Real-time PCR studies of genotypes, mutations and replication of hepatitis B virus

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ABSTRACT

Infection with hepatitis B virus (HBV) is an important cause of liver disease and affects 350 million people worldwide, causing 600,000 deaths/year. Treatment includes interferon and nucleoside analogues (NAs) such as lamivudine, entecavir, and tenofovir. During treatment with NAs, substitutions may arise in the viral genome that confer resistance to treatment, impairing or abolishing the effect. Clinical prognosis and outcome of treatment are affected by viral genotype, and to date there are eight established (A-H) and two putative (I-J) genotypes, as well as several subgenotype strains described.

Levels of viral DNA and surface antigen (HBsAg) in serum are used to monitor the course of infection and the response to treatment. It is however not clear to what extent mechanisms that inhibit transcription of the pregenomic RNA (pgRNA), contribute to suppression of viremia, which mainly occurs in parallel with loss of HBeAg from blood. Likewise, it is unclear how the excessive production of HBsAg is regulated.

The aims of this thesis were to develop methods for genotyping and resistance mutation analysis, to investigate the impact of genotypes on clinical outcome, and to investigate the role of the regulation of viral transcripts for replication and HBsAg production.

Two real-time PCR based assays were designed and evaluated. The first focused on amino acid positions 180 and 204 in the viral polymerase enzyme, which are important for resistance against treatment with the NA lamivudine. The second aimed to include all established genotypes in a multiplex genotyping assay for accurate and rapid analysis. It was not possible to find

one single genomic segment that could be used for amplification and identification of all genotypes. Instead, we chose to target a number of segments in different parts of the genome, and for genotypes A-C two segments each were targeted, to obtain reliable accuracy. Both methods showed high accuracy and concordance with earlier methods, adding the possibility to identify mixed infections and assign relative proportions to the strains in the mixture.

Genotype impact on virological outcome was investigated after 9.2 years in 124 chronically infected adults. HBV DNA levels declined in patients carrying genotype A, B, and D, among whom HBeAg loss was observed in 92%. Genotype A and D showed 36% and 11% loss of HBsAg. In contrast, viral activity and aminotransferase elevation persisted in genotype C infections.

In the final study, real-time PCR was used to analyse the levels of cccDNA and viral RNA in biopsies and cell lines with focus on differences between HBeAg positive and negative stage. Patients negative for HBeAg had 2.15 log lower levels of cccDNA in liver tissue, 4.84 log lower serum levels of HBV DNA and 1.45 log lower serum levels of HBsAg, than HBeAg-positive patients. The pgRNA in liver tissue correlated strongly with cccDNA (R^2=0.87) and HBV DNA levels in serum (R^2=0.81). The S-RNA/pgRNA ratio was higher in HBeAg-negative patients, which may reflect specific down-regulation of pgRNA, or enhanced S-RNA production. Transcription efficiency was lower *in vitro* than in biopsies, and was not influenced by HBV core promoter mutations in transfected Huh7.5 cells.

Keywords: hepatitis B virus, real-time PCR, lamivudine resistance, genotypes, replication

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SAMMANFATTNING PÅ SVENSKA

Hepatit B-virus (HBV) är ett mycket litet DNA-virus som sprids via blod eller sexuella kontakter. Det infekterar leverceller och kan orsaka akut eller kronisk inflammation i levern. Infektionen utgör ett globalt hälsoproblem och enligt WHO har så många som 2 miljarder människor varit smittade med HBV. Det finns runt om i världen 350 miljoner kroniska bärare av viruset och komplikationer så som levercancer och skrumplever orsakar varje år mer än en halv miljon dödsfall. I norra Europa förekommer kronisk HBV-infektion hos mindre än 0,5 % av befolkningen medan andelen i vissa områden, framför allt i östra Asien, Sydostasien och Afrika söder om Sahara kan ligga över 10 %. I dessa länder överförs viruset ofta vid födseln eller i de tidiga barnaåren och orsakar då i regel kronisk infektion med stor risk för leverkomplikationer.

Hepatit B-virusets arvsmassa byggs upp av DNA och är mycket kompakt, bestående av endast 3200 nukleotider. Det har fyra gener: S, C, P och X, som är delvis överlappande och som ger upphov till sju proteiner. För varje virion, infektiös partikel, som produceras under infektion, produceras 10.000 gånger fler tomma så kallade subvirala partiklar. De består av värdcellens lipidhölje och virusegna ytprotein, dvs samma hölje som omger virionen, men har inget innehåll av DNA. Orsaken till överproduktionen av dessa tomma partiklar är okänd.

Vid virusförökning uppkommer genom misstag av virala polymerasenzymet virus med mutationer som ibland kan vara livsdugliga, och under vissa förutsättningar ha överlevnadsfördel, genom att undgå immunangrepp eller motstå antiviral behandling. Det senare kallas antiviral resistens och kan leda till att läkemedel efter en tid slutar att fungera och måste bytas ut.

Under evolutionen har större förändringar uppkommit i HBV-virusets DNA, vilket har lett till att de olika stammarna i slutet av 1980-talet grupperades i genotyper. Idag känner man till genotyperna A till H och ytterligare två (I och J) är på förslag. Många delar av världen har sina typiska genotyper, även om migration och resandet har gjort att fördelningen idag är mer uppblandad. Genotyperna har också visat sig ha olika inverkan på sjukdomens förlopp och förmåga att svara på behandling. Genotyperna C och D har i jämförelse med A och B visat sig vara värst i dessa avseenden.

Den huvudsakliga metodiken i denna avhandling har varit realtids-PCR, en teknik som bygger på användningen av ett polymerasenzym som tål höga temperaturer. Enzymet blandas med byggstenar för DNA-kopiering och så kallade primrar som är komplementära till det DNA man vill påvisa. Blandningen utsätts för stigande och sjunkande temperaturer och resulterar i en exponentiell ökning av DNA, vars mängd under analysens gång detekteras med hjälp av fluorescens och visas som en kurva.

I delarbete I och II utvecklades två metoder för att påvisa genetiska skillnader i HBV-virus. Den första fokuserade på två resistensmutationer, positioner i det virala genomet som ofta förändras vid behandling med nukleosidanalogen lamivudin och som drastiskt försämrar effekten av läkemedlet. Genom att analysera patientprover kan en förestående förändring upptäckas och behandlingen bytas ut för att undvika leverskada orsakad av stigande virusnivåer. Den andra metoden avsåg att genotypsbestämma det infekterande viruset för att på så sätt kunna anpassa behandling och uppföljning utifrån de kunskaper om genotypernas inverkan på behandlingssvar och prognos som finns.

I delarbete III undersöktes genotypernas inverkan på långtidsförloppet hos 124 patienter med kronisk HBV-infektion av genotyp A, B, C eller D. Alla genotyper utom C visade på avtagande virusaktivitet. Vid infektion orsakad av genotyp C däremot, och vid en del orsakade av genotyp D, kvarstod hög virusreplikation, vilket innebär risk för att utveckla leverkomplikationer.

I delarbete IV använde vi oss återigen av realtids-PCR för att kvantifiera det mellansteg i HBV-replikationen som kallas cccDNA (cirkulärt kovalent slutet DNA), samt RNA som uttrycks av den virusinfekterade värdcellen då nya viruskopior tillverkas. Resultaten antyder att transkriptionsreglering endast i liten grad kan förklara den stora minskning i DNA-nivåer som sker vid så kallad HBe-serokonversion, eller den överproduktion av ytantigen (HBsAg) som kännetecknar HBV-infektionen.

Sammanfattningsvis beskriver denna avhandling utveckling och utvärdering av metoder för resistensmutationspåvisning och genotypning som kan användas i virologisk rutindiagnostik. Den belyser också genotypers inverkan på infektionsförloppet, och bidrar med kunskap för förståelsen av två karakteristika för HBV-infektion, den minskade virusproduktionen vid HBeAg-omslag och den höga HBsAg-nivån i blodet.

LIST OF PAPERS

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

I. Malmström S, Hannoun C, Lindh M.
Mutation analysis of lamivudine resistant hepatitis B virus strains by TaqMan PCR.
Journal of Virological Methods 2007; 143: 147-152.

II. Malmström S, Berglin-Enquist I, Lindh M.
Novel method for genotyping hepatitis B virus on the basis of TaqMan real-time PCR.
Journal of Clinical Microbiology 2010; 48: 1105-1111.

III. Malmström S, Eilard A, Larsson SB, Hannoun C, Norkrans G, Lindh M.
Genotype impact on long-term virological outcome of chronic hepatitis B.
Submitted.

IV. Malmström S, Larsson SB, Hannoun C, Lindh M.
Hepatitis B virus RNA levels in human liver biopsies and in transfected and non-transfected hepatoma cell lines.
Submitted.

CONTENT

ABBREVIATIONS

BCP	basal core promoter
bp	base pair
cccDNA	covalently closed circular DNA
gt	genotype
HBcAg, HBc	core antigen
HBeAg, HBe	e antigen
HBsAg, HBs	surface antigen
HBV	hepatitis B virus
HBx	X protein
HCC	hepatocellular carcinoma
IFN	interferon
kb	kilobase
LHBs	large sized hepatitis B surface antigen
MGB	minor groove binder
MHBs	medium sized hepatitis B surface antigen
NA	nucleoside/nucleotide analogue
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
pgRNA	pregenomic RNA
rcDNA	relaxed circular DNA
RFLP	restriction fragment length polymorphism
RFMP	restriction fragment mass polymorphism
RT, rt	reverse transcription/reverse transcriptase
SHBs	small hepatitis B surface antigen
SVP	subviral particle

1 INTRODUCTION

Hepatitis B virus (HBV) is an important cause of liver disease, and accounts for 50% of hepatocellular carcinoma and 30% of cirrhosis cases worldwide. While approximately two billion have been transiently infected with hepatitis B virus, chronic infection affects about 350 million people and an estimated 600,000 persons die each year due to consequences of infection [WHO 2008]. Liver damage is considered to be caused by the reaction of the immune system upon infection, rather than by the cytopathic effect of the virus. Infected children experience poor activation of the specific immune system and consequently only minor damage of the liver is seen in early stage, even with highly active infection.

During the early stage of infection, almost all hepatocytes (approximately 10^{11} cells) are infected, secreting into the blood an estimated 10^{11}-10^{12} viral particles per day. The resulting viremia is measured in clinical practise by quantification of HBV DNA, and this has proven the most important test for assessment of prognosis and response to treatment for hepatitis B. Infected cells also secrete two viral proteins into the blood, the surface antigen (HBsAg) and e antigen (HBeAg). HBsAg is by unknown mechanisms produced in great excess and appear in the blood as subviral particles at concentrations up to 10^{14} copies/mL. Thus, HBsAg is a very useful marker for HBV infection and is detectable even if HBV DNA is lost from the blood. HBeAg is produced in lower quantities and typically becomes undetectable when viremia declines below 10^{6} copies/mL. Therefore, HBeAg is a useful surrogate marker for viral activity, and loss of HBeAg is very important for staging of infection, and is thought to reflect mounting of effective immune responses. Loss of HBeAg is usually paralleled by a 4-5 log decline of viremia. Only part of this decline can be explained by eradication of infected liver cells, and it is postulated that so-called non-cytolytic mechanisms also are important. It has been proposed that these mechanisms may comprise immune-mediated actions that down-regulate transcription of pregenomic RNA, a key intermediate in the replication of HBV.

Due to the error-prone DNA polymerase enzyme that is responsible for reproduction of virus, in combination with selective pressure, mutations accumulate in the viral genome. This adaptation has led to the evolution of genotypes and the same mechanism accounts for the emergence of point mutations that may confer resistance against treatment. Lamivudine has been widely used and is still in use in East Asia despite a high risk for selection of resistance mutations. Early identification of such resistance is important in

order to avoid harmful reactivation due to waning therapeutic effect, and accumulating data suggest that genotypes affect both prognosis and treatment response. These features call for diagnostic methods both for genotyping and resistance mutations identification.

The first part of this thesis engages in the development of well-suited methods for genotyping and resistance mutation analysis. The second part analyses the impact of genotypes on long-term outcome of chronic infection, and by quantification of different species of HBV DNA and RNA investigates the role of the regulation of viral transcripts for replication and HBsAg production.

1.1 Hepatitis B Virus

1.1.1 The Viral Particle

HBV is often referred to as the prototype virus of the Hepadnaviridae family. It has icosahedral nucleocapsid symmetry with a circular, partially double-stranded DNA genome. The Hepadnaviridae family has two genera that infect mammals (orthohepadnaviruses) and birds (avihepdnaviruses) respectively, each genus containing several related viruses. Homology between members of orthohepadnaviruses may be as high as 83% on nucleic acid level, whereas avihepdnaviruses are more distant relatives of HBV with an inter-homology of only 40% [Schaefer 2007]. All members of the Hepadnaviridae family have narrow ranges of hosts, as exemplified by the duck hepatitis virus (DHBV), which does not infect all species of duck. Structural differences between the two genera are found in genomic length, double-strandedness, variety in surface proteins, and presence of X protein.

