

Hepatitis B virus replication and integration

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UNIVERSITY OF GOTHENBURG

Gothenburg 2014

Cover illustration: The future's so bright, I gotta wear sunglasses.

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ISBN 978-91-628-8986-9

ISBN 978-91-628-8988-3 (PDF)

<http://hdl.handle.net/2077/34851>

Printed in Gothenburg, Sweden 2014
by Aidla Trading AB/Kompendiet

Till mamma

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ABSTRACT

Chronic infection with hepatitis B virus (HBV) affects 240 million people worldwide and may cause liver disease including hepatocellular carcinoma (HCC). Initially patients have high levels of HBV DNA in their blood, no liver disease and express the e antigen (HBeAg). At some point an immune response is mounted and the viral load decreases with several \log_{10} copies/ml, they lose HBeAg and severe liver damage may follow if the virus is not cleared efficiently. Meanwhile, the circulating levels of the surface antigen (HBsAg), not bound to viral particles, remain high. In the liver, the viral DNA might integrate in the genome of hepatocytes. This has been proposed as a mechanism potentially promoting cancer development.

The aims of this thesis were to investigate the mechanisms behind the great decline in HBV DNA at loss of HBeAg while the HBsAg levels remain relatively stable, to evaluate the utility of quantification of HBsAg in serum as a marker for liver damage, and to assess the extent of integrated HBV DNA in liver biopsies.

The main methods used in this thesis are various types of polymerase chain reaction (PCR). The material was blood samples and liver biopsies from chronic carriers of HBV. Viral load in the liver, both DNA and RNA, was quantified by real-time PCR and integrated HBV DNA sequences were identified using Alu-PCR.

Serum levels of HBV DNA and HBsAg correlated with intrahepatic levels of covalently closed circular DNA (cccDNA), the template for new viral particles and antigens. By comparing viral load between patients positive or

negative for HBeAg it was found that the 3-5 log₁₀ decline of HBV DNA at HBeAg seroconversion mainly is explained by decrease in cccDNA and reduced transcriptional efficiency of pregenomic RNA (pgRNA), the template for virus DNA. However, retention of viral particles and decreased half-life of virions seem to have an additional impact.

By comparing results of serum levels of HBsAg and histological examination of liver biopsies it was concluded that a cut-off of <3.0 log₁₀ IU/ml of HBsAg and <4.0 log₁₀ copies/ml could identify patients with low liver damage with a specificity of 96%.

With Alu-PCR integrated sequences were detected in 36 of 48 liver biopsies examined. In total 45 integrated sequences were analysed from 32 different patients. Integration of HBV DNA was thus a very common event in the chronic HBV carriers.

In summary, this study contributes to the understanding of the replication and integration of the hepatitis B virus.

Keywords: hepatitis B virus, HBV DNA, HBsAg, replication, integration

ISBN: 978-91-628-8986-9

<http://hdl.handle.net/2077/34851>

SAMMANFATTNING PÅ SVENSKA

Runt om i världen finns mer än 240 miljoner människor som bär på hepatit B-viruset (HBV). Det är ett litet virus som infekterar leverceller och vars arvsmassa består av DNA. Om man smittas som vuxen, till exempel vid blodtransfusion eller sexuella kontakter, får man i 95 % av fallen en akut infektion med gulsot och blir sedan frisk och immun mot viruset. Smittas man däremot vid födseln, om ens mamma är infekterad, eller under tidiga barnaår, är risken mycket stor att infektionen blir kronisk och sjukdomen kan då i princip finnas kvar hela livet. Hos många pågår infektionen under lång tid utan att de märker av den, men på sikt kan den kroniska inflammationen i levern leda till ärrvävnad, skrumplever och i värsta fall levercancer.

När viruset har tagit sig in i levercellen transporteras det till cellkärnan där arvsmassan bildar den en slags minikromosom, cccDNA (covalently closed circular DNA), som finns kvar i cellkärnan under mycket lång tid. Från cccDNA bildas så kallade RNA-transkript, som utgör mallen för att producera virusproteiner. Ett av transkripten fungerar också som mall för att skapa nytt virus-DNA till de nya viruspartiklarna. På virusets yta finns ett speciellt protein, ytantigenet HBsAg, som behövs bland annat för att viruset ska kunna binda till och ta sig in i levercellerna. Dock finns det också HBsAg som cirkulerar i blodet i stora mängder men inte sitter på viruspartiklar. Varför det är så vet man inte riktigt men en funktion skulle kunna vara att ”trötta ut” immunförsvaret. När någon blir frisk från HBV-infektionen försvinner HBsAg från blodet och istället kan man påvisa antikroppar, anti-HBs. Det är sådana antikroppar man får om man vaccinerar sig mot HBV.

Utöver HBsAg finns ett annat protein i blodet hos HBV-patienter, e-antigenet (HBeAg). Dess funktion är inte helt klargjord men troligen har den någon slags inverkan på immunförsvaret. Det man vet är dock att HBeAg finns i blodet hos patienter med höga virusnivåer medan det på längre sikt försvinner och patienten istället bildar antikroppar, anti-HBe.

Alla delarbeten i den här avhandlingen har utgått från samma patientmaterial, både blodprover och leverbiopsier (vävnadsprover). Den huvudsakliga metodiken som använts är olika varianter av polymerase chain reaction (PCR). Det är en metod med vilken man kan skapa en stor mängd kopior av en viss DNA-sekvens så att den blir mätbar. På så sätt kan man få reda på hur mycket det fanns av just den sekvensen i provet eller hur dess sammansättning av DNA:s byggstenar, nukleotiderna ser ut.

I delarbete I och III kunde vi visa att både virusmängd och HBsAg-nivå i blodet avspeglar hur mycket virus det finns i levern genom att analysera prover från 84 patienter. Dock är sambandet inte lika tydligt i patienter som inte har HBeAg. Vi såg att hos personer utan HBeAg verkar det som att en del viruspartiklar hålls kvar inuti levercellerna istället för att utsöndras till blodet. Detta kan vara en förklaring till att virusnivån i blodet är lägre hos dessa patienter jämfört med dem som har HBeAg i blodet.

I delarbete II tittade vi på HBsAg-nivå och leverskada (bedömd på leverbiopsier av en patolog) hos 160 HBV-patienter. Det visade sig att de patienter som hade låg HBsAg-nivå i blodet också hade liten eller ingen leverskada. Man skulle alltså kunna använda HBsAg-nivån för att identifiera patienter med låg risk för problem med levern.

HBV DNA kan integreras i kromosomernas DNA i levercellerna, vilket man framförallt sett i levercancer. Man tror att detta kan bidra till cancerutvecklingen. Några studier har också visat förekomst av integrerat HBV DNA hos patienter som inte fått cancer. I arbete IV undersökte vi förekomsten av integrerade sekvenser av HBV DNA med hjälp av Alu-PCR. Denna PCR-metod kan man använda när man inte vet var i arvsmassan HBV DNA-sekvensen hamnat och man ”chansar” då på att den sitter i närheten av en sekvens man känner till, Alu-sekvensen. Denna sekvens finns utspridd på miljoner platser i vårt DNA. Med denna metod kunde vi hitta integrerat HBV DNA hos 32 av 48 patienter. Vår slutsats blev att integrering av HBV DNA är väldigt vanligt och sker troligen hos alla patienter med HBV-infektion.

Sammanfattningsvis har denna avhandling gett ny kunskap om HBV-infektionen genom att vi kunnat göra analyser både på blodprover och leverbiopsier.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. Malmström S, Larsson SB, Hannoun C, Lindh M. **Hepatitis B viral DNA decline at loss of HBeAg is mainly explained by reduced cccDNA load – down-regulated transcription of pgRNA has limited impact.** *PLoS One*. 2012;7(7):e36349
- II. Larsson SB, Eilard A, Malmström S, Hannoun C, Dhillon AP, Norkrans G, Lindh M. **HBsAg quantification for identification of liver disease in chronic hepatitis B virus carriers.** *Liver International*. 2013 Oct 1. doi:10.1111/liv.12345.
- III. Larsson SB, Malmström S, Hannoun C, Norkrans G, Lindh M. **Reduced serum levels of hepatitis B virus DNA and HBsAg by suppression of cccDNA and pgRNA but not of S-RNA.** *Submitted*.
- IV. Larsson SB, Raimondo G, Norkrans G, Hannoun C, Lindh M, Pollicino T. **Integration of hepatitis B virus DNA in chronically infected patients.** *Manuscript*.

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ABBREVIATIONS

ALT	Alanine amino transferase
AUROC	Area under receiver operating characteristic
cccDNA	covalently closed circular DNA
ENH	e-antigen negative hepatitis
EPH	e-antigen positive hepatitis
HAI	Histology activity index
HBeAg	Hepatitis B virus e antigen
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
IC	Inactive carrier
ihDNA	Intrahepatic DNA
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
RT PCR	Reverse-transcriptase PCR
UDG	Uracil-DNA glycosylase
ULN	Upper limit of normal
WHO	World Health Organization

1 INTRODUCTION

In 1970 Dane et al. described a virus-like particle about 42 nm in size that was found in patients with Australia-antigen-positive hepatitis (Dane, Cameron, and Briggs 1970). Five years earlier, Blumberg had discovered the Australia antigen in a group of leukemia patients (Blumberg, Alter, and Visnich 1965). The association of this antigen with viral hepatitis had already been made and the virus is today known as the hepatitis B virus.

Chronic infection with hepatitis B virus (HBV) is usually acquired at birth, when the virus is transmitted to a child from an infected mother, or during early childhood (Lok and McMahon 2009). The virus infects the hepatocytes of the liver. According to the World Health Organization (WHO) there are more than 240 million persons chronically infected by the virus and every year about 600 000 people die due to consequences of HBV infection (World Health Organization 2013). Chronic HBV infection is the major risk factor for hepatocellular carcinoma (HCC), and accounts for about 50% of cases worldwide (Sherman 2010). The HBV is most common in East Asia and sub-Saharan Africa, where 5-10% of the population are chronically infected (B. K. Kim, Revill, and Ahn 2011).

Initially the infection affects almost all hepatocytes in the liver ($\sim 10^{11}$) and the levels of HBV DNA in the blood are very high, 10^9 - 10^{10} copies/ml. Quantification of serum levels of HBV DNA is currently the method of choice to monitor the disease and also functions as a prognostic marker (European Association For The Study Of The Liver 2012; Lok and McMahon 2009; Liaw et al. 2012). Identification of the surface antigen, HBsAg, is usually seen as the hallmark of HBV infection and in recent years quantification of HBsAg has been proposed as a complement to HBV DNA (Martinot-Peignoux et al. 2013). Both are surrogate markers for covalently closed circular DNA (cccDNA), a viral mini-chromosome present in the nucleus of hepatocytes as template for new viral particles.

In addition to HBsAg, the e antigen (HBeAg), is found in the blood of patients with high levels of HBV DNA (Magnius and Espmark 1972). During the course of infection the levels of HBeAg decrease and antibodies to HBeAg become detectable. This is accompanied by a great decline (3 - $5 \log_{10}$ copies/ml) of HBV DNA, whereas the levels of HBsAg remain relatively stable. Cure from the disease is usually defined as loss of HBsAg and detection of antibodies to HBsAg.

If a patient has symptoms or signs of liver damage, treatment might be considered. Two main strategies are used: Interferon, which has complex antiviral effects and boosts the immune system, or nucleotide/nucleoside analogues, blocking viral replication (European Association For The Study Of The Liver 2012). With the former option some patients may be cured (i.e. lose HBsAg) but only 20-40% respond to treatment. Blocking viral replication decreases serum viral load to undetectable levels but the virus is still present in the liver and very few patients get rid of the virus completely.

In the present thesis, different aspects of chronic HBV infection and viral replication were studied with a focus on analysis of liver biopsies from infected patients. First, an introduction to the HBV field, both from a virological and a clinical point of view, will be given. Secondly the results from four papers dealing with viral replication, clinical staging and investigation of integrated HBV DNA sequences will be discussed.

1.1 The hepatitis B virus

The hepatitis B virus (HBV) is a partially double stranded DNA virus, with a complete minus strand and an incomplete plus strand, belonging to the Hepadnaviridae family (Figure 1)(Block, Guo, and Guo 2007). This family is further divided in genera, the, Orthohepadnaviridae, including human and other mammal hepadnaviruses, for example chimp or woodchuck HBV, and the Avihepadnaviridae, such as Duck hepatitis virus (Locarnini et al. 2013). Human HBV only infects humans, chimpanzees and Tupaia Tree shrew as the virus is dependent of attachment to its receptor, the sodium-taurocholate cotransporting polypeptide (NTCP), for infection to occur (Y. Ni et al. 2013).

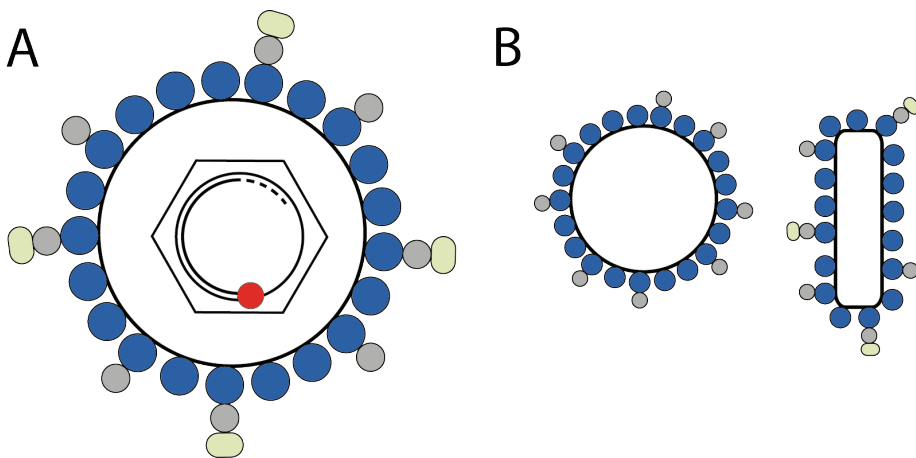


Figure 1. The hepatitis B virus, 42 nm small, with L-, M- and S-proteins attached to a lipid bilayer. Inside is the capsid containing the partially double-stranded relaxed circular DNA including the polymerase (red circle). B. Subviral particles, 22 nm long in the form of spheres and filaments. Only the filaments contain the L-protein.

The HBV genome is relaxed circular partially doublestranded DNA, template for five different transcripts. The genome has four open reading frames: the preCore/Core, for the precore and capsid proteins, Pol, for the polymerase, PreS/S, for surface antigens, and X, for the X protein (Figure 2). The longest transcript, precore, is 3.5 kb and translated into the e antigen, which is secreted to the blood stream and is thought to have an immune-tolerogenic function. Only 33 bp shorter, the core transcript is the template for capsid proteins but also for the polymerase involved in reverse transcription including the RNase H activity. Two transcripts are translated into the surface proteins. The longest, 2.4 kb, is translated into the L-protein whereas the 2.1 kb transcript encodes the M and S proteins. The X-transcript, 0.8 kb, is

template for the X protein, which is suggested to have a transactivating function on many targets as well as involvement in carcinogenesis.

