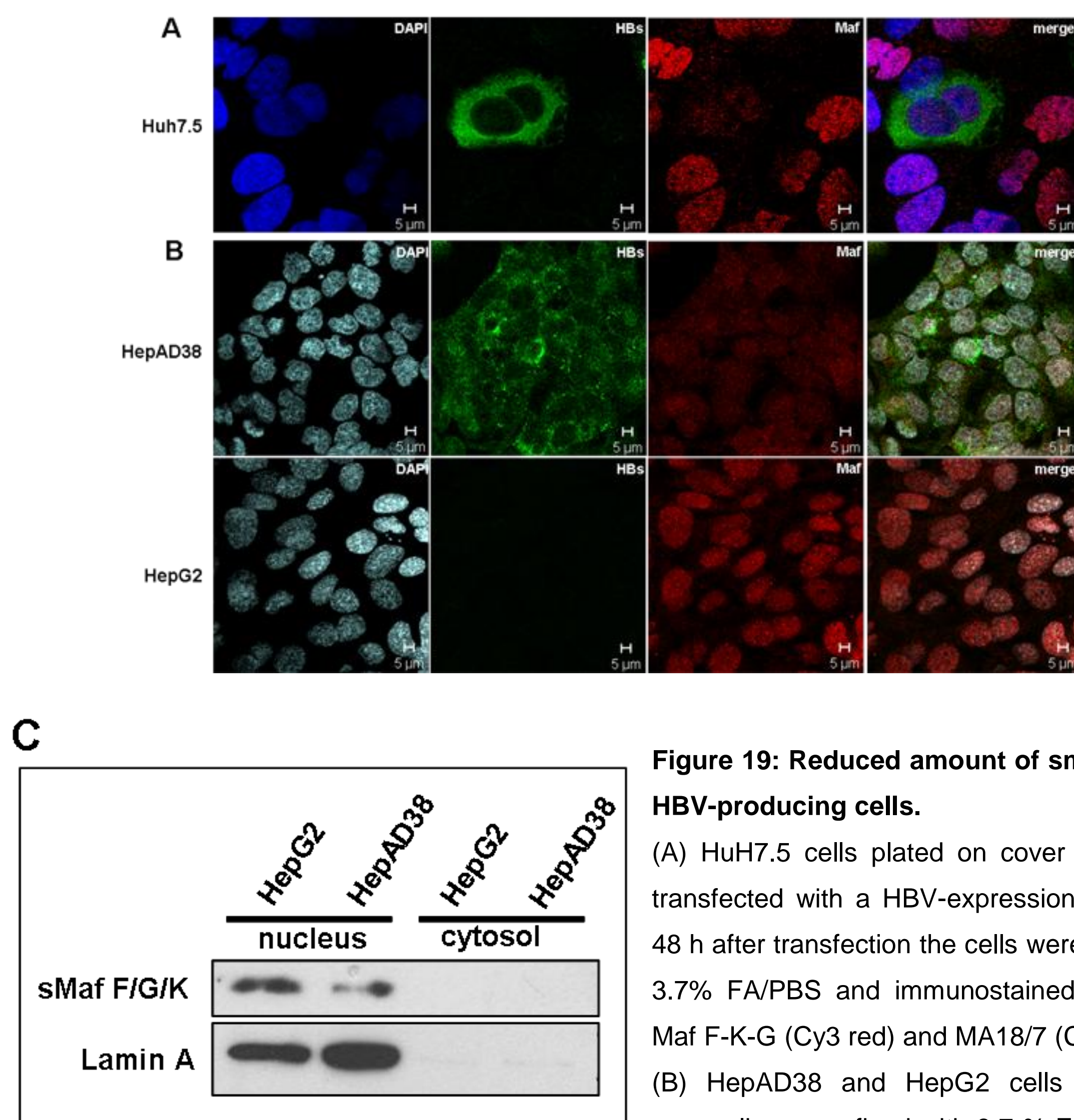


Figure 19: Reduced amount of small Maf in HBV-producing cells.

(A) HuH7.5 cells plated on cover slips were transfected with a HBV-expression construct. 48 h after transfection the cells were fixed with 3.7% FA/PBS and immunostained with anti-Maf F-K-G (Cy3 red) and MA18/7 (Cy2 green). (B) HepAD38 and HepG2 cells grown on cover slips were fixed with 3.7 % FA/PBS and immunostained with anti-Maf F-K-G (Cy3 red) and MA18/7 (Cy2 green) to detect LHBs. (C) Western Blot comparing nuclear and cellular fractions of HepAD38 and HepG2 cells using an anti-Maf F-K-G specific antibody. Lamin A served as loading control and identification control for the nuclear fraction.

5.5 HBV induces ARE-regulated genes via the regulatory proteins HBx and PreS2 activator LHBs

HBx and the PreS2 activator LHBs representing the two viral regulatory proteins induce the cellular c-Raf signaling cascades (Benn & Schneider, 1994; Hildt *et al.*, 2002; Hildt *et al.*, 1996a). Hence, to characterize the relevance of the regulatory proteins for the HBV-dependent induction of ARE-regulated genes HepG2 cells were cotransfected with expression vectors for HBV HBx (pHBx) or LHBs (pSVLM-S-) (Bruss *et al.*, 1994; Hildt *et al.*, 1996a) and pNQO1-Luc. 48 h after transfection cell lysates were analysed by reporter gene assays. HBx as well as LHBs activated reporter gene expression comparable to results obtained for full length HBV genome. Furthermore, coexpression of a tdn Nrf2 mutant abrogated the LHBs- or HBx-induced reporter gene activation indicating that functionality of Nrf2 is essential for LHBs- and HBx-dependent gene induction (Figure 20).

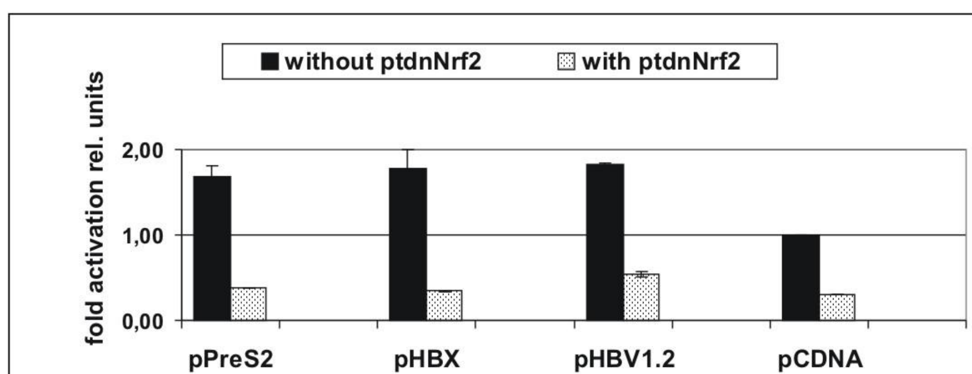


Figure 20: HBV regulatory proteins HBx and LHBs induce ARE-regulated genes.
HepG2 cells were cotransfected with expression vectors for HBx (pHBx) or LHBs (pPreS2 = pSVLM-S-) and 48 h after transfection cells were analyzed by luciferase reporter assay. Nrf2 was knocked out by coexpression of a tdn mutant (ptdnNrf2). Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation.

5.6 Compared to control cells HBV-replicating cells perform better protection against oxidative damage

Since it is well known that Nrf2 plays an important role in the cellular defense against oxidative stress, the question was whether HBV-dependent activation of ARE-regulated genes entails a benefit towards oxidative damage in HBV-replicating cells. To answer this question the HBV-positive cell line HepAD38, as well as HBV-negative HepG2 cells, were treated with H_2O_2 or glucoseoxidase and analyzed by OxyBlot™ protein oxidation kit detecting carbonyl groups introduced into proteins by oxidative reactions. The procedure is based on derivatization of carbonyl groups on the protein side chains to 2,4-

dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). In untreated HepG2 and HepAD38 cells no difference in protein oxidation was observed but after treating the cells with H_2O_2 or glucoseoxidase the HBV-replicating cells showed less oxidative damage of proteins as compared to the HBV-negative control cells (Figure 21A). Coexpression of the tdn Nrf2 mutant which abolishes the HBV-induced activation of ARE-regulated genes abrogated the protection against H_2O_2-induced oxidative damage in HBV-positive cells (Figure 21B).
Taken together these data suggest that HBV-positive cells perform better protection against oxidative damage as HBV-negative cells.