The infectious virion of HBV, the so-called Dane particle, appears in electron microscopy as a sphere with a diameter of 45 nm (Figure 1). Its outer shell consists of host-derived lipids and three viral proteins, which are related through their origin in the same open reading frame (ORF). They are varying in length, translated from the same ORF but with different start codons, and designated small (SHBs), medium sized (MHBs), and large (LHBs) surface protein. The nucleocapsid is formed by core protein particles (HBcAg) and hosts one copy of the relaxed circular (rc) DNA genome and, attached to it, a polymerase enzyme with reverse transcriptase and ribonuclease H activity. Under the right conditions the capsid and its content assemble spontaneously.

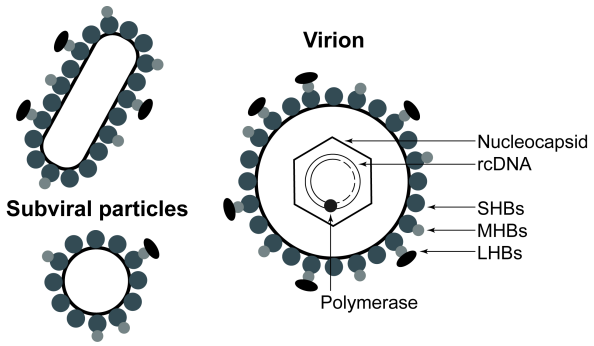

Figure 1. The HBV virion and subviral particles, shaped as a spheres and filaments.

Besides their presence in the virion, the surface proteins also form empty, non-infectious subviral particles (SVP). They are 22 nm in diameter and appear either as spheres or filaments depending on the content of MHBs and LHBs, apart from SHBs. The SVPs are produced in great excess, and are present in the blood at concentrations greater than 10,000 times that of virions.

1.1.2 Viral Genome and Replication

HBV has a compact genome consisting of 3182 to 3248 bases depending on genotype (Table 1). Since the genome is circular, a unique EcoRI restriction site has been designated the starting point for numbering. HBV comprises four partially overlapping open reading frames labelled P (polymerase), S (surface), C (core), and X (HBx protein) in descending length order (Figure 2). Only the minus strand is complete, whereas the plus strand varies in length. Although HBV is a DNA virus, it replicates through an RNA intermediate, which is transcribed by the reverse transcriptase activity of the viral polymerase.

Table 1. Genomic length and features of HBV genotypes.

Genotype	Length (bp)	Features
A	3221	INS core: 6 bp
B	3215	
C	3215	
D	3182	DEL pre-S1: 33 bp
E	3212	DEL pre-S1: 3 bp
F	3215	
G	3248	INS core: 36 bp; DEL pre-S1: 3 bp
H	3215	

INS, insertion; DEL, deletion.

The infection is initiated by the virion binding to an unknown receptor on the hepatocyte. Studies have shown that LHBs is responsible for specific binding, with the pre-S1 domain of the viral genome the being crucial for the attachment [Glebe et al. 2005; Schulze et al. 2010]. HBV then enters the cell either through endocytosis or by fusing its envelope with the cellular membrane, but the exact mechanism is not known (Figure 3). Upon entry, the nucleocapsid is delivered to the nucleus where the viral genome is completed

and transformed from the relaxed circular form into a double-stranded covalently closed circular (ccc) DNA episome. The cccDNA is organised as a minichromosome with chromatin-like structure [Bock et al. 2001; Levrero et al. 2009] and functions as the blueprint for production of five RNA transcripts. Two transcripts are longer than genome length and consist of about 3500 bases (Figure 2). The pregenomic (pg) RNA is the mRNA for further production of DNA, core protein (HBcAg), and polymerase enzyme, and will assemble to become new viral particles, whereas the slightly longer precore RNA is translated into secreted HBe protein (HBeAg). Precore RNA has an additional 33 nucleotides in the 5' end, as compared to pgRNA, and the translated protein is guided to the endoplasmatic reticulum for further processing in both ends. Due to different synthesis and processing HBc becomes the capsid protein and HBe is secreted into the blood, although a 90% homology on amino acid level. Three other transcripts are shorter than genome length, represented by 2.4 kb, 2.1 kb, and 0.7 kb RNAs. The ORF S contains three internal start codons (AUG) and the longest of the subgenomic transcripts translates into LHBs, whereas the 2.1 kb mRNA translates into MHBs or SHBs. The ORF X results in the shortest transcript, which translates into the regulatory X protein.

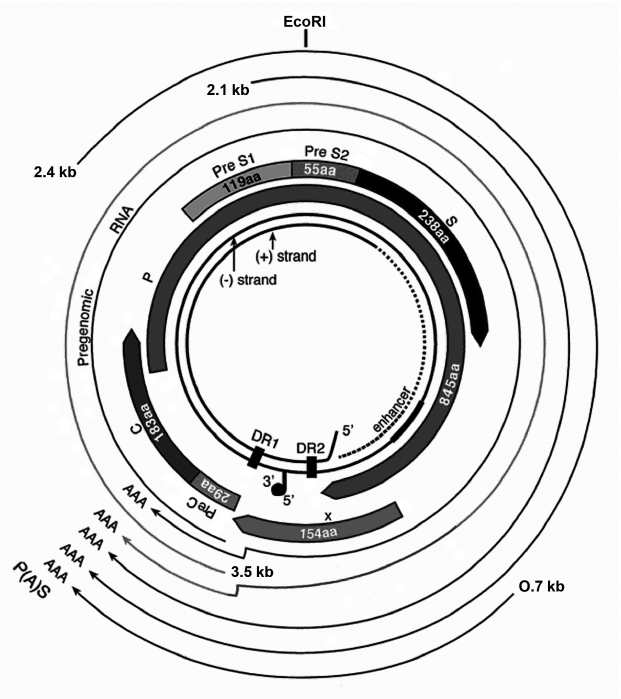

Figure 2. Genomic map of HBV, depicting rcDNA, ORFs, and transcripts.

When core protein and one copy each of pgRNA and polymerase enzyme have self-assembled into a new viral nucleocapsid, the reverse transcription of pgRNA occurs and is followed by plus-strand synthesis, to form the rcDNA genome. Mature nucleocapsids are either enveloped and secreted from the cell as new infectious virions, or re-imported into the nucleus to become part of the cccDNA reservoir.

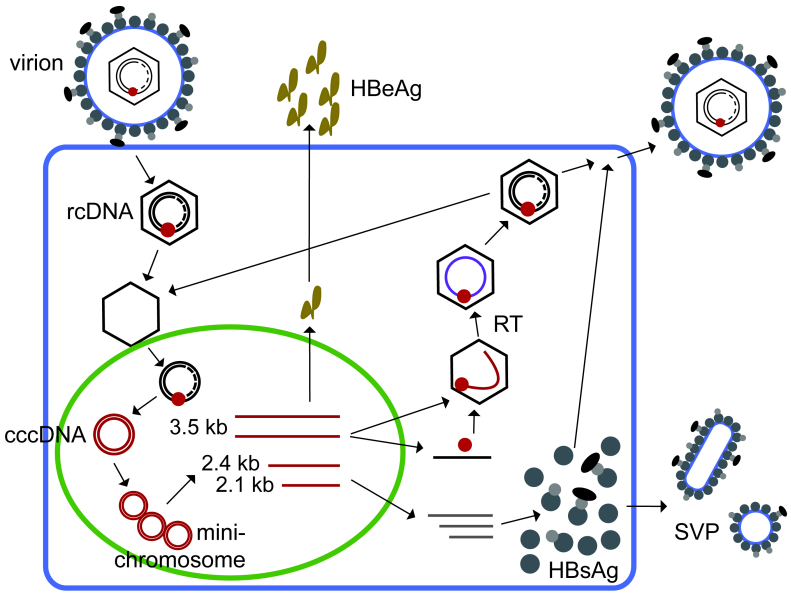

Figure 3. Schematic diagram of the life cycle of HBV.

1.1.3 Genotypes and Subgenotypes

HBV was initially categorised into nine different serotypes, which were based on amino acid composition in antigenic epitopes present on the surface protein, HBsAg. The classification system reflects serological reactivity rather than the phylogenetic relationships between strains [Ohba et al. 1995; Okamoto et al. 1988] and was eventually replaced by genotyping, as introduced by Okamoto et al. in 1988 [Okamoto et al. 1988]. They proposed a new way of grouping cognate strains into four genotypes, A through D, with an inter-genotype divergence of more than 8% in the complete genome sequence and of more than 4% in the S gene [Norder et al. 1992]. To date an additional four genotypes, E through H, have been described [Arauz-Ruiz et al. 2002; Norder,

Courouce, and Magnius 1994; Stuyver et al. 2000] and two propositions have recently been made for genotypes I and J [Huy, Trinh, and Abe 2008; Tatematsu et al. 2009].

The tentatively proposed genotype I have already been the subject of a report in 2000 by Hannoun et al. [Hannoun, Norder, and Lindh 2000], however considered a recombination of segments originating from genotype C and an unknown genotype. Because of proofs of recombination and the reported deviation from genotype C being less than 8%, the designation as genotype has been questioned [Kurbanov et al. 2008; Simmonds and Midgley 2005]. The suggested genotype J strain was recovered from an 88-year-old patient in Japan, who had been in Borneo during World War II. Without any significant evidence of recombination, and diverging with more than 10% from know genotypes, this HBV strain assigns to a phylogenetic position in-between human and ape genotypes, however closer to ape strains.

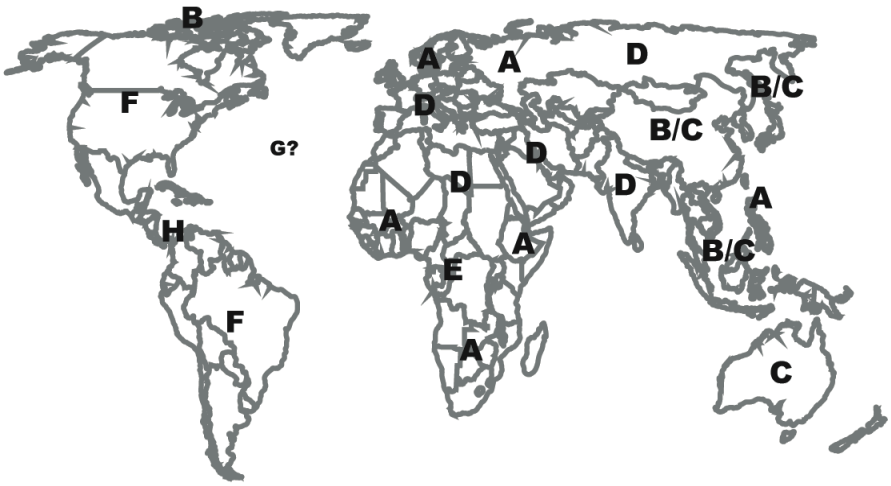

Figure 4. Original distribution of HBV genotypes.

Genotypes reflect genetic divergence that developed in a distant past. The original geographical distribution (Figure 4) of HBV was probably a result of early human migration, and more recent migration has resulted in a more complex genotype map. All genotypes are represented in Europe and North America with a predomination of A and D in Europe, as well as in Central Asia. In the Middle East and India genotype D is in great majority and in

Eastern and Southeastern Asia genotypes B and C prevail. Australia shows an equal distribution between genotypes A, B, C, and D whereas in Africa A, D, and E predominate in the sub-Saharan regions, northern parts, and on the west coast respectively. Genotypes F and H are closely related and almost exclusively found in the Americas. Thus, genotype F can be found in South and Central America whereas genotype H is present in Central America and Mexico. Genotype G has been found primarily in the USA and France [Kramvis, Kew, and Francois 2005; Kurbanov, Tanaka, and Mizokami 2010]. Some of the genotypes have also been subdivided into subgenotypes based on an intra-genotype difference of more than 4% on the nucleotide level of the complete genome [Norder et al. 2004]. To date there are slightly more than 40 subgenotypes in genotypes A, B, C, D, and F, and further subdivisions into clades within subgenotypes have been proposed. Also a change of the point defining the genotypes, decreasing it from 8% to 7.5%, has been put forward to better suite the collected knowledge on HBV strains and genotypes [Kramvis et al. 2008; Kurbanov, Tanaka, and Mizokami 2010].

1.1.4 Basal Core Promoter Mutations

The basal core promoter (BCP) region is an element in the HBV genome that initiates the transcription of pgRNA and precore RNA [Yuh, Chang, and Ting 1992]. The most frequent mutations emerge in two adjacent nucleotide positions, A1762T and G1764A, interfering with a transcription factor-binding site in this region [Okamoto et al. 1994]. They are seen irrespectively of HBeAg status in patients with active liver disease, but are less frequent in HBeAg-negative patients without signs of liver disorder. [Gunther 2006] At their emergence, transcription of precore RNA is reduced as well as the production of HBeAg [Buckwold et al. 1996; Laras, Koskinas, and Hadziyannis 2002]. The occurrence of substitutions in the BCP region has also been shown to increase viral replication, even in presence of lamivudine resistance [Tacke et al. 2004]. In addition to the mutations at nt 1762 and nt 1764, others appear at nt 1753 and nt 1766. An *in vitro* study has shown that adding other substitutions in the same region up regulates viral DNA replication even more [Jammeh et al. 2008; Tong et al. 2005]. The double mutation in the BCP region is more frequent in genotype C than genotype B and has been associated with more severe liver inflammation [Lindh et al. 1999] as well as cirrhosis and hepatocellular carcinoma (HCC) [Fang et al. 2002; Kao et al. 2003; Takahashi et al. 1998]. Despite the high frequence of BCP mutations, in particular of mutations at positions 1762 and 1764, and their strong association with more severe liver damage the mechanism for their selection

remains uncertain. The observation that these mutations evolve in parallel with immunologic activity indicates that they represent some form of escape. Of note, they usually precede precore mutations and often appear in HBeAg-positive phase, indicating that their selection is driven by other mechanisms than precore mutations, even if they have been proposed to represent an alternative way of down-regulating synthesis of HBeAg.

