Functional Analysis of Hepatitis B Virus Variants with Mutations in the Envelope Proteins

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Für Frank

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Zusammenfassung

Lebererkrankungen, die durch Infektionen mit Wildtyp-Hepatitis-B-Virus (HBV) hervorgerufen werden, sind vermutlich ausschließlich auf Immunsystem-vermittelte Mechanismen zurückzuführen. Bei der fulminanten Hepatitis B (FHB), einer besonders schweren Lebererkrankung mit häufig tödlichem Verlauf, scheinen jedoch direkt zytotoxische HBV-Varianten und deren besonders gute Erkennung durch das Immunsystem eine wichtige Rolle zu spielen. Ein Klasse von HBV-Varianten, die man im Serum eines Patienten gefunden wurde, ist durch Defizite in der Viruspartikelsekretion gekennzeichnet. Um mehr Informationen über solche HBV-Varianten zu generieren, wurden in dieser Dissertationsarbeit HBV-Genome eines Patienten mit FHB sowohl strukturell als auch funktionell charakterisiert.

Zunächst wurde untersucht, ob HBV-Varianten mit identischer Sequenz in einer Leberbiopsie des gleichen Patienten vorkommen, wie sie zuvor im Serum dieses Patienten gefunden worden waren. Die Sequenzanalyse offenbarte die gleiche Art und eine Vielzahl von Mutationen in beiden Proben, ein Indiz für ihre mögliche Relevanz für das pathogenetische Geschehen bei der FHB. Eines der klonierten Genome mit der Bezeichnung “Variante 5a” erwies sich als replikationskompetent, sekretierte jedoch sehr viel weniger Viruspartikel (<2%) als das ebenfalls durch Transfektion von Hepatomazellen getestete Wildtyp-HBV. Durch Mutations- sowie Funktionsanalysen konnten als Hauptursache für den Sekretionsdefekt ein Austausch von Cystein zu Arginin an Position 138 in der Sequenz des kleinen Hüllproteins ausgemacht werden. Dieses Protein wird mit HBs bezeichnet, die gleiche Sequenz ist jedoch auch als carboxy-terminale Domäne im großen Hüllprotein L vorhanden und wird daher auch als S-Domäne bezeichnet. Die amino-terminale Domäne des L-Proteins wird mit PreS1 bezeichnet. Die Einführung der gleichen Mutationen in die S-Domäne des L-Proteins, die beim HBs Viruspartikelnsekretion verhinderten, hatten keine Blockade der Sekretion von Viruspartikeln zur Folge. Dies impliziert, dass die Funktion von Mutationen in der S-Domäne sowohl vom Sequenzkontext als auch von der Konformation des entsprechenden Proteins abhängt. Die Reversion der Mutation von Arg138 zu Cys an Position 138 bei der HBV-Variante 5a löste den Sekretionsblock für subvirale Partikel und Virionen nur teilweise auf (ca. 50% Wildtypeffizienz). Die Einführung der Mutationen Cys138Arg und Cys138Ala im Wildtype HBV-Sequenzkontext reduzierte die Sekretion von subviralen Partikeln um 90% bzw. 70% und hatte darüber hinaus eine komplette Blockade der Sekretion von Virionen zur Folge. Letzteres spricht für eine essentielle Funktion von Cys138 bei der Virionenbildung und Sekretion. Diese Ergebnisse, Domänenaustausch- sowie Kommplementations-experimente ergaben zudem Indizien sowohl für negative Einflüsse von Mutationen außerhalb der Hüllproteinsequenzen auf die Viruspartikelsekretion als auch für eine Chaperon-ähnliche Wechselwirkungen zwischen HBs und dem L-Protein. Intrazellulär wurde trotz des Sekretionsdefekts keine Akkumulation von Viruspartikeln der HBV-Variante 5a beobachtet, es zeigte sich jedoch verstärkte L-Proteindegradation durch Proteasomen. Beide Faktoren könnten zur ausreichenden Virusproduktion und der Persistenz der HBV-Variante 5a als auch für dessen vermutlich verstärkte Immunerkennung im Vergleich zum Wildtyp-Virus beitragen. In Immunfluoreszenzstudien wurde bei

der HBV-Variante 5a eine aberrante subzelluläre Lokalisation der Hüllproteine in einer Zellkernrandzone beobachtet. Dies könnte eine Konsequenz oder Auslöser der Sekretionsdefekts sein sowie zum pathogenetischen Geschehen bei der FHB beitragen.

Zusammengenommen liefert diese Studie neue Erkenntnisse über die Struktur und Funktion von sekretionsdefekten, aber replikations- und komplementationskompetenten HBV-Varianten, die wahrscheinlich eine wichtige Rolle bei der Hepatopathogenese der FHB spielen.

Summary

Liver inflammation caused by infection of hepatocytes with Wildtype Hepatitis B Virus (HBV), designated hepatitis B, appears to be mediated exclusively by immune-system mediated mechanisms. In fulminant hepatitis B (FHB), a particular severe and often lethal course of HBV infections, directly cytotoxic HBV variants and their enhanced immune recognition are believed to play an important role. One class of variants found in patients with FHB are defective in viral secretion for unknown reasons. In order to get more information on the structure and function of such variants, HBV genomes derived from serum and liver of an FHB-patient were characterized in this thesis.

First, it was analysed whether HBV variants identical in sequence to those previously reported to occur in serum of this patient are also present in a liver biopsy taken from him. The same complex mutations were detected in both samples arguing for their possible pathogenetic relevance in FHB. One of the cloned full-length genomes, designated variant 5a, was found to be competent in replication but secreted much less viral particles than Wild type (<2%) when tested by transfection of hepatoma cells. By mutational analysis the secretion deficit was demonstrated to be primarily due to a Cys138Arg mutation in the small envelope protein sequence. This protein is designated HBs or alternatively S-domain in the large envelope protein L, which has in addition an amino-terminal domain, designated PreS1. The same mutations in the S-domain at the carboxyl-terminal end of the large envelope protein of variant 5a did not interfere with viral particle secretion, indicating that its function depends both on the sequence context and conformation of the protein. Reversion of the Arg138 to Cys in genome 5a partially restored secretion of subviral particles and virions (about 50%) whereas mutation of Cys138Arg and Cys138Ala in Wild type context reduced viral particle secretion to 10% and 30%, respectively. In the Wild type context virion secretion was abrogated by both mutations to undetectable level suggesting an essential role of Cys138 in virion envelopment and secretion. These data, gene domain swapping and complementation experiments provided also evidence for negative contributions of mutations outside of the envelope protein coding genes to the secretion phenotype of variant 5a and chaperon-like interactions between the small and large envelope proteins. Variant 5a exhibited no increase in intracellular viral particles but enhanced large envelope protein degradation by the proteasome. Both factors are presumably important for virus production sufficient for persistence and enhanced immune recognition of variant 5a. By immune fluorescence staining the mutant envelope proteins of variant 5a were localized aberrantly to a specific perinuclear zone, a possible consequence or inducing principle of the secretion deficit and may have contributed to virus-induced cell stress and FHB pathogenesis.

This study provides novel knowledge on the structure and function of secretion deficient, but replication and complementation competent HBV variants, which probably play an important role in the pathogenesis of FHB.

1. Abbreviations

Aa	Amino acid
anti-HBe	Anti-hepatitis B e antibody
anti-HBs	Anti-hepatitis B surface antibody
Brefeldin A	BfA
C-	HBV core-
Ci	Curie
CccDNA	Covalently-closed-circular DNA
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EndoH	Endoglycosidase H
ER	Endoplasmic reticulum
ERGIC	ER Golgi intermediate compartments
FHB	Fulminant hepatitis B
HBcAg	Hepatitis B core antigen
HbeAg	Hepatitis B e antigen
HbsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x protein
Hsc or Hsp	Heat-shock cytoplasm or protein
Kb	Kilo-base (DNA-size)
KDa	Kilo-Dalton (protein-size)
L-protein	HBV large surface protein
M-protein	HBV middle surface protein
Mabs	Monoclonal antibodies
MRNA	Messenger RNA
Nt	Nucleotide
nm	Nanometer
ORF	Open reading frame
P-	HBV polymerase-
Pab	Polyclonal antiserum
PAGE	Polyacrylamide gel
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PEG	Polyethylenglycol
PgRNA	Pregenomic RNA
PhCo	Phase-Contrast
PNGaseF	Peptidoglycanase F
pSM2-cs	Plasmid pSM2-controls
RcDNA	Relaxed-circular HBV-DNA
Rpm	Revolutions per minute
RNA	Ribonucleic acid
RNase H	Ribonuclease H
SEAP	Secreted alkaline-phosphatase
SDS	Sodium dodecylsulfate
S-protein	HBV small surface protein
SVPs	Subviral particles
U	Enzymatic units of reaction
Wt	Wild-type HBV genome

2. Introduction

2.1 Hepadnavirus infection and treatment

Hepatitis B virus (HBV) is the hepadnaviruses prototype, first identified in 1967 as a cause of hepatitis (Blumberg et al., 1967). It is a rather small and enveloped deoxyribonucleic acid (DNA) containing virus that causes acute and chronic liver diseases in men. Infection and replication takes place primarily in hepatocytes, the major parenchyma cell of the liver. The host range of HBV is limited to humans, chimpanzees (great apes) (Barker et al., 1975), and a specific strain of tupaias (Walter et al., 1996). Cell lines permissive for HBV infection are not available, and there are restricted possibilities of employing primary human hepatocytes (Ochiya et al., 1989). The major tools for investigation of this virus are the expression with full-length HBV-DNA, and the separate expression of single viral gene products in heterologous expression systems, using transfection of cloned HBV-DNA into liver- and hepatoma-derived cell lines or, alternatively, the use of animal models.

The most closely related viruses to HBV identified in non-primates are found in woodchucks (Summers, Smolec, and Snyder, 1978) and ground squirrels (Marion et al., 1980). Woodchuck and ground squirrel hepatitis viruses together with HBV have been grouped into the subfamily of Hepadnaviridae designated as orthohepadnaviruses. Infection with these viruses is associated with acute and chronic liver diseases and the development of hepatocellular carcinoma. These animal models have been used to study extrahepatic replication (Korba et al., 1986), mechanisms leading to chronic liver disease, and pathogenesis (Popper et al., 1987). These studies with animal models provided a wealth of very interesting information but not all of them may be relevant for HBV because several aspects are unique for animal hepadnaviruses (Tolle et al., 1998).

Most distant in evolution to HBV are the viruses found in domestic (Pekin) ducks and geese. These are grouped in the avihepadnavirus *genus*. The duck hepatitis B virus (Summers and Mason, 1982) model created the opportunity to define molecular aspects of the viral life cycle, since it is possible to transmit this virus directly to adult and embryos, and to infect primary hepatocytes or transfect cloned viral DNA into an avian hepatoma cell line. The duck model has also been particularly useful to study antiviral drug activity in vivo and in vitro, and to examine viral clearance. The duck hepatitis B virus and all its features are reviewed elsewhere (Funk et al., 2007).

In addition, the HBV transgenic mouse (Farza et al., 1988) has been introduced for understanding the mechanisms that lead to virus clearance (Wieland et al., 2003) or persistence of infection including cytotoxicity (Yu et al., 1999), and fulminant hepatitis B (FHB) (Ando et al., 1993). The HBV transgenic mouse model is also reviewed in detail elsewhere (Chisari, 1996).

HBV infection can result in acute, self-limited or fulminant hepatitis (5-10%), or become chronic. Patients with a so-called silent infection lack all HBV serological markers.

Chronic HBV infection in humans proceeds through several stages, which are diagnostically determined mainly by the viral markers: hepatitis B surface, e and core antigens (HBsAg, HBeAg and HBcAg, respectively), and the viral HBV-DNA. The first stage can last for periods of up to several decades, and is frequently characterized by an immune tolerance phase, the presence of HBsAg and HBeAg in serum, a high virus load and absence or minimal inflammatory liver disease (Chan et al., 1994; Ni et al., 1993). The second stage is often characterized by seroconversion from HBeAg to the corresponding antibody (anti-HBe) (Hsu et al., 1987). Seroconversion to anti-HBe is associated in most patients with low viremia and a lower risk to suffer from inflammatory liver disease, and a better long-term prognosis (Hsu et al., 1987). Subsequently, there is a seroconversion from HBsAg to the corresponding antibody (anti-HBs), which results in a further reduction of the viral load and resolution of liver disease, and in most patients it is followed by viral clearance. In some patients, liver disease is ongoing despite seroconversion to anti-HBe, in this cases a HBs seroconversion is often not observed. Patients with long-standing active liver disease are at a high risk of developing end-stage liver disease, liver cirrhosis, or hepatocellular carcinoma (Beasley et al., 1981). HBV is considered a major etiological factor in the development of human hepatocellular carcinoma.