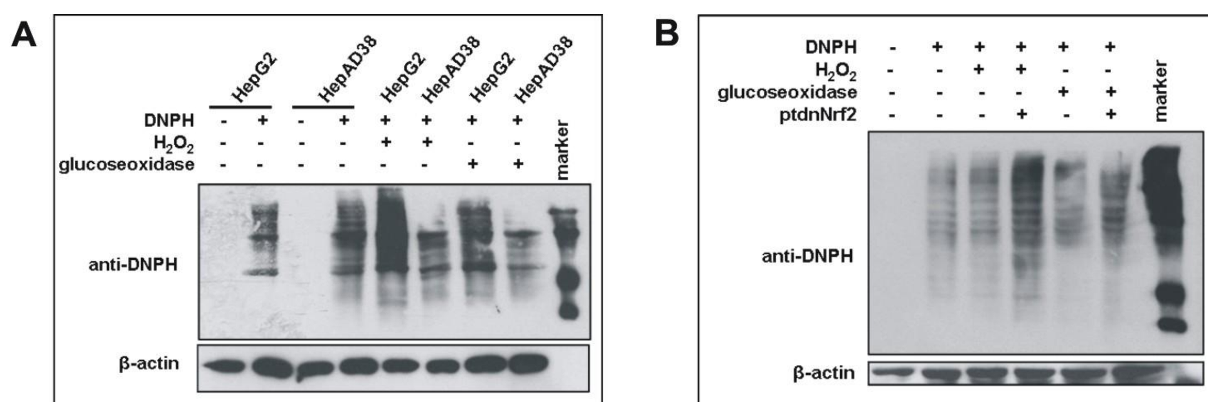


Figure 21: HBV-positive cells are better protected against oxidative damage as compared to control cells.
(A) HepAD38 and HepG2 cells were treated for 30 min with 2 mM H_2O_2 or for 4 h with 20 mU glucoseoxidase to induce oxidative damage. (B) HepAD38 cells were treated for 30 min with 2 mM H_2O_2 and additionally Nrf2 was knocked out by coexpression of a tdn mutant (ptdnNrf2). After stimulation total cell lysates were analyzed by OxyBlot™ to visualize the extent of protein oxidation with a DNPH-specific antibody. β-Actin was used as loading control.

5.7 HBV-replicating cells show modified proteasome activity

5.7.1 Increased constitutive proteasome activity in HBV-positive cells

Besides its role in cellular defense mechanisms the Nrf2/ARE signaling pathway participates as well in regulating the expression of proteasomal β-subunits 1, 3 and 5 which was described recently in detail for the proteasomal subunit PSMB5 (β5) (Kwak & Kensler, 2006). To investigate the effect of HBV on PSMB5 reporter gene assays were performed using luciferase reporter constructs harboring the full length promoter of the proteasomal subunit PSMB5 (p3.4kb-Luc), or reporter constructs containing a 5'-deleted promoter region (p1.1kb-Luc, p0.2kb-Luc). The PSMB5 promoter contains at its 5'-region three xenobiotic response elements (XRE) and at its 3'-region two ARE sequences (Kwak & Kensler, 2006; Kwak *et al.*,

2003). Hence, the p3.4kb-Luc reporter construct contains three XRE sequences (5'-region) and two ARE sequences (3'-region), the p1.1kb-Luc reporter construct two ARE-sequences and the p0.2kb-Luc construct harbors only one ARE-sequence (Figure 22A). The luciferase reporter assay of pHBV1.2- and PSMB5-Luc-cotransfected HepG2 cells revealed a significant HBV-dependent induction of the different PSMB5-Luc reporter constructs. Since the 0.2kb-Luc construct containing only one ARE-sequence was activated comparable to the 3.4kb-Luc construct it can be concluded that the ARE sequence plays an essential role for the HBV-dependent induction of the PSMB5 promoter (Figure 22B). In accordance to this coexpression of a tdn mutant of Nrf2 abolished the HBV-dependent reporter gene activation (Figure 22C).

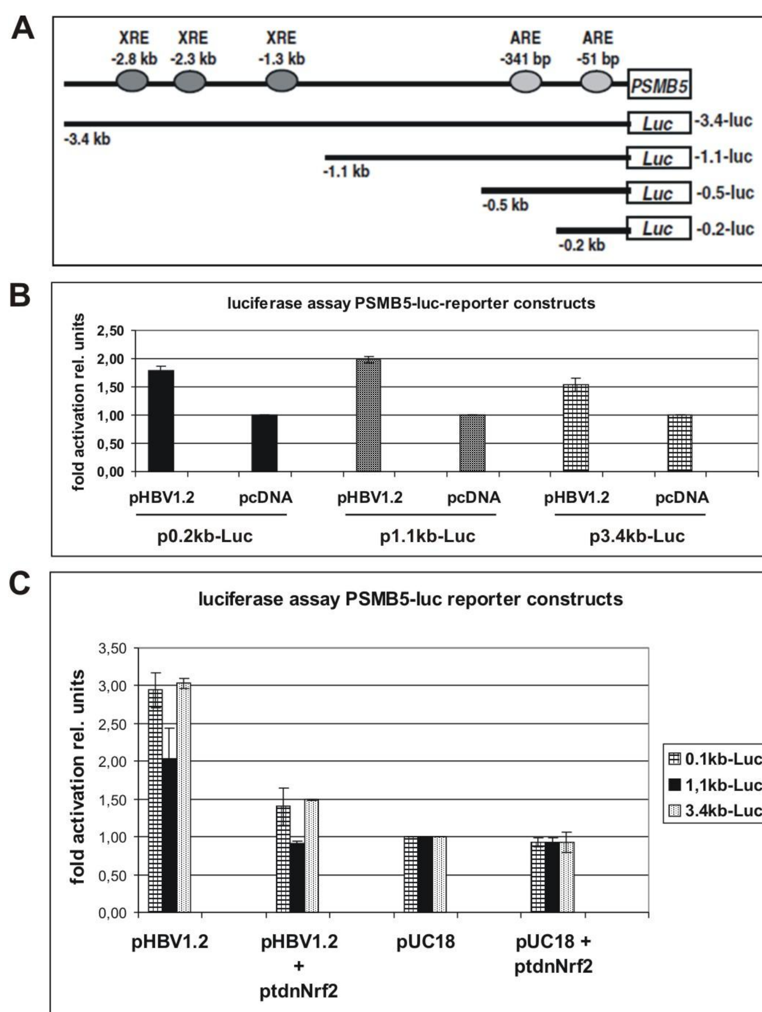


Figure 22: HBV-dependent activation of PSMB5-luciferase reporter gene constructs.

(A) Structural scheme of the PSMB5-Luc reporter constructs. Figure modified from (Kwak & Kensler, 2006). (B) HepG2 cells were cotransfected with pHBV1.2 or pUC18 as control and a PSMB5-luciferase reporter construct (p3.4kb-Luc, p1.1kb-Luc, p0.2kb-Luc). 48 h after transfection cells were analyzed by luciferase reporter assay. (C) Additionally, Nrf2 was knocked-out by coexpression of a tdn mutant. Error bars represent standard deviation. Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation.

In the next set of experiments it was investigated whether the HBV-dependent activation of the PSMB5 promoter is reflected by an increased PSMB5 protein amount in HBV-replicating cells as compared to the HBV-negative control cells. Therefore, purified proteasomes were isolated from HBV-negative HepG2 cells and the HBV-positive cell lines HepAD38 and HepG2.2.15. Western Blot analyses indeed showed an increased amount of PSMB5 in HBV-producing cells as compared to control cells (Figure 23).
In the immune proteasome, among other subunits, the PSMB5 (β5-subunit) is replaced by its interferon-γ inducible form PSMB5i (β5i = LMP7) to achieve an altered proteasomal specificity. Furthermore, according to recent data the protein level of PSMB5 and PSMB5i which is a subunit of the immune proteasome is reversely regulated (Jung *et al.*, 2009). Based on this, the question arose whether the increased amount of PSMB5 comes along with a decreased PSMB5i level in constitutive proteasomes of HBV-positive cells as compared to HBV-negative cells. Western Blot analyses of isolated purified proteasomes of HBV-replicating cell lines HepAD38 and HepG2.2.15 clearly demonstrated a lower PSMB5i protein amount as compared to HepG2 control cells (Figure 23).

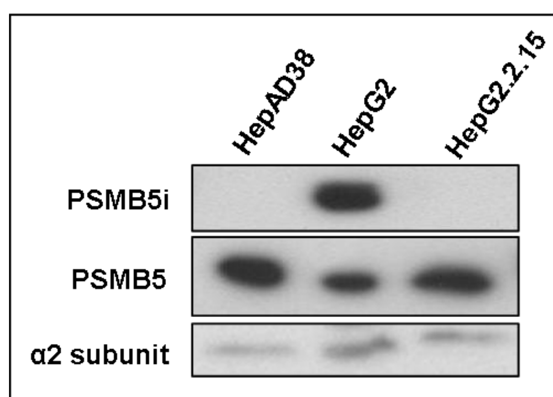


Figure 23: HBV-dependent activation of PSMB5-protein expression.
Proteasomes isolated from the HBV-negative cell line HepG2 and the HBV-positive cell lines HepAD38 and HepG2.2.15 were analyzed by Western Blot using PSMB5- and PSMB5i-specific antibodies. Equal loading was controlled by detection of the proteasome subunit α2.

