X: On the molecular biology of the hepatitis B virus X protein
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Chapter 1

General introduction
A brief history of infectious hepatitis

Infectious hepatitis was already known by Hippocrates, who described "epidemic jaundice" around 400 B.C. The first attempt to combat infectious hepatitis was made around the 8th century A.D., when Pope Zacharias ordered to quarantine individuals with jaundice to prevent its spread throughout Rome. The greatest breakthrough in our knowledge of infectious hepatitis came in 1963, when Blumberg first described the "Australian antigen" (AuAg), a protein in serum collected from an aboriginal that precipitated in an Ouchterlony reaction with serum from a haemophiliac patient. Not long after, in 1968, it was confirmed by two independent groups that the AuAg was specifically found in patients with "serum hepatitis".

These discoveries led to the first antiviral tests to prevent a transfusion related viral infection, initially by using the Ouchterlony reaction, soon followed by other serological tests. Amongst others, the availability of these tests led to a great reduction in transfusion-associated hepatitis, from 30% to 10% of multiply transfused patients during the 1970s. The discovery of AuAg, which was later characterized as the hepatitis B virus surface antigen (HBsAg), led to the development of a human serum-derived HBV vaccine. This vaccine was approved by the FDA in 1982 and effectively included in national vaccination programs in the US and other countries in the early 1980s. People infected with HBV have a cumulative risk of about 25% of developing liver cancer during their lifetime. It has been estimated that the number of HBV infections averted by serological testing of blood products and vaccination have caused the greatest reduction in the prevalence of a human cancer in history. In 1976, Blumberg received the Nobel Prize in Physiology or Medicine for his work on HBV.

Prevalence of hepatitis B virus infection

In adults, acute HBV infection is mostly asymptomatic and generally cleared by an adaptive immune response within a year without therapeutic intervention. In a small subset of adults infected with HBV, about 10%, the adaptive immune response fails to eradicate the virus and the infection becomes chronic. Conversely, in children, the majority of HBV infections become chronic. Worldwide, an estimated 240.000.000 people are chronically infected with HBV (Fact sheet N°204, WHO). Most of these chronically infected people were infected as children, and have been infected for decades. Each year an estimated 780.000 people die as a consequence of HBV infection (Fact sheet N°204, WHO); the majority of these deaths are due to long-term, chronic HBV infections. Worldwide, HBV prevalence is highest in Southeast Asia, Sub-Saharan Africa and South America, where chronic HBV prevalence is higher than 8% (Figure 1).
A brief history of infectious hepatitis

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**Figure 1. Worldwide prevalence of HBV infections and distribution of the major HBV genotypes.** Chronic HBV rates: High: ≥8%, Moderate: 2-7%, Low: <2%. Permission obtained from Elsevier Ltd © The Lancet, 384, 2053–2063.

**HBV in the Netherlands**

The first cases of “serum hepatitis” were described in Bremen (Germany) in 1885, in workers that had received a smallpox vaccination containing human lymph fluid. In the Netherlands, blood donations have been serologically tested for the presence of the HBV surface antigen (HBsAg) since 1973. Since 2008, donations are also tested for the presence of HBV DNA. In the Netherlands, HBV prevalence is low and mainly restricted to risk groups such as immigrants from highly endemic areas, men having sex with men, and intravenous drug users. Because of the low and restricted HBV endemicity in the Netherlands, the HBV vaccine was initially not included in the universal childhood vaccination programme. Instead, in 2002, a vaccination program was introduced directed at risk groups such as commercial sex workers, intravenous drug users and men having sex with men. In 2011, based on a cost-effectiveness analysis, universal childhood vaccination was introduced in addition. The vaccination of risk groups, in combination with the introduction of HBV screening of blood donations has reduced HBV prevalence to a nadir of 0.8 per 100,000 persons in 2013. To further increase the safety of blood donations, standard HBV testing was extended with testing for antibodies against the HBV core protein in 2011, primarily to detect so-called “occult” HBV infections, in which HBsAg, and often also HBV DNA, are undetectable.