HBV infection can cause acute hepatitis after the first establishment of infection or by spontaneous reactivation of viral replication during chronic HBV infection (Meyer and Duffy, 1993). Acute hepatitis is defined as a transient liver disease that can resolve spontaneously (acute self-limited hepatitis) or is associated with different degrees of liver inflammation up to a severe, life threatening form (fulminant stage) (Gocke, 1971). FHB occurs in about 1% of patients with acute hepatitis (Lee, 1993).

Hepadnaviruses are blood-borne and poorly transmittable infectious agents, except when transmitted by sexual contact or when persons are in contact with the blood or blood-contaminated products from an infected individual. In areas of high endemicity, perinatal transmission is the major transmission route (about 90% of the cases), and this frequently

results in chronic infection (Stevens et al., 1985). Up to now, HBV infection remains one of the most deadly infectious diseases in the world, as chronic carriers is estimated to be about 350 million worldwide with an associated annual mortality rate of around two million.

Immunization with vaccines containing HBsAg, prepared from plasma or by genetic engineering techniques, has been effective in preventing the establishment of chronic HBV infections (Fortuin et al., 1993), and the development of new, even more effective vaccines is ongoing (Avdicova et al., 2002). However, vaccination can lead to the emergence of HBV-genomes with specific mutations, designated escape variants (Basuni and Carman, 2004). This may make the development of new type of vaccines against these viruses necessary in the future. Additionally, therapeutic vaccination of chronically infected HBV patients with hepadnaviral antigens is an ongoing approach which aims at the breakage of immune tolerance to the virus (Michel and Loirat, 2001; Michel et al., 2001). Although successful induction of an antiviral immune response has been achieved occasionally, only future attempts will show whether this can be developed into an effective therapy for chronic hepatitis B.

Treatment of chronic hepatitis has had limited success. Two major classes of antiviral therapeutic agents that have been approved for treatment of chronic hepatitis are immunomodulating agents (like interferons) and nucleoside analogs (like lamivudine). Therapy with the immunostimulatory cytokine interferon alpha is only partially effective in inducing virus elimination in HBV carriers: it is ineffective in more than 50% of the carriers (Hoofnagle and di Bisceglie, 1997), it is expensive, it must be administered by injection, and there are severe side effects. In addition, HBV has developed resistance against interferon alpha treatment. The most successful nucleoside analogue against HBV treatment is lamivudine, which is a potent inhibitor of the viral reverse transcriptase with a sustained inhibition of viral replication. Interferon plus lamivudine achieves a higher viral suppression than either treatment alone (Tillmann, 2007).

Saving the life of patients with severe liver diseases, such as those with end-stage liver cirrhosis, severe FHB or liver carcinoma, is often only possible by liver transplantation (Fujiwara and Mochida, 2002). In order to prevent infection of the transplanted liver antiviral agents like nucleoside analogues are often used (Henkes et al., 2002). Furthermore, a strong immune response after organ transplantation (liver, kidney, heart, bone marrow) in HBV-infected patients is intentionally downregulated by immunosuppressive drugs to avoid graft rejection. These patients are in addition routinely treated with anti-HBs immunoglobulins to prevent reinfection.

2.2 Virion structure and genome organization.

The virion of HBV is a 42-nanometer (nm) particle, consisting of a 27-nm icosahedral nucleocapsid containing core- and polymerase-proteins (C- and P-proteins, respectively) surrounded by an envelope consisting of a lipid membrane of cellular origin, into which the viral surface proteins are inserted. The small size of the HBV genome, about 3.2-kilobases (kbs), allows the expression of a small repertoire of proteins in overlapping open reading frames (ORF) (Fig 2.2.1).

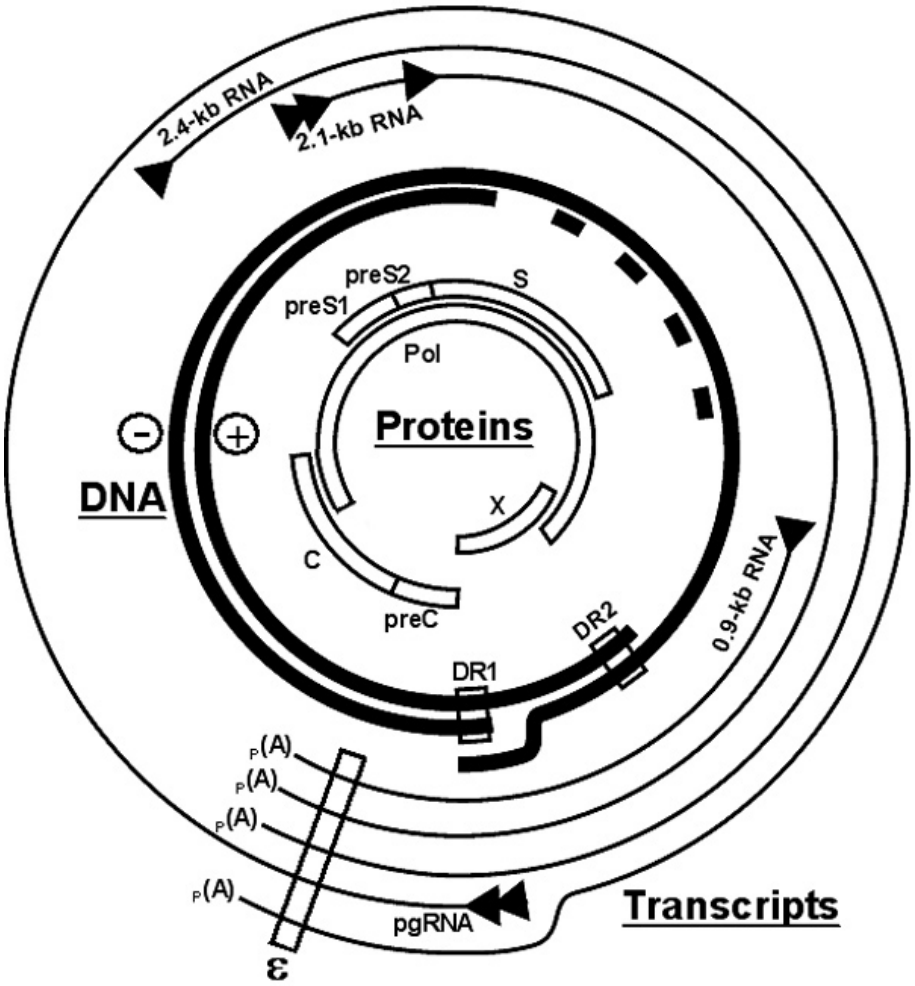


Fig. 2.2.1: **Genome organization, genome structure and transcription map of HBV**. HBV has a circular partially double-stranded DNA (thick circles), which contains two direct repeat regions (DR1 and DR2). The inner open arrows stand for the ORF encoded by the (-)-strand DNA. The outer wavy lines symbolize the major transcripts, the stem-loop region is depicted by epsilon (ε), and the common poly-adenylation site is indicated by $_{P}$(A).

The function of the structural genes is apparent. The C-gene encodes the C-protein with molecular mass of 21-kilo Dalton (kDa), the 183 amino-acid (aa) long capsid protein, also known as HBcAg. The only known chemical modification of the HBcAg is phosphorylation in the carboxy-terminal basic region, which might be involved in regulating the different functions of the HBcAg in replication or viral uncoating during entry (Yu and

Summers, 1994). Assembly of C-proteins into the icosahedral nucleocapsids requires its high intrinsic propensity to form dimers, stabilized through two disulfide-bonds, and promoted by two separate regions of the HBcAg (Konig, Beterams, and Nassal, 1998). The first region, a 144-aa of the amino-terminal domain, directs the assembly, mostly via a disulfide-bridge between two Cys61 residues. The second region, the carboxy-terminal residues, is also easily oxidized, resulting in disulfide-bridges cross-linking neighbouring dimers into a polymeric network. The carboxy-terminal region contains an accumulation of basic residues, which constitute an apparently non-sequence-specific nucleic acid binding domain, and mediate nucleic acid encapsidation. Two separate regions of the HBcAg are exposed to the surface: the major HBcAg epitope is located around aa 80 and the region encompassing HBcAg aa 127-133. The properties of the HBcAg have been reviewed elsewhere (Seifer and Standring, 1995).

The P-ORF, which extends almost along the whole HBV genome, codes for the P-protein (approximately 90-kDa). This protein contains four domains from amino- to carboxy-terminal: the 5'-terminal protein region, the spacer region, reverse transcriptase/DNA polymerase domain, and ribonuclease H (RNase H), respectively. Functionally, the P-protein has three enzymatic activities: it is a ribonucleic acid (RNA) dependent DNA polymerase (reverse transcriptase), a DNA dependent DNA polymerase and has RNase H activity (Radziwill, Tucker, and Schaller, 1990; Toh, Hayashida, and Miyata, 1983). The P-protein is also essential for encapsidation of the pregenomic RNA (pgRNA) which is mediated by its binding to a specific stem loop structure at the 5’-end of the pgRNA designated epsilon (Bartenschlager and Schaller, 1988). The P-protein functions also as a primer for the viral (-)-strand DNA synthesis. The spacer region of the P-protein is not essential for any of its known activities (Chiang et al., 1990).

Two additional gene products expressed during natural infection are still of largely unknown function, the hepatitis B x protein (HBx) and the HBeAg. HBx appear to be required for establishment of the infection in vivo (Chen et al., 1993), but dispensable for virus replication in transfected cells (Blum et al., 1992). Despite the association of multiple activities with HBx, none of them appear to provide a hypothesis regarding its biological function.

HBeAg is not necessary for HBV replication and secretion (Tong et al., 1991), but seems to be a modulator of the host response to viral infection (Milich et al., 1997). The 17-kDa HBeAg is produced by cotranslational amino and carboxy-terminal proteolytic processing from its 25-kDa precursor C-protein. This precursor protein is targeted into the

cell's secretory pathway due to its amino-terminal preC-signal-sequence that inserts the protein into the endoplasmic reticulum (ER). This signal sequence as well as the carboxy-terminal sequence of the preC-protein are cleaved off during the synthesis and secretion process of the mature HBe, as a soluble, non-viral particle associated protein. HBe differs from the nucleocapsid protein by a short additional amino-terminal sequence and lack of carboxy-terminal sequences. The mechanism to prevent dimer formation of HBe and assembly into a nucleocapsid-like structure is controlled by intramolecular dimer formation of Cys61 (Nassal and Rieger, 1993).