Since in HBV-replicating cells an increased PSMB5 gene expression and protein amount was observed it was analyzed whether these alterations are also reflected by a higher activity of the constitutive proteasome as compared to HBV-negative cells. To answer this question proteasomes were isolated from HepG2 cells (HBV-negative), HepAD38 (HBV-positive) and HepG2.2.15 cells (HBV-positive) to analyze their proteasomal activity using LLVY-AMC as substrate. The activity assay indeed clearly reveals an increased constitutive proteasomal activity in HepAD38 and HepG2.2.15 cells as compared to HepG2 cells (Figure 24). Moreover, this increased activity was abolished by coexpression of the tdn mutant of Nrf2 confirming the relevance of Nrf2 for the HBV-induced activation of the constitutive proteasome (Figure 24).

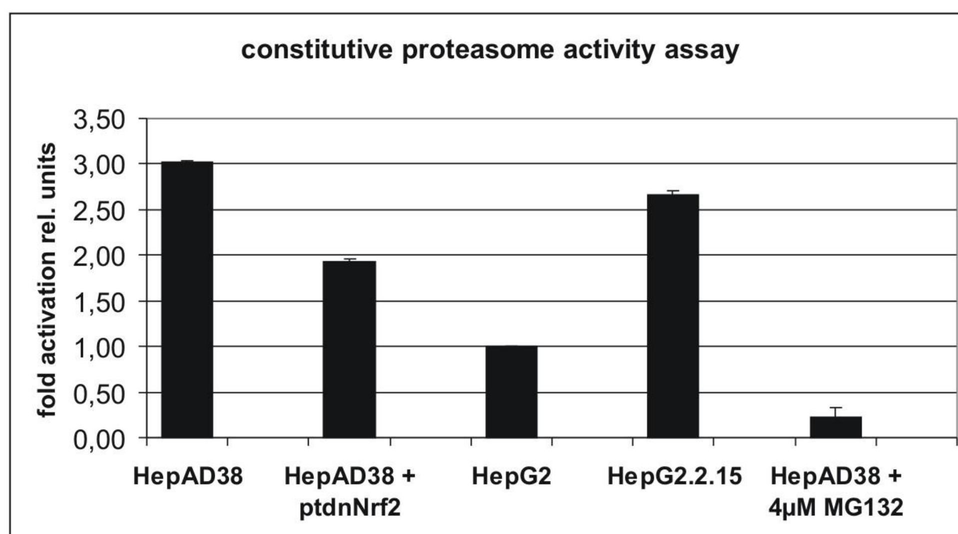


Figure 24: HBV-dependent increase of the constitutive proteasome activity.
Purified proteasomes isolated from the HBV-negative cell line HepG2 and the HBV-positive cell lines HepAD38 and HepG2.2.15 were analyzed by proteasome activity assay using LLVY-AMC as substrate. Coexpression of the tdn mutant was used to knockdown Nrf2 in HepAD38 cells. Constitutive proteasome activity was inhibited by incubation with 4 µM MG132 for 15 min. Constitutive proteasome activity was measured using LLVY-AMC as substrate. Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation.

5.7.2 Decreased immune proteasome activity in HBV-positive cells

The immune proteasomes are involved in antigen processing and the presentation of these non-host antigens to induce an appropriate immune response. The protein fragments produced by the immune proteasome have an average length of 8 - 10 aa and are optimized for the presentation via MHC-I on the cellular surface. To compare immune proteasome activity of HBV-positive and HBV-negative cells lines proteasomes were isolated from HepG2 (HBV-negative), HepAD38 (HBV-positive) and HepG2.2.15 (HBV-positive). Immune proteasome activity was measured using the carboxy-terminal 4 amino acids of the HBV-polymerase (803-811) derived peptide substrate Cbz-SPSV-AMC (Robek *et al.*, 2007). The activity assay shows decreased immune proteasome activity in HBV-replicating cells compared to HBV-negative HepG2 cells. Surprisingly, coexpression of the tdn Nrf2 mutant led to an increased immune proteasomal activity in HBV-producing cells (Figure 25).

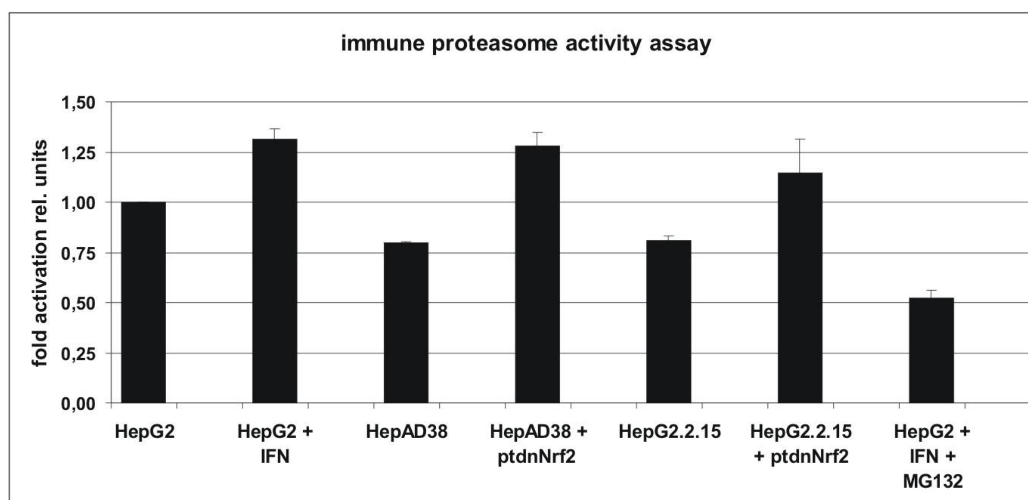


Figure 25: Decreased immune proteasome activity in HBV-positive cells.

(A) Proteasomes were isolated from the HBV-positive cell lines HepAD38 and HepG2.2.15 and the HBV-negative cell line HepG2 and analyzed by proteasome activity assay using Cbz-SPSV-AMC as substrate. Coexpression of the tdn mutant was used to knockdown Nrf2 in HepAD38 and HepG2.2.15 cells. As positive control HepG2 cells were stimulated with 1000 U IFN-γ for 24 h prior to lysis. For inhibition of the immune proteasome, lysates of IFN-γ-stimulated HepG2 cells were incubated with 4 µM MG132 for 15 min. Immune proteasome activity was measured using Cbz-SPSV-AMC as substrate. Activities, shown as multiples of induction, are mean values from three independent experiments. Error bars represent standard deviation.

6 Discussion

Although an effective vaccine to prevent hepatitis B virus infection has been available since 1986, HBV remains a major health problem with an estimated 400 million chronic infections worldwide (Hollinger & Liang, 2001). HBV is a major risk factor for hepatocellular carcinoma representing one of the most common tumors worldwide (Lupberger & Hildt, 2007). Intensive research has therefore been done regarding the molecular mechanisms of HBV-induced HCC and the clinical correlates of HCC development (Chemin & Zoulim, 2009; Lupberger & Hildt, 2007). Especially the interactions between cellular signaling pathways induced by viral proteins, the immue response and clinical symptoms still remain unclear. Currently, chronic HBV infections are treated with pegylated interferon-alpha or nucleoside/nucleotide analogs (Buster & Janssen, 2006; EASL, 2009). Although in the majority of cases this treatment successfully suppresses the viral replication there is a very low cure prognosis for chronical HBV infection. Moreover the appearance of HBV escape mutants that are resistant to nucleos(t)ide-analogs after long term treatment display another problem. This emphasizes the need for further elucidation of open questions and additional therapy strategies to increase the success rate of the current chronic HBV treatments (Buster & Janssen, 2006). Nrf2 plays an important role in the regulation of cytoprotective gene expression and is therefore crucial for the cytoprotection of the liver as one of the most important detoxifying organs (Aleksunes & Manautou, 2007). In accordance to this the liver of Nrf2-/- mice displayed a decreased ability to eliminate free radicals (Hirayama *et al.*, 2003) and it was shown that Nrf2 plays a crucial role in the protection of hepatocytes from ethanaol (Gong & Cederbaum, 2006), bile acid toxicity (Tan *et al.*, 2007) and acetaminophen (Chan *et al.*, 2001). Regarding HCC development chronic inflammation of the liver is a major factor since permanent cell death due to immne response mechanisms and subsequent cell proliferation for liver regeneration could increase the occurrence of genetic alterations and therefore the risk of cancer development (Lupberger & Hildt, 2007). Interestingly, it has been reported recently that Nrf2-/- mice showed an impaired liver regeneration (Beyer *et al.*, 2008). Besides the increased hepatocyte turnover chronic inflammation also leads to an increased ROS formation which as well can cause DNA damage and promote cancerogenesis (Chemin & Zoulim, 2009). Moreover it is suggested that accumulation of viral surface proteins in the ER inducing oxidative stress also contributes to HCC development (Chemin & Zoulim, 2009).