**The hepatitis B virus**

The hepatitis B virus is the archetype of the “hepadna” (for hepatotropic DNA viruses) family of viruses. Endogenised sequences of HBV ancestors (eHBVs) have been identified in zebra finches, snakes, crocodiles and turtles. The oldest eHBVs endogenised >207 million years ago, indicating that the hepadnaviruses have been around at least since the Early Mesozoic years ago, pointing to an origin in an ancient avian ancestor. Several orthohepadnaviruses are known that replicate in a specific mammalian host and only infect species related to their natural hosts; in the case of HBV the virus covalently closed circular DNA (cccDNA). This relaxed coiled DNA (rcDNA) is repaired by host topoisomerase enzymes. The cccDNA is wrapped around histones and thus forms a chromatin template that is generally considered the genetic status of the cccDNA determines the replicative capacity and viral persistence. Virus particles and the much more prevalent HBV multimers in the plasma of HBV infected patients not express an X protein, which is required for the replication of all known orthohepadnaviruses. A remarkable feature of both orthohepadnaviruses and avihepadnaviruses is that the latter species do not express an X protein, which is required for the replication of all known orthohepadnaviruses. Recent studies have shown that the X protein of HBV is not strictly necessary for viral replication, but plays a role in the regulation of the transcription of the main viral ORFs.

The HBV replication cycle is summarised in the flowchart. From the pgRNA, the HBV Core- and polymerase (Pol) proteins are translated. The pgRNA 12

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The hepatitis B virus

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years ago, indicating that the hepadnaviruses have been around at least since the Early Mesozoic. Hepadna viruses have a narrow host range and can only infect species related to their natural hosts; in the case of HBV the virus can only replicate in humans and nonhuman primates. Hepadna viruses that infect mammalian hosts are called orthohepadnaviruses. Several orthohepadnaviruses are known that replicate in a specific mammalian host. These viruses are called after their respective hosts, e.g. the Woodchuck Hepatitis Virus (WHV) and the Beechy Ground Squirrel Hepatitis Virus (GSHV). Recently, an orthohepadna virus species was discovered in bats. A phylogenetic distinct genus of hepadna viruses, the avihepadnaviruses, infects avian hosts. A remarkable difference between orthohepadnaviruses and avihepadnaviruses is that the latter species does not express an X protein, which is required for the replication of all known orthohepadnaviruses. In humans, 10 different putative HBV genotypes (A-J) with more than 8% difference in their genome sequence have been identified. Also various recombinants and subgenotypes (4-8% nucleotide difference) have been observed. The prevalence of these genotypes differs worldwide; while the majority of HBV infections in Asia are of either genotype B or C, and the majority of infections in Europe and the middle East are of the A and D genotype (Figure 1). The clinical characteristics of natural infections with different HBV genotypes differ, for instance infections with genotype C more commonly cause liver cirrhosis and HCC.

The HBV replication cycle

The hepatitis B virus consists of an enveloped core particle that contains a partially double stranded DNA genome. This 3,200 base pair genome is the smallest known genome of a virus that infects humans and consists of four largely overlapping open reading frames (ORFs); the Core, Polymerase, Surface and X ORF, respectively (Figure 2). HBV DNA containing virus particles are 42 nm in diameter and can be distinguished by electron microscopy. Complete HBV virions are also called Dane particles, after D.S. Dane, who first described such particles and made a distinction between these particles and the much more prevalent HBsAg multimers in the plasma of HBV infected patients. HBV infection is initiated by binding of HBsAg to heparan sulfate proteoglycans on a hepatocyte. Subsequently, HBV enters the cell via an incompletely understood process that involves interaction between HBsAg and the sodium taurocholate cotransporting polypeptide (NTCP). After release in the cytoplasm, the core particle is actively transported to the nuclear pore, where the core particle is degraded and the partially double stranded DNA genome is released into the nucleus. This relaxed coiled DNA (rcDNA) is repaired by host enzymes to generate fully covalently closed circular DNA (cccDNA). The cccDNA is wrapped around histones and thus forms a chromatin template that is generally considered to act as a “minichromosome”, and that is subject to epigenetic modification. The epigenetic status of the cccDNA determines the rate of HBV RNA transcription. The cccDNA contains four promoters and two enhancers, which regulate the transcription of the main viral RNAs. The HBV core promoter regulates the transcription of the longest two viral RNA, the preCore RNA and the slightly shorter pregenomic RNA (pgRNA). From the PreCore RNA, the e antigen (HBeAg) is translated. From the pgRNA, the HBV Core- and polymerase (Pol) proteins are translated. The pgRNA
also serves as the template for viral DNA synthesis (reviewed in \textsuperscript{37}). Upon Pol translation, the newly synthesized protein interacts, preferably in cis, with a specific region in the pgRNA called the epsilon (ε, from encapsidation) loop \textsuperscript{38,39}. The interaction between Pol and the ε loop triggers a conformational change in Pol which initiates the encapsidation of the Pol-pgRNA complex in a new core particle \textsuperscript{38,39}. Concomitantly, the presence of the ε loop is required and sufficient to initiate the encapsidation of heterologous RNA \textsuperscript{38,40}. The ε loop adopts an extensive tertiary structure called stem-loop, and this structure is required for encapsidation \textsuperscript{41}.