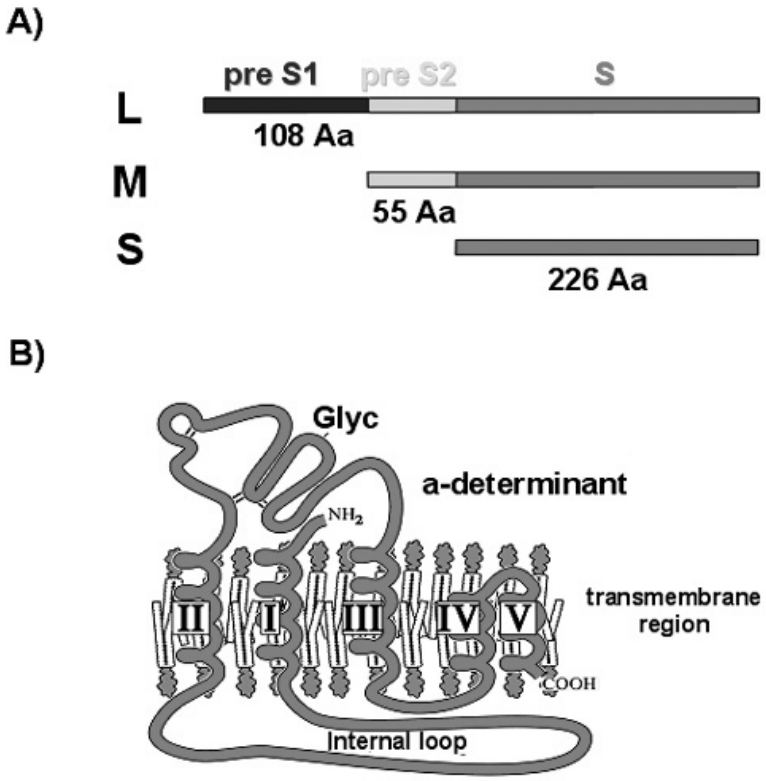


Fig. 2.2.2: **HBV envelope proteins and S-topology**. In panel A, the three products of the S-gene are represented as the large (L), the middle (M), and the small (S) envelope proteins. All of them share a common S-domain translated as the S-protein, whereas the M-protein contains an additional preS2-region, and the L-protein an additional preS1-domain. In panel B, the transmembrane regions of the S-envelope protein are depicted with numbers (I-V), which separate two hydrophilic domains. The first hydrophilic domain is a cytoplasmic-loop (internal loop), and the second is probably a luminal-domain, which contains the main antigenic region for the HBsAg (the a-determinant) and the glycosylation site (Glyc). This Fig B was kindly provided by M. Kann and W.H. Gerlich.

The viral envelope carries three different but related virus-encoded membrane proteins: the small (S), the middle (M), and the large (L) protein. Although these are encoded by a common gene (see Fig. 2.2.1), the L-protein is translated from a 2.4-kb messenger RNA (mRNA), and the M- and S-proteins mainly from a set of about 2.1-kb mRNAs. The S-protein can also be translated from a 2.4-kb mRNA by internal translation initiation at the S-domain initiation codon according to *in vitro* translation experiments (Sheu and Lo, 1992). The envelope gene consists of three in-phase ORFs, termed in 5'- to 3'-direction preS1-, preS2-

and S-domains with a common stop codon. The S-domain is responsible for the expression of the S-protein (226-aa); cotranslation of preS2/S-region yields the M-protein (281-aa containing the first 55-aa from the preS2-region), the entire preS1/preS2/S-gene expresses the L-protein (389-aa, where the first are responsible for 108-aa the preS1-region) (Fig 2.2.2-A). Consequently, the S-, M-, and L-proteins share a carboxy-terminal domain and differ in their increasing amino-terminal extensions. Length differences between different genotypes exist and are described in chapter 2.6.

HBV surface proteins are posttranslationaly modified, glycosylation occurs partially in the S- and L-proteins, with apparent molecular masses of p24-/gp27-kDa, and p39-/gp42-kDa, respectively. On the other side, the M-protein is normally mono- or di-glycosylated when secreted (gp33-/ggp36-kDa, respectively), but is detectable in a non-glycosylated form (p30-kDa) in cellular extracts. Additionally, the amino-terminus of the L-protein is myristylated (Prange, Clemen, and Streeck, 1991; Gripon et al., 1995).

2.3 Subviral particles and surface protein function

A characteristic for hepadnaviruses is that an excess of envelope proteins, not incorporated into virions, self-assembles into 22-nm spherical or, less abundant filamentous particles, which are found in infected patients and are named subviral particles (SVPs) (Eble, Lingappa, and Ganem, 1986). This is common to a few virus types (Shaw et al., 2003) and differs from the particle formation of the closely related retroviruses (Garoff, Hewson, and Opstelten, 1998). In all of the HBV viral particles, the S-protein is the predominant species. Spheres are estimated to consist of up to 100% of S-proteins and very little L-proteins (maximal 5%). In contrast, L-proteins are enriched in filaments (approximately 20-50%). In both form of SVPs the M-protein constitutes 5-10% of the total protein mass. The exact protein composition is difficult to measure and may vary with the source of viral particles.

Typical particle numbers in the serum of highly viremic chronic carriers are in the range of 10^{13}/ml for spheres, 10^{11}/ml for filaments and up to 10^{9}/ml for virions, but these values vary in different patients (Gerlich, Heermann, and Lu, 1992). Taking into account that 100-fold large excess in secretion of spherical particles is detected compared to filaments, and that spheres are mainly composed of S-protein, the 22-nm spherical particles of HBV are of historical interest as they represent the overwhelming majority of the classical ‘Australia antigen’ or HBsAg, measured by enzyme-linked immunosorbent assay (ELISA). For that reason, detection of filaments and virions might be preferably done by techniques using anti-preS1 monoclonal antibodies (MAbs) or DNA hybridisation techniques in case of virions.

The biological function of the SVPs is not clear; they could act as decoys that trap neutralizing antibodies in unproductive interactions or their production may enhance viral infection efficiency (Bruns et al., 1998).

The main role of the surface proteins in an infected cell is to act as a transport vector for viral particles out of the cell. Although having a common carboxy-terminal domain, the S- but not the L-protein, promotes secretion of virions and SVPs through the secretory pathway (Eble, Lingappa, and Ganem, 1986; Gerlich, Heermann, and Lu, 1992; Prange, Nagel, and Streeck, 1992). Moreover, L-protein overexpression inhibits the S-protein secretion (Persing, Varmus, and Ganem, 1986). On the other hand, the M-protein has no vital function in secretion of viral particles neither *in vitro* (Bruss and Ganem, 1991) nor *in vivo* (Fernholz et al., 1993). For virion formation, L-protein is essential, implying a matrix protein-like function of the preS1-domain for envelopment.

A well-defined role of HBsAg is its involvement in host immunity. A highly antigenic site in HBsAg is located between position 124 to 147 and this region is defined as the a-determinant. But antibodies are also produced against other sites of the surface proteins (anti-HBs, anti-preS1, and anti-preS2), reviewed elsewhere (Rehermann and Nascimbeni, 2005). The protective immunity seems to be important for virus clearance that is almost always associated with seroconversion from HBsAg to anti-HBs. This immune response seems to be activated in acute hepatitis but not in chronic hepatitis (Ferrari et al., 1990).

According to current knowledge, at least two determinants appear essential in the L-protein for virion attachment to receptor proteins of the host cell and virion internalisation: a amino-terminal preS1-sequence has been shown to block infection already at picomolar concentration and a modification of the protein by myristylation was shown to be essential either for attachment or entry or subsequently within the host cell fusion of viral and cellular membranes in endosomes (Engelke et al., 2006; Gripon et al., 1995).

The topology of these proteins (Fig 2.2.2-B) is achieved by protein signal sequences, by disulfide-bridge formation and by their capacity to oligomerize. All surface proteins are integral membrane proteins with more than one postulated transmembrane region (Stirk, Thornton, and Howard, 1992), in contrast to most viral glycoproteins (Doms et al., 1993). The presence of N-linked glycosylation in the surface proteins suggests an initial targeting into the membrane of the ER. An amino-terminal signal sequence (aa 10-20 hydrophobic regions called ‘signal I’) is responsible for ER targeting and directs the polypeptide into the membrane (High and Dobberstein, 1992). Complete translocation of the polypeptide into the ER lumen would occur in the absence of a so-called stop-transfer sequence (High and

Dobberstein, 1992). Subsequently, a cytoplasmic loop of about 50-aa follows. A second hydrophobic region ('signal II', aa 80-100) has also been shown to be embedded into the membrane; it contains a reverse translocation signal. This fact allows a stretch of some 70-aa to be luminally exposed, which carry the antigenic a-determinant, located from aa 124 to 147 within the S-domain, and contains the potential glycosylation site at Asn146. The following 50-aa residues are again hydrophobic and may be arranged into two more transmembrane domains (Stirk, Thornton, and Howard, 1992).

The carboxy-terminal transmembrane topologies of the M- and L-proteins are assumed to be similar to that of the S-protein. The N-terminal preS2-containing domain is translocated into the ER lumen by the downstream 'signal I', as the residue Asn4 of secreted M-protein is always glycosylated. In accordance with this, the preS1-domain that is essential for attachment to the target cell was also predicted to expand into the lumen.

Recent evidence, however, suggests that the membrane topology of the HBV envelope proteins might be more complex. First, the partial glycosylation of all envelope proteins at position Asn146 and protease-protection experiments with S-proteins integrated into microsomal membranes (Prange and Streeck, 1995) suggests that the second hydrophobic region in the carboxy-terminus of all three proteins seems to be translocated into the ER lumen only in a fraction of polypeptides. Second, the preS1-domain is required on the particle surface for cell attachment, while for virion formation the preS1-sequence is also required in the cytoplasm. PreS1/preS2-domains of the L-protein are initially cytoplasmic (Bruss et al., 1994; Ostapchuk, Hearing, and Ganem, 1994), while preS2-domain of the M-protein is cotranslationally translocated. The dual topology of L-protein is also confirmed by the lack of glycosylation in the potential preS1- and preS2-sites of the L-protein. The chaperone heat-shock cytoplasm (Hsc) 70 or Hsc70 seems to retain or delay the stay of the preS-region in the cytosol to help in proper folding (Loffler-Mary, Werr, and Prange, 1997). Translocation of preS-domains is likely to occur in a posttranslational fashion, and promoted by a preS-specific sequence which is located in the carboxy-terminal region of the preS1-domain (Bruss and Thomssen, 1994; Loffler-Mary, Werr, and Prange, 1997). The same region is essential for interaction with the nucleocapsid (Bruss, 1997; Bruss et al., 1996). The heterogeneity in preS1/preS2 topology is maintained in virions, where some of the preS1/preS2-domains are hidden (Loffler-Mary, Werr, and Prange, 1997).

2.4 The hepadnaviral life cycle

Schematically, hepadnavirus replication takes place though a series of steps shown in Figure 2.4.1. HBV virions contain nucleocapsids with a single partially double-stranded relaxed-circular HBV-DNA (rcDNA) genome, which infect mainly hepatocytes but extrahepatic infection has also been reported (Hadchouel et al., 1988; Yoffe et al., 1986). Tissue specificity of HBV is controlled by a still unidentified hepatocyte-specific receptor. The preS1-domain is probably most important for attachment of HBV to human hepatocytes (De Meyer et al., 1997). Additionally, an amino-terminal determinant of the L-protein contributes also to the host range of HBV (Engelke et al., 2006). In addition, hepatotropism is also manifested on the level of viral gene expression.

The route and mechanism of viral DNA transport into the nucleus are unknown, but the phosphorylated C-protein may play a role (Yu and Summers, 1994). Immediately after the successful transport of the genome into the nucleus, the rcDNA is converted into the covalently-closed-circular DNA (cccDNA). Using the minus-strand of the cccDNA as a template, a terminally redundant pgRNA and the three subgenomic mRNAs (Fig. 2.2.1) are synthesised by the cellular RNA polymerase II, processed and polyadenylated at the common polyadenylation site in the amino-terminal region of the C-gene with the help of the cellular enzymatic machinery.

HBV sequence elements that display promoter (preS1-, S-, X- and C-promoters) and/or enhancer activity (enhancer-I and II) as well as hepatotropic-specific liver cell factors are involved in regulation of viral gene transcription. Effective and proper transcription of the redundant genome involves in addition two regulatory sequences designated PET- (positive effector of transcription for double round) and NET-sequences (negative effector of transcription for termination after the second round), respectively (Beckel-Mitchener and Summers, 1997; Huang and Summers, 1994). Expression of the different surface proteins seems also be regulated by the mRNA levels in form of a feedback mechanism, where accumulation of the L-protein increases the synthesis of the S- and M-proteins, which might favour the synthesis of a proper ratio of envelope proteins for viral particle budding and secretion (Xu, Jensen, and Yen, 1997).