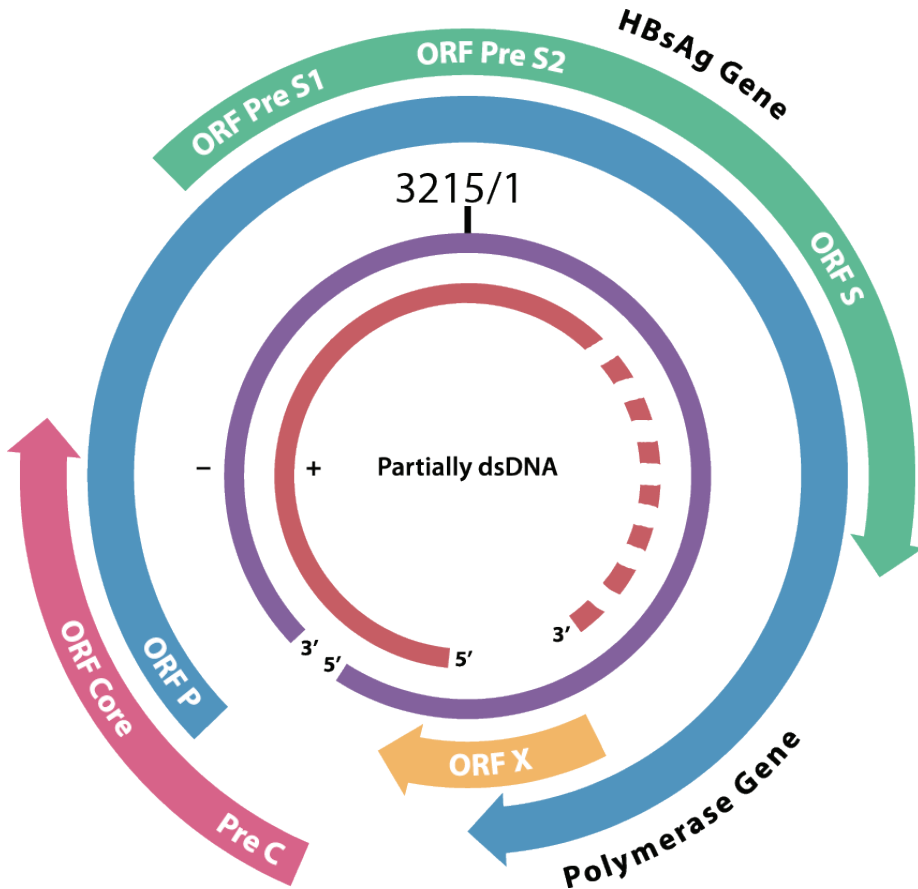


Figure 2. The partially double stranded HBV-genome with the four open reading frames indicated. Numerotation of nucleotides start at the EcoRI-site (the genome is ~3.2 kb).

1.1.1 Replication

The receptor for hepatitis B virus was recently discovered to be the sodium-taurocholate co-transporting polypeptide (NTCP) (Yan et al. 2012). There is a high affinity between the preS1-peptide and NTCP, and this receptor is only expressed in hepatocytes, located to the sinusoidal/basolateral surface, explaining the liver tropism for HBV (Baumert et al. 2014). After binding to

its receptor, the HBV enters the cell through endocytosis and fuses to the endosome, thus releasing its nucleocapsid (Figure 3).

The capsid is transported along microtubules to the nucleus, where the genome is released. Inside the nucleus the plus strand is completed and the genome becomes fully double stranded. Coupled with histones this forms a mini-chromosome, called covalently closed circular DNA (cccDNA), which is the template for the different viral transcripts (Sohn, Litwin, and Seeger 2009).

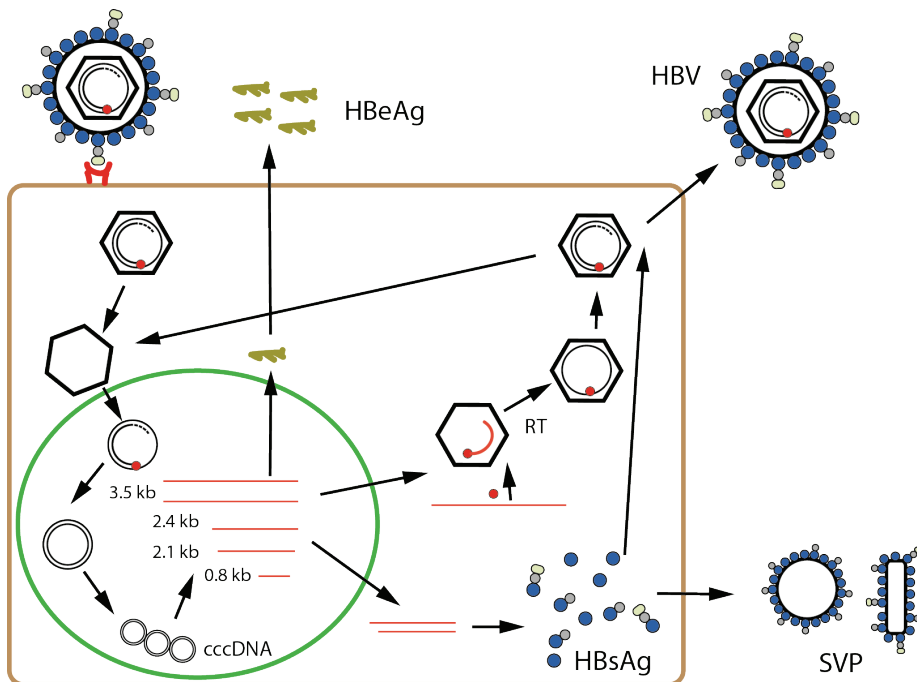


Figure 3. The hepatitis B virus replication takes place in the hepatocytes. The partially double stranded DNA genome is released in the nucleus where it is completed by cellular mechanisms to form the covalently closed circular minichromosome, cccDNA.

The pregenomic RNA (pgRNA) is a more than full-length copy of the genome. It contains regions of direct repeat, DR1 and DR2, 10 bp each, which are important during the reverse transcription. Near both the 5' and the 3' ends an encapsidation signal is present, where the former enables encapsidation of RNA into virus nucleocapsids. The reverse transcription takes place inside the capsid (Beck and Nassal 2007).

The viral polymerase binds to the 5' end of the pgRNA. After an initial three nucleotides priming, the polymerase translocates to the DR1 sequence near the 3' end and synthesis of the negative strand by reverse transcription is followed by degradation of the pgRNA. An 18 bp RNA sequence escapes RNaseH digestion, is translocated to DR2 of the negative DNA strand where it primes plus strand synthesis.

Some capsids are transported back to the nucleus to replenish the pool of cccDNA. To form new virions most capsids containing newly synthesized HBV DNA are transported to the endoplasmic reticulum (ER) where they are enveloped in lipid bilayer containing surface proteins. The virions are further processed through the Golgi apparatus before leaving the cell.

1.1.2 Viral antigens

The HBV has four different kinds of antigens: precore, core, surface and X antigen. The core antigen (HBcAg) is the main component of the capsid.

The precore protein, the precursor of the e antigen (HBeAg), is produced from its own transcript, 33 nt longer than the core transcript. The precore is directed to the ER by means of a leader sequence in its precore part, where it is cleaved before secretion as HBeAg (Milich and Liang 2003).

The three different surface proteins, Large, Middle and Small (L, M, S), are translated from two different transcripts. They all contain the S domain but M proteins also contain the preS2 domain and the L protein an additional preS1 domain. The latter domain is also the ligand to the HBV receptor, NTCP. These proteins are present on the surface of virions, but there are also two forms of subviral particles, spherical and filamentous, that lack capsid and hence viral DNA. These are produced in great excess and outnumber the viral particles with an order of 10^4 - 10^5 (Figure 1B)(Chai et al. 2008).

The small X protein has been attributed many potential functions (Feitelson, Bonamassa, and Arzumanyan 2014). It seems to be required for initiation and maintenance of virus replication after infection (Lucifora et al. 2011) and it has been suggested to have transactivating functions on transcription (H. Tang et al. 2005) as well as carcinogenic effects (L. Yang et al. 2008).

1.1.3 Immunology in hepatitis B virus infection

The hepatitis B virus seems to have several mechanisms to escape or downregulate the host's immune response. Even though extensive research has been done in this field many questions remain to be answered (Bertoletti

and Gehring 2006; Bertoletti and Ferrari 2013; Bauer, Sprinzl, and Protzer 2011). Infection at birth or during the first years of life results in a chronic infection in more than 90% of cases whereas an infected adult clear infection in >95% of cases. It is reasonable to think that the innate immune response in HBV infection is weak or absent. Inefficient triggering due to the peculiar replication mechanism with intranuclear cccDNA and a reverse transcription step inside the viral capsids seems to be one reason. The other might be active HBV-mediated suppression of the innate immunity. As an effective immune response is absent especially during the HBeAg-positive phase, an immune-tolerogenic role has been attributed to this antigen (Milich et al. 1990). One idea is that this secretory form of the core antigen has a tolerizing effect resulting in low levels of core-specific T-cells (M. T. Chen et al. 2004). A more recent study suggested that HBeAg targets and suppresses activation of the Toll-like receptor signalling pathway, modulating both NF- κ B and IFN- β activation (Lang et al. 2011).

The adaptive immune response, on the other hand, is dependent on a strong T cell response. In chronic HBV infection this T-cell response is dysfunctional, probably due to exposure to large amounts of viral antigens, both viral particles and the excess amount of circulating HBsAg and HBeAg particles (Boni et al. 2007). On the other hand, hepatocytes express low levels of MHC-class I molecules and the amount of antigen required to stimulate equivalent numbers of virus-specific CD8⁺ T cells is a 100-fold higher than other antigen presenting cells. This might explain how HBV infected hepatocytes can avoid recognition by T cells when the virus production decreases and HBeAg is lost (Gehring et al. 2007).

Clearance of the virus is dependent on the production of anti-HBs antibodies. Efforts are being made to find therapeutic strategies with immunomodulatory compounds to boost the immune response, in addition to the traditional treatment options; nucleos(t)ide analogues and interferon (Block et al. 2013). Clearly, the key to controlling the HBV infection lies in an adequate immune response.

1.1.4 Genotypes

Originally HBV was divided into different subtypes according to surface antigen determinants (Bouvier and Williams 1975). The major determinant, a, is present on all HBV species. Additional subtypes were identified with one or a combination of d/y and w/r determinants, where subtype w can be further divided in w1-w4. An additional q determinant has been identified and is expressed by most HBV strains (Magnius et al. 1975).

With accumulating data from DNA sequencing of different HBV strains, genotyping based on differences in nucleotide sequences was introduced (Okamoto et al. 1988). The initial four genotypes, A to D, were distinguished by a an intergenotype difference of >8% and an intragenotype difference of <4%. Later, genotypes E and F (Norder et al. 1992), genotype G (Stuyver et al. 2000) and genotype H (Arauz-Ruiz et al. 2002) were discovered. Furthermore, a recombination between genotype C and an unknown genotype was discovered (Hannoun, Norder, and Lindh 2000). Later this recombinant has been classified as genotype I (Huy, Trinh, and Abe 2008), but this designation has been questioned (Kurbanov et al. 2008). An HBV strain from a single Japanese patient diverging from all other human genotypes was assigned the letter J (Tatematsu et al. 2009).

Traditionally the different genotypes had a characteristic geographical distribution, in some way reflecting human migration in ancient and more recent times (Figure 4) (Norder et al. 2004). Roughly, genotype A is found in Northern Europe, Sub-Saharan Africa and Western Africa, B is found in East Asia and Alaska, C is found in East and South East Asia as well as Polynesia and Australia, D is found in Northern Africa, Europe, Central Asia, Mediterranean countries and India, E In West Africa, F in Central and South America and H in Central America. No geographic origin has been identified for genotype G. The migration patterns of today of course changes the distribution of genotypes and in a country like Sweden, where HBV is not endemic, patients from almost all genotypes are found.

Differences in disease profiles have been observed, such as earlier HBeAg seroconversion and less severe liver disease in genotype B as compared to C, and to some extent differences between genotype A and D (Lindh et al. 1999; McMahon 2009b).

1.1.5 Hepatitis B virus variability

Due to the error prone reverse transcriptase, nucleotide substitutions frequently occur during replication leading to an estimated 10^{-5} substitutions per site per year (H.-Y. Wang et al. 2010). As up to 10^{11} virions are produced per day, it can be deduced that every possible mutation arises several times every day. Endogenous or exogenous factors drive the selection of the strains that best fit the environment in the hepatocyte. Immune response, replication fitness of virus variants and the number of cells infected are examples of endogenous factors whereas antiviral or immune-based therapies are exogenous factors influencing the selection of a specific quasispecies.

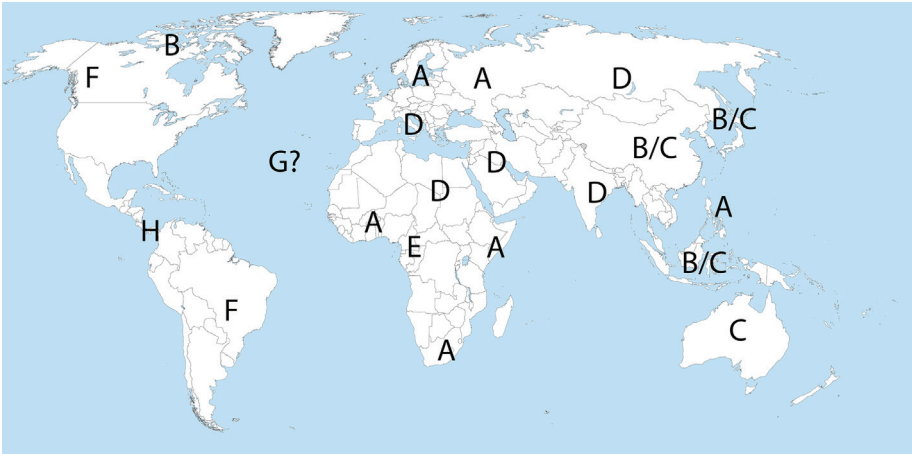


Figure 4. Map of the original distribution of HBV genotypes probably reflecting early human migration. The changing migration patterns during the last centuries have resulted in a more complex distribution.

Two important naturally occurring HBV mutations have been identified. The most common is located to position 1896 in precore region, changing G to A, and thus creating a stop-codon in the precore transcript (Carman et al. 1989). This mutation abolishes the production of HBeAg and frequently emerges in parallel with loss of HBeAg, explaining why HBeAg does not reappear in the blood even if viremia increases to levels above 5-6 log₁₀ copies/ml. This mutation is not common in genotypes A and C1, which usually carry a C at position 1858 creating a weak basepairing at the stem-loop in this region of the precore mRNA (Li et al. 1993).

In the core promoter region, which regulates the transcription of both precore and core RNA transcripts, several mutations have been reported. The most common are located to positions 1762 and 1764 where A to T and a G to A mutations are frequent (Okamoto et al. 1994). Core promoter mutations are of unknown reasons associated with genotype C (Lindh et al. 1999), and with more necroinflammation (Lindh et al. 1999), cirrhosis (Chu et al. 2012) and HCC (C.-J. Liu et al. 2006). In combination with less prevalent mutations in this region they cause downregulation of precore mRNA transcription, thus affecting the level of HBeAg (Jammeh et al. 2008). These mutations are often found prior to the precore mutation and appear years before HBeAg seroconversion. The frequent emergence of the precore and core promoter mutations suggests that at some point during chronic HBV infection the

expression of HBeAg becomes a disadvantage to the virus and mutants expressing less of this antigen are selected.