1.1.5 Precore Mutations

The most common precore mutation is a G replacing A at position 1896, and was first described in 1989 [Carman et al. 1989]. G1896A induces a TAG stop codon in the precore region, which completely eliminates the production of HBeAg. The discovery of the precore mutation helped giving an explanation to the fact that some HBV infected, although HBeAg negative experienced active liver disease and high HBV DNA levels. It also confirmed that HBeAg is not required for viral replication. Later the precore mutation was found to be very common also in HBeAg negative patients that had low viremia. Other positions of relevance for elimination of the production of HBeAg were found in the beginning of the precore region, with a mutated start codon or the second codon changed to a stop codon [Lindh et al. 1996].

Appearance of the G1896A substitution is dependent of the configuration on position 1858. The two nucleotides form a stabilising base pair in the pregenomic RNA loop, which is a part of the encapsidation signal and essential for replication. Consequently, the precore mutation occurs only in strains with T-1858, to maintain the stability of the stem structure. Genotype A (and some genotype C and F) strains carry C-1858, which prevent the G1896A substitution and thus may interfere with seroconversion to anti-HBe [Gunther 2006; Li et al. 1993; Lindh et al. 1995]. It has been argued that severe chronic liver disease is associated with precore mutations, but compiled prevalence data from regions with high endemicity did not show any correlation between liver disease and prevalence of the precore mutant [Gunther 2006].

1.1.6 *In Vitro* Model Systems

Cell lines have been developed for *in vitro* studies of the HBV life cycle, since suitable animal models are lacking. HBV does not efficiently infect cultured cells but viral DNA can be transported into the nucleus by means of transfection, in either stable manner or transient. The cell lines HepG2 and Huh7, both of human origin, have been extensively used for transfection-based molecular virology. None of them contain integrated DNA, which means that any expressed transcripts related to HBV can be assigned to the transfected DNA. Both HepG2 and Huh7 cell lines are suitable for studies of HBV replication, influence of mutation, and the effect of antivirals.

1.2 Infection and Treatment of Hepatitis B

1.2.1 Infection

HBV is transmitted sexually or through contact with contaminated blood. In low endemic areas the virus is mainly horizontally spread, between injection drug abusers or through unprotected sex. In high endemic areas vertical transmission, from mother to child during pregnancy or delivery, and horizontal transmission among preschool children, are the common routes of infection. The virus is not cytopathic by it self, but the liver damage is a result from a vigorous immune response. Infection with HBV may resolve spontaneously, progress to fulminant hepatitis with liver failure, or develop into a chronic state.

The first stage of the infection, lasting 10 to 30 years, is the immune tolerance phase, characterised by viral replication at high level but without immune response and hence minimal liver inflammation and normal ALT levels. Of the viral proteins, HBsAg is the first to be detected, followed by HBeAg. During the next stage, the immune clearance phase, an immune response is mounted towards the infection that leads to inflammation and damage of the liver. HBV DNA levels are reduced but the immune response is not sufficient to eradicate the virus, which leads to fluctuation in DNA and ALT levels. In the end of the second stage, however, levels of viral DNA decreases and seroconversion from HBeAg to anti-HBe occurs. In the third stage, the inactive carrier state, viral replication continues but on a relatively low level. There is no, or mild, liver inflammation and ALT levels are normalised.

Chronically infected persons run a high risk of developing cirrhosis of the liver and hepatocellular carcinoma (HCC) and especially those with high levels of HBV DNA and presence of HBeAg [Chen et al. 2006]. Also genotype and mutations occurring in the viral genome during infection influence the risk for HCC [Han et al. 2011; Lin and Kao 2011]. It has been reported that one half of HCC cases worldwide are caused by infection with HBV, whereas in high-endemic regions HBV infection can make up 70-80% of the cases [Nguyen, Law, and Dore 2009].

1.2.2 Clinical Relevance of Genotypes

The majority of studies that investigate the effect of genotype on disease progression have been conducted in East Asia, where genotypes B and C prevail. Because of the distinct genotypic division, in Asian and Western countries, most comparisons cover genotypes B and C or genotypes A and D respectively. Thus, several reports bring out that persons infected with genotype C face worse prognosis in relation to those infected with genotype B, regarding severity of liver inflammation, being positive for HBeAg, and having high HBV DNA levels. Longitudinal studies from Taiwan have demonstrated that HBeAg and HBV DNA are the most important predictors of long-term complications [Chen et al. 2011; Chen et al. 2006; Yang et al. 2002]. Also, genotype C strains more often have mutations in the core promoter, whereas genotype B strains more often have mutations in the precore region. Persons with genotype C have been associated with lower rate of HBeAg loss and higher risk for cirrhosis and HCC [Chan et al. 2002; Chu, Hussain, and Lok 2002; Kao et al. 2000; Lindh et al. 1999; Livingston et al. 2007].

Less is known about how genotypes A and D, which prevail in northern and southern Europe, influence the course of HBV infection. A study from Spain indicate that genotype A has a more favourable course than genotype D, because loss of HBsAg was more frequent in genotype A infected patients, which also had higher decrease-rate in HBV DNA levels [Sanchez-Tapias et al. 2002]. Also, genotype D has been associated with more severe liver disease than genotype A, but one study comparing genotype A and D in Europe found no difference in liver damage [Kao 2002; Rodriguez-Frias et al. 2006].

The clinical importance of HBV genotype has also been studied in treatment settings, showing that patients with genotype A and B respond better to interferon treatment than those infected with genotype C or D [Buster et al. 2009; Janssen et al. 2005].

1.2.3 Treatment

Chronic HBV infections are treated with two classes of drugs and the aim is to reduce viral replication, attain seroconversion to anti-HBe, and reduce the risk for liver damage. The utmost goal, which is rarely achieved, is the loss of HBsAg [Carosi et al. 2011; Wong and Lok 2006].

Interferon (IFN) is naturally produced in cells during viral infection and induces resistance against viral replication as well as modulation of the

immune system. IFN is administered as injections of pegylated IFN alpha for a limited period of time and can result in durable response, with conversion from active to subclinical state, or even cure the patient. The success rate is however below 40% and the flu-like side effects of interferon may greatly impair the patient's everyday life during treatment [Wong et al. 1993].

Nucleoside/nucleotide analogues (NAs) are synthetic compounds that perform their act through inhibition of the polymerase (reverse transcriptase) enzyme, which is crucial to viral replication, termination of the nucleotide chain during DNA synthesis. They are administered orally and are safe and well tolerated. However, due to relapse of replication after termination of therapy, prolonged therapy is usually necessary for this drug class. Selection of resistance mutations may occur in various extents, which leads to abolition of the antiviral effect and reduced suppression of viral replication. For lamivudine resistance mutations were present in about 50% of the patients after 3 years of treatment [Lai et al. 2003]. The second drug to reach the market was adefovir, which showed slower induction of resistance and a different pattern of resistance mutations. Because of the risk for resistance or poor antiviral potency, lamivudine and adefovir have to a large extent been replaced by newer compounds such as tenofovir and entecavir, which are highly potent and have effective resistance barriers.

There is also a recombinant vaccine available for effective immunisation of non-infected. It is composed of subunits of the virus, mainly HBsAg, and has had great impact on carrier rates and prevalence of cancer. The vaccine has been incorporated in the immunisation program in more than 90% of the world's countries [Kane 2012].

1.2.4 Antiviral Resistance Mutations

A serious drawback to nucleoside analogues in treatment of HBV is the selection of mutations appearing in the viral genome, causing viral strains that are resistant to the administered drug and potentially also to related drugs through cross-resistance. A number of factors are associated with selection of antiviral resistance, among these the selective pressure of drugs and the nature of the viral polymerase; like HIV reverse transcriptase, HBV polymerase is very error-prone and lacks the 3'-5' exonuclease (proofreading) activity.

Two types of mutations are selected in the viral genome by treatment with NAs. Primary resistance mutations reduce the susceptibility to the antiviral

drug, by means of amino acid substitutions, but may also impair the viral fitness or even make the viral strain replication-incompetent. Secondary compensatory mutations emerge to partially or fully restore the viral fitness, by means of additional amino acid substitutions [Lok et al. 2007]. As the resistant strains replace wild-type virus, viral breakthrough occurs, defined as a rapidly increasing viral load after a period of continuously suppressed replication.

The NAs can be divided into three chemical groups (examples in parentheses): L-nucleosides (lamivudine), acyclic phosphonates (adefovir, tenofovir), and D-cyclopentanes (entecavir). Primary resistance to an individual drug may confer cross-resistance, to various extents, to other drugs in the same group. Susceptibility against members of other groups may also be affected.

Lamivudine

The primary mutation in lamivudine resistance is located at amino acid 204 in the conserved YMDD motif, considerably decreasing the sensitivity to lamivudine by exchanging methionine (M) for either valine (V) or isoleucine (I) [Allen et al. 1998; Li et al. 2005; Ling et al. 1996]. These substitutions in the catalytic site have been suggested to create steric hindrance between the mutated amino acid and lamivudine [Das et al. 2001]. Also serine (S) has been reported, due to a double mutation (ATG→AGT), as a putative but rare amino acid substitution [Bozdayi et al. 2003]. A compensatory mutation occurs at amino acid 180, changing a leucine (L) to methionine. Typically, rtM204V appears together with rtL180M, whereas rtM204I may occur alone or in association with rtL180M. Other compensatory mutations include the rtL80I, rtV173L, and rtA181T/V (alanine to threonine or valine) substitutions [Delaney et al. 2003; Lok et al. 2007; Ogata et al. 1999].

Adefovir

Adefovir is related to substitutions at rtN236T (asparagine to threonine) resulting in a lower level of resistance than for lamivudine, and rtA181V, which also may emerge during lamivudine treatment [Fung et al. 2011; Zoulim 2004].

Tenofovir

No primary mutations have been reported for tenofovir. One position (rtA194T) has shown reduced susceptibility *in vitro*, in combination with substitutions selected for resistance against lamivudine and adefovir. In patients, however, tenofovir appears to be effective even in strains with rtA194T [Fung et al. 2011].

Entecavir

For entecavir four new amino acid substitutions have been reported. Alone, rtM250V causes minor decreases in susceptibility, whereas rtI169T, rtT184G (threonine to glycine), and rtS202I changes have little effect. Although resistance requires a combination of three or four substitutions to occur, the presence of rtM204V and rtL180M would require only another one or two to give effect. Thus, in strains with presence of lamivudine resistance the risk of developing entecavir resistance is increased [Fung et al. 2011; Tenney et al. 2004].

1.3 Detection of Genotypes and Mutations

Numerous assays for the identification of resistance related mutations, and for genotyping, have been described. As new antiviral agents become available and used for treatment, the number of resistance related positions in the viral genome grow and also the complexity of many assays. Some focus on positions relevant for a few antiviral agents whereas others attempt to embrace all reported substitutions. Genotyping assays also differ in complexity, some including all reported genotypes and others omitting those that rarely exist in the area for which is has been designed and evaluated [Bartholomeusz and Schaefer 2004; Guirgis, Abbas, and Azzazy 2010].

DNA sequencing

DNA sequencing allows for all genotypes and mutations to be detected, including any secondary mutation that may emerge and recombination between genotypes [Mallory, Page, and Hillyard 2011]. Yet it is a relatively cumbersome and time-consuming technique, with low sensitivity for minor populations. Sequencing requires several steps and much hands-on time, and may therefore not be adapted for high throughput screening.

Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) assays are usually more sensitive than sequencing in detecting minor populations, and may also be used for quantification [Allen et al. 1999; Chayama et al. 1998; Jardi et al. 1999; Lindh, Andersson, and Gusdal 1997; Lindh et al. 1998; Mizokami et al. 1999; Zeng et al. 2004]. Some mutations may destroy cleavage sites, others even create new, which may affect method sensitivity. For each substitution or genotype at least one suitable restriction enzyme and matching primers have to be chosen. Like sequencing, RFLP is associated with several steps and relatively much hands-on time.

Restriction Fragment Mass Polymorphism

Restriction fragment mass polymorphism (RFMP) is a technology in which the DNA is digested into oligonucleotide fragments containing sites of variation. The molecular weight of the fragments is then measured in a MALDI-TOF MS instrument [Ganova-Raeva et al. 2010; Hong et al. 2004]. RFMP, like RFLP, contains steps of PCR amplification and enzyme digestion, and it requires a set of primers for each analysed position. Furthermore, the essential mass spectrometer instrument may not be available in every

laboratory. RFMP has, however, high sensitive and may be used for early detection of resistance mutations or infections with mixed genotypes.

Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) technology allows fast and sensitive analysis in one step with high throughput. The assay may be designed in singleplex or multiplex format, but due to a limitation in detection channels the number of different targets that can be analysed simultaneously is limited. Depending on which real-time PCR system is used, the fluorescent probes are constructed in different ways. Taqman utilises a dual labelled probe, with reporter and quencher in either end of an oligonucleotide, whereas Light cycler make use of two probes, each one with either a reporter or a quencher attached to it. Non-specific detection of double-stranded DNA is also possible, with the use of SYBR green I. Several assays have been described, some with supplementary steps following the amplification step: Taqman [Wong and Lok 2006], Light cycler, in combination with melting point analysis [Cane et al. 1999; Liu et al. 2006; Yeh et al. 2004], and a combination with peptide nucleic acids (PNA) [Hige et al. 2010].

Other Technologies

Recently an assay for the detection of resistance mutations against lamivudine and adefovir treatment, based on a multi-analyte suspension array (Luminex), was described. The assay is divided in two steps where the first is an ordinary PCR with subsequent biotinisation of the PCR product. The second step includes hybridisation to specific probes attached to polystyrene beads before identification of nucleotide substitutions with dual lasers [Liu et al. 2011].

An oligo microarray assay is similar to one based on multi-analyte suspension arrays, including PCR amplification followed by hybridisation of labelled amplicons to specific probes. Instead of the beads used by the Luminex technology, a microarray assay utilises slides onto which the specific probes have been immobilised [Gauthier et al. 2010; Song et al. 2006].

2 AIMS

The general aim for this thesis was to develop and evaluate assays, based on real-time PCR for the analysis of relevant facets of hepatitis B virus.

The specific aims were:

Paper I

To describe and validate an in-house real-time PCR method for the detection of resistance mutations, which during lamivudine treatment appear at high rate at codons 180 and 204 in the viral genome.

Paper II

To describe and validate an in-house multiplex real-time PCR method for the detection of all published viral genotypes.

Paper III

To investigate the association between viral genotypes A to D and the long-term virological outcome in chronically infected.

Paper IV

To study hepatitis B virus in liver tissue and *in vitro* to elucidate how viral replication and the production of surface antigen may be influenced by transcription efficiency and cccDNA load.

3 MATERIALS AND METHODS

Patients and samples

Paper I. Stored serum samples from five patients with genotype A through D, and increasing amounts of HBV DNA in blood were studied. They were selected as being representative for the three main resistance patterns on viral codons 180 and 204, and in total 27 samples were analysed.

Paper II. A total of 184 stored serum samples from equally many patients representing genotypes A through G, and a pUC57 plasmid carrying a segment of genotype H, were included in the study. All serum samples had been genotyped by restriction fragment length polymorphism and/or sequencing in addition to real-time PCR analysis.

Paper III. This study was performed as a follow-up on patients that during the years of 1993 to 1995 were included in a cross-sectional study describing HBV DNA levels and liver histology. Out of 160 patients included in the original study, 124 with genotypes A through D were chosen for follow-up studies.

Paper IV. Frozen liver biopsies from 19 patients with Caucasian origin who participated in a study of viremia levels were investigated. They were infected with genotype A or D.

Serological tests and quantification of serum HBV DNA

HBsAg and HBeAg in serum were analysed by Axsym or Architect assays (Abbott, IL). HBV DNA in serum was analysed by Cobas Amplicor HBV Monitor (Roche Diagnostic systems, NJ) or by Cobas Taqman (Roche Diagnostic Systems).

Nucleic acid extraction and DNase treatment

Prior to PCR and real-time PCR, DNA and RNA were extracted from patient sera, biopsies and harvested cell cultures. Depending of the type of sample, different commercially available kits (all from Roche Applied Science, Germany) were used in a Magnapure LC robot (Roche Applied Science). Thus, the DNA I kit was used to extract DNA from serum samples (paper I and II) whereas the Total NA kit was used on harvested cells to extract both DNA and RNA (paper IV). Biopsies underwent homogenisation in a

Magnalyser instrument (Roche Applied Science) before extraction of DNA and RNA using the DNA II tissue kit (paper IV).

All extracted material intended for RNA analysis was exposed to DNase-treatment with the Turbo DNA-free kit (Ambion Inc, TX) before reverse transcriptase (RT) real-time PCR.

Polymerase chain reaction

The heart of the traditional polymerase chain reaction technique is a heat stable DNA polymerase enzyme, also known as Taq DNA polymerase. It originates from the bacterium *Thermus aquaticus*, hence the denomination, which lives and thrives in thermal habitats around the world.

In conventional PCR the Taq DNA polymerase utilises two oligonucleotide primers defining a segment of interest in the DNA, to synthesise exponentially increasing copies of the targeted segment. The procedure is driven by thermal cycling in a repetitive manner of the reaction mixture, including denaturation of the double stranded DNA, annealing of primers to the DNA strands, and extension by Taq DNA polymerase of the annealed primers. The product is visualised by exposure to UV light, as defined bands, after electrophoretic separation on agarose gel containing fluorescing and DNA binding ethidium bromide. A DNA ladder containing a set of fragments of known sizes is also loaded on the gel before electrophoresis, as a reference. Samples below detection limit after PCR, and invisible on the gel, were subjected to a second round of PCR with one or both primers replaced by others internal to those used in the first round, i.e semi-nested or nested PCR.

The melting temperature of an oligonucleotide is roughly dependent on the length (number of bases) and the proportion of A/T and G/C base pairs. If there is as mismatch present between a primer and the strain of interest, their affinity and the melting temperature of the primer will decline. By extending the primer, if possible, the melting temperature can be restored, as in the amplification-created restriction site (ACRS) technique in preparation for RFLP. In that case the PCR may still work and produce amplicons, as long as the mismatch is positioned in the middle of the primer or towards the 5' end. A mismatch in the 3' end is, however, detrimental for elongation of the primer.

PCR and nested PCR were run with 6 μL of sample in a total reaction volume of 50 μL, containing 0.2 μM of each primer. An initial denaturation at 95 °C for 5 min was followed by 40 cycles of denaturation at 95 °C for 45 s,

annealing at 60 °C for 45 s, and extension at 72 °C for 45 s, with an extension of 3 s per cycle. The last two steps were adjustable and depending on the primers' melting temperatures and the length of the amplicon.

In the present thesis conventional PCR was used in paper I, to prepare for mutation analysis with the RFLP technique by creating an artificial cleavage site with partially mismatched forward primers; in paper II, as a preparation for direct sequencing; and in papers II and IV, before genotyping with RFLP. Primers are listed in Table 2.

Real-time PCR

The basic principle for real-time PCR is identical to that for traditional PCR, with a primer pair delimiting a target sequence and a Taq DNA polymerase that synthesises multiple copies of the targeted segment using nucleotides as building blocks. The process, however, is not followed by any gel electrophoresis but the visualisation is achieved by means of fluorescent oligonucleotides, so-called probes, and the emitted light is read once every cycle of the PCR. The TaqMan approach adopted in the present thesis utilises a single, specific and complementary, dual-labelled probe. In the 5' end of the probe a fluorescent reporter (e.g. FAM, NED, VIC) is attached, which is excited by the PCR instrument. In the intact probe, instead of being emitted as fluorescence, the energy from the excited reporter is passed on to the quencher (e.g. TAMRA, BHQ) in the 3' end, via Förster resonance energy transfer (FRET). With that, a transformation occurs, and the energy absorbed by the quencher is emitted either as heat by the black hole quencher (BHQ), or as background fluorescence by the traditional TAMRA moiety. During the annealing step the probe hybridises to the amplicon in the same way as primers. When the polymerase reaches the probe during extension, the 5' end with the reporter attached is excised from the rest of the probe by the 5'-3' exonuclease activity of the polymerase, and both the reporter and quencher are released into solution. With increasing distance between them quenching is no longer possible and emission from TAMRA or BHQ decreases while reporter-fluorescence is increased.

The attachment of a minor groove-binding (MGB) moiety to the 3' end of the probe increases its affinity for double stranded DNA and by that its stability. The main advantage of this modification is that the probe's nucleotide portion can be constructed having a lower melting temperature and thus be shorter than in regular probes. This makes it suitable for analysing single nucleotide polymorphisms where high sensitivity for single base mismatches is crucial, as was the case in papers I and II.

Table 2. HBV primers and probes used for PCR, direct sequencing, and real-time PCR.

Designation	Sequence (5'→3')[a]	Range (nt)[b]	Modification[c]	In paper
Primers				
256 F	GTGGTGGACTTCTCTCAATTTTC	256-278		II, III
593 F	TGCACCTGTATTCCCATCCCATC	593-615		I
635 F	TTCCTATGGGAGTGGGCCTC	635-654		I
709 F	GCTTTCCCCCACTGTYTGGCTTTCAGTCAT	709-738		I
711 F	TTTCCCCCACTGTYTGGCTTTCAGTAATAT	711-740		I
796 R	CGGTAWAAAGGGACTCAMGAT	796-776		I-III
805 R	GGTAACAGMGGTAWAAAGGGACTCA	804-780		II
824 R	CCAAAGACAAAAGAAAATTGGTAACAGC	823-796		I
1022 R	GRGCAGCAAADCCCAAAAGACC	1023-1003		I
L180 F	GGCCTCAGYCCGTTTCACY	649-667		I
M180 F	GGGCCTCAGYCCGTTTCACA	648-667		I
Cane F[d]	TACTAGTGCCATTTGTTCAGTGG	678-700		I
Cane R[d]	CACGRTGYTGTACAGACTTGG	781-761		I
A_S F	CATCTTCTTRTTGGTWCTTCTGGAT	427-451		II, III
A_S R	GCAKGGTCCCGTRCTGGTT	517-499		II, III
A_C F	AAATGCCCCTATCTTATCAACACTTC	2305-2330		II, III
A_C R	TGCGAGGCGAGGGAGTTCT	2399-2381		II, III
B_S F	AGACTCGTGGTGGACTTCTCTCA	250-272[e]		II, III
B_S R	CCAGGACAAATTGGAGGACAAC	366-345[e]		II, III
B_{pS} F	GCATGGGGACAAATCTTTCTGTC	2879-2901[e]		II, III
B_{pS} R	AATCTGGATTKTCTGAGTTGGCTTT	2974-2950[e]		II, III
C_S F	GTATGTTGCCCGTTTGTCCTCTAC	459-482[e]		II, III
C5&X/C F	GTATGTTGCCCGTTTGTCCTCTA	459-481[e]		II
C_S R	GGARTCGTGCAGGTCTTGCA	534-515[e]		II, III
C_{pS} F	TGCACCGAACATGGAGATCAC	145-165[e]		II, III
C_{pS} R	TCTGTGGTATTGTGAGGATTCTTGTC	245-220[e]		II, III
D F	CTCATTTTGTGGGTCACCATATTC	2804-2827[e]		II, III
D R	GGTCGGGAAAGAATCCCAGA	2896-2877[e]		II, III
E F	CCTCATTTTGTGGGTCACCWTATTC	2803-2827[e]		II, III
E R	CCATTCGAGAGGGACCGTC	2880-2862[e]		II, III
FH F	CCGACTATTGCCTCTCTCACATCA	95-118[e]		II, III
FH R	GGGGTCCTAGGAGTCCTGATGT	188-167[e]		II, III
F R	GCCAGGACACCCGGGTAKTC	304-285[e]		II
H R	CCAGGACACCCGGGTGGTA	303-285[e]		II
G F	GAAACCGCCATGAACACCTCT	1611-1631[e]		II, III
G R	CCGGTTGTTGACATAACAAACAGT	1693-1670[e]		II, III
S-RNA F	TCCTCCAAYTTGTCCTGGTYATC	349-371		IV
S-RNA R	AGATGAGGCATAGCAGCAGGAT	431-410		IV
pgRNA F	GGTCCCCTAGAAGAAGAACTCCCT	2367-2390		IV
pgRNA R	CATTGAGATTCCCGAGATTGAGAT	2454-2431		IV
cccDNA F	CCGTGTGCACTTCGCTTCA	1575-1593		IV
cccDNA R	GCACAGCTTGGAGGCTTGA	1882-1864		IV

Table 2, continued.

Probes[f]				
YxDD (S)	TAGGGCTTTCCCCCACTGTTTGGCT	705-729	FAM, TAMRA	I
YMDD (AS)	CACATCATCCATATAACT	750-733	VIC, MGB	I
YVDD (AS)	ACATCATCCACATAACT	749-733	FAM, MGB	I
YIDD (AS)	CACATCATCAATATAACTG	750-732	NED, MGB	I
A_S (S)	CTCTAATTCCAGGATCMACA	477-496	FAM, MGB	II, III
A_C (AS)	CTCKGTCYCGTCGTCTAA	2363-2346	FAM, MGB	II, III
B_S (S)	CCAAATCTCCAGTCACTC	319-336[e]	VIC, MGB	II, III
B_{pS} (S)	CCCTGGGATTCTTC	2909-2922[e]	VIC, MGB	II, III
C_S (S)	CAGGAACATCAACTACCAGC	486-505[e]	NED, MGB	II, III
C5 (S)	CCAGGAACATCCACAACAAGCACGG	485-509[e]	FAM, BHQ-1	II
X/C (S)	CCAGGATCCTCGACCACCAGTACGG	485-509[e]	FAM, BHQ-1	II
C_{pS} (S)	ACCCCTGCTCGTGTTA	184-199[e]	NED, MGB	II, III
D (S)	CAGAATCTTTCCACCAGCA	2854-2872[e]	FAM, MGB	II, III
E (AS)	AGCCCCATGATGTAGC	2855-2840[e]	VIC, MGB	II, III
FH (S)	CCCTGCTATGAACATGGA	142-159[e]	NED, MGB	II, III
G (S)	TCTGCCAAGGCAGTTAT	1637-1653[e]	FAM, MGB	II, III
Q (S)	CAGGTCCCCTAGWAGA	2365-2380	NED, MGB	II, III
S-RNA (AS)	ATGATAAAACGCCGCAGACACATCCARC	399-372	FAM, BHQ-1	IV
pgRNA (S)	TCTCAATCGCCGCGTCGCAGA	2408-2428	FAM, BHQ-1	IV
cccDNA (S)	CATGGAGACCACCGTGAACGCCC	1607-1629	FAM, BHQ-1	IV

[a] D, not C (A, G or T); K, G or T; M, A or C; R, A or G; W, A or T; Y, C or T.