After synthesis the RNAs are transported to the cytoplasm, where translation, viral particle assembly and DNA synthesis takes place. Expression of the P-protein and HBcAg might be co-regulated because both proteins are translated from a common template, the pgRNA, although not in the same reading frame. Each molecule of the pgRNA may serve as a

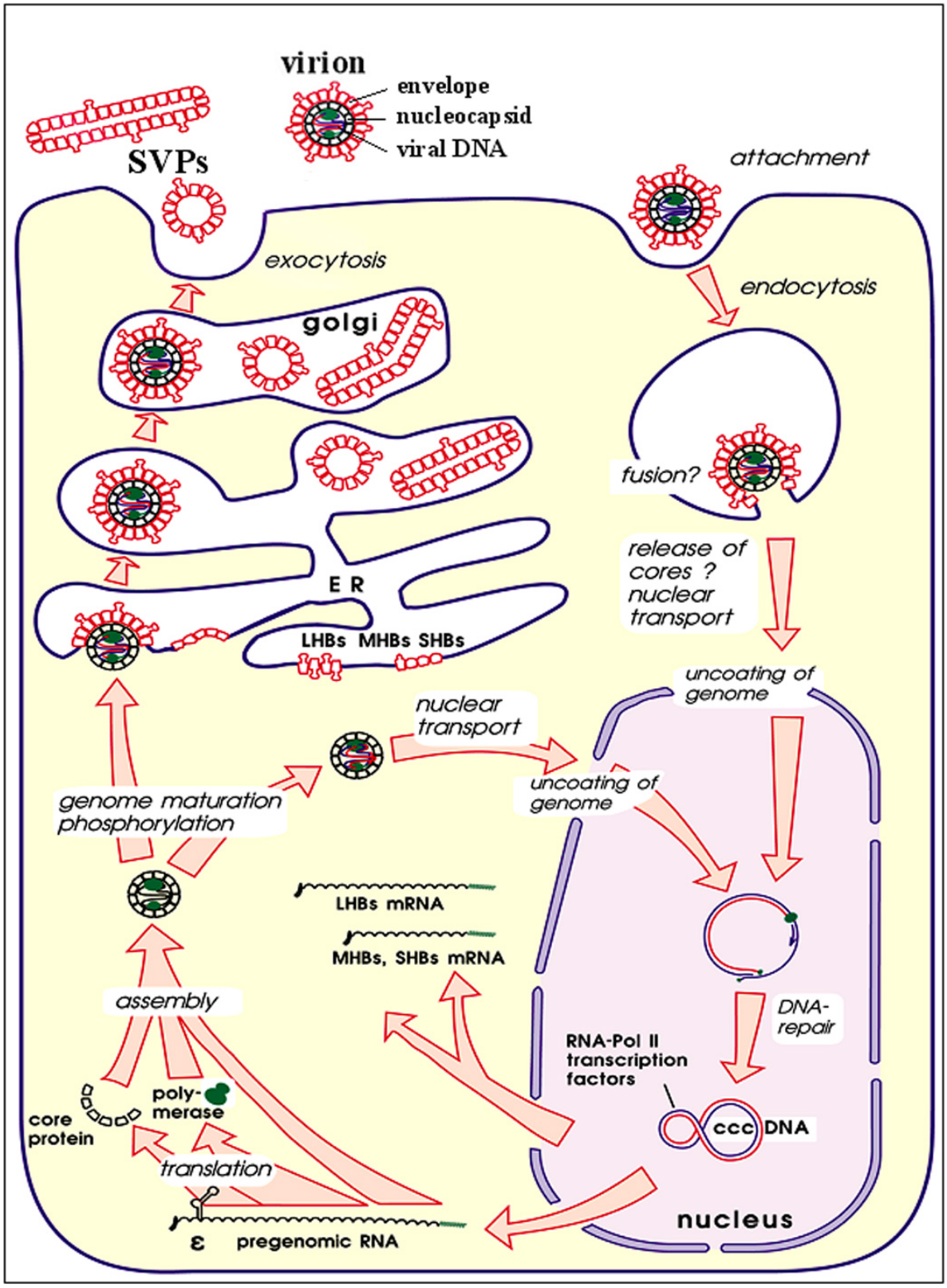


Fig. 2.4.1: **HBV life cycle**. Attached virions are internalised and probably fuse with internal cellular membranes. Viral DNA is then transported to the nucleus and repaired to form the circular HBV-DNA depicted as cccDNA. The pgRNA and the mRNAs are transcribed, and transported to the cell cytoplasm, where translation takes place. Direct interaction of pgRNA with the P-protein directs its encapsidation by C-protein oligomers. Nucleocapsids can be retransported to the nucleus and add to the cccDNA pool or interact with envelope proteins for virion secretion. Additionally, surface protein expression lead to the secretion via Golgi-apparatus of HBV-DNA free-particles, spheres and filaments, named generally SVPs. This figure was kindly provided by M. Kann and W.H. Gerlich.

mRNA for the translation of approximately 200-300 molecules of HBcAg before allowing the translation of a single P-polypeptide. The P-protein binds to the 5'-end of its own mRNA to initiate reverse transcription and packing (Bartenschlager, Junker-Niepmann, and Schaller, 1990). Synthesis of the P-protein is sufficient to stop further translation of the pgRNA and promote encapsidation.

Replication of the viral genome occurs in nucleocapsids, a ribonucleoprotein complex that is present in the cytoplasm of infected cells. First, the HBcAg forms dimers, facilitated by Cys61 disulfide-bridge formation, and with threshold accumulation 120 dimers assemble into icosahedral capsids. The ribonucleoprotein complex formation also depends on the P-protein, the presence of cis-acting sequences on pgRNA, the stem-loop epsilon (Bartenschlager and Schaller, 1992), and cellular factors like Hsp90 (Hu and Seeger, 1996). Following complex formation, (-)-strand DNA is reverse-transcribed by a protein-priming mechanism. The epsilon RNA-hairpin serves as a template for the synthesis of the primer, the short DNA oligomer is transferred to the 3'-end of pgRNA, the direct repeat 1 sequence (-)-strand DNA is synthesized, and the RNA is concomitantly degraded by a RNase H activity of the P-polypeptide (Radziwill, Tucker, and Schaller, 1990). When (-)-strand DNA synthesis has been completed, (+)-strand DNA synthesis initiates with the help of a RNA primer, which is derived from the 5'-end of the pgRNA and created by cleavage mediated by the viral RNase H activity. After translocation, the RNA primer hybridises with sequences near the 5'-end of (-)-strand DNA. Most (+)-strands DNA are half genome length in the rcDNA and this is the predominant species in nucleocapsids. However, small fractions of HBV virions also contain linear genomes.

The HBV DNA containing nucleocapsids may have two destinies: they can either be secreted in the form of virions after they have been enveloped, or they are transported to the nuclear pores where they disassemble and deliver the rcDNA into the nucleoplasm. The rcDNA is then repaired in order to increase the nuclear cccDNA-pool.

2.5 Secretion of hepatitis B virus particles

Like corona viruses, hepadnaviruses need structural and envelope components for virion secretion, and thus follow a secretion pathway type I (Garoff, Hewson, and Opstelten, 1998). The export might be via the normal secretory pathway, as suggested by the characteristic modifications of the glycan-chains of its envelope proteins. Cytoplasmic virions with mature rcDNA as well as SVPs are assembled and secreted only when sufficient S- and

L-proteins are present in ER membranes. The M-protein seems to be dispensable (Fernholz et al., 1993), but it participates in the protein composition of virions and SVPs.

Different to rhabdoviruses or retroviruses, the apparent dependence of nucleocapsid budding on the presence of the L-protein implies that HBV envelopment relies on specific interactions. In addition, viral particle formation requires precise molecular interactions of their surface proteins. The fact that phenotypic mixing of surface proteins is allowed within closer related mammalian viruses (HBV-woodchuck hepatitis virus), but not between mammalians and avian hepadnaviruses (HBV-duck hepatitis B virus) (Gerhardt and Bruss, 1995; Chang et al., 1994) demonstrate the precision of these interactions. The translocation signals in the S-domain have sequence identities of 66 and 38% in the aa-level, respectively, and they are supposed to have similar topology.

The mechanism of the envelopment signal for virions is unknown, but is thought to involve a conformational change at the surface of the capsid that facilitates interaction with the envelope proteins. A region in the carboxy-terminal preS1-domain interacts with the nucleocapsid. In addition, newly synthesized L-protein interacts with the chaperone Hsc70 (Loffler-Mary, Werr, and Prange, 1997), which is found in the inner part of virion particles. It is tempting to speculate that the interaction with Hsc70 might induce inhibition of L-protein translocation. The meaning of the L-protein retention and inhibition of translocation might enhance virion production versus SVPs in vivo, but is not a prerequisite for virion formation in transfected cells (Bruss and Thomssen, 1994). In addition to L-specific interaction, S-domains of envelope proteins could be involved in interaction with nucleocapsids and therefore have an influence in virion formation.

In vitro studies have demonstrated that the S-protein alone, even after expression with heterologous promoters such as simian virus 40- or cytomegalovirus (CMV)-promoters, can drive the HBsAg assembly process for formation of spherical SVPs (Aden et al., 1979). In the presence of an excess of the L-protein, particles are also assembled into filamentous SVPs (see Fig. 2.4.1).

All hepadnaviral surface proteins also contain abundant Cys residues, which are extensively used for intramolecular and intermolecular disulfide-bridges (Mangold et al., 1997). Correct disulfide-bonding is essential for correct protein structure, particle formation and further secretion (Mangold and Streeck, 1993; Mangold et al., 1995; Mangold et al., 1997). The mechanism signalling envelopment of SVPs is unknown, but at least two steps are well investigated. First, the surface proteins generate dimers by disulfide-bridge formation. In the presence of enough dimers, particles are assembled. The first step occurs in ER-

compartments, which contains the ER-resident protein disulfide isomerase (PDI), while the second step occurs in compartments devoid of PDI (Huovila, Eder, and Fuller, 1992).

Several lines of evidence show that viral particles bud into the pre-Golgi compartments (Huovila, Eder, and Fuller, 1992). First, the lipid composition of viral particles resembles more pre-Golgi membranes than plasma membrane, however, phophatidylinositol and cholesterol in HBsAg membranes do not correlate in the ER lipid compositions (Satoh et al., 2000). Second, biochemical analysis showed that intracellular surface proteins are sensitive to endoglycosidase-H (endo-H) while the extracellular forms are resistant. The endo-H enzyme digests only immature, high mannose-type glycans (Patzer, Nakamura, and Yaffe, 1984). This indicates that budding might occur prior to the pre-Golgi compartments and the rate-limiting step of export might lie prior to the medial Golgi.

2.6 Hepatitis B virus genotypes and variants

Lack of the 'proof reading' activity of P-protein during reverse transcription and DNA synthesis might be the main reason for the appearance of single point mutations, similarly as described for retroviruses (Girones and Miller, 1989). Assuming an error rate of the HBV reverse transcriptase similar to that of the retroviral ones (Roberts, Bebenek, and Kunkel, 1988), a large amount of genomes with *de novo* mutation could be produced per day (about 10%). In addition to the appearance of single mutations, deletions and insertions can occur during reverse transcription, or be induced by nonhomologous recombination. Sequence changes are also likely to result from co-infection of one cell with different strains, genotypes or subtypes.

Phylogenetically, the natural variability of the HBV genome is constituted by subtypes and genotypes, which cluster geographically. The classification of a subtype is determined by the difference in recognition of HBV surface proteins with antibodies. In addition to the known, relatively well-conserved a-determinant common to all subtypes, there are the mutually exclusive determinants d/y and w/r. Consequently, there are four major serological subtypes: adw, adr, ayw, ayr (Mimms et al., 1990). The difference in antigenicity has a genetic basis: d/y by Lys/Arg at position 122, and w/r by Lys/Arg at position 160 (Okamoto et al., 1987) of the HBsAg coding region. Besides, from the classification of genotypes determined by sequence heterogeneity, HBV is currently divided into eight genetic groups, the so-called genotypes A-H (Norder et al, 2004). There are some characteristic differences between the genotypes: genotype A HBV genomes are characterized by a 6-nucleotide (nt) insertion at the 3'-end of the C-gene, and genotype D genomes lack 33-nts in the preS1-

region, leading to an amino-terminally truncated L-protein and a deletion in the P-protein. Geographically, genotype A HBV genomes are prevalent in USA, in Northern and Middle Europe, and in South Africa; genotype B and C in the Far East; genotype D in the Mediterranean area and in the Near and Middle East; genotype E in Africa; genotype F in South and Central America (preferentially among Amerindians). Genotypes F and H are described elsewhere (Norder et al, 2004). HBV genomes named Wild-type (Wt) are rather arbitrarily often designated according to the sequence of the first HBV genome discovered for each genotype. For genotype D, the Wt sequence was assigned by Galibert and colleagues (Galibert et al., 1979). However, there are still major gaps in knowledge, it is currently considered that genotypes play a role in outcome of infection (Schaefer, 2005).