Taken together, the crucial role of Nrf2 in liver regeneration and in protection against oxidative damage justifies to look closer at the Nrf2/ARE signaling pathway regarding a potential crosstalk between HBV and the Nrf2/ARE system which could lead to further understanding of HBV-dependent HCC development and present further strategies for chronic HBV treatment.

6.1 HBV activates Nrf2/ARE-regulated gene expression

Antioxidant response element (ARE)-regulated genes are involved in a variety of cellular functions including antioxidant defence or drug metabolism and have been identified as down-stream targets of the transcription factor Nrf2 (Nguyen *et al.*, 2003b). Examples are NAD(P)H quinione oxidoreductase 1 (NQO1), gluthathione peroxidase 1 (GPx1), or γ-glutamate-cysteine ligase catalytic subunit (γGCLC) (Aleksunes & Manautou, 2007; Mates, 2000).

Initially it was demonstrated that HBV induces the expression of ARE-dependent genes (Figure 13A + B). These *in vitro* results were verified *in vivo* by immune histochemistry stainings of consecutive sections of tissue samples from chronic and acute HBV-infected patients (Figure 13C) confirming that the transcriptional activation of these cytoprotective genes does not only occur in cell culture based experiments but also during the viral life cycle in HBV-infected patients. Due to the fact that Nrf2/ARE-regulated genes contribute to the protection against oxidative damage (Chan, 2001; Gong & Cederbaum, 2006; Tan *et al.*, 2007) and fibrosis (Xu *et al.*, 2008) and are crucial for liver regeneration (Beyer *et al.*, 2008) the HBV-dependent activation of these genes could function as guarantee for host tissue survival and therefore for viral persistence. A recent report charcterized the overexpression of ARE-regulated genes in hepatocellular carinomas (HCCs) that might result in a growth advantage to these tumors (Lau *et al.*, 2008) because overproduction of cytoprotective proteins probably protects tumor cells from cellular elimination mechanisms that are based on increased levels of radicals or electrophiles. Consequently the contribution of the HBV-induced activation of ARE-dependent gene expression for the treatment of HCC has to be further characterized.

Since the transcription factor Nrf2 is the key regulator regarding regulation of ARE-dependent gene expression (Nguyen *et al.*, 2003b) it was suggested that Nrf2 is as well relevant for the HBV-induced activation of ARE-regulated genes. Luciferase reporter assays with HepG2 cells cotransfected with a tdn mutant of Nrf2 and a plasmid coding for HBV (pHBV1.2) indeed clearly showed that coexpression of the tdn mutant and subsequent inhibition of the transcription factor abrogated the HBV-dependent induction of the target genes (Figure 14A and B). Comparable results were obtained by Northen Blot hybridization using NQO1- and GPx1-specific probes (Figure 14C). These data therefore reveal the first report about the modulation of the transcription factor Nrf2 and its downstream target genes by HBV.

Regarding the interaction of virus and Nrf2 a recent report demonstrated that the HIV-1 Tat protein enhanced the cellular gene expression and protein level of Nrf2 and activated the expression of ARE-driven genes (Zhang *et al.*, 2009). Moreover it was reported that infection

of wt mice with respiratory syncytical virus (RSV) resulted in a significant induction of Nrf2 target genes as compared to Nrf2-/- mice (Cho *et al.*, 2009).

In the next set of experiments it was asked whether overexpression of ARE-induced genes have an impact on HBV-replication. Transfection experiments using expression plasmids coding for NQO1 and GCSc clearly demonstrated no effect of NQO1- or GCSc-overexpression on HBV-replication as shown by Western Blot analysis (Figure 15C), HBsAg- and HBeAg-ELISA (Figure 15A and B), real time PCR (Figure 16C) and immunofluorescence microscopy (Figure 15D - F). Comparable results were obtained for general inhibition by a trans-dominant negative Nrf2 mutant (tdnNrf2) or activation of ARE-regulated genes a constitutive active Nrf2 mutant (caNrf2). In order to verify these results *in vivo* primary hepatocytes isolated from from Nrf2-/- (Chan & Kan, 1999) or from the appropriate wt C57BL/6 mice and infected recombinant adenovirus encoding for 1.3 fold HBV genome (adeno-HBV) (Ren & Nassal, 2001). Supernatant was collected for subsequent analysis by HBsAg- and HBeAg-ELISA which revealed no difference between Nrf2-/- and wt mice. This is in accordance to the observation that none of the HBV promoters contains an ARE-sequence. Taken together it can be concluded from these experiments focusing only on the impact on HBV-replication that further observed effects regarding modulation of Nrf2/ARE-pathway are not due to altered HBV-replication but due to an HBV-dependent effect. Moreover, from the observation that HBV-replication thereby does not depend on Nrf2 or Nrf2/ARE-regulated genes *in vitro* it cannot be concluded that the Nrf2/ARE-system is relevant for HBV-replication *in vivo*.

6.2 C-Raf/MEK signaling pathway is crucial for HBV-dependent activation of Nrf2

Activation of Nrf2/ARE-regulated genes can occur on the one hand by increased cellular radical levels (Mates, 2000) or on the other hand they can be activated in a c-Raf-dependent manner as reporter recently (Kensler *et al.*, 2007; Shen *et al.*, 2004). A previous report characterizing the activation of Nrf2/ARE-regulated gene expression in a murine model of respiratory syncytical virus (RSV) disease by enhanced pulmonary oxidative stress in infected cells (Cho *et al.*, 2009). There are as well reports describing an accumulation of the large envelope proteins of HBV in the ER inducing ER stress (Chemin & Zoulim, 2009; He, 2006) whereas ER stress is caused by the incapability of the ER to secrete unfolded or misfolded proteins proteins finally leading to an accumulation in the ER. This induces intracellular signaling cascades which are designated as unfolded protein response (UPR)

(Ron & Walter, 2007). Besides the activation of various cellular processes UPR leads to the induction of oxidative stress (Haynes *et al.*, 2004) which could as well mediate the HBV-dependent activation of cytoprotective ARE-regulated genes.
However, luciferase reporter gene assays of HepG2 cells treated with increasing concentrations of the radical scavenger N-acetylcystein (NAC) demonstrated that even high NAC concentrations induced only a small decrease of HBV-induced expression of ARE-dependent genes (Figure 18A). Therefore the present data suggest that the HBV-dependent modulation of the Nrf2/ARE-pathway is not mediated by HBV-induced oxidative stress but finally leads to increased expression of cytoprotective genes which could contribute to protection against oxidative damage.

The relevance and presence of HBV-induced formation of ROS is still enigmatic. On the one hand there are reports demonstrating that viral protein expression in transgenic mice results in an increased ROS formation and subsequent in increased oxidative DNA damage (Hagen *et al.*, 1994; He, 2006). In human liver tissues of patient with chronic HBV infection hepatocytes showing mutated PreS proteins were referred to as “ground-class” hepatocytes (Winckler *et al.*, 1976). It is suggested that especially mutations in the PreS region of the large HBV envelope protein result in an accumulation of LHBs which inhibits secretion of the envelope proteins from the ER (Huang & Yen, 1993; Xu & Yen, 1996). Subsequent accumulation of viral proteins in the ER induces ER stress finally resulting in the induction of oxidative stress and oxidative damage (Hsieh *et al.*, 2004). However it was reported that oxidative DNA damage is less significant in chronic hepatitis B infected patients as compared to chronic HCV infected patients (Fujita *et al.*, 2008) and characterization of HBV-related HCC patients revealed that the PreS2 mutation in the HBV genome associated to ER stress and DNA damage has no pathophysiological relevance (Gwak *et al.*, 2008). Moreover it was as well demonstrated that HBV antigens only induce modest oxidative stress *in vivo* (Yang *et al.*, 2008). The present data clearly demonstrated that HBV-induced ROS-formation has no impact on ARE-dependent gene activation although ROS represents an important stimulus for Nrf2/ARE-regulated gene expression (Mates, 2000). As a consequence the present data argue against the hypothesis that HBV strongly induces the production of ROS subsequently leading to oxidative stress. In contrast the data described have demonstrated that HBV-replicating cells are better protected against oxidative damage as compared to the HBV-negative controls. In comparison thereto, the HIV-1 Tat-induced activation of Nrf2/ARE-driven gene expression was demonstrated to be mediated by ROS (Zhang *et al.*, 2009). The authors proposed that Tat-triggered ROS formation might act as a signaling molecule to affect the expression of the Nrf2 mRNA. In accordance with these findings HIV-1-induced

AIDS is proposed to be caused by an increased production of ROS by the HIV-1 proteins gp120 and Tat (Pocernich *et al.*, 2005).