[b] Nucleotide position in genotype A genome unless otherwise indicated.

[c] MGB, minor groove binder; FAM, 6-carboxyfluorescein; VIC and NED, fluorophores proprietary to ABI; BHQ-1, Black hole quencher-1; TAMRA, 6-carboxytetramethylrhodamine.

[d] Primers described previously by Cane et al. [Cane et al. 1999].

[e] Nucleotide position in genotype B, C, D, E, F, G, or H genome in conformity with designation.

[f] (S), sense; (AS), antisense.

Multiplex PCR refers to a variant of real-time PCR where several sets of primer pairs and their corresponding probes are combined in the same reaction vessel, to allow amplification and reading of multiple targets simultaneously. Designing a multiplex PCR system is more complex than a singleplex assay and requires precise optimisation of how to combine different PCR sets to avoid false negative or positive results. There is an increased risk for hybridisation between primers and probes in a multiplex assay and in some combinations the normal PCR reaction can even be wholly or partially inhibited. Advantages of a well-optimised multiplex real-time PCR assay include the increased number of analyses that can be made on the same sample volume, reducing both hands on-time and the running time, but also the deviation caused by handling, such as a fluctuating pipetting volume, equalising the reaction conditions between targets.

Real-time PCR on DNA was run with 10 μL of sample in a total reaction volume of 50 μL, containing 25 μL Universal PCR master mix (Roche Molecular Systems, NJ), 0.2 μM or 0.9 μM of each primer and 0.2 μM of each probe. In paper IV the volumes were halved. After uracil DNA glycosylase activation at 50 °C for 2 min and initial denaturation at 95 °C for 10 min, 45 cycles of either a two-step (95 °C, 15 s; 60 °C, 1 min) or a three-step program (95 °C, 15 s; 60 °C, 1 min; 72 °C, 10 s) was run.

For reverse transcriptase real-time PCR analysis of RNA the Superscript III platinum one-step RT-PCR kit supplemented with ribonuclease inhibitor (Invitrogen, CA) was used. Total reaction volume was 25μL with 5μL of sample and primer and probe concentrations of 0.2 μM. Except for an initial RT step on 48 °C for 30 min followed by 95 °C for 10 min, the same three-step program as mentioned above was run.

In the present thesis real-time PCR was used for mutation analysis (paper I), for genotyping with a multiplex assay (paper II), and for DNA and RNA quantification (paper IV). All primers and probes are listed in Table 2.

Restriction fragment length polymorphism

Restriction fragment length polymorphism RFLP utilises the ability of restriction enzymes to distinguish between variants of homologous DNA sequences. Each enzyme has a more or less defined target sequence and enzymal action on its cleavage site will result in fragments of predictable lengths, which after agarose gel electrophoresis are visualised with UV light. The fragment lengths, i.e. the patterns produced on the gel, are typical for the enzyme and its target and can for example help distinguish between wild-type and mutated strains.

If a cleavage site does not natively exist in the DNA sequence, ACRS can be used to introduce such a site by the use of PCR and partly mismatching primers. In paper I this was attained with two forward primers, 709 F and 711 F (Table 2), bearing a single incorrect nucleotide each towards the 3' end. The introduction of these incorrect nucleotides in either a wild-type or a mutated strain produced cleavage sites for one or two of the restriction enzymes F*ok*I, *Nde*I, *Nla*III, and *Ssp*I. After digestion and gel electrophoresis it was possible to distinguish between one wild-type and two differently mutated HBV strains. RFLP was also used in papers II and III for genotyping with the *Hin*fI and *Tsp*509I enzymes.

Sequencing

Direct sequencing was performed on amplification products, with the same primers that were used in PCR or nested PCR. After cycle sequencing using the Terminator v1.1 cycle sequencing kit (Applied Biosystems, CA), and ethanol precipitation, both sense and antisense sequences for each amplicon were read in an ABI Prism 310, 3100 or 3130 automated capillary sequence reader (Applied Biosystems, CA). Sequence Navigator (Applied Biosystems, CA) or Sequencher (Gene Codes Corp., MI) softwares were used to process the recorded sequences. Sequencing was used to verify the real-time PCR-based mutation analysis described in paper I, as well as the assay for genotyping described in paper II.

Cloning

In paper I, the real-time PCR-based mutation analysis of lamivudine resistant HBV strains was validated with cloning. Using One Shot TOP10 competent *E. coli* cells and the TOPO TA Cloning kit for sequencing (Invitrogen Corp., CA) cloning was performed according to the instructions provided by the manufacturer. Colonies were suspended in Super-Q water, heated to 99 °C for 10 min and centrifuged at 6000 × *g* for 5 min, to lyse cells and remove debris before PCR and sequencing.

Cell cultures and transfection

Both cell lines used in paper IV were cultured in Dulbecco's modified medium, with supplements specific for each cell line, at 37 °C in 5% CO_2. The initial cell count was 480 000 cells/mL and 1 mL was added to each well in the 12-well culture plate. After 24 h the Huh7.5 cell line was subjected to transfection with 0.625 μg of plasmid DNA, using Metafectene Easy (Biontex Laboratories, Germany). Both plasmids used in transfection contained genotype A genomes of full length, one with wild type sequence at position 1762-64 in the basal core promoter and the other with a double mutation (AGG→TGA) inserted by mutagenesis at the same position. The PLC/PRF/5 cell line was cultured for 72 h before harvest, without any transfection performed.

Statistical methods

The statistical methods used for analysis of data in paper III and IV included the Mann-Whitney U test, Kruskal-Wallis test, paired t test, Student's t test, Fischer's exact test, Kaplan-Meier analysis, and linear and multiple linear regression analysis. The Statview (SAS Institute, NC) and Prism (GraphPad, CA) softwares were used for statistical analyses and visualisation. All reported p-values are two-sided and those below 0.05 were considered significant.

4 RESULTS AND DISCUSSIONS

4.1 Paper I

The nucleoside analogue lamivudine inhibits viral reverse transcriptase, an enzyme that is essential for the reproduction process. Lamivudine is well documented and has a good safety profile. The antiviral effect is strong and the price is low and by contrast with interferon, lamivudine has also been reported to reduce the risk of developing HCC [Liaw et al. 2004; Lok 2004; Matsumoto et al. 2005]. Prolonged administration is however often necessary, due to risk of replication relapse, and during monotherapy with lamivudine rapid selection of resistance mutations occur, leading to lost antiviral effect. With resistance developing in 15-32% of the treated patients each year lamivudine should not be prescribed as first-line therapy [Hadziyannis et al. 2000; Osborn and Lok 2006].

The main positions of interest in emergence of resistance mutations against lamivudine are amino acids 180 and 204 [Allen et al. 1998; Ling et al. 1996]. The primary mutation is at position 204, considerably decreasing the sensitivity to lamivudine by exchanging methionine (M) for either valine (V) or isoleucine (I). A secondary mutation from leucine (L) to methionine is seen at amino acid 180.

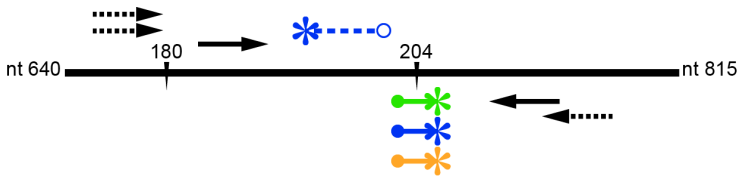

Figure 5. Schematic diagram of primer pairs and probes used for identification of nucleotide variations on amino acid positions 180 and 204.

In this real-time PCR based assay we made use of three reaction mixtures, and combined both primer and probe specificity, to identify the variations in

focus, as outlined in Figure 5. Two mixtures were dedicated to codon 180, each containing a specific forward primer bearing the distinctive nucleotide difference on the last position in the 3' end. The third mixture carried three MGB probes to distinguish between the wild-type and mutated variants at position 204.

Mutation patterns as identified by this method, as well as DNA level and treatment regimen from three patients are compiled in Figure 6. Each panel A through C respectively depict the most common mutation patterns: rtL180M+rtM204V, rtM204I, and rtL180M+rtM204I. In general, high pre-treatment viremia is followed by notable reduction after initiation of treatment. Due to missing samples, this is not evident in the patient depicted in XB. Next, resistance mutation occurs followed by raise in HBV DNA level. After cessation of treatment, reversion to wild-type may occur due to replication advantages in the wild-type genome, as seen in XA. Following reversion to wild-type the virus again becomes sensitive to lamivudine, but minor fractions of mutated strains may persist. Finally, exchanging lamivudine for another drug with lower rates of drug resistance would be beneficial for the decrease of HBV DNA levels.

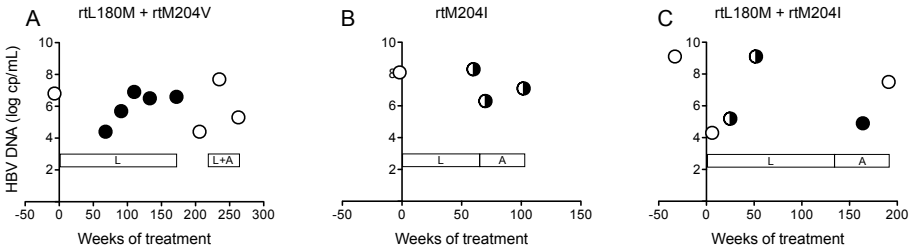

Figure 6. DNA levels, treatment and mutation status of three patients with typical mutation patterns. Open circles, wild-type in both positions; half-filled circles, mutant in one position and wild-type in the other; filled circles, mutant in both positions.

In a context where lamivudine is still used despite high risk of developing resistance mutations, a simple assay for detection of mutations connected to lamivudine treatment is relevant to prevent viral breakthrough and disease progression [Yuen et al. 2001]. This real-time PCR method presents a rapid

alternative to other methods, based on more time consuming and less sensitive techniques such as direct sequencing and RFLP [Allen et al. 1999; Chayama et al. 1998; Jardi et al. 1999]. Other methods identify only the YMDD motif [Cane et al. 1999; Wang et al. 2006], whereas our assay also include codon 180. However, this method has some limitations in detecting minor fractions in mixtures of wild-type and mutant strains. Typically, strains that could not be detected in such mixtures were present in 10-30% minorities as estimated by RFLP interpretation or by sequencing after cloning.

4.2 Paper II

There are presently eight genotypes (A through H) of hepatitis B described and acknowledged. The classification is based on nucleotide diversity of more than 8% in the entire genome, and was first described with 18 full genome strains of various subtypes that phylogenetically clustered into four groups [Okamoto et al. 1988]. An additional two (I through J) have been proposed, but their states as genotypes have not yet been confirmed. Further subdivision into subgenotypes and clades follows upon the recurrent disclosure of new strains.

Initially, genotypes pointed to geographic and ethnic origin and were associated with different regions of the world [Lindh, Andersson, and Gusdal 1997; Norder et al. 2004; Norder et al. 1993], but as a consequence of migration and traveling that linkage has been reallocated. Thus, essentially all genotypes are prevalent in multi-ethnic societies such as North America and Australia. Genotypes A and D are found worldwide but Eastern and Southeastern Asia are predominated by genotypes B and C. In addition to A and D, genotype E is common in Africa and C in India. Genotypes F and H are restricted to South and Central America.

A great many studies have been performed to elucidate the connection between genotype and clinical outcome, demonstrating that genotype affects both prognosis and treatment response. However, most of the studies have been performed in regions predominated by either genotypes A and D or B and C and thus lack comparisons with other less common genotypes. Genotype C has been identified as a risk factor for the development of HCC [Chan et al. 2004; Yin et al. 2008; Yu et al. 2005], but genotype B has also been associated with HCC, especially in younger people. Genotype B has also been identified as an independent risk factor for recurrence of HCC after surgical treatment [Yin et al. 2008].

The most straightforward way of designing a genotyping system based on real-time PCR would be to find one region distinguishing all genotypes, which could be amplified with one pair of primers. Using internal probes with specificity for each genotype would then reveal the genotype of the analysed sample. However, finding such a region proved difficult and therefore several PCR sets, consisting of a primer pair and a probe, were designed mainly in the S- and pre-S-regions of the genome (Table 3). The PCR sets were organised into multiplex reaction mixtures, each containing

two or three primer pairs and probe, to restrict the number of wells needed for each sample to four.

Table 3. Coverage of PCR sets for genotyping.