Naturally occurring mutated HBV genomes without direct phylogenic relation with genotypes are assigned as variants (Bichko et al., 1985; Blum, 1993). Although the immune-system plays an essential role in determination of virus clearance or pathogenesis, a large number of variants are described, and some of them are sometimes related with a specific outcome of the infection (Carman, 1997; Gunther et al., 1999). However, strains isolated from nocosomial (blood-infection) outbreaks, fulminant outbreaks or family member transmission were highly homologous (Liang et al., 1991; Petrosillo et al., 2000).

HBV variants can coexist with Wt in the same patient (Jeantet et al., 2002; Kalinina et al., 2001), and can be selectively transmitted and survive in newly infected human beings (Raimondo et al., 1993; Santantonio et al., 1997). The emergence of drug resistant variants has been one of the handicaps for successful HBV treatment of patients, immunosuppressed patients or infected patients after vaccination (Buti et al., 1998; Gunther et al., 1992; Gunther et al., 1998; Gunther et al., 1996; Xiong et al., 2000). For instance, the G145R S-gene escape variant is selected after long-term polyclonal anti-HBs immunoprophylaxis (Santantonio et al., 1999) or the most commonly described drug-resistant strain YMDD-variant is described to emerge after long-term lamivudine therapy (Chayama et al., 1998). Such variants impair HBsAg seroconversion and clearance of the infection.

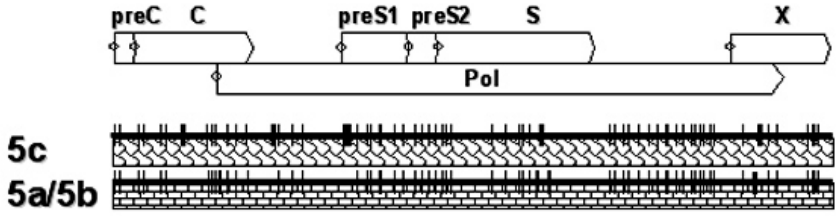
2.7 Variants from a patient with fulminant hepatitis

Acute hepatitis with fulminant stage is characterized by the combination of liver cell necrosis and encephalopathy (disturbances in mental function), while in acute hepatitis the mental function is normal. FHB is associated with high mortality, with more than half of the patients dying despite receiving liver transplantation (Williams, 1996). Patients develop signs of multiple organ failure and often die of a secondary infection syndrome, like bacterial

infection. Unfortunately, few models are available for studying the pathogenesis of FHB: murine hepatitis virus strain-3 (Levy, 2000) is one, a transgenic model of HBV infection (Ando et al., 1993) is another one, and studies of clinical cases.

Several factors induce fulminant hepatitis: alcohol, drugs, and viral infections. The main viral agents are hepatitis A (31%), hepatitis B (29.2%), other hepatitis viruses rarely cause this stage (like hepatitis delta virus) (O'Grady and Williams, 1993), non-hepatic viruses can also cause fulminant-stage of hepatitis. The pathology of virally caused hepatitis with fulminant consequences remains unclear, the immune system seems to play the major role in pathogenesis, but viral factors are believed to play a role, too.

A)



B)

HBV nt	Wt	Serum	5a	5c
1	T	C	C	C
25	G	A	A	A
53	T	C	C	C
96	T	.	C	C
110	C	A	A	A
112	T	A	A	A
128	T	A	A	A
346	T	C	C	C
499	A	C	C	C
520	G	A	A	A
528	T	C	C	C
533	A	C	C	C
543	G	.	A	.
554	T	.	.	C
566	T	.	C	.
852	G	A	A	A
882	T	C	C	C
925	A	G	G	G
930	A	T	T	T
961	A	G	G	G

Fig. 2.7.1: **Serum population and variant HBV genomes**. In panel A, HBV full-length genome is represented with the preC/C-, preS1/preS2/S- P- and X-genes. Genomes 5c as well as the identical genomes 5a and 5b are presented below with single mutations represented by vertical bars, compared to Wt sequence genotype D. Mutations were detected after direct HBV genome amplification from the serum of patient 5, and the variants 5a and 5c are listed in the table for the HBV region between nt number 1 to 961, corresponding to the S-gene. The table in panel B was obtained from Sterneck and colleagues (Sterneck et al., 1998).

Evidences point to the fact that emergence of variants can influence the course of viral hepatitis B infection and FHB development. Several cases of reactivation of HBV infection and/or *de novo* FHB have been described (Ehrmann et al., 1996; Meyer and Duffy, 1993; Sterneck et al., 1996). Occasional transmission of HBV variants from a single origin (one patient), designated as outbreaks, has caused concomitant FHB in several patients (Liang et

al., 1991; Petrosillo et al., 2000; Tanaka et al., 1995). In addition, FHB has been connected by functional analysis to the presence of HBV variants of specific phenotypes: C-gene mutations and loss of secretion of HBeAg (Alexopoulou et al., 1996; Friedt et al., 1999; Hasegawa et al., 1991; Laskus et al., 1995; Ogata et al., 1993; Sterneck et al., 1998; Stuyver et al., 1999), high replication variants (Hasegawa et al., 1994; Sterneck et al., 1998; Stuyver et al., 1999), or preS2-defective variants (Pollicino et al., 1997). Mechanisms by which these mutations can lead to pathogenesis are reviewed elsewhere (Liu, 2001).

Recently, a novel phenotype connected with variants isolated from two patients with FHB was found. Several variants showed a viral particle secretion deficiency according to an investigation by ELISA (Sterneck et al., 1998). In the serum of one patient (designated patient 5), several variants, designated here 5a, 5b and 5c, presented mutations along the whole HBV genome (Fig. 2.7.1-A) (Sterneck et al., 1998). Variants 5a and 5b were identical, and therefore only variant 5a was further investigated in the current study.

2.8 Aims of this thesis

The aim of this work was to characterize functionally HBV variants with a viral particle secretion defect which were previously isolated from serum of a patient, designated with number 5, suffering from FHB. Specifically the following questions were addressed:

-Are the S-gene mutations naturally occurring in the liver of the patient or are they an artefact of the PCR-amplification?

-Do these variants represent a dominant population in the liver?

-Do these variants have a specific phenotype?

-If yes, which mutations of the variant genomes are responsible for these phenotypes?

-Does reversion of these mutations reverse the phenotype?

-Is secretion of virions and SVPs equally affected by mutations in the surface proteins?

-Could these mutations be implicated in nucleocapsid interactions?

-Are the intracellular surface protein pools of the variants present in different amounts than those of the Wt-virus?

-Is variant HBV genome expression cytotoxic in cell culture after transfection in contrast to Wt-virus?

-Is there a relation between variant 5a phenotypes and severe pathogenesis?

-Can the variant with a defect in viral particle secretion be rescued by complementation by Wt-virus?

3. Results

3.1 Analysis of HBV variants from patient 5

The immune system plays an essential role in the pathogenesis after hepadnaviral infection, and can be responsible for the emergence of a large number of variants. Some of these variants are related with failure of treatment or vaccination or with a specific outcome of infection (Carman, 1997; Gunther et al., 1999). The presence of HBV variants from a patient suffering fulminant end-stage in the liver, their prevalence and the functional analysis of these variants were studied in this chapter, in order to understand the hepatitis B viral factors of these variants leading to pathogenesis.

3.1.1 Mutations in HBV genomes from a liver biopsy of patient 5

Variants might be mainly created due to the lack of 'proof-reading' activity of the hepadnavirus reverse transcriptase or may be due to recombination between viral subspecies within infected hepatocytes. Competent variants might be secreted and circulate in the serum. Consequently, viral population present in the liver and in the serum was initially supposed to be similar. However, strong immune response or specific phenotypes, like silent serological markers, can lead to drastically different viral population in both compartments (Lugassy et al., 1987). In these cases, analysis of variants in the liver is required.

In the serum of patient 5, several variants were previously detected, named 5a and 5c. The variant 5a contained a nt mutation, which lead to a substitution of the aa Cys in position 138 of the a-determinant to Arg (Sterneck et al., 1996; Sterneck et al., 1998). However, after HBV-DNA genome amplification from the serum of patient 5, direct sequence analysis of the DNA of this HBV population did not show this aa-exchange in position 138 of the S-domain (Fig. 2.7.1-B, column serum). This suggested that variant 5a existed either only as a minor population in the serum or only in the liver. Alternatively, mutants with the Cys138Arg mutation in the S-domain might be detectable in HBV-amplified products from a liver biopsy only as mixed virus population.

A paraffin-embedded piece of liver-tissue from the patient 5 in the FHB stage, an HBV-positive paraffin-embedded tissue from another patient, an HBV-negative paraffin-embedded tissue, and a serum of an HBV-infected patient were investigated. The HBV-positive tissue and the HBV-positive serum were used as positive controls for HBV-DNA extraction and purification, respectively, while the HBV-negative tissue was used for the

exclusion of contamination. In order to isolate total DNA from the samples, small slices of about 25mg of tissue were dewaxed and digested with proteinase K. A *tissue DNA purification* kit was then used for DNA purification of dewaxed tissues and of an aliquot of HBV-positive serum.

HBV variants from the infected liver were amplified by full-length HBV polymerase chain reaction (PCR) (Gunther et al., 1995a). The efficiency of the PCR was measured by amplification of pSM2-plasmid (a HBV-dimer), ranging from 10^{3}, 10^{1}, 10^{-1}, 10^{-3}, 10^{-4}, and 10^{-5}pg/µl of plasmid concentration, designated later as pSM2-controls (pSM2-cs). The lowest pSM2-DNA concentration of 10^{-5} pg/µl corresponds to approximately 1 HBV DNA molecules in 1µl. Full-length PCR (Fig 3.1.1.1) was performed with 1µl for pSM2-cs and DNA samples isolated from HBV-positive tissue, HBV-negative tissue, and HBV-positive serum. PCR products were detected neither for the HBV-positive nor the HBV-negative tissues (Fig 3.1.1.1, lanes 8 and 10, respectively), whereby the sensitivity of amplification was about 10-genomes of the pSM2-cs (Fig 3.1.1.1, lanes 1-6). In addition, HBV-DNA amplification of the HBV-positive serum yielded a significant band at the position of 3.2-kb (Fig 3.1.1.1, lane 9). The amplification of a full length HBV genome of about 3-kb from the tissues was unsuccessful, probably due to DNA fragmentation and cross linking which occurred during paraffin embedding and fixation.

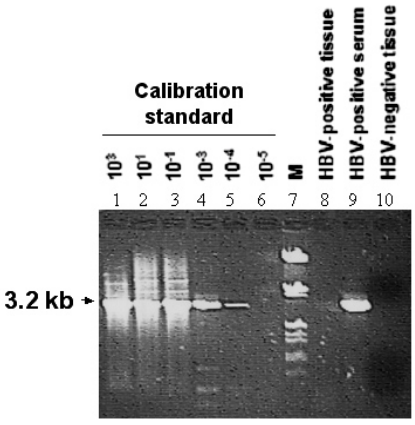


Fig. 3.1.1.1: **Amplification of full-length HBV variants from the liver**. HBV-DNA was amplified by full-length PCR protocol, and DNA-fragments were separated and detected in an ethidium bromide stained 1% agarose gel. pSM2-cs of 10^{3}, 10^{1}, 10^{-1}, 10^{-3}, 10^{-4} to 10^{-5}pg/µl (lanes 1-6, respectively) were used for determination of the efficiency of the reaction. An HBV-positive serum (lane 9) and HBV-positive as well as an HBV-negative paraffin-embedded tissues (lanes 8 and 10, respectively) were subsequently analysed. Lane 7 corresponds to the DNA molecular weight markers 21226, 5148, 4268, 3530, 2027, 1907, 1884, 1375, 947, 831, and 567 in base pair-sizes (M).