In addition to activation through ROS the Nrf2/ARE-regulated genes can as well be induced via the cellular c-Raf signaling pathways (Kensler *et al.*, 2007; Shen *et al.*, 2004). The molecular details of this activation mechanism are still unknown and needs to be further investigated but it is well established that the c-Raf signaling cascade is as well modulated by the viral proteins PreS2 and HBx (Benn & Schneider, 1994; Hildt *et al.*, 2002; Hildt *et al.*, 1996a). Consequently the relevance of c-Raf signaling pathways for HBV-dependent gene induction was analyzed by luciferase repoter assays. The analysis of these cotransfection experiments showed that inhibition of c-Raf either by the small molecule inhibitor Nexavar® or by coexpression of the tdn mutant Raf-C4 abolished the HBV-induced activation of the reporter gene (Figure 18B). Comparable results were obtained after inhibition of MEK with PD98059. In contrast, inhibition of neither p38 MAPK nor PKC activity had an impact on HBV-dependent induction of the ARE-reporter gene construct (Figure 18B). Since recently it was shown that suppression of p38 MAPK resulted in inhibition of HBV-replication, these results demonstrating that inhibition of p38 MAPK did not affect the HBV-induced activation of ARE-regulated genes confirm that inhibition of HBV replication is not relevant for the observed HBV-dependent induction of Nrf2/ARE-regulated genes (Chang *et al.*, 2008).
Since the regulatory proteins of HBV induce the cellular c-Raf signaling pathways (Benn & Schneider, 1994; Hildt *et al.*, 2002; Hildt *et al.*, 1996a) the relevance of HBx and the PreS2 activator LHBs for the HBV-induced ARE-dependent gene expression was characterized using expression vectors for HBx or LHBs. The luciferase reporter gene assays demonstrated that both HBx and LHBs induced expression of the target gene to a comparable extent as it was obtained for full length HBV genome. Moreover coexpression of the tdn mutant of Nrf2 abolished the LHBs- or HBx-induced reporter gene activation confirming the necessity of Nrf2 for activation of ARE-regulated gene expression. Furthermore it can be concluded that the HBV-dependent modulation of Nrf2/ARE-driven gene expression is mediated by the viral regulatory proteins PreS2 and HBx. To further characterize the role of HBx and PreS2 activator LHBs for the induction of Nrf2/ARE-regulated gene expresission it certainly would be also helpful to use expression vectors containing functional knock-out mutations in both HBx and PreS2 but since either HBx or PreS2 is mandatory for HBV replication (Schädler & Hildt, 2009) it is not possible to use this experimental approach.

Besides the interaction between the c-Raf signaling cascade and the HBV-induced activation of ARE-regulated gene expression c-Raf might also be involved in the regulation of Nrf2-

activity by the amount of small Maf proteins. Regarding Nrf2 regulation it is known that on the one hand small Maf proteins heterodimerize with Nrf2 in the nucleus and on the other hand increased levels of small Mafs inhibit Nrf2-dependent activation of ARE-regulated genes (Dhakshinamoorthy & Jaiswal, 2000). Therefor the impact of HBV on the amount of small Mafs was analyzed. Confocal immunofluorescence microscopy of HBV-positive cells showed a decreased amount of small Mafs as compared to HBV-negative control cells (Figure 19A and B) which was confirmed by Western Blot analysis comparing nuclear fractions of HBV-positive and HBV-negative cells (Figure 19C). These data give first hints about an impact of the HBV-activated expression of ARE-regulated genes on small Mafs but concerning the role of c-Raf in the altered amount of small Mafs has still to be elucidated.

Taken together the present data suggest that the HBV-dependent modulation of the Nrf2/ARE-pathway triggered by HBx and PreS2 is not mediated by HBV-induced oxidative stress but in a c-Raf/MEK dependent manner finally leading to an increased expression of cytoprotective genes which could lead to a benefit against oxidative damage.

6.3 HBV-replicating cells are better protected against oxidative damage

There are many reports describing ROS as important factors for HBV-dependent induction of HCCs (Chemin & Zoulim, 2009). As already mentioned above, ER overload because of an increased production of HBV envelope proteins is suggested to induce the formation of ROS. Additionaly the immune system as well produces intracellular radicals within the antiviral immune response trying to eliminate HBV-positive cells. Concerning this matter upregulation of cytoprotective genes inactivating intracellular radicals could contribute to host cell survival on the one hand but on the other hand it can therefore also maintain the continuity of the viral life cycle and the establishment of the viral infection.

However, a previous report demonstrated that HBx gene expression in HepG2 cells resulted in a survival benefit against oxidative stress which could have implications for HCC development (Severi *et al.*, 2007). Moreover a recent study demonstrated that Nrf2/ARE-induced expression of antioxidant genes plays a crucial role in airway protection and rescues mice from ROS-induced pathogenesis (Cho *et al.*, 2009).

In accordance with these studies HBV-replicating cell line HepAD38 showed less protein oxidation after treatment with H_2O_2 or glucoseoxidase as compared to HBV-negative HepG2 control cells whereas in untreated cells no difference was observed between HBV-positive and HBV-negative cells (Figure 21A). Carbonyl groups introduced into proteins by oxidative

reactions were detected by OxyBlot^{TM} protein oxidation kit. Coexpression of the tdn mutant of Nrf2 which abrogated the HBV-dependent activation of ARE-regulated genes abolished the protection against H_2O_2-induced oxidative damage in HBV-positive cells (Figure 21B).
The HBV-induced oxidative stress and its contribution to HCC development are still controversely discussed. On the one hand it was reported that LHBs overexpression in transgenic mice results in liver damage, regenerative hyperplasia and an increased oxidative DNA damage in these mice finally leading to HCC development (Hagen *et al.*, 1994) and mutations within the PreS-region of the LHBs are suggested to lead to intracellular accumulation and promote HCC development (Wang *et al.*, 2006). In contrast to these data a recent study analyzing the correlation of HBV PreS mutation with ER stress and cellular oxidative damage in HBV-associated HCC patients indicated that PreS mutation-associated ER stress and oxidative damage had no impact on hepatocarcinogenesis (Gwak *et al.*, 2008). Moreover comparison of oxidative DNA damage in the liver of patients with chronic hepatitis B and C showed that hepatic oxidative DNA damage was siginificantly higher in chronic HCV infection as compared to chronic HBV infection (Fujita *et al.*, 2008). In accordance with this, HCV was found to decrease Nrf2/ARE-regulated gene expression (Carvajal Yepes *et al.*, 2009 submitted) whereas the present work demonstrated an HBV-dependent activation of Nrf2/ARE-regulated genes. Furthermore, the fact that in natural infections LHBs is unlikely to accumulate to such an extent as observed in transgenic mouse models and the missing functional difference between the ER and the cytoplasmatic localized PreS2 activators as well argues against the ER-overload hypothesis (Hildt *et al.*, 1995; Seeger & Mason, 2000). Consequently HBV-induced ROS formation might contribute to viral pathogenesis in a significantly less extent as compared to the ROS-production induced by the host immue reponse since HBV-replicating cells activate the expression of cytoprotective genes by triggering the Nrf2/ARE signaling pathway.

In conclusion, the present data suggest that the HBV-induced activation of the Nrf2/ARE-pathway and subsequent upregulation of cytoprotective Nrf2 target genes results in a better protection against oxidative damage which also argues against the hypothesis suggesting a significant pathological relevance of HBV-induced ROS. Since chronic HBV infection is a major cause of HCC development (Lupberger & Hildt, 2007) it can be speculated that this could allow the infected cell to be less vulnerable by the immune system. Consequently the HBV-infected cell might have a survival benefit and accumulate mutations finally leading to the development of a tumor cell. Therefore the HBV-dependent modulation of Nrf2/ARE signaling pathway might represent a first cross-talk between virus-modulated cellular signaling cascades and the HBV-associated pathogenesis.

6.4 HBV-replicating cells show modified proteasome activity

Aside from playing an important role in cellular antioxidant response mechanisms the Nrf2/ARE signaling pathway is as well involved in the regulation of the expression of proteasomal β-subunits 1, 3 and 5 which has been recently characterized in detail for the proteasome subunit PSMB5 (β5) (Kwak *et al.*, 2007; Kwak & Kensler, 2006; Kwak *et al.*, 2003). This raised the question whether HBV as well modulates the expression of proteasomal β-subunits. Indeed, luciferase reporter assays using reporter constructs containing the full length promoter of the PSMB5 subunit (p3.4kb-Luc) or constructs harboring a 5'-deleted promoter region (p1.1kb-Luc, p0.2kb-Luc; for details see Figure 22A) demonstrated a significant activation of the different PSMB5 luciferase reporter constructs in HBV-replicating cells (Figure 22B). Moreover, the HBV-dependent induction was comparable for the full length PSMB5 reporter constructs containing three XRE sequences (5'-region) and two ARE-sequences (3'-region) and the 5'-deleted reporter constructs harboring only two ARE sequences (p1.1kb-Luc) or one ARE sequence (p0.2kb-Luc) which confirmed the crucial role of ARE sequences for the HBV-mediated induction of the proteasomal subunit PSMB5. Furthermore, in accordance to previous results coexpression of the tdn Nrf2 mutant abolished the HBV-dependent reporter gene activation (Figure 22C). The HBV-dependent impact on PSMB5 expression was as well reflected by an increased PSMB5 protein amount as compared to control cells (Figure 23) as shown by Western Blot analysis of purified proteasomes isolated from HBV-positive and -negative cells.