Reverse transcription is initiated by interaction between HBV polymerase and a bulged region of ε, which results in the generation of a short oligonucleotide covalently linked to a tyrosine residue in Pol\textsuperscript{42}. This short oligonucleotide translocates to a reverse complementary region at the 3’end of the pgRNA called direct repeat 1 (DR1) and serves as a primer for the initiation of reverse transcription. After reverse transcription, Pol translocates to the 3’end of the newly synthesized negative strand and partially synthesizes the positive strand to form the rcDNA found in mature HBV particles. Reverse transcription is exclusively initiated and completed after encapsidation of the pgRNA/Pol complex and requires several cellular chaperones\textsuperscript{43}. The ε terminus of the core protein interacts with nucleic acid, and disruption of this domain critically interferes with reverse transcription without affecting encapsidation\textsuperscript{44}. Vice versa, the nucleic acid within the core particle also affects core particle structure. Core particles containing pgRNA or incompletely reverse transcribed rcDNA are called immature core particles and are phosphorylated on several residues\textsuperscript{45}. Only after reverse transcription is completed, the core particles “mature” and are dephosphorylated\textsuperscript{45,46}. Subsequently, these mature core particles can either shuttle back to the nucleus to increase the pool of cccDNA, or they are enveloped and excreted as new virions. Several factors determine which fate the rcDNA containing core particles undergo. It has been observed that cccDNA accumulation is negatively regulated by the amount of viral surface proteins available, suggesting that only when sufficient amounts of envelop proteins are produced, HBV DNA containing core particles are enveloped and excreted as new virions\textsuperscript{47}. As a result of cccDNA accumulation, the amount of cccDNA copies in the infected hepatocyte ranges from 5-50 copies/cell\textsuperscript{48}. Intranuclear cccDNA accumulation contributes to the preservation of cccDNA during cell division\textsuperscript{49}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Organisation of the HBV genome. (Permission obtained from MDPI © Minor, M. M. and Slagle, B. L. Viruses, 6, 4683-4702 (2014)\textsuperscript{50}).}
\end{figure}
HBV proteins

The HBV genome is extensively used. Beside the high degree of overlap of the four viral ORFs, functionally different proteins are produced from different start codons in the structural surface- and core genes. The different variants of the surface proteins are all structural proteins that are embedded in the viral envelope. Translation of the core ORF from the precore start codon generates an immune regulatory protein called the e antigen. Beside its structural genes, HBV encodes a polymerase to reverse transcribe its genomic material, and an accessory protein called X (Figure 2).

Core

The core protein is transcribed from the pregenomic RNA. Dimers of the core protein accumulate in the cytoplasm and the nucleus of the HBV infected hepatocyte. The archetypical function of the core protein is the formation of the core particle that contains the rcDNA. Such core particles can have two sizes, which contain either 240 or 180 core proteins, respectively. In the nucleus, core protein dimers interact with the cccDNA and facilitate transcription.

HBeAg

The HBV e antigen is translated from the preCore mRNA. The HBeAg ORF is completely in frame with the HBV core protein ORF, but its transcription is initiated 63 bases upstream of the core protein start codon. The n-terminal 21 amino acids (aa) of the resulting HBeAg precursor serve as a signalling peptide that direct it to the ER, where the n-terminal 11 aa are cleaved of. The HBeAg precursor is transported to the golgi apparatus, where the c-terminal 44 aa are removed to form HBeAg, which is subsequently excreted. Because of different folding, the tertiary structure of the HBeAg differs significantly from the HBV core antigen. HBeAg production correlates with high levels of virus production during the first phases of the infection. When a sufficiently strong adaptive immune response does develop, the viral population is replaced by HBV mutants that do not produce HBeAg. The most commonly observed mutation that abolishes HBeAg expression is the G1896A mutation, which results in a premature stop codon in the HBeAg ORF. As G1896 is part of the stem loop region of e, the mutation is often compensated by the C1858T mutation, which restores base pairing in e. This and other mutations that cause premature stop codons in the HBeAg ORF are concomitantly called precore (PC) mutations. PC mutations are always observed in the first 63 bases the PreCore mRNA, which are not translated from the pregenomic RNA and therefore PC mutations do not affect HBV core production. Beside PC mutations, nucleotide substitutions are also commonly observed in the adjacent basal core promoter (BCP) region in HBeAg negative HBV infections. Although the BCP is a part of the core promoter, BCP mutations occurring in vivo interfere with HBeAg expression but do not negatively affect HBV core protein production.
Polymerase