Deletion in the C-gene is the only type of mutation associated with the highly viremic immune tolerant phase, where the immune response is suppressed (Gunther 2006). The mutant core protein is defective and these mutant strains coexist with wild-type strains that can provide functional core protein to form new capsids (Marinos et al. 1996). C-gene deletion mutants accumulate due to a replication advantage compared to wild-type HBV strains but they often disappear after HBeAg seroconversion and appearance of anti-HBe.

There are some mutations in the S gene that have been shown to reduce HBsAg binding to its antibody. The most common is a glycine to arginine substitution at codon 145 of the S protein. This mutation has been reported to escape vaccination (Carman et al. 1990).

Deletions in the preS1/S2 regions have been associated with a more severe liver disease in HBeAg-negative patients (B.-F. Chen et al. 2006; C.-H. Chen et al. 2007). These regions are important as epitopes for B and T cells, for binding to the HBV receptor, viral assembly and secretion. In combination with mutations in the core promoter region these deletions predict a worse clinical outcome than wild-type strains. Accumulation of Large S inside the hepatocytes has been associated with pre-S deletions and has been proposed to explain a typical histological finding named ground glass hepatocytes (GGH) (H.-C. Wang et al. 2003). Recent data report a growth advantage of those hepatocytes accumulating pre-S2 mutants GGHI, and the distribution pattern of these cells could be clinically relevant (Mathai et al. 2013).

Other mutations occur as a result of antiviral treatment, in particular with nucleos(t)ide analogues. These drugs interfere with the viral polymerase. The first drug in this category to be used in the treatment of chronic HBV infection was lamivudine (Dienstag et al. 1995). It is effectively reducing serum viral load but it has a high rate of resistance with up to 70 % of patients after 4-5 years of treatment. Additional drugs in this category, with lower rates of resistance, have been developed, including tenofovir, which has not been associated with resistance mutations in HBV from patients. An important factor influencing development of resistance is the patient's compliance to therapy.

1.1.6 HBV DNA integration

While the hepatitis B virus replicates via an RNA-intermediate and reverse transcriptase, it is not dependent on integration for the replication process.

However, it has been known since more than thirty years that HBV DNA can integrate into the human genome (Marion et al. 1980; Bréchet et al. 1980; Edman et al. 1980). In these early studies integrated forms of HBV DNA was first detected in a cell line, PLC/PRF/5, derived from a patient with liver cancer. Soon thereafter integrations were also detected in tumour tissue and in liver biopsies from patients with HBeAg-negative chronic hepatitis (Bréchet, Hadchouel, Scotto, Fonck, et al. 1981). It is believed that a linear form of the normally relaxed circular genome of HBV is the precursor for integration (W. Yang and Summers 1999). This form of the viral DNA is imported into the nucleus preferentially during early phases of infection or during periods of extensive cell turnover and reinfection. A preferential site of integration in the HBV genome has been found, namely the region between DR1 and DR2 (Z. Jiang et al. 2012; Sung et al. 2012; Takada et al. 1990). In an extensive study on HCC from HBV patients Sung et al found that 40% of integrations occurred at a breakpoint within the 1800-bp region, close to the DR1.

Different methods have been used to detect integrated sequences of HBV DNA. The first studies used cloning, restriction enzymes and hybridisation with Southern blot (Edman et al. 1980). Since the PCR method was introduced, several modifications of this technique have been applied to detect viral-host junctions These include inverse-PCR (Tsuei et al. 1994), Alu-PCR (Minami et al. 1995) and cassette-ligation-mediated PCR (Tamori et al. 2003). The PCR-based methods are more specific, but an important limit is that only the sequence of one viral-host junction is detected. In recent years, next-generation sequencing has facilitated the search for viral-host junctions (Sung et al. 2012). Many new integration points have been discovered using this technique but so far only tissue from explanted livers have been analysed, probably due to a lack of liver biopsies.

The accumulating data of integrated HBV DNA sequences suggest that the integrations occur randomly across all human chromosomes (S. Jiang et al. 2012). From analysis of liver tumours with integrated HBV DNA there are findings of recurring integration at genes connected to carcinogenesis, such as TERT and MLL4 (Sung et al. 2012; Saigo et al. 2008; K.-W. Tang et al. 2013; Paterlini-Bréchet et al. 2003; Murakami 2005). This is most probably due to these integrations driving a cancer development since integration sites are more diverse in non-tumorous tissue (Sung et al. 2012; Z. Jiang et al. 2012).

1.2 Chronic hepatitis B virus infection

Chronic infection with the hepatitis B virus is most often acquired at birth or during early childhood. An adult who is exposed to HBV most often develops acute hepatitis, which resolves within months. The persistence of HBsAg in blood for more than six months without detectable anti-HBs is defined as chronic hepatitis B virus infection (McMahon 2009a).

Vertical transmission of HBV usually occurs by passage over the placenta at delivery. Infection through horizontal transmission is often acquired through blood transfusions, other medical procedures, sexual intercourse, or between small children by unknown mechanisms. HBV DNA has been detected at high levels in various body fluids such as urine, sweat, saliva, tears (Komatsu et al. 2012) and to some extent even cerumen (Eftekharian et al. 2013). It is therefore likely that there are several possible routes of transmission among children.

1.2.1 Natural history

The chronic HBV infection is initially characterised by high levels of virus, HBeAg positivity, normal alanine aminotransferase (ALT) levels and minimal histological damage (Table 1). This Immune tolerant phase may last for 20-30 years. At some point an Immune clearance phase is initiated with a range of immune responses coupled with more or less damage to the liver. During this stage the patients lose HBeAg from serum and the HBV DNA levels decrease (Realdi et al. 1980; McMahon 2009a; Hadziyannis 2011).

The HBeAg negative patients have been divided into two groups and a patient may shift from one group to the other during the course of infection. The patients in the inactive carrier group have low levels of HBV DNA and normal ALT levels during a minimum follow-up of one year. However, this stage might be followed or preceded by the HBeAg-negative hepatitis phase. This is a late immune reactive phase characterised by recurring periods of fluctuating HBV DNA levels and elevated ALT with active liver damage and thereby a higher risk of fibrosis and eventually cirrhosis or hepatocellular carcinoma.

Patients in whom HBsAg is no longer detectable in the blood belong to the HBsAg-negative stage. Some of these patients have cleared the infection and no longer have HBV DNA detectable in the blood or in the liver, and have anti-HBs in the blood. Others have “occult” HBV infection, with some residual viral replication that is detectable as HBV DNA in the liver, but HBV DNA is only rarely found in serum (Raimondo et al. 2012).

HBsAg-negative patients have a favourable outcome in terms of much lower risk for cirrhosis and HCC than HBsAg-positive patients (J. Liu et al. 2013). The remaining risk for HCC probably is due to previous oncogenic steps, including integration of HBV DNA, but possibly also to some persistent replication in the remaining infected cells.

Table 1. Characteristics of the different phases of chronic HBV infection.

	Immune tolerant	HBeAg+ hepatitis	HBeAg- hepatitis	Inactive carrier	HBsAg neg
Serum HBV DNA	High	Elevated	$>10^4$	$<10^4$	-(+)
ALT	$<2xULN$	$>2xULN$	$>2xULN$	$<ULN$	$<ULN$
HBeAg	+	+	-	-	-
HBsAg	+	+	+	+	-
anti-HBs	-	-	-	-	+
Intrahepatic HBV DNA	+	+	+	+	+/-

HBV DNA: copies/ml; ULN: Upper limit of normal

1.2.2 Monitoring

In Sweden, patients with chronic HBV infection are usually admitted to the clinics of Infectious diseases. Depending on disease stage the patients are followed every 3 to 12 months with measurement of ALT levels and usually HBV DNA levels are also repeatedly measured. Patients in the inactive carrier phase are sometimes referred to the primary health care.

The degree of viral replication influences the immune mediated liver damage, and accordingly liver inflammation is more pronounced in patients with higher HBV DNA, in particular if the levels in the HBeAg negative phase are greater than $5 \log_{10}$ copies/ml (Lindh et al. 2000; Papatheodoridis et al. 2008). Accordingly, recommendations to treat have long been guided by HBV DNA quantifications in addition to ALT measurements and histological evaluations. During the last few years HBV DNA quantification has become even more used, because in a large longitudinal study in Taiwan it was shown that HBV DNA levels $>4-5 \log_{10}$ copies/mL were associated with higher risk for hepatocellular carcinoma (HCC) (C.-J. Chen et al. 2006). One should keep in mind that these results were obtained from Chinese patients, probably infected at birth and older than 30 years at inclusion.

In recent years, quantification of HBsAg levels in serum has come into focus as an alternative to quantifying HBV DNA (Su et al. 2010; Nguyen et al. 2010; Thompson et al. 2010). This marker tends to be more stable during the course of infection, whereas HBV DNA levels may fluctuate, especially during the immune clearance and HBeAg-negative hepatitis phases. Quantification of HBsAg could also be useful when monitoring patients under antiviral treatment, with undetectable levels of HBV DNA in serum (Gramenzi et al. 2011). It could also be used to evaluate the effect of interferon treatment where absence of decline in HBsAg levels during the first 12 weeks in combination with a small decline in HBV DNA has been proposed as a stopping rule for further treatment (Rijckborst et al. 2012).

A liver biopsy gives information on liver fibrosis (stage) and inflammation (grade), and is mainly considered in cases where ALT is repeatedly elevated or if HBV DNA levels are more than 4-5 log₁₀ copies/ml in the HBeAg negative phase (Lok and McMahon 2009). Based on the histological examination decision on treatment can be made.

1.2.3 Treatment options and Vaccination

Since the early 1980ies there is an effective vaccine against HBV (Szmunes et al. 1980). The vaccine consists of HBsAg (Small S), and is now a recombinant protein produced by yeast cells. HBsAg vaccination has been implemented in the general immunization program in most countries (however not yet in Sweden). In Taiwan, where vaccination was also given directly after birth to children born by HBsAg positive mothers (guided by HBsAg screening of pregnant women), the prevalence of HBV and incidence of HCC have decreased dramatically (Y.-H. Ni et al. 2012).

The two main treatment options in HBV infection is natural or pegylated interferon, which is given as subcutaneous injections and oral antivirals (nucleoside/nucleotide analogues) (European Association For The Study Of The Liver 2012; Liaw et al. 2012; Lok and McMahon 2009). Interferon treatment has antiviral and immunomodulatory effects that lead to decreasing levels of HBV DNA and in some cases cure of the disease, defined as loss of HBsAg. The side effects are often pronounced with flu-like symptoms (fever, chills, headache, muscle aches, malaise), neutropenia, trombocytopenia, depression and deterioration of liver function in patients with cirrhosis. Some people do not respond to treatment at all, and only 20-40% have satisfactory and sustained responses.

The nucleos(t)ide analogues were introduced in the early 1990s and were a great success as they reduced the risk for hepatic decompensation and the risk of HCC (Dienstag et al. 1995; Liaw et al. 2004; Yuen et al. 2007). Still, the risk for HCC remains higher in patients on long-term treatment with NA as compared with those in inactive stage (Cho et al. 2014). The first drug, Lamivudine, was effective in reducing the HBV DNA levels but was prone to developing resistance (Tipples et al. 1996). Newer drugs such as entecavir and tenofovir show little or no tendency to resistance (Tenney et al. 2009; Kitrinovs et al. 2014). With antiviral treatment the reverse transcriptase step is hampered to nearly 100%. Consequently, the viral load in serum rapidly decreases with several \log_{10} copies/ml and reaches undetectable levels within 6-12 months. Meanwhile, the inflammation of the liver decreases and even fibrosis improves. However, if treatment is stopped the HBV DNA levels increase again, often to pre-treatment levels, because the pool of cccDNA is not removed with this kind of treatment. Therefore, guidelines from Europe and the US suggest that therapy should preferably not be discontinued in HBeAg-negative patients, unless HBsAg is lost (European Association For The Study Of The Liver 2012; Lok and McMahon 2009). The Asian guidelines however advocate treatment discontinuation after 2 years of undetectable HBV DNA in serum (Liaw et al. 2012). As one year treatment with a nucleoside analogues costs roughly 60 000 SEK, cost issues might influence the latter guideline.

1.2.4 Hepatocellular carcinoma

The identification of HBV as a causative agent of HCC was first based on epidemiological observations, i.e. similar geographic distribution of HBV and HCC, increased prevalence of serum markers of HBV in serum of patients with HCC and that HBV infection precedes HCC (Maupas et al. 1975). Further evidence for this hypothesis came with detection of HBV DNA in tissue from HCC, both as free form (Summers et al. 1978) and integrated DNA (Bréchet et al. 1980). The relationship between HBV and HCC was established and it became clear that HBV was the most important risk factor for HCC world wide, accounting for 50% of cases (Sherman 2010; El-Serag 2011; El-Serag 2012).

It is believed that HBV contributes to development of HCC in several ways. The chronic inflammation leads to an increased turnover of liver cells. With time, this accelerated regeneration of hepatocytes might alter the architecture of the tissue. Fibrous tissue bands appear, expanding the portal fields and later forming bridges between them. Bridging fibrosis in combination with the formation of regeneration nodules is classified as cirrhosis, which usually

is accompanied by impaired liver function test (thrombocytopenia, reduced albumin in serum, increased bilirubin in serum). Adenomatous foci may form within the cirrhotic nodules and subsequently develop into HCC (Idilman et al. 1998). Secondly, the hepatitis B X-antigen plays some role in carcinogenesis, but whether it is through activation of signalling cascades, DNA damage through generation of reactive oxygen species or other mechanism(s) remains to be elucidated (Xu et al. 2013). PreS deletion mutants have also been suggested to participate in carcinogenesis (B.-F. Chen et al. 2006). An intracellular retention of HBV surface proteins is believed to increase stress of the endoplasmic reticulum leading to liver damage and eventually HCC. Finally, integration of HBV DNA in the genome of hepatocytes may have a tumorigenic effect as the integrated sequence might upregulate or downregulate host genes involved in cell division or tumor suppression (Z. Jiang et al. 2012; S. Jiang et al. 2012; Pollicino et al. 2013).

The above mechanisms surely act together and the risk to develop HCC is also affected by environmental factors such as exposure to aflatoxins, alcohol, smoking and diet.

2 AIMS

The overall aim of this thesis was to analyse blood samples and liver biopsies from patients with chronic hepatitis B virus infection in order to learn about viral replication, the natural history of the disease and the clinical utility of different biomarkers.

Paper I

A study of hepatitis B virus in liver tissue and in vitro to evaluate molecular methods and investigate the viral replication and correlations between hepatic viral load, serum markers and clinical stage.

Paper II

To investigate the correlation between HBsAg levels in serum and histological liver damage to assess whether this serum marker can reflect clinical stage and liver disease.

Paper III

To investigate possible mechanisms explaining the drastic decrease of serum HBV DNA between HBeAg-positive and HBeAg-negative patients by quantifying intrahepatic levels of viral DNA and RNA.