Since an increase in the expression of the proteasomal subunits is supposed to be accompanied by an increase in proteasome activity (Jung *et al.*, 2009) it was assumend that the HBV-induced augmented PSMB5 expression could be reflected by an increased activity of the constitutive proteasome. Actually, the activity assay with purified proteasomes isolated from the HBV-positive cell line HepAD38 and HepG2.2.15 and the HBV-negative cell line HepG2 clearly showed an increased constitutive proteasome activity in HBV-replicating cells as compared to control cells using LLVY-AMC as a substrate (Figure 24). Coexpression of the tdn nrf2 mutant abolished the HBV-induced increase in proteasome activity which verified the crucial role of Nrf2 for the HBV-dependent activation of the constitutive proteasome (Figure 24). Regarding the relevance or consequence for both the infected cell and the virus it can be speculated that an increase in constitutive proteasome activity could result in removal of misfolded or unfolded proteins to prevent cellular accumulation and resulting intracellular stress and therefore to prevent oxidative stress.

In the immune proteasome the PSMB5 (β5) subunit is substituted by its interferon-γ-inducible forms PSMB5i (β5i = LMP7) to obtain an altered proteasomal specificity which is optimized

for the production of small peptides (8 - 10 aa) for the presentation by MHC-I on the cell surface. Thereby the protein amount of PSMB5 and PSMB5i representing the immune proteasomal subunit are contrary regulated (Jung *et al.*, 2009). According to this, the increased amount of PSMB5 in HBV-replicating cells should be accompanied by a low level of PSMB5i as compared to HBV-negative control cells which indeed could be demonstrated by Western Blot analysis using purified proteasomes isolated from the HBV-positive cell line HepAD38 and HepG2.2.15 and the HBV-negative cell line HepG2. The analysis clearly revealed an increased protein amount of PSMB5i in the HBV-negative HepG2 cells (Figure 23). In addition immune proteasome activity assay with purified proteasomes from HBV-positive and HBV-negative cell lines showed a decreased immune proteasome activity in HBV-replicating cells as compared to HBV-negative control cells. As substrate the previously described carboxy-terminal 4 amino acids of the HBV-polymerase (803-811) derived peptide substrate Cbz-SPSV-AMC was used (Robek *et al.*, 2007). Interestingly coexpression of the tdn mutant of Nrf2 resulted in an elevated immune proteasome activity in HBV-replicating cells (Figure 25).
In summary these data suggest that the activity of the immune proteasome is decreased in HBV-replicating cells whereas the constitutive proteasome activity is increased.

Since one of the most important assignment´s of the immune proteasome is the production of optimized oligopeptides for antigen presentation by the MHC-I on the cell´s surface (Jung *et al.*, 2009) reduced immune proteasome activity in HBV-replicating cells could lead to a reduced antigen presentation and consequently to a decreased immune response. Therefor it would be meaningful to characterize a potential impact of the HBV-dependent activation of Nrf2/ARE-signaling cascades on MHC-I antigen presentation. Up to date it is well established that an appropriate MHC-I-mediated cytotoxic T cell (CTL) response plays an important role in clearance of an acute HBV infection (Shi & Shi, 2009) but the molecular mechanisms mediated by HBV finally leading to the establishment of a chronic HBV infection are still subject of intensive research. It can be speculated that the HBV-dependent modulation of immune proteasome activity might also play a role regarding an insufficient MHC-I-mediated CTL-response resulting in clearance failure of the viral infection.
Besides the better protection against increased ROS formation primarily induced by the immune system this would present an additional survival benefit for the virus as less HBV-infected cells would be eliminated by the host immune response. Moreover this would describe a crosstalk between the HBV-induced signaling pathways and the immune response.
However, it is well established that one of the major factors in the process of HBV-associated HCC development is the immune system (Chisari, 2000; Rehermann, 2003; Rehermann &

Nascimbeni, 2005) and that the CTL-mediated immune response and the removal of infected hepatocytes is as well supposed to contribute to the HBV-induced immunopathogenesis (Chang & Lewin, 2007). It can be speculated that despite the reduced immune proteasome activity there is sufficient immune system-mediated chronic liver inflammation, elimination of infected cells and as a consequence an increase in cell proliferation which could result in accumulation of critical mutations and finally the development of HCC (Chisari, 2000; Chisari *et al.*, 1985; Ferrari *et al.*, 2003; Visvanathan & Lewin, 2006). It will be worthwhile to further investigate the cross-talk between the HBV-induced signaling cascade and the cellular immune response and to analyze in detail the relevance of Nrf2 as therapeutical target for the treatment of HBV infections.

Especially, new strategies for the treatment of HBV-associated HCC are imperative. The most promising routes of cancer chemoprevention might be on the one hand protecting cells against distinct cancerogenic molecules emerged from cellular metabolism or from exogenous sources or on the other hand preventing that endangered cells and cancer cells benefit from cytoprotective mechanisms in order not to facilitate a prosurvival phenotype. During the past years the modulation of reactive species elimination through phase II detoxifying enzymes in cancer chemoprevention has moved more and more in the focus of interest. There are several reports highlighting a protective role for the induction of cytoprotective enzymes in chemoprevention, especially via the Nrf2/ARE signaling cascade (Fahey *et al.*, 2002; Henderson *et al.*, 1998; Iida *et al.*, 2004; Ramos-Gomez *et al.*, 2001) and based on its antioxidant function, Nrf2 has become a potential molecular target for cancer chemoprevention (Jeong *et al.*, 2006). Up to date oltipraz (4-methyl-5-(2-pyrazinyl)-3-dithiolethione) represents one of the most potent inducers of the Nrf2/ARE pathway which has entered into clinical trials. Several studies confirmed an increased expression of cytoprotective enzymes and subsequent positive effects on cancerogenesis in humans after oral dosing (Gupta *et al.*, 1995; O'Dwyer *et al.*, 1996; Sofowora *et al.*, 2001).

However, besides the positive role of Nrf2 in cancer protection, recent reports demonstrate also a “dark” side of Nrf2 in cancer. There are several *in vivo* studies demonstrating that overexpression of Nrf2 resulting in an increased expression of cytoprotective genes can lead to a benefit for cancer cell survival and can cause resistance to chemotherapies (Hayes & McMahon, 2009; Ikeda *et al.*, 2004; Nioi & Nguyen, 2007; Ohta *et al.*, 2008; Singh *et al.*, 2006). Consequently, either induction or attenuation of Nrf2 activity is the key to match the desired effect, to prevent cancerogenesis (Kensler & Wakabayashi, 2009). Regarding the HBV-dependent activation of Nrf2/ARE signaling cascades and its role for hepatocarcinogenesis it can be speculated that from the viral point of view activation of the cellular Nrf2/ARE pathway is supposed to ensure host cell survival and the establishment of the viral infection. Infected hepatocytes could thereby develop cancerogenic properties and

benefit from the increased expression of cytoprotective genes finally resulting in the development of HCC. Hence accurate attenuation of Nrf2 activity in chronic HBV infection might represent a new strategy to prevent HCC development and to combat HCC.

7 Summary

The Hepatitis B virus (HBV) is an enveloped DNA virus that belongs to the family of *Hepadnaviridae*. The 3.2 kb partial double-stranded DNA genome codes for seven viral proteins, amongst others the two regulatory proteins HBx and LHBs. Hepatitis B is an inflammatory liver disease caused by HBV. HBV infection is a severe worldwide health problem and one of the major risk factors for the development of hepatocellular carcinoma (HCC). Although an effective vaccine is available about 400 million people are chronically infected with HBV having a very low cure rate. Interactions between HBV-induced cellular signaling pathways, the immune response and clinical symptoms are still not fully understood and above all additional treatment options to improve the current chronic HBV therapy are imperative.

The transcription factor Nuclear Factor-Erythroid 2-related Factor 2 (Nrf2) plays an important role in cellular defense mechanisms against oxidative stress by triggering the expression of cytoprotective genes as NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx) or glutamate-cysteine synthetase catalytic subunit (GCSc). The expression of these genes depends on a short *cis*-acting sequence in their promoter, the antioxidant response element (ARE) that is recongnized by Nrf2.