The HBV polymerase is the enzyme that converts the HBV pregenomic RNA into partially double stranded rcDNA. The HBV Pol protein is translated from the pregenomic RNA. Pol translation is initiated from a start codon downstream from the core protein start codon, in a cap independent manner$^{58}$, probably by translation reinitiation$^{59}$. The Pol protein has three functional domains; a reverse transcription (RT) domain that reverse transcribes the HBV RNA into DNA, an RNase H domain that degrades the viral RNA after it has been reverse transcribed, and an N-terminal terminal protein (TP) domain that is involved in Pol-mediated protein priming activity. In between the TP and RT domains Pol contains a spacer region of about 150 nucleotides that does not appear to be involved in Pol activity and can be deleted without functional consequence$^{60}$. This region in Pol completely overlaps with the critical PreS region of the surface ORF. Thus, the tolerance of Pol to mutations in this region partially relieves the constraint overlapping open reading frames put on viral evolution$^{61}$. The 5’ end of the Pol ORF overlaps with the 3’ end of the core ORF.

The HBV surface proteins

The HBV envelope contains three variants of the HBV surface protein (HBsAg), which are translated from two different viral RNAs that are driven by the S1 and S2 promoters, respectively. The HBsAg ORFs completely overlap with the Pol ORF. Based on their size, the different HBsAg proteins are named the small (S), middle (M) and large (L) HBsAg. The large HBsAg (LHBs) contains the PreS1 domain (108 or 119 aa, depending on genotype), the PreS2 domain (55 aa) and the S domain (226 aa). The S2 promoter regulates the transcription of the RNA that encodes the middle- (MHBs) and small (SHBs) surface proteins, which consist of the PreS2 and S domains, and the S domain only, respectively. Viral entry is initiated by an interaction between two positively charged residues in the antigenic loop (AGL, also called “a” determinant or antigenic domain) of the S protein with heparan sulfate proteoglycans (HSPGs) on a hepatocyte$^{62-64}$. Next, the first 48 amino acids of the PreS1 domain, which are present in the LHBs only, interact with the sodium taurocholate cotransporting polypeptide (NTCP)$^{62}$. This interaction is essential for viral entry, albeit the subsequent steps by which NTCP interaction leads to membrane fusion are largely elusive.

The majority of HBsAg produced in the infected hepatocyte is excreted as subviral particles (SVPs); multimers composed of all three HBsAg variants that do not contain viral DNA. These multimers form spheres of 25 nm in diameter and filaments (also called rods) of 22 nm and a variable length. These SVPs are excreted in a 1000- to 100,000 fold excess relative to infectious Dane particles$^{65}$.

HBV splice-generated protein (HBSP)

Several spliced HBV RNAs have been described$^{66,67}$. At least one protein called HBV splice-generated protein (HBSP), which consists of a part of the HBV Pol protein and a new ORF
generated by the splicing event, is translated from spliced RNA during natural infection.\textsuperscript{66,68,69} HBV spliced RNA translation products seem to be involved in evasion of innate immune responses and may contribute to the carcinogenic effect of HBV infection.\textsuperscript{70,72}

**Protein X**

The hepatitis B virus protein X (HBx) is the only accessory protein in the HBV genome. HBx is the smallest HBV protein, and is translated from an mRNA that is driven by the HBV X promoter. HBx-like proteins are highly conserved amongst all known orthohepadna viruses, and their expression is a perquisite for the replication of these viruses.\textsuperscript{73-76} HBx has a size of \textasciitilde17 kDa and does not have significant homology to other known proteins. No crystal structure of HBx is available. It has been suggested that HBx is highly unstructured and only obtains secondary structure under specific conditions.\textsuperscript{77}

HBx can transactivate RNA transcription and is critically involved in initiating and maintaining RNA transcription from the HBV cccDNA.\textsuperscript{36,74,78-85} HBx interacts with various cellular components, and thereby affects cellular functions and processes (Figure 4). It is unclear how the cellular processes and functions modulated by HBx and the transcriptionally transactivated state of the HBV cccDNA are related to each other.