The study was then focused on amplifying the viral DNA comprising the a-determinant of the S-domain, since it is well described in the literature that mutations in the HBV surface proteins can cause intracellular protein retention and change in antigenicity

(Mangold and Streeck, 1993; Mangold et al., 1995) and, in this region, an aa-exchange cluster in the variant genomes was observed (Sterneck et al., 1996).

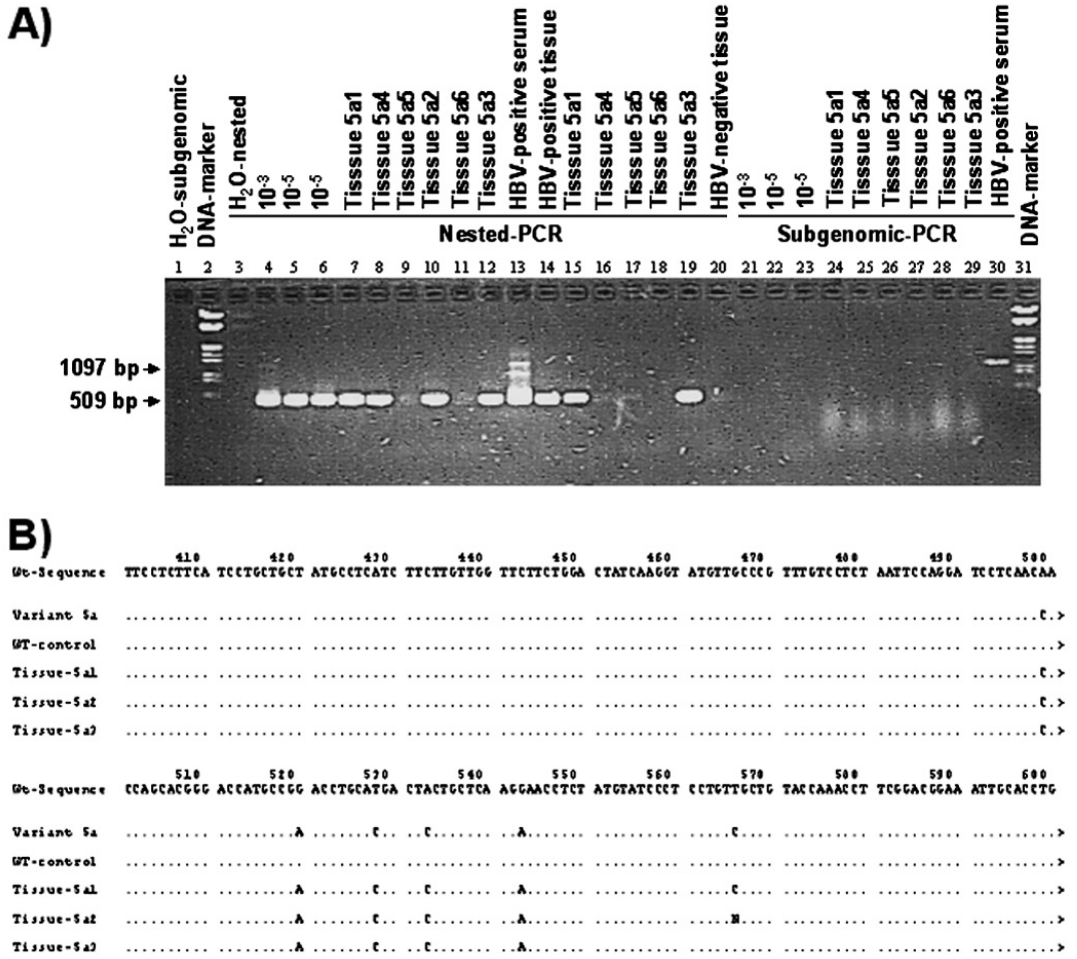


Fig. 3.1.1.2: **Analysis of the viral HBV population from an infected liver by subgenomic-PCR**. In panel A, the presence of HBV-DNA was determined by subgenomic-PCR (lanes 1 and 21-30) and nested-PCR (lanes 3-20). Six independent small slices of liver tissue of patient 5, named 5a1 (lanes 7, 15, and 24), 5a2 (lanes 10 and 29), 5a3 (lanes 12, 19, and 29), 5a4 (lanes 8, 16, and 25), 5a5 (lanes 9, 17, and 26), and 5a6 (lanes 11, 18, and 28) were used. As positive controls, subpopulations in an HBV-positive tissue (lane 14) and an HBV-positive serum (lane 13) were also amplified. As a negative control, HBV-negative tissue (lane 20) was assayed. As PCR-mock control, water (H_2O) was used both for the nested-PCR (lane 3), and as well as for the subgenomic-PCR (lane 1). The efficiency of the PCR was controlled by amplification of pSM2-cs of plasmid with 10^{-3} pg/µl (lanes 4, and 21) and 10^{-5}pg/µl of *DN*A (lanes 5-6 and 22-23), respectively. Lanes 2 and 31 correspond to the DNA molecular weight markers (DNA-marker). The sequences (panel B) were obtained from PCR-amplified products of tissue 5a1, tissue 5a2, and tissue 5a3 and aligned to Wt from the data base (Wt-sequence). Two pSM2-cs (corresponding to the products in panel A, lanes 5-6) had identical sequences (designated here Wt-control), which were identical to Wt genotype D (designated Wt-sequence). Variant 5a sequence (designated Variant 5a) was included for alignment (Sterneck et al., 1996).

For sequence analysis, the presence of HBV-DNA in fixed liver-tissue was first investigated after 30-cycles of subgenomic-PCR (Fig. 3.1.1.2-A, lanes 1 and 21-30). Several combinations of primers were tested with the pSM2-cs, whereby the combination +67 and –1164 yielded the best result. However, amplification by subgenomic-PCR was not successful for HBV-DNA from paraffin-embedded tissues. Amplification of such a low titer of HBV-DNA required a high number of PCR cycles. Subsequently, nested-PCR (Fig. 3.1.1.2-A, lanes

3-20) was performed with the primers +191 and –700 from the products of the subgenomic-PCR.

To avoid the possibility of introduction of artificial mutations, a Hifi-polymerase was used for both subgenomic- and nested-PCR amplifications. This Hifi-mixture has a three-fold lower error rate than normal *Taq*-polymerases (see Material and Methods section). In addition, the error in this experiment was also controlled by amplification (Fig. 3.1.1.2-A, lanes 5-6) and sequencing (Fig. 3.1.1.2-B, Wt-control) of two independent pSM2-cs samples (DNA concentration of 10^{-5}pg/µl). In these experiment artificial mutations were not detected, and both amplified sequences corresponded to Wt genotype D, as expected. Therefore, the efficiency of the combined-PCR (subgenomic- and nested-PCRs) was about 1-genome per amplification, because these two independent amplifications of pSM2-cs (Fig. 3.1.1.2-A, lanes 5-6) were positive.

Six purifications (5a1-5a6) from the tissue of patient 5 suffering from FHB were subjected to HBV-DNA specific PCR under the same conditions as for the controls mentioned above (Fig. 3.1.1.2-A). Three samples were positive: 5a1-5a3 after nested-PCRs (Fig. 3.1.1.2-A, lanes 7 and 15; 10 and 12; and 19, respectively), but not after subgenomic-PCR amplification (Fig. 3.1.1.2-A, lanes 24, 27 and 29, respectively). Positive PCR products from each single tissue sample were collected and mixed together for sequencing with primers in the S-domain. Alignment of the sequences (Fig. 3.1.1.2-B) showed appearance of specific mutations in nt positions 499, 520, 528 and 533. These mutations are characteristic for both variants, 5a and 5c (Fig. 2.7.1-B).

In addition to the common mutations, two additional nonsynonymous (aa-altering) mutations at nt positions 543 (Gly130Glu) and 566 (Cys138Arg) were specific only for the 5a variant genome and detected neither in variant 5c nor in the serum population. While the first mutation (nt 543: Gly130Glu) was present in the total viral population isolated from the liver biopsy of patient 5, the exchange Cys138Arg was only present in some amplified products. In tissue 5a1, sequence analysis showed the presence of a mutation leading to a Cys138Arg exchange. In contrast, this mutation was combined with Wt sequence in the second piece of tissue, 5a2. The third piece of tissue investigated, 5a3, showed sequence corresponding to Wt, a Cys in position 138. In summary, the HBV variant with an aa-exchange Cys138Arg represented over 30% of the total liver viral population. Therefore, it is a natural occurring mutation of HBV genomes in liver and serum of patient 5.

Surprisingly, aa-exchange Tyr134His (nt T554C) of variant 5c (variant 5c sequence genome see Fig. 2.7.1) was not present in an HBV-population of liver specimen of patient 5.

This mutation could have been introduced by the Hifi-PCR system during amplification of HBV-DNA from the serum of the patient 5. Alternatively, Tyr134His could represent a minor HBV population in the piece of liver specimen investigated, which was not detected by this PCR amplification-sequencing method.

The mutations of the HBV genomes present in the liver specimen were almost the same as those present in the serum of the patient 5. The appearance of no additional mutations suggests a lack of recombination between the Wt-genome and variant genomes during PCR amplification and hints to missing PCR contamination.

3.1.2 Functional analysis of the variants 5a and 5c

Only a few studies have been performed to analyse the functional phenotype or behaviour of HBV variants. The lack of a proper HBV-infection model has also impaired the understanding of the postulated capacity of variants to assess their pathogenesis. Cell lines for in vitro studies which can be infected by HBV are reported (Gripon et al., 2002), but infection efficiency is too low for analysis of the functional phenotype, or cells lines are not available. The late stages of HBV replication can be investigated by transfection of the investigated HBV genomes or variants in hepatoma cell lines and the functional phenotype of these genomes can be compared to an arbitrary Wt HBV genome.

For functional analysis, Wt or variant HBV full-length genomes 5a or 5c (designated pHBV-SapI constructs in Material and Methods) were excised from their corresponding vectors with the enzyme Sap I. This restriction digestion allows the formation of a HBV monomer with cohesive-ends containing only HBV homologous sequences. This monomer can recircularise in transfected cells and serve as transcriptional template for the initiation of HBV replication (Gunther et al., 1995a). As variant 5a has more homology to genotype D, HBV genomes were compared functionally with Wt genome of this genotype. The transfection efficiency was measured by determining the amount of secreted alkaline phosphatase (SEAP) expressed from the cotransfected SEAP-plasmid. Similar values were obtained for all constructs (data not shown).

Full-length HBV DNA Wt or variants 5a and 5c were transfected into Huh-7 cells. In order to allow sufficient time for replication of the HBV genomes after transfection, cells were harvested 4-days post-transfection. DNA from intracellular nucleocapsids was isolated, separated and detected by Southern blot analysis with a HBV specific digoxigenin-labeled probe. Three independent experiments were performed, and several exposition times were analysed, one shown in Fig. 3.1.2.1-A. According to this analysis, Wt and variant 5a HBV

genomes replicated well, as shown by the appearance of HBV specific single-stranded DNA inside the nucleocapsids (Fig. 3.1.2.1-A, lanes 1 and 3). Additionally, HBV specific double-stranded DNA and the rcDNA forms were detectable. On the other hand, single-stranded DNA was not detected for the variant 5c, indicating that this HBV genome is incompetent in virion formation. The presence of double-stranded DNA in variant 5c transfected cells could derive from linearized input-DNA.