Multiplex reaction mixture/PCR set	Reporter	Range (nt)[a]
1		
A_S	FAM	427-517
B_{pS}	VIC	2879-2974
C_{pS}	NED	145-245
2		
E	VIC	2803-2880
FH	NED	95-188
G	FAM	1611-1693
3		
A_C	FAM	2305-2399
B_S	VIC	250-366
Q	NED	2305-2399[b,c]
4		
CS	NED	459-534
D	FAM	2804-2896
Supplements		
C5		459-534
X/C		459-534
F		95-304
H		95-303

[a] Nucletide position according to corresponding genotype

[b] Q probe covers gt A through F and uses A_C primers

[c] Nucletide position according to genotype A

All clinical samples used for evaluation of this genotyping assay had been genotyped by RFLP and/or sequencing. They totalled 184 samples, with all eight genotypes represented. The majority, however, were of genotype A, B, C or D. Genotype H was not covered for by any clinical sample. Instead a synthetic plasmid containing the genotype H-segment relevant for this assay was used.

Genotypes B, F, G, and H were fully covered by the real-time PCR method, whereas D had two deviating samples. One sample, previously assigned genotype D, could with our method be revealed as a mixture of genotypes A and D. One each of genotypes A and D had low viral concentration and could

not be detected by the real-time PCR method. One genotype E sample was detected only by the general Q probe and could therefore not be genotyped by this assay. Similarly, three samples of subgenotype C5 (not detected by C) and six of the X/C recombinant strain, for which a specific primer set was previously missing, were only detected by the Q probe. After upgrading with probes specific for C5 and X/C, those samples could be correctly genotyped (Table 4).

Table 4. Results of genotyping by sequencing/RFLP and real-time PCR.

Sequencing/RFLP genotype (n)	Real-time PCR genotype (A-H) correspondence		
	Consistent (%)	Inconsistent (%)	Note
A (24)	96	4	1 undetected
B (26)	100	0	
C (38)	92	8	3 by Q probe only (gt C5)[a]
D (79)	97	3	1 A/D mix, 1 undetected
E (8)	88	12	1 by Q probe only
F (2)	100	0	
G (1)	100	0	
H (1)	100	0	Plasmid (no sample available)
X/C rec. (6)	NA	NA	6 by Q probe only (no specific PCR for X/C)[b]
Supplements			
[a] C5 (3)	100	0	Upgrade: probe for C5[c]
[b] X/C rec. (3)	100	0	Upgrade: probe for X/C[c]

[c] Supplementary probes use additional forward primer C5&X/C and original reverse primer C_S.
NA, not applicable.

Since the presentation of this method several subgenotypes have been discovered and described. Thus, the genotype A group has increased with four subgenotypes (A4 through A7) [Hubschen et al. 2011; Olinger et al. 2008; Pourkarim et al. 2010] and B with one (B9) [Thedja et al. 2011]. Genotype C has more than doubled the collection of subgenotypes with nine newly discovered (C8 through C16) [Mulyanto et al. 2010; Mulyanto et al. 2009; Mulyanto et al. 2011; Mulyanto et al. 2012], whereas D and F have grown with three (D5 through D7) [Banerjee et al. 2006; Lusida et al. 2008; Meldal et al. 2009] and two (F3 and F4) [Huy et al. 2006] respectively. Genotypes E, G, and H are as yet solitaires in their groups.

	Forward primer	MGB probe	Reverse primer
A_S set	CATCTTCTTRTTGGTWCTTCTGGAT	CTCTAATTCCAGGATCMACA	AACCAGYACGGGACCMTGC
Subgenotype A1	---------A---------------	--------------------	---------------C---
Subgenotype A2	---------A-----T---------	----------------A---	------T--------A---
Subgenotype A3	---------A-----T--------C	----------------C---	------T-----G--T---
Subgenotype A4	---------A-----T--------Y	----------------C---	------------R--Y---
Subgenotype A5	---------------T---------	----------------C---	C-----T------M-----
Subgenotype A6	---------A-----A---------	--------------A-C---	------T--------C---
Subgenotype A7	---------A-----T--K------	----------------C---	M--------------C---
A_C set	AAATGCCCCTATCTTATCAACACTTC	TTAGACGACGRGACMGAG	AGAACTCCCTCGCCTCGCA
Subgenotype A1	-------------Y------------	----------A--YC---	-------------------
Subgenotype A2	-------------------------	----------G---C---	-------------------
Subgenotype A3	-------------------------	----------A---C---	-------------------
Subgenotype A4	-------------------------	---------------C---	-------------------
Subgenotype A5	-------------------------	-----Y-W-----YC---	M----------R-------
Subgenotype A6	-------------------------	--------A-G---C---	-------------------
Subgenotype A7	-------------------------	--------M-G---C---	-------------------
B_S set	AGACTCGTGGTGGACTTCTCTCA	CCAAATCTCCAGTCACTC	GTTGTCCTCCAATTTGTCCTGG
Subgenotype B1, B2, B4, B6, B9	-----------------------	-------------------	----------------------
Subgenotype B3, B5, B7, B8	-----------------------	-------------------	----------G----------
B_{pS} set	GCATGGGGACAAATCTTTCTGTC	CCCTGGGATTCTTC	AAAGCCAACTCAGAMAATCCAGATT
Subgenotype B1	-------------Y---------	-------------	-------------A----------
Subgenotype B2, B6	-----------------------	-------------	-------------A----------
Subgenotype B3	-------------------Y---	-T---------Y-T	----------C--C-----C----
Subgenotype B4	-----------------------	-M------------	-------------A----------
Subgenotype B5	----------G------------	-G----------T	----------C--C-----C----
Subgenotype B7, B8	-----------------------	-T----------T	----------C--C-----C----
Subgenotype B9	-------------------Y---	-T----------T	----------C--C-----C----
C_S set	GTATGTTGCCCGTTTGTCCTCTAC	CAGGAACATCAACTACCAGC	TGCAAGACCTGCACGAYTCC
Subgenotype C1	------------------------	-------------Y------	----------------T---
Subgenotype C2	------------------------	-------------------	-------------------
Subgenotype C3, C9, C10	------------------------	-------------------	----------------T---
Subgenotype C4	----------------------W-	-----W--W-R--Y------	----RR----------KYM-
Subgenotype C5	-----------------------A	-------------A--A---	----------------C---
Subgenotype C6	------------------------	------------------S-	--------------A-T---
Subgenotype C7, C8	------------------------	--------------------T	----------------T---
Subgenotype C11	------------------------	----------------C-	----G---------A-T---
Subgenotype C12	------------------------	------------W--M---	----------------T---
Subgenotype C13	----------------------M---	-------------------	--------------A-----
Subgenotype C14	------------------------	-------------W------	----------------T---
Subgenotype C15	------------------------	-------------A------	----------------T---
Subgenotype C16	------------------------	--------G---------C-	----G----------T---
Recombinant X/C	----------------------A	-----T-C--G--C-----T	-------------------
C_{pS} set	TGCACCGAACATGGAGATCAC	ACCCCTGCTCGTGTTA	GACAAGAATCCTCACAATACCACAGA
Subgenotype C1, C6	-----------------R---	----------------	--------------------------
Subgenotype C2	---------T-------G---	----------------	--------------------------
Subgenotype C3	--T-A-----------A---	----------------	-----R---------------------
Subgenotype C4	----Y-----------R---	----------------	--------------------W----
Subgenotype C5	-----------------A---	----------------	--------------------G----
Subgenotype C7, C8	-----------------G---	----------------	-----A---------------------
Subgenotype C9	----T-----------A---	----------------	-----A---------------------
Subgenotype C10, C13, C14	-----------------A---	----------------	--------------------------
Subgenotype C11	---------------C-A---	----------------	--------------------------
Subgenotype C12	----------------R-G---	----------------	--------------------------
Subgenotype C15	----T----------C-A---	----------------	-----A---------------------
Subgenotype C16	-----------------G---	----------------	--------------------------
Recombinant X/C	----G-----------A--T	----------------	-----A--------------G----
D set	CTCATTTTGTGGGTCACCATATTC	CAGAATCTTTCCACCAGCA	TCTGGGATTCTTTCCCGACC
Subgenotype D1-D7	------------------------	-------------------	-------------------
E set	CCTCATTTTGTGGGTCACCWTATTC	GCTACATCATGGGGCT	GACGGTCCCTCTCGAATGG
Genotype E	-------------------T-----	----------------	-------------------
FH set	CCGACTATTGCCTCTCTCACATCA	CCCTGCTATGAACATGGA	ACATCAGGACTCCTAGGACCCC
Subgenotype F1-F2	------------------------	--------Y---------	----------------------
Subgenotype F3-F4	------------------------	-------------------	----------------------
Genotype H	------------------------	-------------------	----------------------
G set	GAAACCGCCATGAACACCTCT	TCTGCCAAGGCAGTTATA	CTGTTTGTTATGTCAACAACCGG
Genotype G	--------------------	------------------	-----------------------

Figure 7. Comparison of primers and probes, with their targets in genotype and subgenotype sequences, highlighting positions that differ. All strains are consensus strains except subgenotypes C15 and C16, which denote single strains. The X/C recombinant was first described by Hannoun et al. [Hannoun, Norder, and Lindh 2000].

As can be seen in Figure 7, the new subgenotypes added to genotype D and F do not present any alterations in the actual binding sites of primers and probes, meaning that genotypes D through H will still be detected by the original PCR sets, without any predictable drawbacks. Genotype B is expected to be detectable with similar outcome as before the addition of B9, which was found in Indonesia. Thus, the B_S set can distinguish all nine subgenotypes, whereas the B_{pS} set is predicted to detect only B1, B2, B4, and B6 due to mismatches in both reverse primer and probe. Subgenotypes B3, B5, and B7 through B9 carry three identical contrasting nucleotides in the primer region, which most likely will counteract the PCR amplification. These strains also present at least two mismatches against the probe, whereas those in probe position 2 and 14 (last position) counted from the 5' end are important for the discrimination against all other genotypes. Genotypes A and C both have increased their stocks of subgenotypes more than twofold. The new genotype A strains have been found in Gambia, Haiti, Nigeria, Congo, Rwanda, and Cameroon. Subgenotype A5 is probably not possible to detect with our genotyping system, mainly due to mismatches in the 3' ends of both reverse primers of the A sets. A7 suffers a similar problem, but the A_C set might detect some strains of this subgenotype. For subgenotypes A4 and A6, the two PCR sets are predicted to function in a complementary manner. Thus, A_C may detect A4 and some strains of A6 whereas A_S probably will identify only A6. Genotype C is the most numerous having 16 subgenotypes to date, with fairly irregular differences. Both the two PCR sets, C_S and C_{pS}, should however match sufficiently to detect C1, C2, C7, C8, C10, C13, and C14, as well as most strains of C6, C9, C12, and C16. Subgenotypes C3, C11, and C15 should be identified by one of the two sets, whereas C4, C5, and the recombinant X/C strain were found to be poorly detected by the C sets. Because of that, specific real-time PCR systems were designed for C4, C5, and X/C recombinant strains. C5 and X/C were detected by these additional primers and probes, the C4 set could however not be evaluated due to too little sample material.

4.3 Paper III

In this paper we performed a virological follow-up after 9.2 years of 124 patients whose HBV infection had previously been characterised in terms of viremia, histopathology and ALT levels [Lindh et al. 2000]. The aim was to investigate genotype impact on parameters that reflect the course of infection, including HBV DNA levels, HBeAg and HBsAg status.

Table 5. Baseline characteristics of the 124 patients.

	All	Genotype				
		A	B	C	D	p value
	n=124	n=28	n=21	n=12	n=63	
Mean age (years)	35.8	43.3	32.2	30.5	34.7	0.0004[a]
HBeAg positivity (n=33)	26%	14%	38%	75%	19%	0.0002[b]
Median HBV DNA (log cp/mL)						
All patients	4.46	4.17	4.37	7.72	4.51	0.05[a]
HBeAg+ at baseline	8.60	8.10	8.56	8.12	9.53	> 0.1[a]
HBeAg– at baseline	4.22	3.88	4.08	3.37	4.28	> 0.1[a]

[a] Kruskal-Wallis test; [b] Chi square test.

The mean age was 31.0 years in HBeAg-positive and 37.7 years in HBeAg-negative patients (range 17-67 years). Patients of European origin were older than non-Europeans, and because Europeans often carry genotype A there was an association between genotype A and older age. The higher age in Europeans may reflect epidemiological differences, such as acquisition of HBV infection sexually and later in life, rather than vertically from mother to child or by other routes during early childhood.

At baseline, patients with genotype C infection were more often HBeAg positive and had higher HBV DNA levels (median 7.72 vs. 4.17-4.51 log copies/mL) than those with other genotypes (Table 5). At follow-up the HBV DNA levels had declined in patients carrying genotypes A, B and D, but not in those with genotype C. As shown in Figure 8 (left panel) the decline was most pronounced in patients with genotype A, B and D who lost HBeAg.

Out of the 33 patients who were HBeAg positive at baseline, HBeAg loss was observed in 5 out of 5 with genotype A, 7 out of 8 with genotype B, 4 out of 9 with genotype C, and 11 out of 12 with genotype D. Most of the patients that lost HBeAg also reached HBV DNA levels below 5 log copies/mL, and part of them levels below 4 log copies/mL.