**HBV pathogenesis**

The early phases of chronic HBV infection are asymptomatic, but over decades the infection may lead to liver cirrhosis and the development of hepatocellular carcinoma (HCC). In the natural history of chronic HBV infection, four main stages can be distinguished. These stages can be distinguished by assessing HBV DNA, alanine transaminase (ALT, a measure for liver damage), and the presence of HBeAg. Patients can move from one stage to another in any order, and not all stages occur in all patients (Figure 3).\textsuperscript{86} At a low rate, (<1%/year) patients lose HBV chronicity and clear all markers of viral replication. Patients may also progress into a so called “occult” infection, in which low levels of HBV DNA are present in the serum and liver, but no HBsAg is detectable.

**Stage 1: The immune tolerant stage**

After infection with HBV has occurred, the development of an immune response is often delayed. These infections are called immune tolerant. HBV replication is not cytopathic, and although the viral load is high during this stage, no liver damage is evident. During this phase HBeAg is always detectable in the serum. Immune tolerant hepatitis B is mainly observed in children infected perinatally with HBV, in which case it usually lasts decades.\textsuperscript{87} This stage also occurs in persons infected at later ages, but is generally of much shorter duration.\textsuperscript{88}
Stage 2: The immune clearance stage

During the “immune clearance” stage, adaptive immune responses develop, which suppress viral replication. This phase differs from the immune tolerant phase by elevated and fluctuating ALT levels, as a result of the killing of infected hepatocytes by cytotoxic CD8+ T cells. In chronic HBV infection, the immune responses during the immune clearance stage are insufficiently strong to eradicate the virus\(^9,90\). As a result, multiple “rounds” of immune activity occur, which are accompanied by strong, temporal ALT elevations. Such temporal elevations in ALT are called “flares”. During the immune clearance stage, the HBeAg levels fluctuate and eventually HBeAg disappears from the blood, concomitant with the appearance of anti-HBeAg antibodies\(^9\). The duration of the immune clearance stage, and the age at which HBeAg seroconversion occurs, strongly influence the development of cirrhosis and HCC.

Stage 3: The inactive stage

Although the adaptive immune responses rarely clear all markers of viral replication in chronic HBV infection, they can suppress viral replication to a great extent. The infection is considered to be effectively suppressed when HBeAg has disappeared, anti-HBeAg antibodies are detectable, ALT has normalised, and the viral DNA load has stably dropped. The chance of developing chronic HBV infection-related symptoms is low during this phase, and generally no histological signs of liver damage are evident.

Stage 4: The immune escape stage

Inactive HBV infections reactivate in about 30% of patients. Reactivation can be caused by reduced immunological control or by viral escape mutations that prevent recognition by the adaptive immune system. Reactivation of HBV replication results in an increase in HBV DNA. HBV reactivation can be distinguished serologically from flares during the immune clearance stage by the absence of HBeAg. Usually reactivation is followed by an increase in hepatocyte killing by CD8+ T cells. As a result, both HBV DNA and ALT fluctuate during this phase and, on average, are increased as compared to the inactive phase.

Occult HBV

Adaptive immune responses can also induce “occult” HBV infection. Possibly, occult HBV infection is a stage occurring just before complete clearance of HBV infection. Occult HBV infection is defined by the chronic or intermitted presence of HBV DNA in the blood in the absence of HBsAg. The molecular biology of these occult infections differs. While in some cases of occult HBV infection HBsAg is not produced due to multiple mutations in the HBsAg gene itself\(^91,92\), occult infections can also occur without any HBsAg mutations. HBV genomes cloned from hepatocytes from patients with occult HBV infections in the absence of
HBsAg mutations are completely replication competent in vitro, suggesting that immunological control can induce this state by regulating viral transcription rather than selecting HBV mutants. In line with this observation, when the host immune system is suppressed, occult HBV infections can become overt again. Blood donations from occult HBV donors may cause an HBV infection, largely depending on the recipients’ anti-HBsAg levels.

**Figure 3. The four stages of a HBV infection.** The typical time course of a perinatally acquired chronic HBV infection. Not all stages occur in all patients, and the age at which stages occur and their duration vary. Permission obtained from Elsevier Ltd © Lok, A. S. Gastroenterology 132, 1586–1594 (2007).