In chronic HBV infection persistent inflammation and hepatocyte proliferation to regenerate immune-mediated liver injuries play an important role in the development of HCC (Chemin & Zoulim, 2009; Lupberger & Hildt, 2007). In Nrf2-/- mice an impaired liver regeneration after partial hepatectomy was observed suggesting that Nrf2 plays as well an important role in liver homeostasis (Beyer *et al.*, 2008). Since Nrf2 can be regulated by c-Raf and the HBV regulatory proteins modulate the c-Raf/MAPK signaling cascade (Benn & Schneider, 1995; Kensler *et al.*, 2007; Klein *et al.*, 1999; Shen *et al.*, 2004) a potential interaction between HBV and the Nrf2/ARE signaling pathways can be assumed.

Luciferase reporter assays clearly demonstrated an HBV-induced activation of ARE-regulated promoters which was as well reflected by elevated protein amount of the corresponding genes as demonstrated by Western Blot analysis. Coexpression of a transdominant negative Nrf2 mutant abolished the HBV-induced activation of ARE as shown by luciferase reporter assay and Nothern Blot hybridization confirming the relevance of Nrf2 for the HBV-mediated gene induction. Immune histochemistry stainings of consecutive sections derived from patients with chronic or acute HBV-infection revealed as well an increased protein amount of GCSc in HBV-positive cells in both chronic and acute infection.

In the next set of experiments it was analyzed whether overexpression of ARE-regulated genes affects HBV replication. HBV-replicating cells transfected with expression plasmids

coding for NQO1 or GCSc were therefore analyzed by real time PCR, HBsAg- and HBeAg-ELSIA, Western Blot and immunofluorescence microscopy. All approaches demonstrated that overexpression of both NQO1 and GCSc had no effect on HBV replication. Comparable results were obtained for general activation or inhibition of ARE-regulated genes by coexpression of a constitutive active or a tdn Nrf2 mutant. In addition, quantification of HBsAg- and HBeAg-secretion by ELISA in supernatant of adeno-HBV infected primary mouse hepatocytes isolated from Nrf2-/- and wt C57BL/6 mice displayed as well no difference between Nrf2-/- and wt mice.

Treatment with increasing concentration of the radical scavenger N-acetlycystein (NAC) luciferase reporter assays revealed that radical formation is not relevant for the HBV-induced activation of ARE-dependent genes. By contrast, inhibition of c-Raf either by the small molecule inhibitor Nexavar® or by coexpression of the tdn c-Raf mutant and inhibition of MEK kinase by treatment with PD98059 abrogated the HBV-mediated gene induction.

Since the viral regulatory proteins are known to modulate cellular signaling pathways (Benn & Schneider, 1994; Hildt *et al.*, 2002; Hildt *et al.*, 1996a) the role of HBx and the PreS2 activator LHBs for ARE-regulated gene induction was investigated by means of luciferase reporter gene assay. It was demonstrated that both HBx and LHBs activated ARE reporter gene expression comparable to the full length HBV genome which was abolished by coexpression of the tdn Nrf2.

In the next set of experiments the physiological role of the activation of ARE-regulated genes was characterized. After treatment with H_2O_2 or glucoseoxidase OxyBlot^{TM} analysis of HBV-positive cells showed less oxidative damage of proteins as compared to HBV-negative control cells whereby coexpression of a tdn Nrf2 mutant abrogated the better protection against H_2O_2-induced oxidative damage.

Since the Nrf2/ARE pathway is as well involved in the regulation of the proteasomal subunit β5 (PSMB5) (Kwak & Kensler, 2006) a potential impact of HBV-replication on PSMB5 expression and proteasome activity was investigated. Luciferase reporter assay showed a significant induction of PSMB5-Luc reporter constructs in HBV-positive cells which was impeded by coexpression of a tdn mutant of Nrf2. Furthermore Western Blot analysis of purified proteasomes isolated from HBV-positive and HBV-negative cell lines clearly showed increased protein amounts of PSMB5 in HBV-replicating cells as compared to control cells. Increased gene expression and protein amount was as well reflected by an increased constitutive proteasome activity in purified isolated proteasomes of HBV-positive cells. Coexpression of the tdn Nrf2 mutant significantly reduced the HBV-induced activation. In the immune proteasome PSMB5 is replaced by the IFN-inducible and reversely regulated subunit PSMB5i (Jung *et al.*, 2009). Immune proteasome activity assays with purified isolated proteasomes showed a decreased activity in HBV-replicating cells. Interestingly,

coexpression of the tdn Nrf2 mutant resulted in an increased immune proteasome activity in HBV-producing cells.

Taken together, the present work characterized the HBV-dependent modulation of Nrf2. HBV induces a strong activation of ARE-regulated genes *in vitro* and *in vivo* whereby the functionality of Nrf2 is required. Activation of ARE-dependent target genes by HBV is triggered via the c-Raf/MEK signaling pathway whereas radical formation is not relevant for the HBV-dependent induction of ARE/Nrf2-regulated genes. The HBV regulatory proteins HBx and PreS2 are able to induce expression of ARE-dependent target genes comparable to full length HBV genome. The Nrf2/ARE-mediated activation of cytoprotective gene expression leads to a better protection against oxidative damage in HBV-replicating cells as compared to control cells. In addition, HBV-positive cells show a significantly increased expression of PSMB5 which is accompanied by a decreased level of PSMB5i. In accordance to this HBV-producing cells present an increased constitutive proteasome activity and a decreased immune proteasome activity as compared to control cells.

In conclusion these data suggest that HBV triggers the expression of Nrf2/ARE-regulated genes to ensure the survival of the infected cell and thereby promotes the establishment of the viral infection. Consequently, these observations could be relevant for future therapy concepts for chronic HBV infection and HBV-associated HCC.

8 Zusammenfassung

Das Hepatitis B Virus (HBV) ist ein behülltes DNA Virus, das in die Familie der *Hepadnaviridae* eingruppiert ist. Das 3.2 kb große, partiell doppelsträngige DNA Genom codiert für sieben virale Proteine, unter anderem die zwei regulatorischen Proteine HBx und LHBs. Die Hepatitis B ist eine durch HBV verursachte Infektionskrankheit der Leber, die ein schwerwiegendes, weltweites Gesundheitsproblem darstellt und eine der Hauptursachen ist für die Entwicklung eines hepatozellulären Karzinoms (HCC). Obwohl eine effektive Impfung vorhanden ist, sind ungefähr 400 Millionen Menschen Träger einer chronischen HBV-Infektion, welche nur über eine geringe Heilungschance verfügt. Interaktionen zwischen den HBV-induzierten zellulären Signalkaskaden, der Immunantwort und den klinischen Symptomen sind bis heute noch nicht vollständig aufgeklärt und machen zusätzliche Behandlungsmöglichkeiten zwingend notwendig, um die gegenwärtige Therapie der chronischen HBV-Infektion zu verbessern.
Der Transkriptionsfaktor Nuclear Factor-Erythroid 2-related Factor 2 (Nrf2) spielt eine wichtige Rolle in den zellulären Abwehrmechanismen gegen oxidativen Stress, indem er die Expression von zytoprotektiven Genen wie NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx) oder glutamate-cysteine synthetase catalytic subunit (GCSc) veranlasst. Die Expression dieser Gene ist abhängig von einer cis-agierenden Sequenz in ihrem Promoter, dem antioxidative response element (ARE), welches durch Nrf2 erkannt wird.

In der chronischen HBV-Infektion spielt die permanente Entzündung und Hepatozytenproliferation, welche die immuninduzierten Leberschäden regeneriert, eine bedeutende Rolle in der Entwicklung eines HCC (Chemin & Zoulim, 2009; Lupberger & Hildt, 2007). In Nrf2-/- Mäusen konnte nach partieller Hepatektomie eine beeinträchtigte Regeneration der Leber beobachtet werden, was darauf hindeutet, dass Nrf2 auch eine wichtige Rolle in der Leberhomöostase spielt (Beyer *et al.*, 2008). Da Nrf2 durch c-Raf reguliert werden kann und die regulatorischen Proteine von HBV die c-Raf/MAPK Signalkaskade modulieren, kann eine potenzielle Interaktion zwischen HBV und dem Nrf2/ARE Signalweg angenommen werden.
Luziferase Reportergen Experimente zeigten deutlich eine HBV-induzierte Aktivierung der ARE-regulierten Promotoren, was anhand von Western Blot Analysen durch eine erhöhte Proteinmenge der entsprechenden Gene ebenfalls bestätigt wurde. Luziferase Reportergen Experimente und Northern Blot Hybridisierung demonstrierten drüber hinaus, dass durch Coexpression von einer transdominant negativen (tdn) Nrf2 Mutante diese Aktivierung aufgehoben wurde, was die Bedeutung von Nrf2 für die HBV-induzierte Aktivierung von