Paper IV

To investigate integration of HBV DNA in the genome of hepatocytes in different stages of chronic infection by means of the Alu-PCR method.

3 PATIENTS AND METHODS

Patients and samples

Between 1993 and 1995, 160 patients with chronic HBV infection attending the outpatient clinic at Östra hospital, Gothenburg, were included in a cross-sectional study focusing on histology and HBV DNA levels (Lindh et al. 2000). None of the patients were co-infected with hepatitis C or D viruses or HIV and none had received antiviral treatment before entering the study. The patients represented different genotypes and phases of chronic HBV infection. The regional ethics committee board approved the study and written informed consent was obtained from each patient before blood sampling and liver biopsy were obtained.

The study described in Paper I included 19 of 84 patients, from whom a piece of frozen liver biopsy was available. The patients represented genotypes A and D, and nine were HBeAg-negative and ten HBeAg-positive. In addition to the 19 liver biopsies two different cell lines were used for separate experiments. The first was the PLC/PRF/5 or Alexander cell line, known to contain several integrated sequences of HBV DNA, the other a Huh7.5 cell line that was used for transfection with a plasmid carrying HBV DNA originating from serum of a patient with genotype A.

Paper II focused on HBsAg levels and different stages of disease. All 160 patients were included in this paper as well as data on intrahepatic load of cccDNA in all the 84 patients from whom stored liver biopsies were available. The patients represented genotypes A to E; 36 patients were HBeAg-positive and 124 were HBeAg-negative.

Paper III investigated intrahepatic viral load, both DNA and RNA in liver biopsies from the 84 patients in paper II. The patients represented genotypes A-D; 16 patients were HBeAg-positive and 68 were HBeAg-negative.

Paper IV focused on analysis of integrated sequences of HBV DNA and to this purpose 48 patients of the 84 patients in paper III mentioned above, were included.

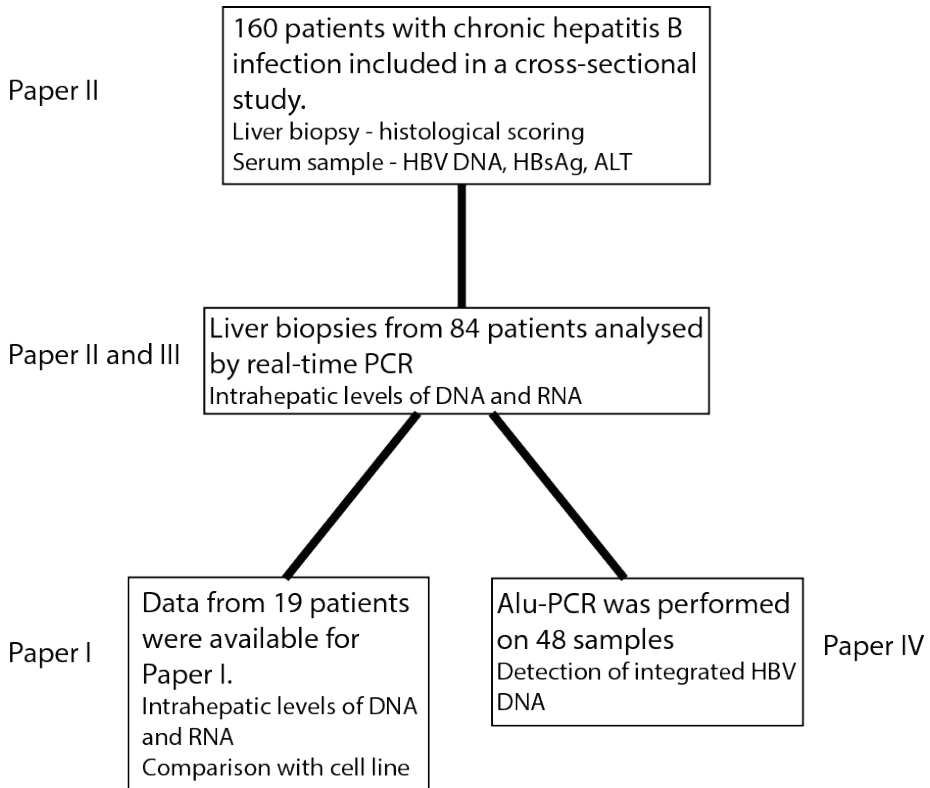


Figure 5. Flowchart of patients included in the different papers.

Serum analyses

Serum HBV DNA was analysed by Cobas Amplicor HBV Monitor (Roche Diagnostic Systems, Branchburg, NJ). HBsAg was quantified using the Architect assay (Abbott, Abbott Park, IL). Alanine aminotransferase (ALT) levels were recorded and values were expressed as ALT divided by the Upper Limit of Normal (ULN).

Histological analysis

A liver biopsy was taken in addition to the serum sample. An experienced pathologist examined the samples and the histology activity index (HAI) was scored as described by Knodell et al (Knodell, Hepatology, 1981). The inflammation score was obtained as the sum of the component scores for

piecemeal necrosis, lobular inflammation and portal inflammation. The fibrosis score was analysed separately.

Extraction of nucleic acids and DNase treatment

To extract DNA and RNA from liver biopsies, a small piece of liver, approximately 5 mg, was first homogenised in a MagNA Lyser instrument (Roche Applied Science). The homogenate obtained was diluted 1:2 and half of the material was saved for future analyses. The remaining half was inserted in a MagNA Pure robot (Roche Applied Science) and the DNA II tissue kit was used to extract both DNA and RNA. For RNA analysis, DNA was then degraded with DNase (Ambion Inc, TX). Nucleic acids from cells used in paper I was extracted using the same instruments but with the Total NA kit.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was developed in the mid eighties and is a method for the synthesis of specific DNA sequences (Saiki et al. 1985). The method was further developed by using a polymerase enzyme isolated from *Thermus aquaticus*, a thermophilic bacterium (Saiki et al. 1988). This enabled higher specificity, yield, sensitivity and length of products as the amplification reaction could be run at higher temperatures.

In this reaction, two oligonucleotide primers flanking the DNA segment to be amplified are mixed with the Taq polymerase, reaction buffer and a DNA containing sample. Repeated cycles of denaturation of DNA by heating to 95 °C, annealing of primers at 55-65 °C and extension of the annealed primers by Taq polymerase at 70-72 °C enables an exponential amplification of the selected DNA sequence, potentially a duplication with each cycle (Figure 6). With PCR it is possible to amplify even small quantities of DNA, for instance viral DNA in a small concentration.

The amplified product can then be used in several ways such as gel electrophoresis to separate bands of different lengths, sequencing, cloning or Southern blot.

In this thesis, conventional PCR was used in paper IV in a modified form called Alu-PCR.

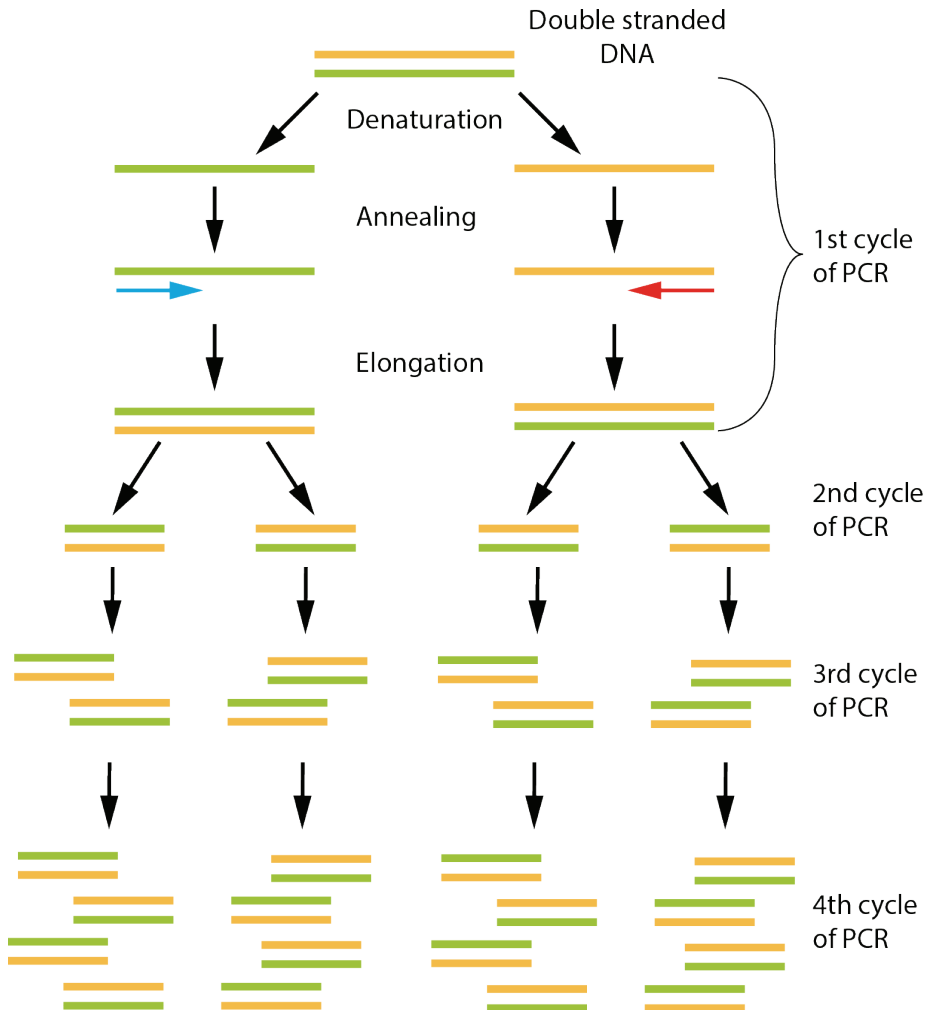


Figure 6. The principle of the polymerase chain reaction (PCR). The double-stranded DNA is denaturated at 95 °C. Annealing with specific primers at 55-65 °C. Elongation from primers at 72 °C. A new cycle starts by denaturation of newly synthesized double stranded DNA. An additional 35-45 cycles is performed, ideally with a duplication of the selected sequence each cycle.

Alu-PCR

The Alu-PCR was first described as a method to amplify human sequences specifically from hybrid cells in mouse (Nelson et al. 1989). Later, this method was adapted to search for integrated HBV DNA sequences in human DNA (Minami et al. 1995; Murakami 2005). Alu sequences are the most abundant repeat sequence in the human genome, accounting for 10% of the DNA (Batzer and Deininger 2002). They are around 300 bp each and their sequence is highly conserved. The idea of Alu-PCR is to anneal one primer to an Alu-sequence and the other primer to a potential adjacent sequence, in our case integrated HBV DNA. To make the reaction specific it is carried out in three nested amplification steps, with three different HBV primers and two Alu-specific primers. With a bit of luck the HBV DNA sequence is in the vicinity (<3000 bp) of an Alu-repeat and there will be an amplification of this hybrid sequence, containing one part of HBV DNA and the other human DNA. To confirm the specificity of the assay Southern blot or direct sequencing may be performed.

In paper IV the Alu-PCR method was used following the protocol of Murakami et al. (Figure 7)(Murakami 2005). Five µl of sample was added to a PCR mix containing High Fidelity Enzyme, MgCl₂, dNTPs, one HBV primer and one Alu-primer with a tag sequence. These primers contained dUTP. After ten cycles of PCR, the UTP containing primers were digested with Uracil-DNA glycosylase (UDG) and a new pair of primers were added, a nested HBV primer and a primer specific for the tag-sequence introduced in the first round of PCR. This was followed by 20 cycles of PCR, where the annealing temperature was initially 65 °C and decreased in a step-wise manner of 1 °C every two cycles followed by 20 cycles with an annealing temperature of 55 °C. To further amplify the PCR products, a nested PCR was performed with almost identical conditions as described above but with a new, nested, HBV primer in combination with the Alu tag-primer. The PCR products from both reactions were then migrated by gel electrophoresis. If multiple bands were detected they were excised and the gel slices were subsequently treated and prepared for sequencing. When only one band was visible the PCR product was instead purified and sequenced.

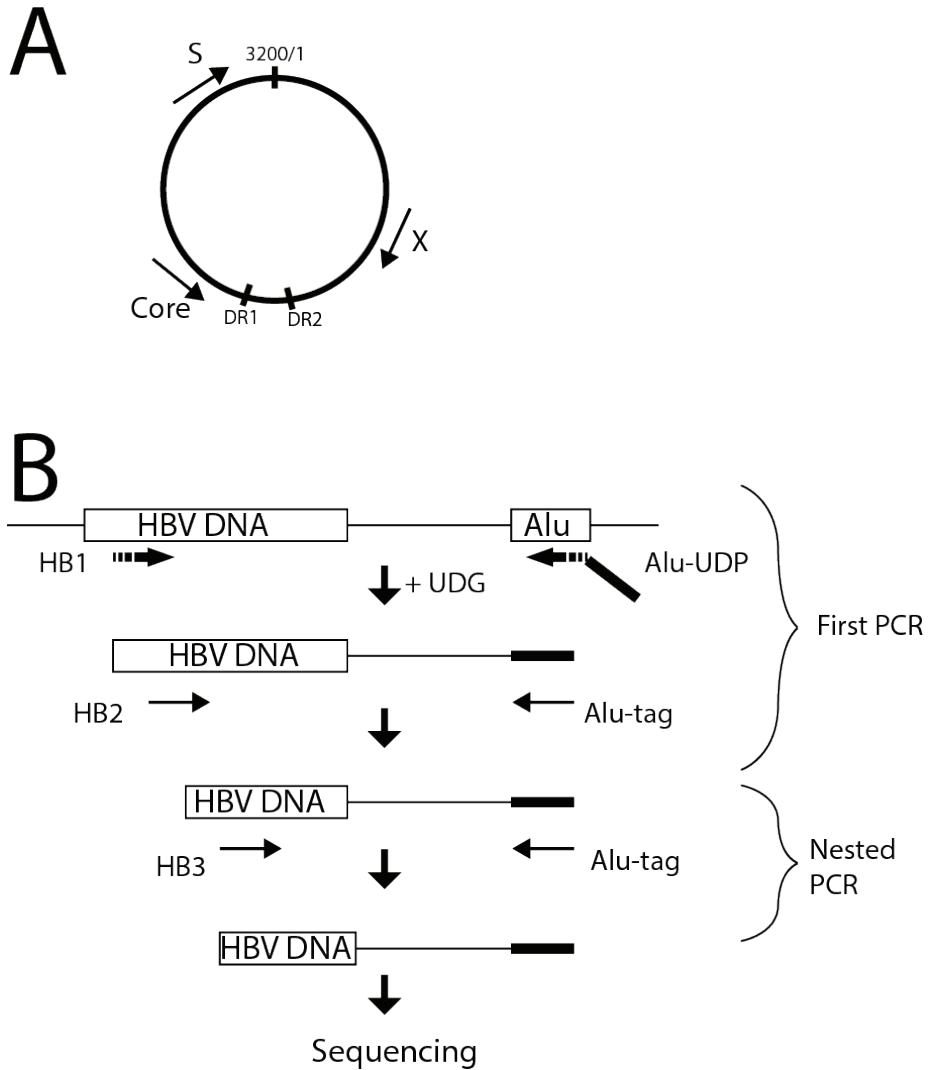


Figure 7. Schematic overview of the Alu-PCR method. A. Position of HBV primers S, X and Core and their relation to the Direct Repeats 1 and 2. B. The Alu-PCR is performed in three steps. First, UDP-containing primers are used for 10 cycles and the Alu-primer introduces a specific “tag-sequence”. The first primer pair is removed with UDG. A second HBV-primer together with an Alu-tag primer is introduced. For a nested PCR a third HBV-primer is used with the Alu-tag primer. The resulting amplicon is subjected to direct sequencing. Thin line: human DNA, thick line: tag sequence, UDG: Uracil-DNA glycosylase.