**Hepatocellular carcinoma**

Hepatocellular carcinoma (HCC) is an aggressive tumour with a poor prognosis, and is the third leading cause of cancer-related death worldwide (Reviewed in). Infection with HBV increases the risk of developing hepatocellular carcinoma (HCC) by about a hundredfold to a cumulative lifetime risk of 25%. The relative risk of developing HCC is increased during all stages of HBV infection. Also occultly infected patients are still at increased risk of developing HCC. In 2002 it was estimated that 60% of all liver cancers in developing countries and 23% of liver cancers in developed countries were directly attributable to HBV infection. Despite the strong correlation between HBV infection and HCC occurrence, no single causative principle has been defined. Instead, several aspects of CHB are independent risk factors for developing HCC. In principle, HBV replication is not cytopathic, and in chronic HBV infections most liver damage is caused by adaptive immune responses during the immune active stages of the infection. The ongoing inflammation during these stages leads to cirrhosis, which is an important risk factor for developing HCC. The killing of HBV infected hepatocytes also causes a large increase in hepatocyte turnover and clonal expansion of single hepatocytes to populations of thousands of clones are observed in the liver in chronic HBV infections. Within such clonal expansions HCC can develop by malignant transformation of hepatocytes. In about 90% of HBV-related HCC cases, HBV DNA is integrated in the host DNA, strongly suggesting that transformation occurs in hepatocytes previously infected with HBV. HBV DNA integration occurs in all
chromosomes, and may affect the activity or function of genes in which the integration occurs. However, no specific cellular genes are consistently affected in HCC and clonal expansion does not correlate with specific integration sites, indicating that disruption of host genes is not a major cause of HBV-related HCC. Integrated HBV DNA is often rearranged. Functional HBx, transactivation competent 3’truncated variants of HBx, and truncated HBsAg variants are frequently expressed from integrated HBV DNA in HCC, suggesting that the expression of these proteins contributes to malignant transformation. Both virus- and host factors contribute to the risk of developing HCC in CHB. For instance infection with genotype C, male gender, higher age, higher viral load, core promoter mutations, specific surface- and X gene mutations, and cirrhosis, are all independent risk factors for developing HBV-related HCC.

**Immune responses against HBV**

The first line of defence against viral infection is the innate immune system. An innate immune response to a viral infection is mounted by a cell when pathogen associated molecular patterns (PAMPs) are recognised by cellular pattern recognition receptors (PRRs), such as the family of toll-like receptors (TLRs), the retinoic acid-inducible gene I-like receptors (RLRs) and the nucleotide oligomerization domain-like receptors (NLRs) (reviewed in). Upon activation by a PAMP, PRRs induce the production of interferon, which locally activates the interferon response by autocrine and paracrine interferon receptor stimulation. Interferon receptor activation induces the production of both directly acting antiviral- and immune regulatory cytokines. Because of the central role of interferon in these responses, these antiviral- and immune regulatory proteins are commonly referred to as interferon stimulated genes (ISGs).

The early stages of acute HBV infections are characterised by a lack of innate immune responses in the infected hepatocyte. HBV may partially avoid TLR activation, for instance by retaining its DNA either shielded off in a core particle or in the nucleus, where it is not detected. HBV replication also interferes with TLR activation and the subsequent activation of signalling pathways required to induce interferon production. HBx interferes with mitochondrial antiviral signalling protein (MAVS), an essential adapter for several PRRs, HBeAg interferes with TLR activation, and HBV polymerase interferes with IRF3 activation. HBV replication probably suppresses the activation of an innate immune response in more, incompletely understood ways. Because of the ability to replicate in the absence of innate immune activation, HBV is considered to be a “stealth” virus.

This stealth capacity may be essential for viral replication, as TLR stimulation strongly suppresses in vivo HBV replication. Several ISGs that are induced upon TLR activation, such as APOBEC, MxA, MyD88, IDO, TRIM22 and IFITs, have been shown to be capable of interfering with HBV replication (reviewed in). Concomitantly, interferons can reduce HBV replication by activating ISGs in the absence of an adaptive immune response. These
findings suggest that avoiding the development of an innate immune response directly benefits HBV replication.

In adults, acute infection with HBV is mostly cleared within a couple of months. Strong CD4+ and CD8+ T cell responses against multiple HBV epitopes are observed during the clearance of acute infection\textsuperscript{133}. HBV specific CD8+ T cells kill infected hepatocytes by cytolitic mechanisms but also produce cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\); which clear HBV via non cytopathic mechanisms\textsuperscript{134-136}. IFN\(\gamma\) production also leads to the recruitment of antigen non-specific immune cells by inducing the production of the chemokines CXCL9 and CXCL10. Beside HBV specific CD8+ T cells, the recruitment of these cells is essential to clear acute HBV infection\textsuperscript{137}(Reviewed in\textsuperscript{138}). A minority of acutely infected adults do not clear HBV infection and progresses into the immune active stage of chronic HBV infection. In children, HBV infection is characterised by an initial lack of both innate and adaptive immune responses against the virus (during the immune tolerant stage). When an adaptive immune response does develop after such an immune tolerant stage, it is not sufficient to control viral replication.