ARE-regulierten Genen bestätigt. Immunhistochemische Färbungen konsekutiver Leberschnitte von Patienten mit chronischer und akuter HBV-Infektion zeigten ebenfalls eine erhöhte Proteinmenge an GCSc in HBV-positiven Zellen in sowohl chronischer, als auch akuter Infektion.
In den darauffolgenden Experimenten wurde untersucht, ob eine Überproduktion von ARE-regulierten Genen die HBV-Replikation beeinflusst. HBV-replizierende Zellen wurden mit Expressionsplasmiden für NQO1 oder GCSc transfiziert und mittels real time PCR, HBsAg- und HBeAg-ELISA, Western Blot und Immunfluoreszenz Mikroskopie analysiert. Alle Methoden zeigten, dass weder eine Überproduktion von NQO1, noch von GCSc einen Einfluss auf die HBV-Replikation hatten. Vergleichbare Ergebnisse wurden für die allgemeinen Aktivierung oder Inhibierung von ARE-abhängigen Genen durch die Coexpression einer konstitutiv aktiven oder einer tdn Nrf2 Mutante erreicht. Zusätzlich zeigten HBsAg- und HBeAg-ELISA mit zellulären Überständen von adeno-HBV-infizierten primären Maushepatozyten, welche aus Nrf2-/- und wt C57BL/6 Mäusen isoliert wurden, keine Unterschiede zwischen Nrf2-/- und wt Mäusen.
Behandlungen mit steigenden Konzentrationen des Radikalfängers N-Acetylcystein (NAC) zeigten in Luziferase Reportergen Experimenten, dass die Radikalbildung für die HBV-abhängige Geninduktion keine Rolle spielt. Im Gegensatz dazu, wurde durch Inhibition von c-Raf durch Nexavar® oder durch Coexpression einer tdn c-Raf Mutante und nach Inhibierung von MEK mit PD98059 die HBV-vermittelte ARE-Induktion aufgehoben.
Da die viralen regulatorischen Proteine zelluläre Signalkaskaden modulieren können (Benn & Schneider, 1994; Hildt *et al.*, 2002; Hildt *et al.*, 1996a), wurde die Rolle von HBx und dem PreS2 Aktivator LHBs für die ARE-regulierte Geninduktion anhand von Luziferase Reportergen Experimenten charakterisiert. Es konnte gezeigt werden, dass sowohl HBx, als auch LHBs die Reportergene vergleichbar zum kompletten HBV-Genom aktivieren konnten, wobei diese Aktivierung durch Coexpression der tdn Nrf2 Mutante wieder aufgehoben wurde.

Im weiteren Verlauf dieser Arbeit wurde die physiologische Bedeutung der Aktivierung von ARE-regulierten Genen untersucht. Nach Behandlung mit H_2O_2 oder Glukoseoxidase zeigte die Analyse von HBV-positiven Zellen durch OxyBlot™ einen geringeren oxidativen Schaden an Proteinen im Vergleich zu HBV-negativen Zellen, wobei der bessere Schutz gegen H_2O_2-induzierte oxidative Schäden durch Coexpression der tdn Mutante wieder aufgehoben wurde. Da der Nrf2/ARE Signalweg auch in der Regulation der Proteasomuntereinheit β5 (PSMB5) involviert ist (Kwak & Kensler, 2006), wurde ein möglicher Einfluss der HBV-Replikation auf die PSMB5 Expression untersucht. Reportergen Experimente zeigten eine signifikante Induktion der PSMB5-Luziferase Konstrukte in HBV-positiven Zellen, welche durch die Coexpression der tdn Nrf2 Mutante wieder rückgängig gemacht wurde. Außerdem

demonstrierten Western Blot Analysen von gereinigten Proteasomen eine deutlich größere Proteinmenge an PSMB5 in HBV-positiven Zellen, als in HBV-negativen Kontrollzellen. Die erhöhte Genexpression und Proteinmenge wurde auch durch eine erhöhte Aktivität des konstitutiven Proteasoms in HBV-replizierenden Zellen widergespiegelt, die wiederum durch Coexpression der tdn Nrf2 Mutante aufgehoben wurde. Im Immunproteasom wird PSMB5 durch das IFN-induzierbare, gegensätzlich regulierte PSMB5i ersetzt (Jung *et al.*, 2009). Immunproteasom-Aktivitätstests mit isolierten Proteasomen zeigten eine verminderte Aktivität in HBV-replizierenden Zellen. Interessanterweise resultierte hier die Coexpression der tdn Nrf2 Mutante in einer erhöhten Immunproteasom-Aktivität in HBV-produzierenden Zellen.

Zusammenfassend wurde in der vorliegenden Arbeit die HBV-abhängige Modulation von Nrf2 charakterisiert. HBV induziert eine starke Aktivierung von ARE-regulierten Genen *in vitro* und *in vivo*, wobei die Funktionalität von Nrf2 erforderlich ist. Die Aktivierung von ARE-abhängigen Zielgenen durch HBV wird über die viralen regulatorischen Proteine LHBs und HBx vermittelt und durch den c-Raf/MEK Signalweg eingeleitet, wobei Radikalbildung für die Induktion der ARE-abhängigen Gene keine Rolle spielt. Die Nrf2/ARE-vermittelte Aktivierung von zytoprotektiven Genen führt im Vergleich zu HBV-negativen Kontrollzellen zu einem besseren Schutz gegen oxidativen Schaden in HBV-replizierenden Zellen. Des Weiteren zeigen HBV-positive Zellen eine signifikant erhöhte PSMB5 Expression, die einhergeht mit einem reduzierten Level an PSMB5i. In Übereinstimmung hiermit präsentieren HBV-produzierende Zellen im Vergleich zu HBV-negativen Kontrollzellen eine erhöhte konstitutive Proteasomaktivität und eine verminderte Aktivität des Immunproteasoms.

Die vorliegenden Ergebnisse weisen abschliessend darauf hin, dass HBV die Expression der Nrf2/ARE-regulierten Gene steuert, um das Überleben der infizierten Zelle zu sichern und fördert dadurch die Etablierung der viralen Infektion. Folglich könnten diese Beobachtungen für zukünftige Therapiekonzepte für die chronische HBV-Infektion und für das HBV-assozierte HCC von Bedeutung sein.

9 Abbreviations

AP-1	Activating protein 1
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BAY43-9006	Raf-kinase inhibitor II (Bayer)
Bcl-2	B-cell lymphoma-2 protein
BSA	Bovine serum albumin
C	Viral core protein
ca	constitutive active
cDNA	Complementary DNA
cccDNA	Covalently closed circular DNA
CTR	C-terminal region
DGR	Double glycine repeat
DHBV	Duck hepatitis B virus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DMEM	Dulbecco's Modified Eagle edium
DTT	1,4-Dithiothreitol
E.coli	*Escherischia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmatic reticulum
ERK	Extracellular-signal-regulated kinase
FCS	Fetal calf serum
GCSc	Glutamate-cysteine synthetase catalytic subunit
GPx1	Glutathione peroxidase 1
GST	Glutathione-S-transferase
HBcAg	HBV core antigen
HBeAg	HBV e antigen
HBs	HBV surface protein
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HBx	HBV x protein
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HepAD38	Human hepatoma cell line stable transfected with 1.2 fold HBV genome
HepG2	Human hepatoma cell line
HepG2.2.15	Human hepatoma cell line stable transfected with 1.2 fold HBV genome
HepG2-4A5	Human hepatoma cell line stable transfected with 1.2 fold HBV genome
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HuH-7.5	Human hepatoma cell line
IFN	Interferon
IgG	Immunoglobulin G
IVR	Intervening region
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LHBs	Large HBV surface protein
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MG132	Proteasome inhibitor
MHC-I	Major histocompatibility complex class I
MHBs	Middle HBV surface protein
NAC	N-acetylcysteine
NF-κB	Nuclear factor-κB
NP-40	Nonidet P-40
NQO1	NAD(P)H:quinine oxidoreductase 1
Nrf2	nuclear factor-E2 related factor 2
NTR	N-terminal region
ORF	Open reading frame
P	Vial polymerase
PAGE	Polyacrylamide gel electrophoresis
pgRNA	Viral pregenomic RNA
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD98059	MEK inhibitor
PD-1	Programmed death receptor
PD-1L	PD-1 ligand
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PSMB5	Proteasomal subunit β5
rcDNA	Relaxed circular DNA
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
mRNA	messenger RNA
RSV	Respiratory syncytial virus

RT	Reverse transcription
SB203580	p38 MAPK inhibitor
SHBs	small HBV surface protein
SDS	Sodium dodecyl sulfate
SVPs	Subviral paricles
tBHQ	tert-butylhydroquinone
tdn	Transdominant negative
TLM	Translocation motif
TNF	Tumor necrosis factor
Tris	Tris(hydroxyamino)methane
U0126	MEK inhibitor
wt	Wild type
XRE	Xenobiotic response element
YFP	Yellow fluorescence protein

10 References

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