Real-time PCR

The real-time quantitative PCR method is used to quantify levels of DNA or, with an initial reverse transcriptase (RT) step, RNA (Chiang et al. 1996; Heid et al. 1996; Gibson, Heid, and Williams 1996). In contrast to traditional PCR where the result is read at end-point, real-time reactions are carried out in a thermocycler that contains a camera that registers fluorescence emitted by amplified products. In our studies we have used the TaqMan approach, developed by Applied Biosystems (Morris, Robertson, and Gallagher 1996). This method uses the 5'-3' exonuclease activity of the Taq polymerase, which then degrades a fluorescent DNA probe, previously hybridised to the sequence to be amplified. The probe contains both a fluorescent reporter and a quencher, which silences the fluorescence. When the polymerase reaches the probe the reporter is excised and with a greater distance from the quencher, its fluorescence is increased (Figure 9). During each cycle the fluorescence is measured and a plot is generated where initially there is an exponential increase in fluorescence until a plateau phase is reached when the reaction runs out of substrates or the enzyme activity is exhausted (Figure 8).

Real-time and RT-real-time PCR was used for amplification of viral DNA and RNA in papers I-III. Human beta-globin was also amplified to standardize the HBV DNA results in terms of copies per human cell equivalent. RNA-measurements were normalized to 18S transcript.

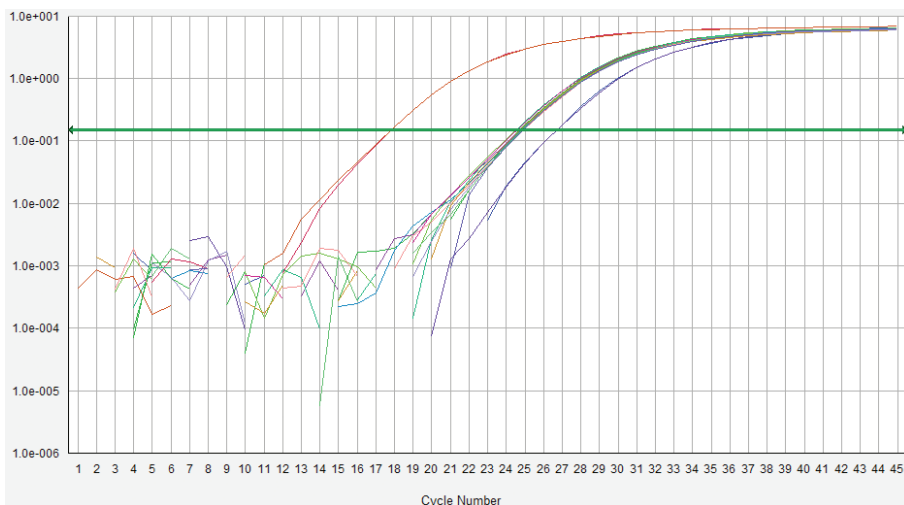


Figure 8. Amplification curves from real-time PCR. The intersection between the green line and the curves is the cycle threshold (Ct) value. Lower value means higher concentration of the target sequence in the sample.

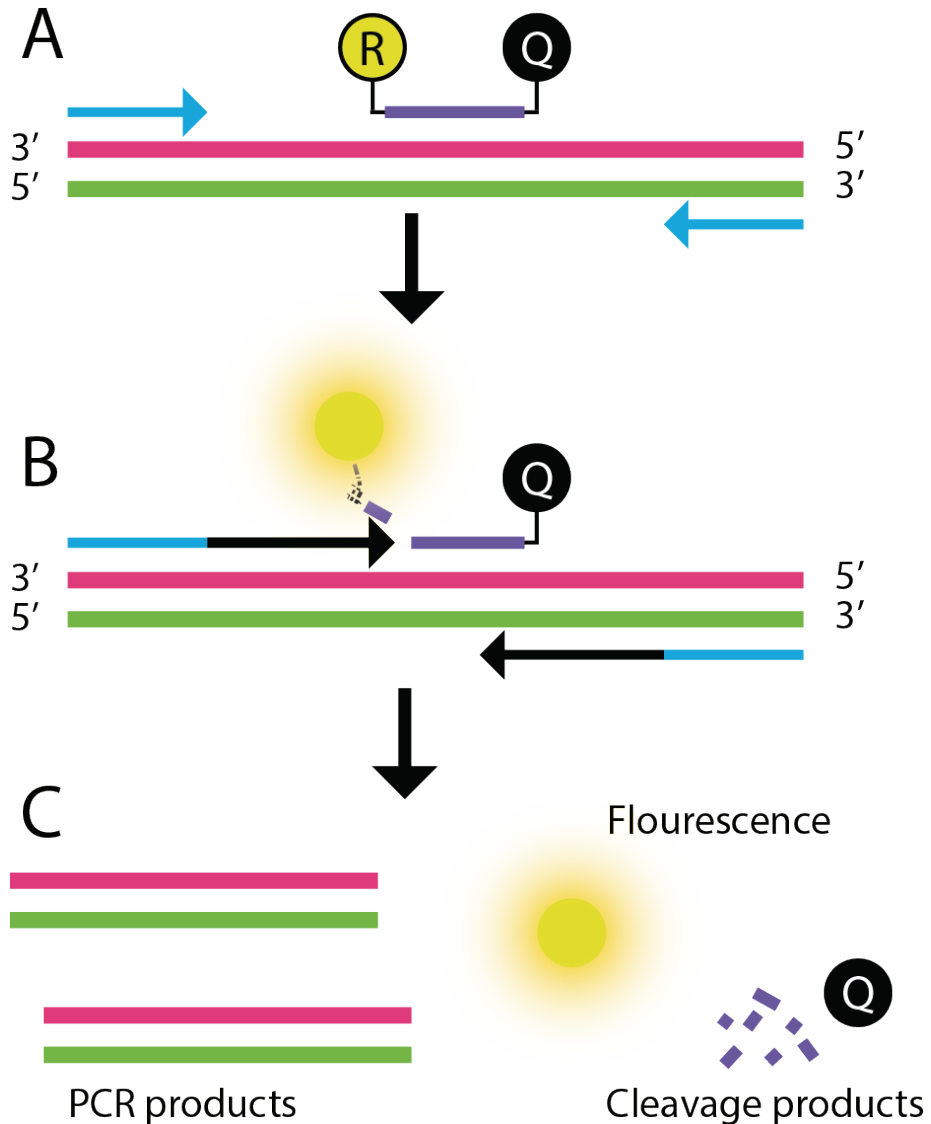


Figure 9. The principle of TaqMan real-time PCR. A. Primers and probe anneal to the target sequence. The quencher is close to the reporter, silencing the fluorescence. B. During the elongation step the reporter is excised from the probe and with a greater distance to the quencher it starts to emit light. C. With every cycle of PCR a duplication of the target sequence is achieved. Simultaneously the fluorescence becomes stronger, proportionally to the amount of PCR product.

Sequencing

The amplified sequences from Alu-PCR were sequenced using Sanger sequencing (Sanger, Nicklen, and Coulson 1977). The PCR product, sometimes as a band extracted from agarose gel, was purified and then prepared for sequencing in an ABI Prism 310 Genetic Analyzer. Analysis of the obtained sequences was performed in the MacVector software (Accelrys, Cary, NC, USA). To verify that the sequences contained both HBV and human DNA the BLAST software was used.

Cell cultures and transfection

PLC/PRF/5 and Huh7.5 cells were cultured in Dulbecco's modified medium, with supplements specific for each cell line. The cells were seeded in 12-well plates with 480 000 cells per well. After 24 h of growth at 37 °C in 5% CO₂, the Huh7.5 cells were transfected with a plasmid containing the genome of an HBV patient with genotype A. After another 24 hours the cells were washed three times and with new medium added the cells were harvested after another three days of incubation. The PLC/PRF/5 cells were harvested after three days, without transfection.

Southern blot

Part of the samples in paper IV were analysed with Southern blot to verify the specificity of the amplification (Southern 1975). After gel electrophoresis the products were blotted on a nylon Hybond N+ membrane (Amersham, Buckinghamshire, England). Hybridization was performed over night with a ³²P random prime-labeled full-length HBV genome probe. After a washing step exposure on X-Omat film (Kodak, Rochester, NY) was performed at -80°C.

Statistical methods

The statistical methods used in Paper I included Mann-Whitney U-test for comparison of groups without normal distribution, correlation by Pearson's correlation analysis and multiple linear regression. In paper II Spearman's test was also used for correlation analysis and diagnostic performance of HBsAg was evaluated by receiver operating characteristic (ROC) curve. In paper III and IV, unpaired t-test was used for comparison of values with normal distribution. The Statview and JMP softwares (SAS institute) were used for statistical analyses.

4 RESULTS AND DISCUSSION

4.1 HBV DNA and RNA quantification

The advent of PCR in 1986, facilitated quantification of genomic elements and with the introduction of quantitative real-time PCR (qPCR) ten years later, relative quantities of known sequences could be assessed with high sensitivity and specificity (Mullis et al. 1986; Heid et al. 1996). The qPCR is now widely used in diagnostics of chronic HBV infection, as a means to monitor disease progression and treatment efficacy (A. Abe et al. 1999). Quantification of HBV DNA levels in serum has been shown to be the best predictor of disease outcome (Iloeje et al. 2006). In the last couple of years, quantification of the HBsAg has been proposed as an alternative to HBV DNA in disease monitoring (Martinot-Peignoux et al. 2013). HBsAg was presumed to be a better reflection of viral load in the liver than HBV DNA, not only during treatment but also during the natural course of infection. Over all, there are few reports on viral load in the liver of patients with chronic HBV infection, probably mainly because such studies require liver biopsies, which now are rarely performed. Thus the interplay and relation between cccDNA, replicative intermediates and HBV DNA in the blood has been poorly understood. (Laras et al. 2006; Volz et al. 2007; Pollicino et al. 2011). With this in mind we investigated by means of real-time PCR several intracellular replication markers in hepatoma cell lines and liver biopsies (Paper I). Following this “pilot study”, where our methods were shown to be reliable, we continued with the analysis of additional liver biopsies and focused on the relations between serum markers, viral transcripts and HBV DNA in the liver (Paper III). First I will discuss the results of our experiments on cell lines followed by a discussion on our results from intrahepatic analyses.

In Paper I we used two different cell lines. The PLC/PRF/5 (Alexander cells) cell line is known to contain several integration sites for HBV and produces HBsAg despite the absence of replicating virus (Edman et al. 1980). To our knowledge, no group had previously quantified the levels of transcripts in this cell line. As HBsAg is produced in this cell line, sequences with an intact S-region as well as promoter region must be present. The levels of transcripts from this region were compared with core transcripts, using region specific primers and probes in a reverse-transcriptase (RT) qPCR.

In addition to the Alexander cells we used another hepatoma cell line, Huh7.5, for a transfection experiment that compared the replicative activity

of two different strains of HBV DNA from genotype A, one strain with a wild-type genome and the other with an AGG → TGA double mutation at position 1762-64 in the BCP region.

In figure 10, levels of transcripts are compared between cell lines and patients. The cccDNA level was almost one per cell equivalent in the Alexander cells, indicating presence of a single integrated sequence spanning the region amplified by the cccDNA primers. This is in line with results from sequencing of the integrated HBV DNA in Alexander cells (Shaul et al. 1984; Koch et al. 1984; Ziemer et al. 1985). In Huh7.5 cells, which do not contain integrated HBV DNA, the cccDNA qPCR probably detected circularised DNA introduced with the plasmid, a rate of 6 copies per cell.

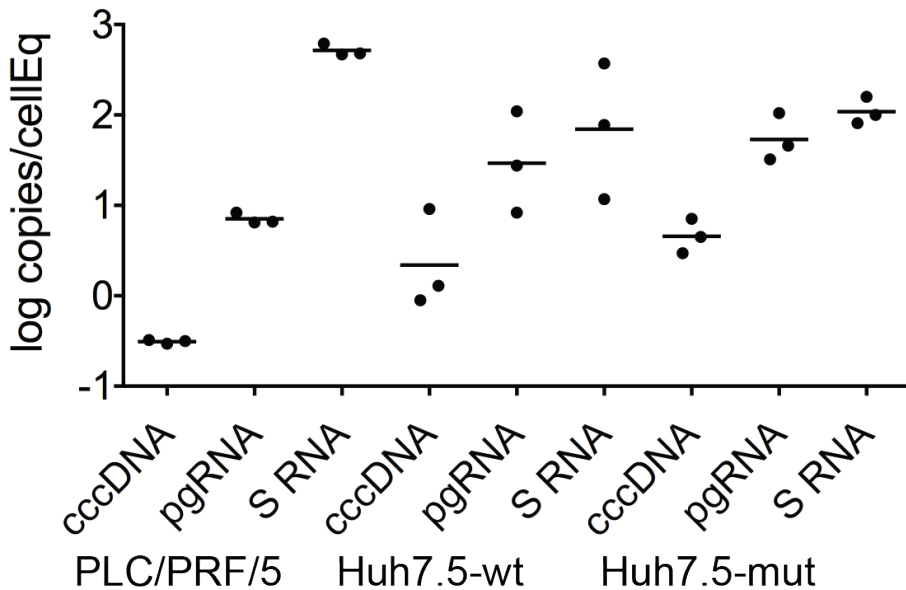


Figure 10. Levels of cccDNA and viral transcripts per cell equivalent in PLC/PRF/5 cells and Huh7.5 cells transfected with either wild type or BCP-mutant HBV strain. PLC/PRF/5 produced a 100-fold more S transcripts as compared to pgRNA transcripts (≈ 2 log difference). In transfected Huh7.5 cells the expression was comparable between wild type and mutant HBV strains with a small difference between pgRNA and S RNA.

Integrated HBV DNA has long been suggested to be a potential contributor to HBsAg that is detected in the blood in chronic HBsAg carriers. In the Alexander cell lines we observed a significant amount of S-RNA transcripts, outnumbering the core transcripts by almost a 100-fold (Figure 10). The

levels of S-RNA per cell were comparable to those found in biopsies from HBeAg-positive patients. These observations suggest that integrated HBV DNA sequences might contribute significantly to HBsAg production also in infected patients. It is however not known at all how large proportion of hepatocytes that has to carry integration in order for their HBsAg production to be significant. Probably their contribution is more likely to be of importance in HBeAg negative patients, in whom cccDNA levels have declined and the proportion of hepatocytes that are HBsAg positive has reached levels below 10%. If a similar fraction of the hepatocytes carry integrated HBV DNA segments carrying the S region and its promoter, it is reasonable to believe that their production of HBsAg could be in the range of what is produced from cccDNA. Such a degree of integration is not unlikely even in a patient without HCC, as discussed later.