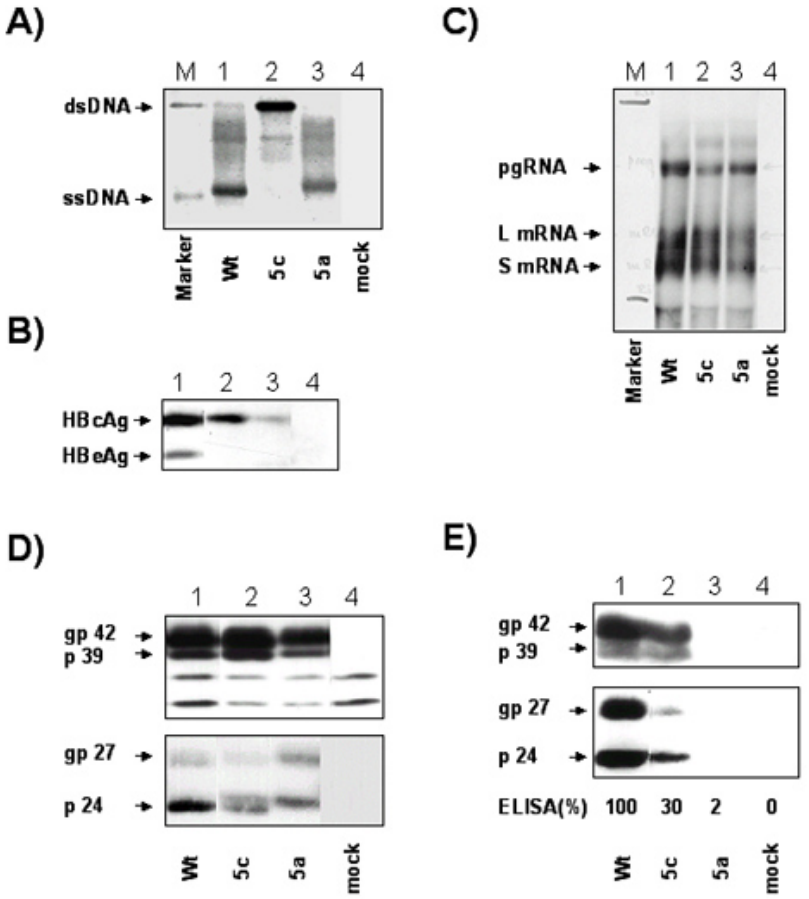


Fig. 3.1.2.1: **Functional analysis of Wt and variant genomes**. The Wt (lane 1), variant 5c (lane 2), variant 5a (lane 3), and mock-control (lane 4) were transfected into Huh-7 hepatocarcinoma cells. In panel A, Intracellular nucleocapsids were purified and replicative intermediates were detected by Southern blot analysis with an HBV-DNA digoxigenin-labeled full-length probe: double-strand (dsDNA), and single-strand DNA (ssDNA) as well as replicative intermediates of virus were detected. The DNA marker corresponds to the product of asymmetric-PCR. In panel B, Western blot analysis from cell extracts detected with anti-C PAb; the upper band corresponds to HBcAg and the lower to HBeAg. In panel C, HBV-RNAs were specifically detected by Northern blot analysis with an x/c-gene RNA probe; the upper band corresponds to pgRNA and the two lower bands correspond to the preS1 (L-mRNA) and preS2/S-messengers (S-mRNA). The ribosomal RNAs were used as markers. Detection of envelope protein by Western blot analysis of cell extract (panel D) and PEG-precipitated particles (panel E) was performed with anti-preS1 (upper-figure) and anti-S MAbs (above-figure). The envelope proteins detected were the L-protein glycosylated (gp42-kDa) and nonglycosylated (p39-kDa), and the S-protein glycosylated (gp27-kDa) and nonglycosylated (p24-kDa).

In addition, expression of the C-ORF was examined by Western blot analysis with an anti-HBc polyclonal antiserum (PAb) (Fig. 3.1.2.1-B) and RNA transcripts were detected by a Northern blot (Fig. 3.1.2.1-C). For protein- and RNA-analysis the cells were harvested 5- and

3-days post-transfection, respectively. The amount of HBcAg, of the pgRNA and of the other mRNAs were slightly lower for the variant 5a than those for the Wt genome. The variant 5c showed similar amounts of HBcAg and RNAs compared to Wt.

The secretion phenotype of HBV Wt and variant genomes was analysed by measuring the amounts of HBsAg and HBeAg from the medium of transfected cells by ELISA. Both variants 5a and 5c were incompetent for secretion of HBeAg, because the corresponding HBeAg-ELISA gave similar values as the mock-control. An about 50-fold reduced amount of HBV surface proteins was found in supernatants for variant 5a compared to Wt, when measured by HBsAg-ELISA, consistent with previously published data (Sterneck et al., 1998). A 60% secretion phenotype was observed for variant 5c, compared to Wt genome, arbitrarily set to 100%. The HBsAg-ELISA measures all three-envelope proteins.

In order to identify specifically which envelope protein was not secreted, the levels of surface protein expression (Fig. 3.1.2.1-D) and secretion (Fig. 3.1.2.1-E) were investigated by Western blot analysis. Cells were lysed, whereas secreted particles were first concentrated by addition of polyethylenglycol (PEG) or by ultracentrifugation. Western blot analysis for the S- and L-proteins was always performed with anti-S and anti-preS1 MAbs, respectively. These antibodies recognized linear peptides of the corresponding proteins and were protein-conformation independent (Mangold and Streeck, 1993). The L- and S-proteins could be detected either in cell extracts or in secreted particles after transfection with Wt genome containing plasmid (Fig. 3.1.2.1-D and Fig. 3.1.2.1-E, lane 1; respectively). In addition, clear S- and preS1-signals were detected using cell extracts and viral particles from the supernatant of cells transfected with the replication incompetent variant 5c (Fig. 3.1.2.1-D and Fig. 3.1.2.1-E, lane 2; respectively). On the other hand, neither L- nor S-protein could be detected in the medium of cells transfected with the variant 5a (Fig. 3.1.2.1-E, lane 3), indicating that this virus has a defect in secretion of viral particles.

One trivial reason for the secretion incompetence of variant 5a could be that the surface proteins were expressed inefficiently. To investigate this possibility, surface proteins were analysed in cell lysates by immunoblotting (Fig. 3.1.2.1-D). The resulting picture shows that both the variant 5a S- and L-proteins could be detected intracellularly for the secretion deficient variant (Fig. 3.1.2.1-D, lane 3). This indicates that the MAbs are able to detect those mutated proteins. Protein levels were slightly reduced compared to Wt. Therefore, it is possible that the proteins are either recognized slightly less efficient or were expressed slightly less efficiently.

To confirm this result and demonstrate the intracellular levels of Wt and variant 5a surface proteins, pulse experiments were performed by immunoprecipitating HBV surface proteins with a PAb against the S-protein (anti-S PAb). First, the capacity of PAbs to immunoprecipitate native mutated-proteins as efficiently as Wt-proteins was tested as a control as similar recognition for natured Wt- and mutant-proteins by the PAbs was supposed. Therefore, cell extracts were divided into two parts; either surface proteins were untreated (Fig. 3.1.2.2, lanes Wt, 5a, and mock), or were specifically immunoprecipitated with anti-S PAb (Fig. 3.1.2.2, lanes Wt-IP, 5a-IP, and mock-IP). After separation, they were detected by Western blot analysis, with anti-preS1 MAb. Similar amounts of envelope proteins were detected in untreated cell extracts after immunoprecipitation, indicating that the efficiency of this process was over 90%, both for Wt and variant 5a. Notably, the two unspecific signals evident when the cell lysates were analysed (Fig. 3.1.2.2, lanes 1, 3 and 5) became undetectable after precipitation with specific antibodies, a good evidence for specific precipitation. Therefore, there might be no difference in recognition for Wt or variant 5a surface proteins by the anti-S PAb.

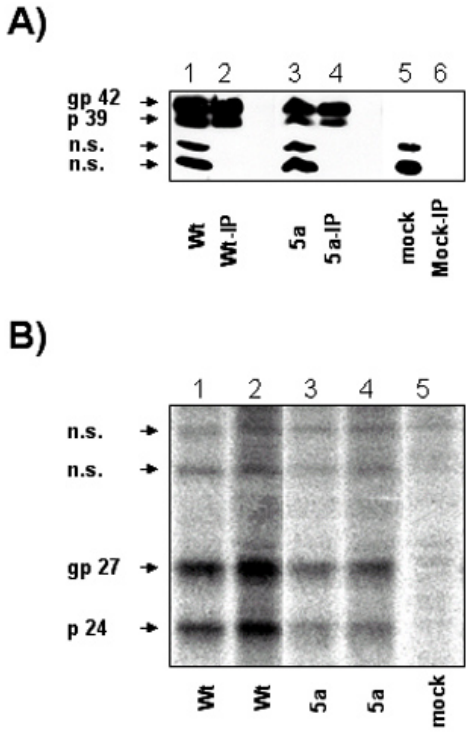


Fig. 3.1.2.2: **Levels of WtS- and 5aS-proteins in pulse experiment**. In panel A, surface proteins in cell lysates transfected with Wt and 5a HBV genomes were immunoprecipitated with anti-S PAb, and detected by Western blot analysis with anti-preS1 MAb (lanes Wt-IP, and 5a-IP, respectively). Cell lysates were also loaded for comparison (lanes Wt and 5a, respectively). In panel B, cells were transfected with Wt (lanes 1-2), or 5a (lanes 3-4) genomes for pulse experiment (duplicates). Labelling was performed two days later with (^{35}S)-Met for 3-hours. HBV surface proteins were immunoprecipitated from cell lysate with anti-HBs PAb, separated by SDS-PAGE and detected by autoradiography. The envelope proteins detected were the L-protein glycosylated (gp42-kDa) and nonglycosylated (p39-kDa), and the S-protein glycosylated (gp27-kDa) and nonglycosylated (p24-kDa). Non-specific proteins were assigned as n.s.

For pulse-experiments, Huh-7 cells were transfected, in duplicates, with Wt (Fig. 3.1.2.2-B, lanes 1-2) or variant 5a HBV-DNA (Fig. 3.1.2.2-B, lanes 3-4). Proteins were metabolically labelled with (^{35}S)-Met for 3-hours two days post-transfection. Immediately thereafter, cells were harvested and disrupted. Viral proteins were then immunoprecipitated with anti-HBs PAb and separated on sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE); the gels were dried and exposed to a screen for autoradiography. Variant 5a S-protein displayed a normal pattern of glycosylation gp27- and p24-kDa. Quantification of the signals by TINA-software showed about two-fold reduced amounts of 5a S- compared to Wt S-proteins. Neither Wt L- nor 5a L-proteins were clearly detected, this fact might probably be caused by the low expression level of the preS- versus S-proteins under the HBV natural promoters.

To summarize, the functional analysis of the variant genomes showed that, while a replication competent variant 5a has a secretion defect of about 50-fold in respect to viral particles secretion, the replication incompetent variant 5c secreted viral particles with an intermediate efficiency compared to Wt HBV genome. The secretion block of variant 5a is not caused by a lack of surface protein expression, although a reduction in intracellular 5a S-protein levels was detected. Intracellular accumulation of viral HBV-DNA or secretion deficient proteins was not observed.

3.1.3 Variant 5a genome does not produce mature surface proteins

Variant surface proteins are expressed and partially glycosylated, therefore translocation might take place in the ER-membrane. In an attempt to further characterize transport defects of the variant proteins, which was partially retained intracellularly, the type of oligosaccharides present in the glycoproteins was investigated. As the carbohydrate modification occurs in specific subcellular compartments along the secretion pathway (Ellgaard and Helenius, 2003), envelope synthesis and transport can be monitored indirectly by determining changes in carbohydrate structure. Proteins that successfully reach the Golgi apparatus acquire endo-H resistance, whereas retained or unfolded proteins remain sensitive to endo-H digestion. Alternatively, the enzyme peptidoglycanaseF (PNGaseF) is always able to digest the N-glycosyl-trees of glycoproteins.

In order to analyse whether there is a difference in endo-H sensitivity, Wt and secretion deficient variant genomes were transfected into Huh-7 cells. Five days posttransfection, cells were harvested for further analysis. Total protein extracts were denaturated and divided into three parts; in the first part proteins were treated with endo-H

enzyme, in the second they were treated with PNGaseF, or they were untreated. The enzyme PNGaseF was used as a complete digestion control, as the enzyme should digest all types of N-glycosyl-trees. Detection was performed by Western blot analysis with anti-preS1 MAb (Fig. 3.1.3.1-B and C).