Especially in immune tolerant HBV infections, the lack of innate immune activation contributes to the failure to mount an effective adaptive immune response. The lack of interferon production hampers the production of cytokines that coordinate the development of an adaptive immune response by attracting immune cells and stimulating their maturation. Interferon production is also important to directly stimulate the activity of dendritic cells, B cells, T cells and NK cells\textsuperscript{139-143}. When an adaptive immune response has been mounted, interferon production would facilitate MHC production and antigen presentation, which enhance the recognition of infected cells by antigen specific T cells\textsuperscript{144,145}.

Another means by which HBV frustrates the development of an adaptive immune response, is the large amounts of HBsAg and HBeAg that is excreted into the blood. How these large amounts of viral proteins have a tolerogenic effect is incompletely understood. The sheer presence of large amounts of antigen in itself has a tolerogenic effect, especially towards CD4+ T cells\textsuperscript{146-148}. However, HBsAg and HBeAg may also contribute to the tolerogenic state by directly interfering with effector functions of both innate and adaptive immune cells. HBeAg interferes with interleukin-18 (IL-18) mediated signalling, interferon-\(\gamma\) expression and inflammatory cytokine production by different immune cells\textsuperscript{149,151}, and HBsAg interferes with dendritic cell function\textsuperscript{152-154}. Taken together, HBV can persistently replicate in people and cause chronic infection by interfering with the innate and adaptive immune responses.

**Therapy**

Currently available drugs are not curative; therapeutic intervention is mainly targeted at preventing progression to cirrhosis and HCC\textsuperscript{155}. As most liver damage is induced during the immune active and immune escape phases of the infection, the idea behind most treatment strategies is to treat during these phases to suppress viral replication sufficiently to go into the immune controlled phase of the infection. Although worldwide the guidelines differ
somewhat, in most countries this strategy is translated into therapeutic guidelines in which antiviral therapy is indicated for patients with a high viral load and elevated ALT.

**Interferon**

The first antiviral therapy that was used to reduce HBV replication was human leukocyte-derived interferon (Interferon-α)\(^{156}\), which was later replaced by the more stable pegylated interferon-α (PegIFNα). PegIFNα mainly affects the lymphoid arm of the immune system. Several markers for functionality of natural killer (NK) cells, which are disturbed in CHB, are partially restored by interferon treatment\(^ {157}\). Conversely, PegIFNα negatively affects the number of CD8+ T cells and does not restore functionality of HBV specific T cells\(^ {157,158}\). This may explain that although a limited, 12-month course of PegIFNα can induce a switch from HBeAg positive to negative in about 25% of patients, PegIFNα does not affect the rate of HBV clearance\(^ {159,160}\). Although a limited course of PegIFNα may sustainably improve HBV status in a fraction of patients, its use is hampered by severe side effects, injection as route of administration and high costs\(^ {161}\).

**Nucleotide analogues**

Currently, the primary therapeutic option for treatment of chronic HBV infection are nucleotide analogues (NAs), of which several are approved for the treatment of chronic HBV infection\(^ {162-165}\). Nucleotides interact with HBV Pol and critically interfere with the conversion of viral RNA into DNA. The main mechanism by which NAs interfere with Pol activity is their incorporation into the viral DNA, which terminates further elongation and thus prevents the production of HBV DNA. Some NAs may also suppress Pol-mediated protein priming\(^ {166}\). For all NAs, mutations have been described that render Pol resistance\(^ {166}\). Typically, these mutations reduce viral fitness and need to be “compensated” by other mutations to fully restore Pol functionality\(^ {166}\). In vivo, there are large differences in the chance Pol will become resistant to a given NA. In HBV infected patients treated with lamivudine monotherapy, resistance develops in 23% of all patients within a year, and in 80% of patients within 5 years. Development of resistance seems to be less of a problem for entecavir and tenofovir. While resistance to entecavir develops after 5 years of monotherapy in only 1.2% of patients, resistance to tenofovir has not been observed in patients\(^ {167}\). NAs prevent the formation of new viral DNA, but do not affect RNA transcription from already existing DNA. Therefore HBV protein production continues unhindered in patients treated with NAs. By preventing cccDNA accumulation and *de novo* infection of hepatocytes, prolonged NA therapy may lead to a complete disappearance of HBV DNA and a significant reduction in viral protein production. NA therapy does not lead to immunological control of the infection and has to be taken life long, as viral replication relapses when treatment is ceased.
Combination therapy and therapeutics under development