No significant differences were found in pgRNA (=core RNA) levels between wild-type and mutant strains, transfected in Huh7.5 cells. A previous study has described suppression of precore mRNA transcripts in patients with the double BCP mutation A1762T/G1764A (Laras, Koskinas, and Hadziyannis 2002). We used the samples from paper I to test this hypothesis using specific primers for precore mRNA. However we did not find any difference in expression between wild type and mutant strains. Another study did not find any influence from BCP mutations at 1762/64 on the precore levels in vitro either, but reported that mutations in other positions had an impact (Jammeh et al. 2008). These diverging results might reflect that conditions in vitro are different from those in patients.

The putative diverging impact on precore RNA and pgRNA was further investigated in tissue samples from patients known to have either precore mutation or BCP mutation. In 18 patients carrying HBV of genotype B or C precore and pregenomic RNA was quantified using the primers of Laras et al. No apparent differences could be found as regards BCP 1762/1764 mutations on the levels of these transcripts, which is in line with the in vitro results of Jammeh et al. The conditions might be different in patients carrying genotypes A or D, but also mutations in other positions could be important.

After the initial results from liver biopsies in paper I we went further, analysing samples from a total of 84 patients. Of these, 16 were HBeAg-positive and 68 were HBeAg-negative. Clinical data on these patients are shown in Table 2. Our main aim with this study was to find explanations to the drastic difference in HBV DNA between HBeAg-positive and HBeAg-negative patients (Maruyama et al. 1998). Previous reports have suggested that this decline is mainly due to a reduction of the cccDNA pool (Werle

Lapostolle et al. 2004; Wong et al. 2004; Laras et al. 2006) and reduced transcription of pgRNA (Volz et al. 2007), and we confirmed these findings in our group of patients. However, the total decline of HBV DNA in serum seems to be greater than the contribution of the above-mentioned factors, and we wanted to investigate what these additional factors could be.

Table 2. Characteristics of patients included in paper III.

	HBeAg+ n=16	HBeAg- n=68	P value
Age, years, median, (range)	27 (16-60)	34 (18-59)	0.02
Gender (M/F)	11/5	40/28	0.57
Serum HBV DNA ^a	8.30 ± 0.38	4.30 ± 0.14	<0.0001
Serum HBsAg ^b	4.40 ± 0.18	3.41 ± 0.11	<0.0001
HBV DNA/HBsAg	3.90 ± 0.29	0.89 ± 0.14	<0.0001
ALT/ULN, median, (range)	1.37 (0.68-8.6)	0.72 (0.24-12.38)	<0.001
cccDNA ^c	-0.70 ± 0.20	-2.67 ± 0.10	<0.0001
S RNA ^c /cccDNA ^c	1.65 ± 0.20	2.73 ± 0.11	<0.0001
pgRNA ^c /cccDNA ^c	2.04 ± 0.12	1.50 ± 0.11	0.02
pgRNA ^c /S RNA ^c	0.39 ± 0.23	-1.22 ± 0.07	<0.0001

^a log₁₀ cp/mL; ^b log₁₀ IU/mL; ^c log₁₀ copies/cEq (cell equivalent), ALT/Upper Limit of Normal. Virological values as mean ± SEM. P values by Mann-Whitney U test for age and ALT, t test for virological parameters and Fisher's exact test for gender.

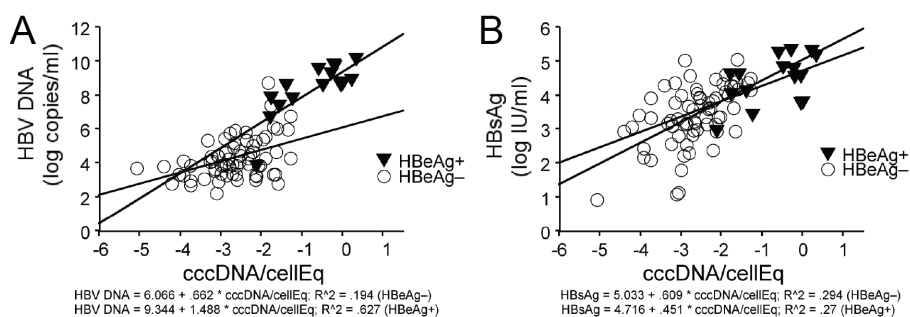


Figure 11. Correlation between serum levels of HBV DNA (A) and HBsAg (B) with cccDNA.

As seen in Figure 11 both HBsAg and HBV DNA showed a good correlation with cccDNA, even though the latter correlation was better in HBeAg-positive than in HBeAg-negative patients. To get a better understanding of the steps from cccDNA to serum marker we quantified the different

intermediates using real-time PCR for HBV DNA and real-time RT PCR for HBV RNA. The results are summarized in Table 2 and Figure 12.

Between HBeAg-negative and HBeAg-positive patients there was a mean difference in cccDNA of about $2 \log_{10}$ copies/cell. This is in line with previous studies (Werle Lapostolle et al. 2004; Volz et al. 2007). The transcriptional efficiencies of the two main actors, pgRNA and S RNA, differ between the two groups. As in a previous report we found that the pgRNA/cccDNA ratio was lower in the HBeAg-negative patients (Laras et al. 2006; Volz et al. 2007). However, what has not been previously shown is the higher level of S RNA/cccDNA we observed in this group. This finding agrees with the observation by us and others that the levels of HBsAg decline to a lesser extent than the HBV DNA levels when serum HBeAg is lost, and may help to explain this phenomenon. The explanation might be that the transcription of S RNA is not down-regulated to the same extent as pgRNA (or that the half-life of S RNA is longer) when HBeAg is lost. Alternatively, as mentioned earlier, there might be a significant transcription of S RNA from integrated HBV DNA sequences explaining this relative increase in HBeAg-negative patients.

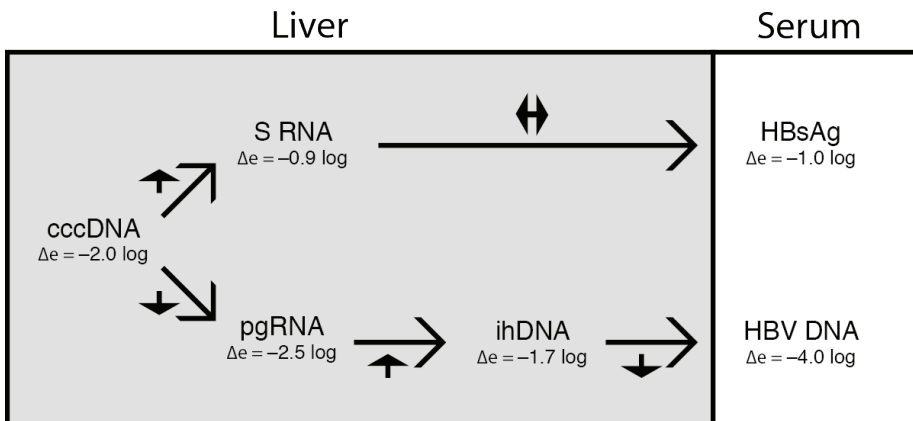


Figure 12. Flowchart of differences in virological parameters between HBeAg-negative and HBeAg-positive patients. Δe =mean difference between groups. Small arrows indicate increase or decrease in ratio between two parameters. The ratio HBsAg/S RNA was unchanged between groups.

A flowchart summarising the differences between HBeAg positive and negative patients for all viral parameters is presented in Figure 12. It specifies the contributions of each step from cccDNA to differences in serum levels of HBV DNA and HBsAg.

As shown in the figure and discussed above, cccDNA levels were 2 \log_{10} lower in HBeAg negative as compared with HBeAg positive patients. At transcript level this difference was bigger for pgRNA (to $-2.5 \log_{10}$), but was smaller for S RNA ($-0.9 \log_{10}$). The latter difference was similar in the blood where serum levels of HBsAg were one \log_{10} lower in HBeAg-negative patients. In contrast, the mean serum levels of HBV DNA were 4.0 \log_{10} copies/ml lower in the HBeAg-negative than in HBeAg-positive patients. This difference is even bigger than observed for pgRNA, suggesting an additional mechanism acting between pgRNA and HBV DNA in serum. To explore this we analysed total HBV DNA in liver tissue (intrahepatic DNA, ihDNA) using the same primers as in the pgRNA PCR, but without the RT step and without digesting DNA by preincubation with DNase. This PCR amplifies all DNA species that contain the targeted segment (nt 2367-2428), including rcDNA (the species in the virions), linear forms and cccDNA. We found that there was a strong correlation in HBeAg-positive patients but not in the HBeAg-negative group. The mean difference between HBeAg positive and negative patients in the level of ihDNA was 1.7 \log_{10} , a difference 0.8 \log_{10} smaller than observed for pgRNA (Figure 12), suggesting that HBV DNA might accumulate in the hepatocytes in the HBeAg-negative phase. The possibility that intrahepatic HBV DNA might accumulate is supported by reports by others that viral particles might be blocked by anti-HBs antibodies that are internalized in hepatocytes (Schilling et al. 2003; Neumann et al. 2010). This proposed mechanism is interesting and could explain our observation, but to our knowledge no one has pursued research on this topic. Another group investigated intrahepatic data in both HBeAg-negative and HBeAg-positive patients and concluded that export rates were similar as they found a significant correlation between serum HBV DNA and ihDNA in the two groups (Volz et al. 2007). The patients in their study all had persistently elevated ALT, whereas the patients in our study had significantly lower levels of ALT, a difference that might contribute to the diverging observation.

As mentioned earlier, Figure 12 describes differences in viral markers between the two groups, HBeAg-negative and HBeAg-positive patients, and thus gives an indication of mechanisms that might explain loss of HBeAg. Alternatively, and possibly more relevant for understanding the processes leading to loss of HBeAg, may be to analyse the correlation between replicative intermediates in HBeAg-positive patients only. Such an analysis is presented in Figure 13.

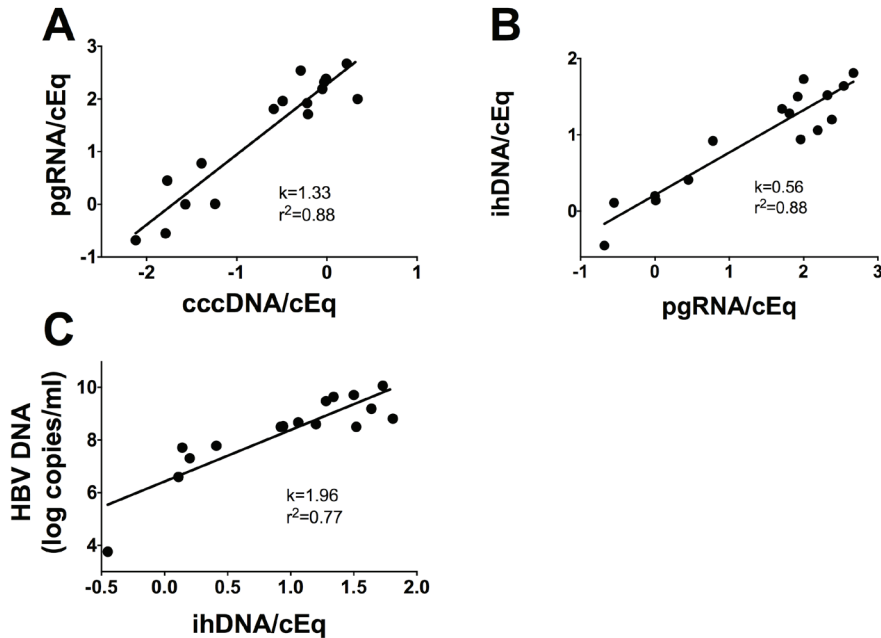


Figure 13. Correlations between different replicative intermediates in HBsAg-positive patients presented in a stepwise manner from the template cccDNA to serum HBV DNA. A. pgRNA vs. cccDNA. B. ihDNA vs. pgRNA. C. Serum HBV DNA vs. ihDNA. cEq=cell equivalent

Firstly it indicates that cccDNA spans over more than 2 \log_{10} units within the HBsAg-positive phase, and that a 2 \log_{10} decline corresponds to $\approx 2.7 \log_{10}$ decline in pgRNA ($k=1.33 \log_{10}$, A). This $\approx 0.7 \log_{10}$ contribution from reduced transcription of pgRNA agrees well with the observation from comparing the HBsAg positive and negative groups. The next graph (B), shows that a difference in pgRNA of $\approx 3 \log_{10}$ only corresponds to a $\approx 1.7 \log_{10}$ decline of ihDNA ($k=0.56$), in agreement with the proposed accumulation of ihDNA. The last graph (C) shows that the span of ihDNA is much smaller than the wide span observed for HBV DNA in serum (4 \log_{10}). This observation (reflected by $k=1.96$), that HBV DNA in serum declines much more than ihDNA suggests that there is an additional mechanism that serves to reduce HBV DNA in HBsAg positive phase, for example an enhanced clearance of free virions leading to a shorter half-life..

Several studies have reported that virion half-life decreases with decreasing levels of HBV DNA and that there is a difference between HBeAg-groups (Dahari, Shudo, et al. 2009; Dandri et al. 2008; Dahari, Cotler, et al. 2009; Ribeiro et al. 2010). The mechanism for this association is not well known, but it seems reasonable to believe that a shorter half-life of virions in the blood is a result of an upgraded immune response towards HBV, and should lead to lower serum levels of HBV DNA.

Taken together, our findings from paper III indicate that in addition to reduced levels of cccDNA and a reduced transcriptional efficiency of pgRNA, a decreased export of virions and a decreased half-life of virions may contribute to reduce the levels of HBV DNA in blood at loss of HBeAg. Furthermore we found higher levels of S RNA per cccDNA in the HBeAg-negative group indicating higher transcription as compared with HBeAg-positive patients or as compared with pgRNA. Whether this can be attributed to transcription from integrated sequences of HBV DNA will be discussed further in section three of this part of the thesis.

4.2 HBsAg quantification for identification of liver disease

It took ten years from the discovery of the Australia antigen until the first assay for quantification of HBsAg was presented (Gerlich and Thomssen 1975). As both HBsAg and HBV DNA are synthesised from the same template, the cccDNA minichromosome, it has been suggested that the former might be useful as a complement or alternative to quantifying HBV DNA as a marker for viral load in the liver. (Sonneveld, Zoutendijk, and Janssen 2011; Liaw 2011; Chan, Thompson, et al. 2011). Serum levels of HBsAg are more stable during the natural history of the infection and the levels decrease with a different pace than HBV DNA over time. HBsAg could also be used to monitor the disease in patients under nucleoside analogue treatment when levels of HBV DNA in serum usually become undetectable while the cccDNA pool in the nucleus of hepatocytes decreases very slowly, as reflected by levels of HBsAg.

In paper II we wanted to investigate the potential role of HBsAg as a marker for liver disease in a group of patients with chronic HBV infection. A total of 160 patients had previously been characterised in a study focusing on histology and HBV DNA levels (Lindh et al. 2000). In addition to serum levels of HBV DNA, histological examination of liver biopsies was made by an experienced pathologist and HBsAg was quantified as well as levels of ALT, as a marker for liver disease. In 84 patients a small part of the liver biopsy had been stored at -70°C and was available for analysis of intrahepatic levels of cccDNA. Patient characteristics are found in Table 3.