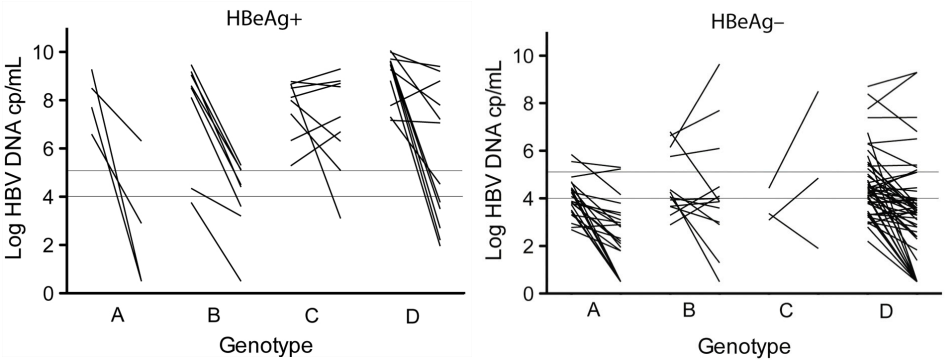

Figure 8. HBV DNA levels at baseline and follow-up for patients who at baseline were HBeAg+ (n=33) or HBeAg– (n=91).

In patients who were HBeAg-negative at baseline, similar genotype related differences at follow-up were seen, with more favourable virologic outcomes in genotype A and D. The overall impact of genotype on the proportion of patients that reached HBV DNA levels below 4 or 5 log copies/mL is summarised in a mosaic plot in Figure 9. This figure also shows the strong correlation between HBV DNA levels at baseline and follow-up.

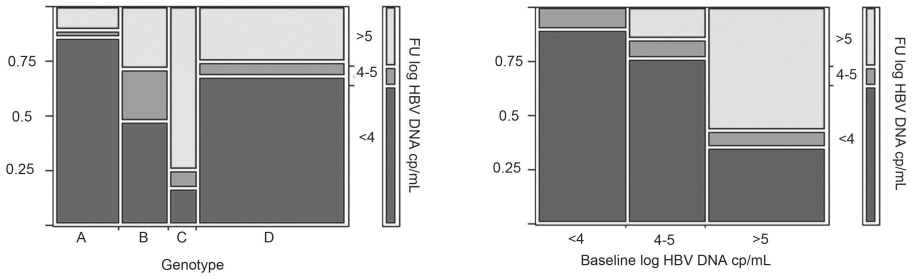

Figure 9. HBV DNA levels at follow-up as a function of genotype and HBV DNA levels at baseline.

Patients with baseline HBV DNA below 4 log copies/mL never had levels above 5 log copies/mL at follow-up, and almost always still had levels below 4 log copies/mL. Conversely, 60% of patients with HBV DNA above 5 log copies/mL still had such levels at follow-up. In particular, such high levels at follow-up were observed in genotype C, but also in genotype D patients with high baseline HBV DNA levels.

Table 6. Virological and biochemical outcome.

	All	Genotype				
		A	B	C	D	p value
	(n=124)	(n=28)	(n=21)	(n=12)	(n=63)	
Median follow-up time (years) (range)	9.22 (4.0-12.6)	9.22 (5.5-11.7)	9.24 (4.1-12.4)	8.76 (4.0-12.1)	9.26 (4.2-12.6)	
Loss of HBeAg	85%	100%	88%	44%	92%	0.004[a]
Loss of HBsAg	13%	36%	4.8%	0%	11%	0.002[c]
Median HBV DNA (log cp/mL)						
All patients	3.50	2.26	4.00	7.01	3.50	< 0.0001[b]
HBeAg+ at baseline	5.10	1.70	4.45	7.32	5.79	0.034[b]
HBeAg– at baseline	3.30	2.26	3.87	4.85	3.40	0.016[b]
Median HBV DNA decline (log cp/mL)						
HBeAg+	2.79	4.70	3.98	–0.30	2.82	0.03 for A, <0.01 for B and D[d]
HBeAg–	0.80	1.38	–0.06	–1.76	0.82	<0.0001[d] for A and D

[a] Fisher's exact test, genotype C vs. non-C; [b] Kruskal-Wallis test; [c] Chi square test; [d] Paired t test.

These genotype related differences also had an impact on loss of HBeAg and HBsAg. There was a higher rate (p=0.004) of HBeAg loss in patients carrying genotypes A (100%), B (88%), and D (92%) as compared with genotype C (44%) (Table 6). As illustrated by the Kaplan-Meier plot in Figure 10, genotype also influenced loss of HBsAg, which was seen in a relatively large proportion of patients with genotype A infection (36%), whereas this fraction was lower (11%) in genotype D and minimal in genotypes B and C. Of note, the only genotype B infected patient who lost HBsAg was a Swedish male who had contracted HBV infection at adult age.

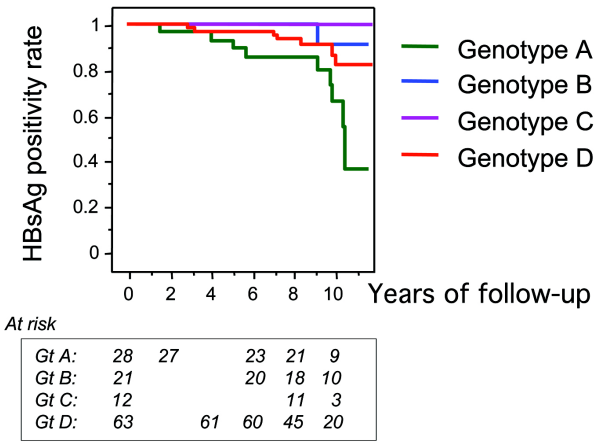

Figure 10. Kaplan-Meier plot showing loss of HBsAg over time.

As shown in Table 7, the four patients who developed complications were infected with genotype B or C, and all had been recognised as risk patients at baseline, because they had high HBV DNA levels, elevated ALT and severe fibrosis at the time of liver biopsy. Two of the patients, both Vietnamese, died from hepatocellular carcinoma. A Swedish man with genotype C infection since childhood was non-responder to sequential treatments, and was transplanted because of liver failure (HCC was identified in the explant). A Chinese man with genotype B was transplanted because of acute liver failure, induced by a resistant strain emerging during lamivudine treatment.

Table 7. Patients with complications.

Diagnosis	Origin	Age	Gender	Gt	Fibrosis score at baseline	HBV DNA at baseline/diagnosis, (log cp/mL)	ALT/ULN at baseline/ diagnosis	Transplanted
HCC	Vietnam	61	F	B	4	5.8 / 6.1	0.7 / 1.0	No
HCC	Vietnam	64	M	C	3	7.4 / 5.1	1.9 / 3.5	Yes
HCC and cirrhosis	Sweden	41	M	C	4	6.3 / 7.2	2.2 / 2.1	Yes
Liver failure, small HCC	China	44	M	B	3	6.1 / 9.6	2.0 / 9.5	Yes

Overall, the results from this study agree with results from earlier studies on the impact of genotype, most of them carried out in East Asia comparing genotypes B and C. In 1999 we observed that patients with genotype C infection had more severe liver inflammation, were more often HBeAg positive with high viremia levels, and that genotype C strains more often had mutations in the core promoter and more rarely had mutations in the precore region [Lindh et al. 1999]. This finding has since then been confirmed and extended. Thus, genotype C has been associated with lower rate of HBeAg loss [Chu, Hussain, and Lok 2002; Livingston et al. 2007], more severe liver inflammation and higher risk for cirrhosis [Chan et al. 2002] and HCC [Kao et al. 2000]. Longitudinal studies from Taiwan have demonstrated that HBeAg [Yang et al. 2002] and HBV DNA [Chen et al. 2011; Chen et al. 2006] are the most important predictors of long-term complications. These studies have confirmed observations from cross-sectional studies that patients with HBV DNA below 4 log copies/mL have mild liver disease, and that those with levels above 5 log copies/mL often have significant liver damage. These studies also reported that even moderate HBV DNA levels (between 4 and 5 log copies/mL) may be associated with an elevated risk for complications [Chen et al. 2006], a finding that has influenced treatment guidelines to be more liberal ["EASL Clinical Practice Guidelines: management of chronic hepatitis B" 2009].

Less is known about how genotype A and D, which prevail in northern and southern Europe, influences the course of HBV infection. A study from Spain indicates that genotype A has a more favourable course than genotype D, because loss of HBsAg was more frequent (16% vs. 8% after 8 years) [Sanchez-Tapias et al. 2002]. In a longitudinal study over 20 years in Alaska, the rate of HBeAg loss was similar in genotype A and D (94% and 93%), but was significantly lower in genotype C (61%) [Livingston et al. 2007]. In Italy, where essentially all patients carry genotype D, 87% of 70 HBeAg-positive patients lost HBeAg after a mean follow-up of 25 years [Fattovich et al. 2008]. In another study from Italy, HBsAg positive subjects identified at blood donor units, were followed for 30 years: 32% lost HBsAg corresponding to an annual rate of 1%, which is close to what we observed in genotype D (11% after 9.3 years) [Manno et al. 2004]. Our results agree with these reports and demonstrate that genotype D infections with low HBV DNA levels have a good prognosis with further reduced viremia levels and more than 1% loss of HBsAg per year. However, viremia levels remained high in half of the patients with HBV DNA above 10^5 copies/mL at baseline.

This agrees with Italian studies of genotype D, which have identified an enhanced risk of progressive liver disease in this group [Brunetto et al. 2002], further underlining the importance of careful follow-up of patients with genotype D infection and high HBV DNA levels.

The clinical importance of HBV genotype is also evident from treatment studies. Patients with genotype A reportedly respond better to interferon treatment than those infected with genotype C or D [Buster et al. 2009; Janssen et al. 2005]. The aim of our study was not to investigate genotype differences on treatment outcome. Instead, treatment was a factor that complicated our analysis. In all, 28 patients (23%) had been treated with either interferon or nucleoside analogues. However, only one of them had achieved a sustained treatment response, identified as loss of HBeAg or HBV DNA levels below 5 log copies/mL within one year post treatment. Because of the poor response, many patients were given long-term nucleoside analogue treatment, either as primary treatment or when hepatitis reactivated after the primary treatment. In these cases, the outcome (HBV DNA, serology and ALT) was assessed at a time point prior to initiation of long-term treatment. Thus, we believe that inclusion of treated patients had limited impact on the results of our study. It should be noted however that although none of the 5 patients with genotype A responded within 1 year (all received 6 months of interferon), 4 (80%) lost HBsAg 3-7 years post treatment. Among the untreated 23 patients with genotype A, 6 lost HBsAg (26%), at a rate that was still higher than for those carrying genotypes B or C (p=0.015).

In summary, this study supports that genotype influences the long-term virologic course of HBV infection, with better outcome in genotype A infection, poorer for genotype C, and variable outcome for genotype D with a poor outcome for those who showed high HBV DNA levels at baseline. The observation that part of the more favourable outcome, for patients with genotype A infection, may be related to epidemiologic factors warrants further study.

4.4 Paper IV

In this study we quantified HBV RNA to investigate if regulation of transcription could explain two important features of HBV infection, the very high production of HBsAg and the drastic reduction of viremia that parallels the loss of HBeAg from the blood.

Viral clearance during HBeAg loss

The HBeAg is used as a marker for high viral loads, and loss of HBeAg from serum is an important clinical event, which typically is paralleled by a decline in viremia from levels around 10^8-10^9 copies/mL to levels below 10^5 copies/mL [Realdi et al. 1980]. It is well known that this process is a result of an effective immune response, but despite extensive research it is not well understood how this is initiated and how such a response reduces viremia [Chisari, Isogawa, and Wieland 2010]. However, during the last years it has become clear that reduction of viral load is the result of an impact at different levels. Thus, immune histostaining for HBsAg in liver tissue has shown that the proportion of infected hepatocytes decreases from around 90% in HBeAg positive stage to less than 10% in HBeAg negative patients with low viremia levels [Wursthorn et al. 2006]. This observation reflects that eradication of infected cells, which probably is mediated by cytotoxic T cells that recognise HBV derived epitopes displayed on the surface of infected cells, only accounts for part (1-2 log) of the 4-5 log decline in HBV DNA that typically accompanies loss of HBeAg. Apparently, other effectors must be involved, and experimental support for such non-cytolytic mechanisms was presented long ago [Guidotti et al. 1994]. These effects are immunologically mediated, but are still poorly understood.

To some extent the non-cytolytic reduction of viral load can be explained by a clearance of cccDNA (the episomal nuclear form of HBV DNA) within infected hepatocytes, and quantification of cccDNA in liver tissue indicates that this process corresponds to a 1 log decline of viral load [Laras et al. 2006; Volz et al. 2007]. Reduced productivity from cccDNA [Volz et al. 2007] and shortened half-life of HBV virions [Dandri et al. 2008] have been shown to contribute to reduced viremia, but it is not known how immune response mechanisms can achieve these effects. The fact that HBV replication comprises an RNA intermediate suggests that reduced pgRNA transcription may be involved in viral suppression. Extensive studies indicate that such

mechanisms, including histone acetylation [Pollicino et al. 2006], interaction with transcription factors [Chou et al. 2007; Gordien et al. 2001], miRNA [Zhang et al. 2011] and degradation of HBV RNA [Tsui et al. 1995], may indeed be important. However, the relative importance (in quantitative terms) of these effects for the viral clearance during loss of HBeAg has not been established. By quantification of HBV RNA in liver tissue and *in vitro* we hoped to increase the understanding of this process.