Several studies have revealed that combining NA therapy with a course of Peg-IFNα can induce more favourable virological and biochemical outcomes at the end of therapy as compared to a course of PegIFNα monotherapy alone. However, this initial difference is mostly lost after 6 months of follow-up (reviewed in168). Due to the disadvantages of PegIFNα therapy, much effort has focused on identifying those patients that will benefit from PegIFNα combined with NA therapy, i.e., those who will sustainably clear markers of viral replication. As it turns out, patients that benefit from PegIFNα therapy may be predicted by the levels of HBV DNA and HBsAg, and the decline in these during the first weeks of therapy169,170, the HBV genotype171, host genetic polymorphisms, such as e.g. IL28B172, IPS1173, SLC16A9174 and HLA-DP175, and the expression of chemokine receptors on NK cells176. Several other strategies to target HBV replication are currently under investigation. An HBV envelope protein derived polypeptide that inhibits HBV entry has recently entered clinical trials177-179. Strategies to interfere with the RNase H domain of the HBV polymerase180, HBV core particle assembly181, apoptosis182 and lymphotxin-β receptor activation183 are in preclinical stages of development.

Figure 4. HBx interacts with various cellular components. HBx modulates the transcription of host proteins180-182, affects several cell signalling pathways183, interferes with cell cycle progression and apoptosis184-186, and interferes with multiple DNA repair pathways187-191.
Scope of this thesis

Expression of the HBV accessory protein HBx is a requirement for viral replication. HBx affects various cellular processes, and thereby enables HBV RNA transcription (Figure 4). Thereby HBx drives the ongoing viral protein production during treatment of chronic HBV infection with currently available antivirals. In this thesis, we investigated how HBx supports HBV replication, and we performed studies to identify its function. In chapter 2 we determined the mutation rate of different parts of the HBV genome by analysing sequences from different time points in a cohort of people chronically infected with HBV. We show that the overlap of reading frames in the HBV genome restricts viral evolution and affects the mutation rate. The mutation rate negatively correlated with viral load, suggesting that when viral replication is suppressed, HBV may increase the mutation rate to facilitate the escape from adaptive immune responses. Because the secondary structure of HBx is not related to any known protein, and no crystallography-based tertiary structure is available, we have predicted the tertiary structure of HBx by ab initio computer modelling in chapter 3. Our model shows that HBx may have significant structural homology with the human thymine DNA glycosylase (TDG). In chapter 4 we show that HBx inhibits TDG-initiated DNA repair. Amongst the HBx interacting proteins described in the literature are various epigenetic modulators. The data we present in chapter 5 show that an important class of these proteins, the histone deacetylases, indeed suppress HBV replication, but that their activity is not affected by HBx. Therefore we applied subtractive mass spectrometry to systematically identify relevant HBx interacting proteins. As described in chapter 6, we identified one HBx interacting protein, talin-1, which was targeted for proteasomal degradation by HBx. Subsequent analysis revealed that talin-1 is a viral restriction factor, and that HBV replication critically depends on HBx-mediated talin-1 degradation. In chapter 7, we explored the possibility to target HBV transcription in HBx dependent- and independent ways. By screening 640 FDA approved drugs, we identified compounds that suppressed HBV replication by targeting HBV RNA transcription. The contents of this thesis are discussed in chapter 8.
chapter 8

replication by targeting HBV RNA transcription. The critically depends on HBx-mediated talin-1 degradation. In Subsequent analysis revealed that talin-1 is a vira interacting protein, talin-1, which was targeted to identify relevant HBx interacting proteins. As desc affected by HBx. Therefore we applied subtractive m modulators. The data we present in DNA glycosylase (TDG). In predicted the tertiary structure of HBx by any known protein, and no crystallography-based ter from adaptive immune responses. Because the seconda viral replication is suppressed, HBV may increase the mutation rate to facilitate the escape mutation rate. The mutation rate negatively correla the overlap of reading frames in the HBV genome res determined the mutation rate of different parts of HBV replication, and we performed studies to identi in infection with currently available antivirals. In t Thereby HBx drives the ongoing viral protein produc affects various cellular processes, and thereby ena

Expression of the HBV accessory protein HBx is a re quirement for viral replication. HBx

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