A few previous studies have used quantification of HBsAg to distinguish active from inactive carriers (Brunetto et al. 2010; Park et al. 2011). Brunetto et al. suggested that $<3 \log_{10}$ IU/ml of HBsAg alone or in combination with low levels of HBV DNA would identify HBeAg negative patients with low risk of liver disease and thus not in need of treatment. Compared with that study our group of patients was more heterogenous and comprised both HBeAg-negative and HBeAg-positive patients. We used a modified form of the classification proposed by EASL (European Association For The Study Of The Liver 2012) to categorise the patients into four groups. The immune tolerant (IT) and e-antigen positive hepatitis (EPH) groups included patients that are HBeAg-positive whereas the Inactive carrier (IC) and e-negative hepatitis (ENH) groups contained the HBeAg-negative patients. Characteristics of the four groups are found in table 4.

Table 3. Characteristics of patients included in paper II.

	All patients (n=160)	HBeAg positive (n=36)	HBeAg negative (n=124)
Age, years	33 (16-66)	27 (16-66)	35 (18-61)
Gender, F/M n	55/105	12/24	43/81
Genotype (A/B/C/D/other ^a)	32/22/14/81/11	28/14/5/68/9	4/8/9/13/2
HBsAg (log IU/mL)	3.70 (0.92-5.46)	4.72 (2.91-5.46)	3.54 (0.92-5.04)
HBV DNA (log copies/mL)	4.43 (2.20-10.10)	8.56 (3.76-10.10)	4.15 (2.20-8.80)
ALT/ULN ^b	0.83 (0.21-8.5)	1.48 (0.24-8.13)	0.70 (0.21-8.50)
Fibrosis score ^c			
0-1	122	23	99
3-4	38	13	25
Inflammation score			
≤6	135	24	111
≥7	25	12	13

^a genotype E, n=2; genotype I, n=1; not detectable, n=8.

^b ULN, upper limit of normal.

^c Fibrosis score 2 is lacking in the Knodell scoring system.

Median values are given for HBsAg, HBV DNA and ALT.

Table 4. Classification of patients used in paper II

	IT	EPH	ENH	IC
HBV DNA (log ₁₀ copies/ml)	>6	Any	>4	<4
ALT/ULN	Normal	>ULN	>ULN	Normal
Inflammation	≤3	≥4	*	≤3
HBeAg	Positive	Positive	Negative	Negative

*ENH was further divided based on inflammation score ≥4 or ≤3, referred to as moderate-severe and mild inflammation respectively.

IT=Immune tolerant, EPH=HBeAg-positive hepatitis, ENH=HBeAg-negative hepatitis, IC=Inactive carrier

In line with observations in previous studies we found differences in the levels of HBV DNA, HBsAg and the ratio of HBV DNA/HBsAg between groups (Jaroszewicz et al. 2010; Nguyen et al. 2010). Both HBV DNA and HBsAg correlated with cccDNA levels in the liver, in a similar manner as described by others (Wong et al. 2004; M. Wang et al. 2012; Chan et al. 2007). The viral productivity, expressed as the ratio of HBV DNA/HBsAg serum levels, was 300 times higher in HBeAg-positive patients as compared with HBeAg-negative patients. This ratio also differed between the different subgroups, however the results are sometimes difficult to compare with previous studies where the log ratio has been confounded with the ratio of log values (Jaroszewicz et al. 2010; Nguyen et al. 2010; Park et al. 2011).

HBsAg correlated with HBV DNA, age and ALT (the latter only in HBeAg-negative patients as the IT patients have high levels of HBsAg but minimal liver damage). Our results indicate that HBsAg could be a useful marker for liver disease. We further investigated this by ROC-curve analysis of the relation between HBsAg levels and histological inflammation score. With the results we tried to find cut-offs suitable for clinical application as a means to distinguish patients with minimal liver damage in the HBeAg-negative group.

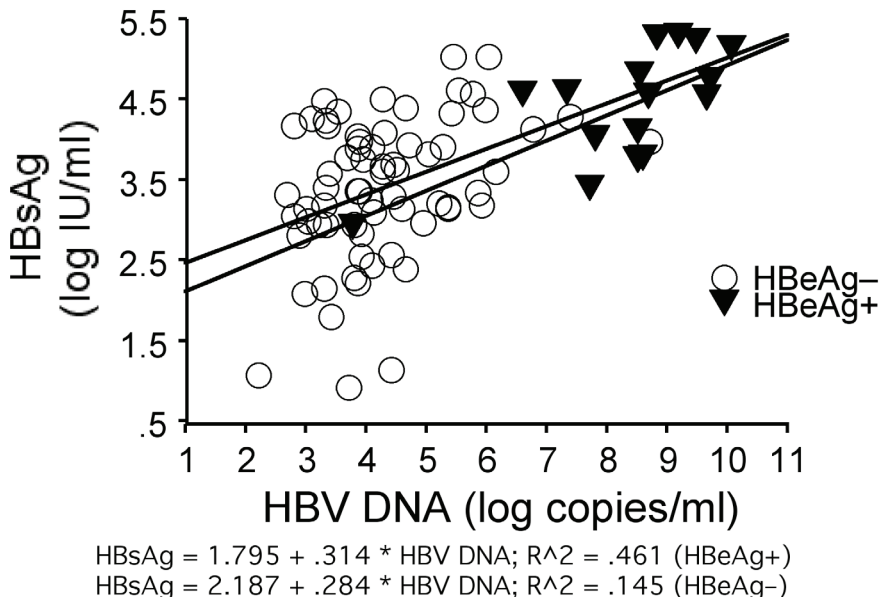


Figure 14. Correlation of HBsAg with HBV DNA in 36 HBeAg-positive and 124 HBeAg-negative patients.

A previous study from Italy used HBsAg-levels to identify inactive carriers among HBeAg-negative patients (Brunetto et al. 2010). They monitored the patients for one year to be able to categorise them accurately. We had cross-sectional data on HBsAg levels and histological score and wanted to evaluate the possibility to use HBsAg levels to identify patients with minimal liver disease. We found that low levels of HBsAg and HBV DNA, alone or in combination, could identify patients with minimal inflammation with high specificity and a good positive predictive value (Table 5). However, the sensitivity is low, meaning that many patients with minimal inflammation had levels of HBsAg and HBV DNA above our cut-off, 3.0 log₁₀ IU/ml and 4.0 log₁₀ copies/ml respectively. The specificity we obtained, 89%, 79% and 96% for HBsAg, HBV DNA and a combination of these, was comparable to the Italian study mentioned but the sensitivity was poorer. Thus, a single time-point measurement of HBsAg is a useful tool to identify patients with minimal inflammation but for patients above the cut-off additional monitoring, including biopsy might be necessary to rule out liver disease.

Table 5. Predictive values for serum levels of HBsAg and HBV DNA for identification of minimal inflammation in 124 HBeAg-negative patients.

	Minimal inflammation (HAI score ≤3)						
	Yes	No	PPV	Sens	NPV	Spec	
HBsAg <3 log IU/mL	Yes	33	3	92%	34%	28%	89%
	No	63	25				
HBV DNA <4 log cp/mL	Yes	49	6	89%	51%	32%	79%
	No	47	22				
HBsAg <3 log IU/mL + HBV DNA <4 log cp/mL	Yes	22	1	96%	23%	27%	96%
	No	74	27				

PPV: positive predictive value; Sens: Sensitivity; NPV: Negative predictive value; Spec: specificity

When comparing genotypes A to D, we did not find any significant difference in levels of HBsAg, neither in HBeAg-positive nor HBeAg-negative patients. We compared previous studies of HBsAg levels and found that patients carrying genotype B or C had lower levels as compared with genotype A or D (Nguyen et al. 2010; Thompson et al. 2010; Chan et al. 2010; Brunetto et al. 2010; Jaroszewicz et al. 2010; Togo et al. 2011; Park et al. 2011; Jang et al. 2011; Y. J. Kim et al. 2011). Genotype might therefore have to be considered when comparing results from patients in different studies.

The utility of quantitative HBsAg has been evaluated in different contexts: as a tool for monitoring patients under nucleoside analogue treatment (Gramenzi et al. 2011; Seto et al. 2013), to introduce a stopping rule for patients under interferon treatment (Rijckborst et al. 2012), to identify inactive carriers (Brunetto et al. 2010), to predict spontaneous HBsAg seroconversion (Ruan et al. 2013; Y.-C. Chen et al. 2012; Chan, Wong, et al. 2011) and identify patients at high risk of HCC in patients with low HBV load (Tseng et al. 2012). HBsAg indeed seems promising as a clinical marker because it is more stable than HBV DNA during the natural course of infection and remains detectable during antiviral treatment. Concerns have however been raised regarding the potential impact on HBsAg quantification by the genetic variability in the preS/S-region, as suggested by dissociation between HBV DNA and HBsAg levels in patients carrying preS/S-variants (Pollicino et al. 2012). This observation indicates that HBsAg titer must be interpreted with caution in patients with chronic hepatitis B virus infection.

The most widely used instrument for serum HBsAg quantification is the Architect robot from Abbott, which was used in the first large-scale study of HBsAg quantification (Deguchi et al. 2004). Since then, the number of published papers on HBsAg quantification has increased on PubMed, with a peak in 2011 of 46 papers. Meanwhile the mean impact factor of journals publishing these papers peaked in 2010 (mean 7.8) and has since declined to “baseline” levels (Figure 15). A recent review summarizes that HBsAg might be useful in identifying inactive carriers and as a tool for evaluating the efficacy of PEG-IFN treatment during antiviral treatment (Martinot-Peignoux et al. 2013). Its usefulness during NA treatment remains to be determined. This could be an important step in creating stop rules for these therapies, today considered to be life-long. Indeed, a recent study found that a HBsAg cut-off value of log 2.31 IU/ml at initiation of treatment predicted HBsAg seroclearance up to six years after cessation of treatment with an AUROC of 0.85 (sensitivity 83.9 %, specificity 81 %) (Ruan et al. 2013). More studies where antiviral treatment is stopped after successful viral suppression and normalisation of ALT levels are needed to confirm the role of HBsAg as a predictor of HBsAg clearance.

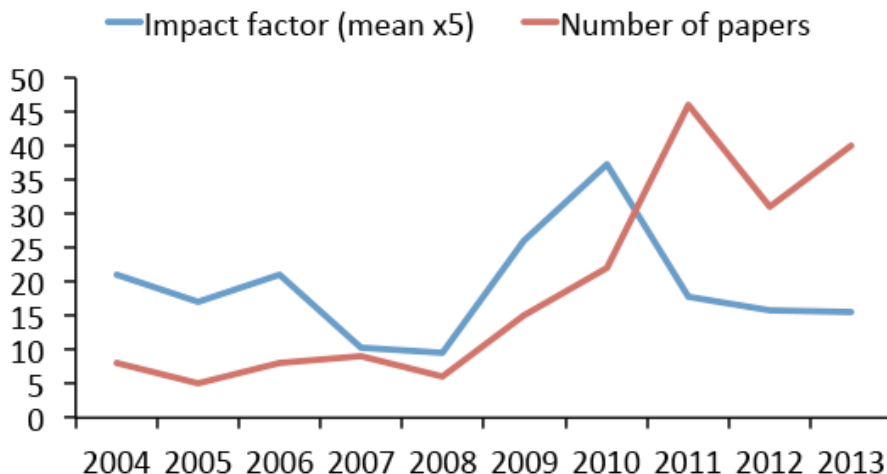


Figure 15. Graph showing the number of papers on quantification of HBsAg indexed by PubMed (red line) and the mean impact factor of these papers (multiplied by five for graphical reasons (blue line)).

As we showed in paper III, HBsAg is a good marker for its template, the S RNA transcript. However, the levels of S RNA seem to be dependent on different factors during different phases of chronic HBV infection. Possibly, the contribution of S RNA from integrated sequences of HBV DNA might increase over time and stay at significant levels even with a general decline in intrahepatic viral load. Thus, HBsAg quantification may reflect the HBV infection better in the HBeAg-positive phase and results in later phases of disease should be interpreted with caution. However, according to our study a group of patients with minimal liver disease can be identified among HBeAg-negative patients using cut-offs for HBV DNA and HBsAg levels. Close monitoring including liver biopsies might be needed in other cases.

4.3 Integration of HBV DNA in chronic HBV carriers

In the mid 1970s, a new cell line was established from tumour tissue of a patient with HCC (Alexander et al. 1976). The cell line, PLC/PRF/5 but commonly called Alexander cells, after one of its discoverers, was shown to produce HBsAg, despite the fact that no virions could be detected (MacNab et al. 1976). Further investigations revealed that these cells contained several integrated sequences of HBV DNA (Edman et al. 1980; Twist et al. 1981; Bréchet, Hadchouel, Scotto, Degos, et al. 1981; Shaul et al. 1984). Since then, integrated HBV DNA sequences have been attributed an oncogenic potential that together with cirrhosis, caused by the host immune response, and effects of the X protein, may promote the development of HCC (Bonilla Guerrero and Roberts 2005). Most studies on integration have focused on tumour tissue from explanted livers, possibly because of the large amount of material available after surgery (Bréchet et al. 1980; Shafritz and Kew 1981; Sung et al. 2012; S. Jiang et al. 2012; Z. Jiang et al. 2012). However, there are also some studies performed on liver tissue from chronic HBV patients who have not developed HCC (Bréchet, Hadchouel, Scotto, Degos, et al. 1981; Shafritz 1982; Fowler et al. 1986; Fagan et al. 1991; Huang et al. 2005)

Different methods have been used for the assessment of integrated HBV DNA. These include cloning and hybridization (Shafritz et al. 1981; Fowler et al. 1986), ligation-cassette PCR (Tamori et al. 2003), Alu-PCR (Minami et al. 1995; Murakami et al. 2004; Pollicino et al. 2013), inverse PCR (Mason et al. 2010; Tsuei et al. 1994; Tsuei et al. 2002; Huang et al. 2005) and recently, next generation sequencing (Sung et al. 2012; Fujimoto et al. 2012; Toh et al. 2013; K.-W. Tang et al. 2013). As described in the Methods section Alu-PCR takes advantage of the most common repeated sequence in the human genome, the Alu-repeat, making it possible to detect HBV DNA sequences in the vicinity of these repeats. Alu-PCR is a suitable method when the amount of material is limited, and because we had only small parts of a liver biopsies, we chose Alu-PCR to characterise the pattern of integration in different phases of chronic HBV infection.

A total of 48 biopsies were included in this study, 14 from HBeAg-positive patients and 34 from HBeAg-negative patients. Clinical data of the patients are shown in Table 6. Integrations were detected in 36 patients as indicated by gel electrophoresis. Direct sequencing was performed in 32 cases, and some of the samples had several integrations giving a total number of sequences of 45. Thus, integrations were found in two thirds of the patients,

indicating that this is a very common event. From a previous study of integrations using next generation sequencing on samples from patients with HCC, it was shown that almost 30 % of the integrations detected were located >3 kb from the nearest Alu-repeat (Sung et al. 2012). This finding combined with the lower level of detection for Alu-PCR (100 copies) suggests that integrations could be present in all patients, even though we could not detect them. Furthermore, one liver biopsy is approximately 5 mg (1/10⁶ part of the liver) making it possible for millions of different integrations to be present in the liver of a chronic HBV patient at a given time point. Our figures are in contrast with the findings from Sung et al, because in non-tumour tissue they found few integration break points. Possibly, this reflects a limitation of the broad next generation sequencing technique used in that study, which requires that cells carrying integrations have to be clonally expanded to a certain level for the integration to be detectable.