Liver biopsies from 10 HBeAg positive and 9 HBeAg negative patients were investigated. As shown in Table 8, these patients represent a wide range of HBV DNA levels, spanning almost 7 logs, with the median at 8.74 log copies/mL in HBeAg positive and 3.90 log copies/mL in HBeAg negative patients. We quantified cccDNA, total HBV DNA, pgRNA and S-RNA in liver tissue and related these findings to HBV DNA and HBsAg levels in serum.

Table 8. HBV DNA and RNA levels in 19 liver biopsies.[a]

	HBeAg+ (n=10)	HBeAg– (n=9)
Intrahepatic		
cccDNA/cEq	–0.92 (–1.79-0.34)	–3.07 (–4.04- –1.65)
pgRNA/cccDNA	2.32 (1.90-2.82)	1.61 (1.03-2.71)
S-RNA/cccDNA	2.56 (2.28-3.80)	3.11 (1.29-4.35)
pgRNA/S-RNA	–0.49 (–1.75-0.10)	–1.64 (–1.92- –0.01)
Serum		
HBV DNA (log cp/mL)	8.74 (6.60-10.10)	3.90 (3.00-5.80)
HBsAg (log IU/mL)	4.57 (3.20-5.21)	3.12 (1.14-4.36)

[a] Median values of log copy numbers, ranges in parentheses.

As shown in Figure 11 there were strong correlations between cccDNA, pgRNA, and HBV DNA in liver and serum. These included a strong link between pgRNA and cccDNA with an R^2 of 0.87, showing that pgRNA levels closely reflect cccDNA and that the difference in pgRNA between HBeAg positive and negative patients mainly was due to decline in cccDNA. The HBV DNA in serum also correlated strongly with cccDNA, and the much lower HBV DNA in HBeAg negative patients was largely a function of reduced cccDNA levels. Our findings agree with observations by others [Laras et al. 2006; Volz et al. 2007]. In agreement with Volz et al. we found that the

correlation between cccDNA and serum HBV DNA was lacking within HBeAg negative patients.

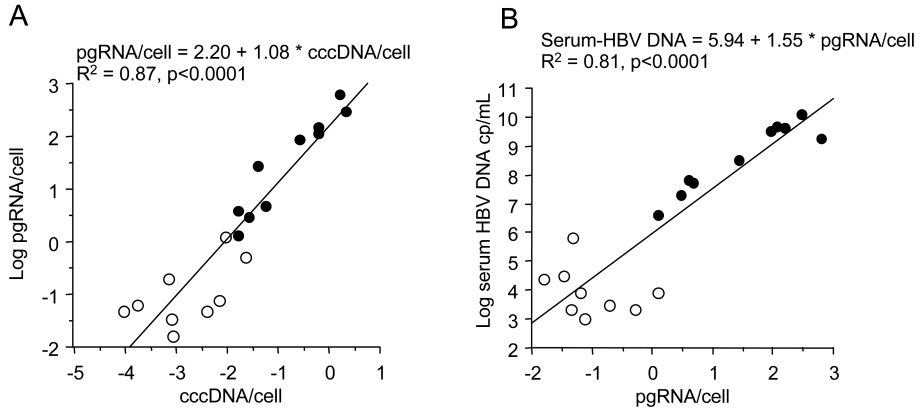

Figure 11. Correlation between cccDNA and pgRNA in liver tissue (A) and between pgRNA and serum levels of HBV DNA (B).

At group level, HBeAg negative patients had 2.15 log lower levels of cccDNA in liver tissue and 4.84 log lower serum levels of HBV than HBeAg positive patients. The transcription efficiency in terms of pgRNA per cccDNA was 0.71 log lower in HBeAg negative than HBeAg positive patients, but a similar difference was not observed for S-RNA.

Taken together these findings indicate that the reduction of HBV DNA seen in HBeAg patients is partly explained by reduced viral load in the liver, in terms of cccDNA. However, reduced cccDNA could only explain approximately 2 log of the 5 log decline between these stages of infection. The reduced pgRNA/cccDNA ratio in HBeAg negative patients point at the possibility that down-regulation of pgRNA transcription may contribute, but that this mechanism is of limited importance for the difference in viremia between HBeAg positive and negative stage. Possibly, regulation of transcription is more important for explaining differences within HBeAg negative patients, because in this group there was a lack of correlation between cccDNA and HBV DNA in serum.

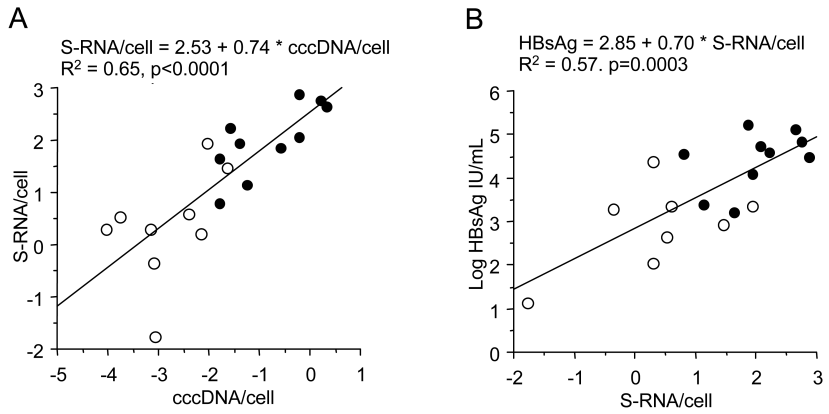

Figure 12. Correlation between cccDNA and S-RNA in liver tissue (A) and between S-RNA and serum levels of HBsAg (B).

Loss of HBeAg is also paralleled by a decline of HBsAg production, and quantification of HBsAg levels in serum has been suggested to be useful for monitoring response to antiviral treatment, or for following the clinical course of infection [Chan et al. 2011]. In our study, the HBsAg levels were 1.45 log lower in HBeAg negative patients (3.12 vs. 4.57 log IU/mL). This difference was smaller than the 2.15 log decline of cccDNA, and much smaller than the 4.84 decline of serum HBV DNA. S-RNA/cell correlated significantly with cccDNA and HBsAg with S-RNA/cell (Figure 12A and B), but the R^2 values were lower than for corresponding values of pgRNA and serum HBV DNA, indicating weaker correlations.

Further correlation analysis (not shown) showed that 1 log reduction of cccDNA corresponded with 0.64 log reduction of HBsAg and with 1.70 log reduction of HBV DNA in serum. Thus, neither HBV DNA nor HBsAg reflected cccDNA levels accurately, and the correlation was weaker for HBsAg (R^2 0.51 as compared with 0.70 for HBV DNA).

Transcription impact on HBsAg excess

Transcription of the S region of the HBV genome is regulated by two promoters and produces three versions of HBsAg, the S (small), the M (medium sized), and the L (large) surface proteins, sized 226, 281 and 400 amino acids. These proteins are embedded in lipid membranes to form either

the envelope of the virion, or so-called subviral particles. The latter, shaped as spheres or filaments, are produced in excess, and are present in the blood at concentrations 10,000 times that of virions. The function of the high levels of HBsAg in serum is not well understood and neither is the mechanism explaining how these levels are achieved. One may presume that at least part of the explanation might be an enhanced transcription of the HBsAg genes. This possibility is supported by observations in some *in vitro* studies, in which S-RNA have been detected at much higher levels than pgRNA/precore RNA, as illustrated by the Northern blot in Figure 13 from [Tacke et al. 2004].

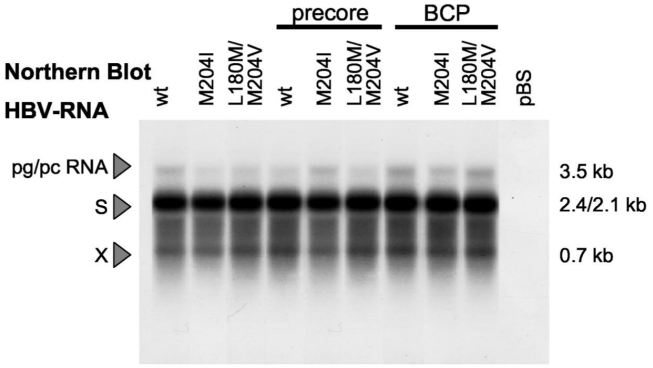

Figure 13. HBV RNA by Northern blot, from [Tacke et al. 2004]. Reproduced with permission from American Society for Microbiology.

In the 19 liver biopsies the levels of S-RNA were approximately 1 log greater than levels of pgRNA, but this ratio was different in HBeAg positive (0.5 log) and negative (1.6 log) patients. The latter difference was also observed (Figure 14A) as a correlation between cccDNA and log pgRNA/S-RNA ($p=0.006$). Thus, our data support that S-RNA is transcribed more effectively than pgRNA and that this difference is greater in HBeAg negative stage. However, the magnitude of the difference in transcription is relatively moderate, and cannot explain the very high levels of subviral particles (HBsAg) in serum, which exceeds HBV DNA levels by a factor of 10,000.

We used two different *in vitro* systems to further explore transcription of HBV. Firstly, we analysed Huh7.5 cells transfected with complete HBV genomes [Gunther et al. 1995] without or with mutations in the BCP. Transcription efficiency in terms of pgRNA per cccDNA was lower in transfected cells as compared with in liver tissue (Figure 14C). The

pgRNA/S-RNA ratio was similar to that observed in liver tissue from HBeAg positive patients, and was not influenced by BCP mutations. This discrepancy between transfected cells and biopsies might reflect that some transcription factors that are present *in vivo* are not active in Huh7.5 cells, and possibly this may be of importance for interpretation of the functional analysis of BCP mutations *in vitro*.

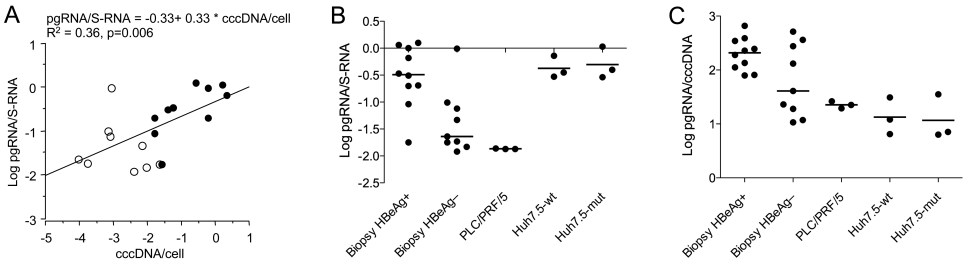

Figure 14. Ratio between pgRNA and S-RNA in liver tissue (A and B) and in vitro (B). Transcription efficiency (pgRNA/cccDNA) was lower in vitro than in liver tissue (C).

We also analysed HBV RNA in a hepatoma cell line with integrated HBV DNA, in which investigations carried out in the 1980ies revealed rearrangements and presence of several integrated segments [MacNab et al. 1976]. It is well known that these cells produce HBsAg (and thus must transcribe S-RNA), but to our knowledge the levels of transcripts have not been quantified. Our analyses indicate that these cells carry a segment (probably 1 copy/cell) that is amplified by the cccDNA PCR (nt 1575-1882). This is the region that contains the BCP and indeed pgRNA was detected. The transcription efficiency (pgRNA per cccDNA) was similar to the one observed in transfected Huh7.5 cells and in some of the biopsies from HBeAg negative patients. The pgRNA/S-RNA ratio was 1.5 log lower (Figure 14B) than in transfected cells (and similar to the ratio in some HBeAg-negative patients). The similar pattern of transcription in cells with integrated HBV DNA and HBeAg negative patients raises the question whether HBsAg production from integrated HBV DNA may be operative *in vivo*. This was previously (in the 1980ies) an accepted idea, but has not been much discussed since it was revealed by PCR in the 1990ies that most HBeAg-negative patients retain some viral replication [Blum et al. 1991; Lindh et al. 1996]. The possibility should be further explored, because it might contribute to explain the frequent observation that patient with undetectable HBV DNA in serum still have relatively high levels of HBsAg in serum.

5 CONCLUSIONS

The replication machinery of hepatitis B virus includes an error-prone polymerase enzyme, which is partly responsible for the plethora of viral strains. Genotypes and treatment-induced quasispecies influence the clinical course and outcome of treatment. During infection the clinical stage and response to treatment are evaluated through the quantification of viral DNA and HBsAg.

The main conclusions in this thesis are:

Real-time PCR is a simple one-step technique, well suited for the identification of nucleotide substitutions in the HBV genome. Assays can be composed in singleplex or multiplex manner to cover several targets in the same reaction mixture.

By combining primer and probe specificity in the design of PCR sets, high accuracy can be reached in the analysis of resistance-related mutations and genotypes. Mixed infections may be detected and their relative proportions revealed.

Infection with genotype C implies worse prognosis and clinical outcome of treatment, as compared with genotypes A, B, and D. Persons with highly active genotype C or D infection showed higher risk for progressive liver damage.

The reduction of viral DNA levels that are observed after HBeAg loss can only partly be explained by down-regulated transcription of pregenomic RNA. Enhanced transcription of S-RNA does not explain the extreme production of HBsAg.

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