Table 6. Characteristics of patients included in paper IV.

	Integration	No integration	p
Age ^a	30.5 (26-40)	38.5 (27-48)	>0.05
Gender (M/F) ^b	22/10	5/11	<0.03
Serum HBV DNA ^a	5.69 (3.84-8.52)	3.89 (3.35-4.44)	0.04
serum HBsAg ^a	3.68 (3.15-4.58)	3.35 (2.98-3.96)	>0.05
ALT/ULNa	0.76 (0.56-1.59)	0.67 (0.56-1.06)	>0.05
Genotype (A/B/C/D)	6/7/2/17	8/0/2/6	
Clinical stage (IT/EPH/ENH/IC)	3/11/11/7	0/2/6/8	
total ihHBV DNA ^c	11.15 ±0.15	10.17 ±0.28	0.002

a = median (range); b = Fisher's exact test; c = mean ±SEM

The group with no detectable integration had a lower level of intrahepatic DNA (ihDNA). This indicates that the higher amount of substrate for integration, i.e. replicative intermediates, the higher the probability for integration.

It has been suggested that a linear form of HBV DNA is the origin of integrated HBV DNA sequences (W. Yang and Summers 1999). Evidence for this is that most breakpoints for integration are found near direct repeats 1 and 2 (Takada et al. 1990; Sung et al. 2012; Z. Jiang et al. 2012). As seen in figure 16, where all sequences containing HBV DNA from our samples are aligned to a reference genome, most of them have one of the ends in this

region. However two samples contained HBV DNA sequences spanning this region and thus other theories of integration are not excluded (Bonilla Guerrero and Roberts 2005).

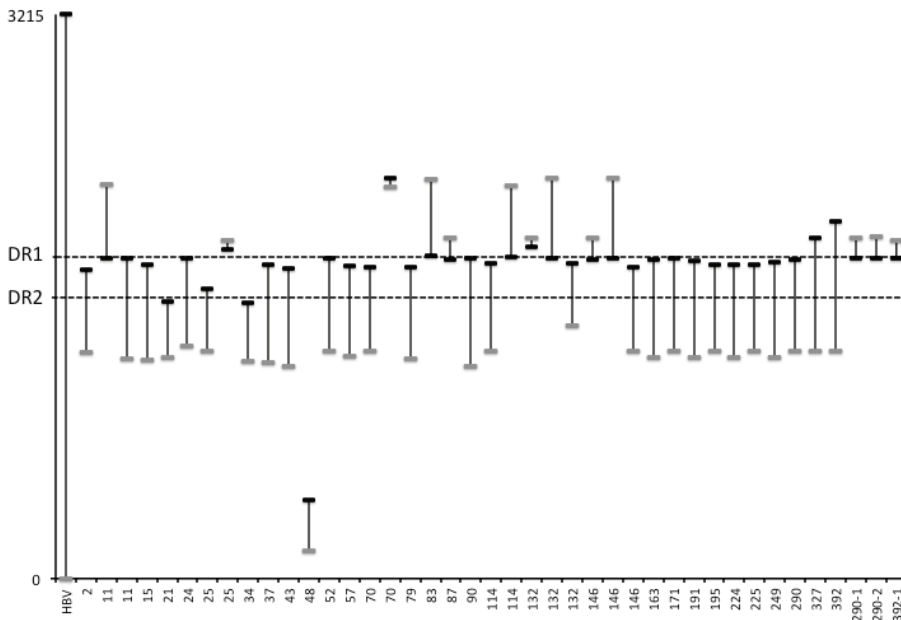


Figure 16. Alignment of integrated sequences containing HBV DNA to a reference genome (3215 bp). Direct repeats 1 and 2 are indicated with a dotted line.

Some studies on HCC have reported preferential integration sites for HBV DNA, such as the MLL4 and TERT genes (Sung et al. 2012; Saigo et al. 2008; K.-W. Tang et al. 2013). In our patients the integrations were evenly distributed on various chromosomes. The findings of specific genes involved in carcinogenesis as targets for HBV DNA might be biased, as these integrations are likely to have contributed to tumour development. We found however that 40% of integrated sequences were located in intragenic regions, somewhat surprising as only 1-5% of the human genome consists of coding regions (Strachan and Andrew 2010). This might be due to the fact that Alu-repeats are more often found in intragenic regions as compared with intergenic regions. (Grover et al. 2004). Whether HBV DNA preferentially integrates in coding regions of the human DNA warrants further study. Taken together our observations support the notion of a random site of integration for HBV DNA.

When submitted to the BLAST alignment tool, two of the sequences, patient 381 and 392, contained rearrangement of the HBV DNA. This has been previously reported by others (Takada et al. 1990). Their explanation was that an illegitimate recombination takes place between two complementary viral strands by means of a nucleotide identity at the junction site in a weakly homologous region (patchy homology). We tested this on our sequences and found similar results (Figure 17). Whether rearrangements are common prior to integration and their possible clinical significance remains to be investigated.

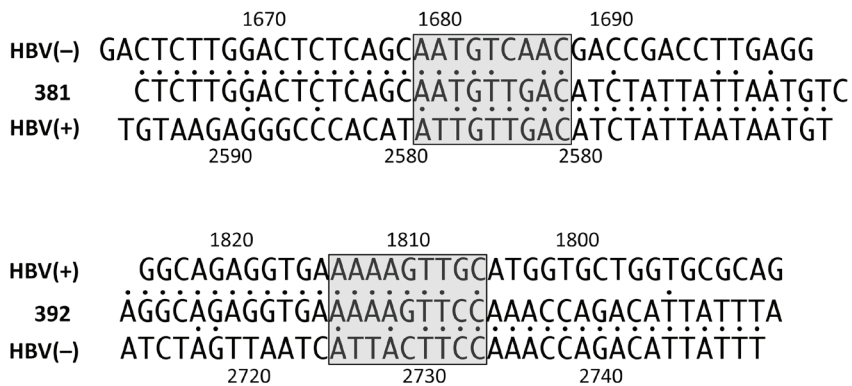


Figure 17. Alignment of integrated sequences from two patients containing possible rearrangements with the + and - strand of the complete HBV genome. Dots indicate homology between strands. Shaded area is the proposed weakly homologous region adjacent to the virus-virus junction.

We developed a method for next generation sequencing of whole genomes of HBV using the Ion Torrent instrument. In this way we could compare the integrated sequences found with Alu-PCR in paper IV with the sequence of the circulating virus at the time of biopsy. Four whole genomes were available for comparison (patients 15, 48, 83 and 290). These sequences showed an almost 100% homology to the ones obtained by Alu-PCR. All four patients were HBeAg-positive and probably the major replicating strains had not undergone any significant changes in the regions that had corresponding integrated sequences. Further comparison of integrations from patients with more advanced disease phase might show differences if integration occurred before mutations arose.

The interest in integrated HBV DNA has been fluctuating over the years. Almost 35 years has passed since the first publications on this topic but still the importance of these events remains to be demonstrated. An early study

from the mid 1980s, published before the PCR method was fully developed found integrations in 5.5% of HBeAg-positive patients and in 16.5% of HBeAg-negative patients (Fowler et al. 1986). Taken into account the low sensitivity of their methods (Southern blot), the result is in line what we and others have found later with more sophisticated methods (Murakami et al. 2004). The study also included patients with HCC and the conclusion was that integration of HBV DNA was not a prerequisite for cancer development. Even though it is well known that integration of HBV DNA occurs even in the chronic carrier, and that it is very common, the actual importance of these integrations, in terms of increased risk for cancer and contribution to HBsAg levels remains largely unknown.

Integration is not necessary for viral replication and may be a coincidence when uncircularised HBV genomes enter the nucleus of hepatocytes and are inserted into chromosomes. Recent papers, where focus has been on whole genome sequencing of liver explants from patients with HCC, have once again put attention on the plausible oncogenic role of integrated HBV DNA (Fujimoto et al. 2012; Sung et al. 2012; Toh et al. 2013). The list of implicated genes is growing longer but the conclusions made by Fowler et al in 1986 remains to be refuted.

A few studies have reported integrations of HBV DNA in children, especially in those with HCC (Yaginuma et al. 1987; Tsuei et al. 2002; Huang et al. 2005). Assuming that all patients with chronic HBV infection must have lots of integrated sequences of HBV DNA already after a few years of infection it is tempting to hypothesise that it is a matter of bad luck if an integration is oncogenic. A question that arises is whether treatment with nucleos(t)ide analogues, which reduce replicative intermediates including the putative substrate for integration, i.e. linear HBV DNA, might lower the risk for cancer. An interesting hypothesis comes from William Mason and colleagues (Mason, Jilbert, and Summers 2005; Mason et al. 2009; Mason et al. 2010). They studied clonal expansion of hepatocytes without detectable core antigen (HBcAg), first in woodchuck and chimpanzees and later in human tissue. They argue that during chronic HBV infection more and more cells somehow become “resistant” to HBV infection by unknown mechanisms and that these cells have a growth or survival advantage as compared to HBV infected cells. They demonstrated that integrated HBV DNA is present in some of these cells suggesting that the relative importance of these integrations increases with time. These findings have lead to a proposal that antiviral treatment should be initiated already as patients enters the immune clearance phase, with serum HBV DNA levels $<10^8$ copies/ml (Zoulim and Mason 2012). This

remains to be demonstrated but could easily be tested in prospective follow-up studies as this group currently does not meet treatment guidelines criteria.

In summary our findings indicate that integration of HBV DNA is a very common event in chronic HBV infection probably occurring already at an early stage of infection and potentially present in all adult patients. The real importance of integrated HBV DNA from a clinical point of view warrants further study.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The hepatitis B virus causes a chronic infection in the liver, and may produce billions of viral particles every day and at the same time escape the immune response of the patient. Many questions regarding this virus remain to be solved. The present thesis focused on factors explaining the 3-5 \log_{10} decline of serum HBV DNA when a patient loses HBeAg while the levels of circulating HBsAg remain high. One explanation to the latter has long been that part of S RNA is transcribed from integrated sequences of HBV DNA.

In the PLC/PRF/5, known to produce HBsAg, the levels of S RNA were found to be similar to the levels in HBeAg-positive patients. The Huh7.5 cell line, transfected with wildtype HBV or virus with an AGG \rightarrow TGA mutation at nucleotides 1762-1764 had lower levels of transcripts without difference between wildtype and mutant.

Quantification of viral DNA and RNA in 84 liver biopsies showed that the decline of cccDNA and a lower pgRNA/cccDNA ratio may explain a great part of the lower levels of HBV DNA in HBeAg-negative patients, in agreement with previous reports. The correlations, in particular among HBeAg positive patients, between pgRNA and ihDNA, and between ihDNA and HBV DNA in serum, suggest that ihDNA accumulate in hepatocytes, and that half-life of free virions decreases during the immune clearance phase. In the HBeAg-negative patients the correlation between cccDNA, replicative intermediates and serum levels of HBV DNA and HBsAg were weaker, indicating that in this stage the mechanisms that suppress the virus are more complex.

In the last couple of years, much attention has been given to the quantification of HBsAg in serum and several suggestions on how to use it in clinical practice have been made. Quantification of HBsAg in 160 patients in different phases of chronic HBV infection, from whom liver biopsies had been taken, indicates that a cut-off at $<3 \log_{10}$ IU/ml of HBsAg might be useful for identifying patients at low risk for liver damage.

Since 1980 it has been known that HBV DNA can integrate into the human genome as integrated sequences were found in a cell line producing HBsAg. Accumulating data suggests that in 80-90% of tumours from chronic HBV carriers with HCC contain integrated HBV DNA. Less is known about the

extent of integration in HBV patients who have not developed cancer. We investigated the rate of integration in 48 chronic HBV carriers. In 32 patients a total of 45 integration events were found by Alu-PCR, a method suitable for identifying known sequences at unknown locations in the human genome. Integration of HBV DNA was thus a very common event, and appear to occur already at early stages of chronic infection. The integration sites were evenly distributed on different chromosomes. Despite the lack of a preferential integration site integrations may contribute to cancer development because some integrations may randomly locate to oncogenic sites.

Almost 50 years after its discovery, the HBV is still an important cause of liver disease worldwide, in particular in Sub-Saharan Africa and South-East Asia. An efficient vaccine is available since more than 30 years and the virus could potentially be eradicated, provided that new-borns to infected mothers receive vaccination at birth.

The recent discovery of the HBV receptor, NTCP, will probably give valuable information, with development of new cell lines permissive of HBV infection or mouse models expressing the NTCP. New techniques, such as next generation sequencing, will provide unprecedented amounts of data, with possibilities to sequence whole HBV genomes from several patients in a matter of hours.

The patents for the nucleoside analogues entecavir and tenofovir will soon expire paving the way for generics at a much lower cost. Some argue that treatment should be given even to patients in the early, highly viremic, phase of the disease, even though no signs of liver damage are present, but the value of such treatment is uncertain. It is largely unknown if and when one can stop treatment with nucleos(t)ide analogues, and with a more liberal prescription of these drugs, many healthy HBV patients might be put on treatment that risks to be life-long. A few studies have shown that it is safe to stop treatment after 4-5 years in some patients. The challenge now lies in finding who these patients are.

In summary, the work presented here has contributed with some answers regarding hepatitis B virus replication and integration. During this project more questions have been raised and more studies are needed to better understand this enigmatic virus.

ACKNOWLEDGEMENT

I would like to thank everyone who supported me in one way or another, and especially (in no specific order):

Magnus Lindh, for being my mentor.

Charles Hannoun, my co-supervisor, for sweet Libanese cookies and interesting discussions regarding research.

Sofia and Sebastian, for introducing me to lab work. Sofia, for being my ally in political discussions with Magnus. Sebastian for being a great friend and many laughs.

Maria Andersson, always in a happy mood, a good friend and of great help in the lab.

Gunnar, Anders, my co-authors.

Giovanni and Teresa, for inviting me to Messina and giving me the opportunity to spend some time doing lab work and eating gelato.

Antonio, Gianluca, Cristina, Marika, Giusy and the others at the Ospedaliera Universitaria Di Messina, for all the help with lab work and all the funny moments in the lab and downtown Messina.

Anna, Joanna, Rickard, Katarina, Beata, Charlotta, James, Jean-Claude, Jesper, Nancy, Kristina, Linn, for sharing the every day life of a PhD student. Nina for technical advice.

Jonas Söderholm for organizing the betting during the European Championship of soccer.

Maria Johansson, Carolina and Anette, for all your help regarding a number of issues.

Everyone else at Virologen, making it the best workplace imaginable.

Caterina Finizia for giving me research leave so I could finish my thesis.

Erik Alestig and Steven Shev, for letting me moonlight at Internetmedicin.

All of my wonderful friends.

Astrid, Anna-Karin and Ingrid – the best sisters in the World.

Eva, Ingalill, Margareta, cousins and other relatives for hosting in Stockholm and other things.

Mats, my role model.

Göte, for many interesting discussions and for introducing me to irony.

Jonna, for love and patience.

Karin, without you none of this would have been possible. I will always miss you.

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