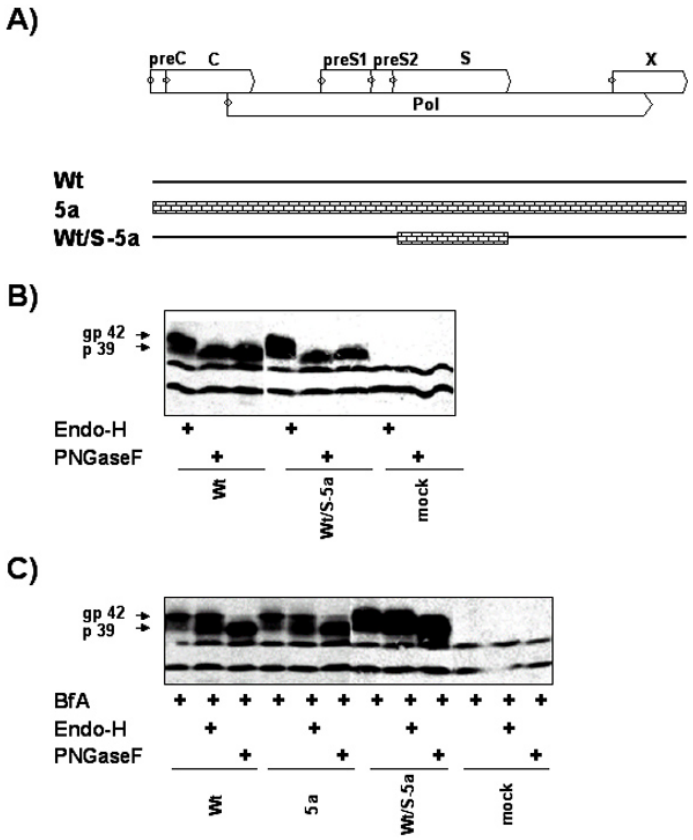


Fig. 3.1.3.1: **Analysis of the N-glycosyl-tree modification of Wt and variant surface proteins by endo-H digestion**. Schematic representation (panel A) of HBV genome with the corresponding ORFs (above) and the constructs used (below). Western blot analysis (panel B) of lysates from cells transfected with Wt, a construct expressing 5aS-protein (Wt/S-5a) or mock. Lysates were divided in three parts: untreated, treated with endoglycosydase-H (endo-H), or treated with peptidoglycanase-F (PNGaseF); detection was performed with anti-preS1 MAb. In panel C, cells were transfected with the constructs named below the picture and treated for 18h with secretion inhibitor brefeldin A (designated here as BfA). Lysates were also divided in three parts: untreated, treated with endo-H or treated with PNGase-F, and detection was also performed with anti-preS1 MAb.

As an additional control, the fate and glycosyl-tree modification of Wt and variant proteins were also analysed by blocking the constitutive secretion pathway with the drug Brefeldin A (BfA), which induces disruption of the Golgi apparatus. The aim was to study the glycosylation pattern of normally secreted proteins by forced ER-retention. Experimentally, Huh-7 cells were transfected with the constructs named in Fig. 3.1.3.1-A. Five days post-transfection, medium was changed and cells were incubated in BfA medium for 18-hours. Immediately thereafter, cells were harvested. For further investigations, cells were lysed, total protein extracts were denaturated, and divided into three parts for glycosyl-tree digestions. First, no enzyme was added; second, digestion was performed with endo-H enzyme and third,

glycosyl-tree digestion was performed with PNGaseF. Proteins were separated by SDS-PAGE and detected by Western blot analysis with anti-preS1 MAb.

The variant 5a surface proteins were uniformly sensible to treatment with endo-H, as predominantly the non-glycosylated forms could be detected (Fig 3.1.3.1-B, lane Wt/S-5a). In conclusion from these data, the block of secretion of the variant occurs before transport to the medial cisternae of the Golgi apparatus. However, Wt secretion competent surface protein was similarly susceptible to endo-H (Fig. 3.1.3.1-B, lanes Wt). Since the secreted glycoproteins are reported to be endo-H resistant (Patzer, Nakamura, and Yaffe, 1984), these proteins might have transversed the Golgi cisternae. A rapid transport through the Golgi apparatus and from the trans-Golgi apparatus to the cell surface is thought to account for the endo-H resistant Wt proteins (Patzer, Nakamura, and Yaffe, 1984).

Surprisingly, treatment of cells with BfA, a potent inhibitor of the vesicle transport from Golgi to cell surface, led to resistance of the surface proteins to endo-H digestion (Fig. 3.1.3.1-C). Possibly, the ER-vesicle-system might allow the retrieval of Golgi-enzymes to the ER, thus inducing the formation of resistant glycoproteins (Ellgaard, Molinari, and Helenius, 1999).

3.1.4 Subcellular localization of variant surface proteins

Mutations in the variant 5a proteins might cause a structural alteration that somehow prevents proper folding and secretion. If transport of these proteins is inhibited, localization might be different from those of Wt. In order to test this possibility, Huh-7 cells were transfected with Wt or variant genomes: 5a and 5c. Four-days posttransfection, cells were split, and coverslips were fixed 24-hours later. Anti-HBs MAb (Fig. 3.1.4.1-A) or anti-HBs PAb (Fig. 3.1.4.1-B) were used for detection of all HBV surface proteins (Fig. 3.1.4.1-A and B). Additionally, anti-preS1 MAb (Fig. 3.1.4.1-B) was used for specific detention of the L-proteins. Phase-contrast (PhCo) microscopy was always additionally performed. To test the hypothesis that surface proteins are assembled and bud as lipoprotein particles in the ER and/or Golgi compartment, MAbs staining for these compartments were used: PDI (Fig. 3.1.4.1-C) or calnexin (data not shown) and 58-kDa Golgi-protein (Fig. 3.1.4.1-D), respectively. A more complete description of these MAbs is described in the Material and Methods section.

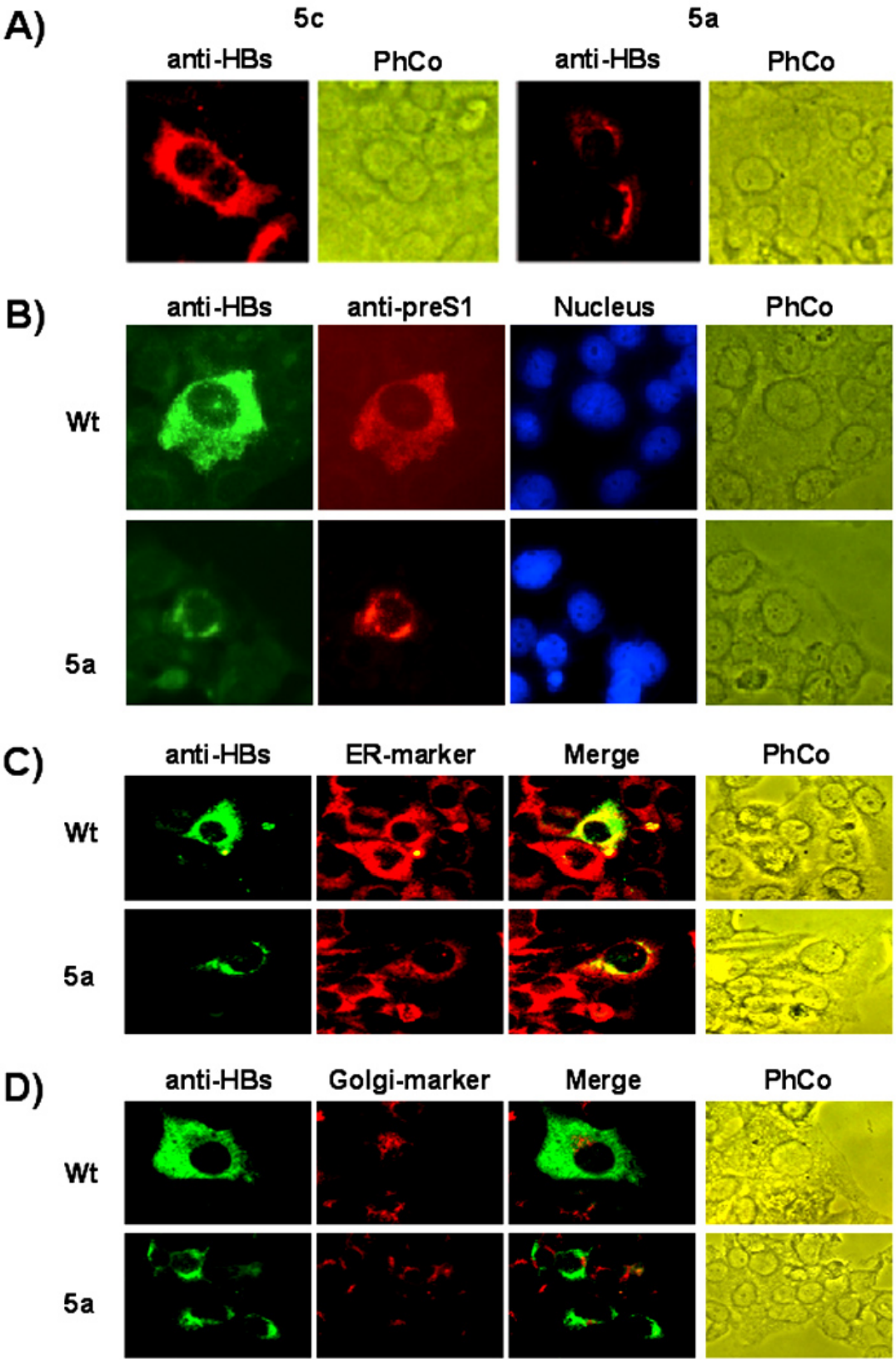


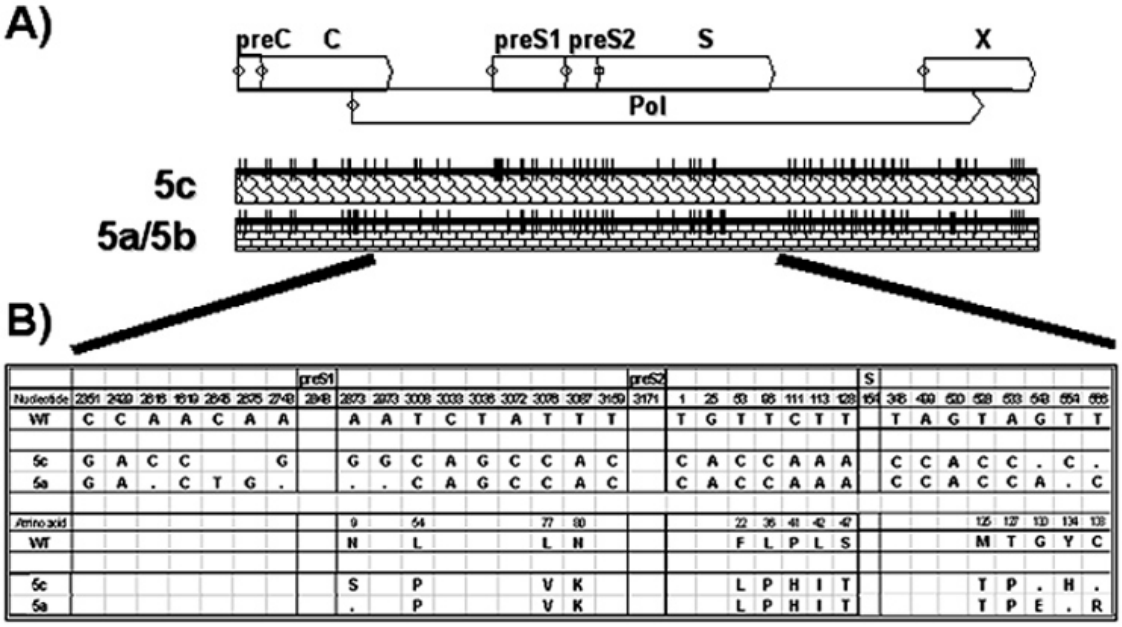
Fig. 3.1.4.1: **Subcellular localization of the variant surface proteins**. Huh-7 cells were transfected with Wt, variant 5a or variant 5c, and 4-days posttransfection cells were split and seeded on coverslips. In panel A, variant 5a and 5c surface proteins were detected with anti-S MAb. In panel B, surface proteins were co-stained with anti-HBs PAb (green) and anti-preS1 MAb (red), and DNA-binding dye (blue) was added for 10-minutes on the last washing procedure. In panel C and D, co-staining of anti-HBs specific PAb was performed with cellular ER-marker (anti-PDI, red) and Golgi-marker (58kDa protein, red), respectively. Co-localization is shown with both antibody combinations (merge). Size and distribution of the cells can be observed within PhCo.

The intracellular surface proteins synthesized by the Wt construct (Fig. 3.1.4.1-A and B, picture Wt) and secretion-efficient variant 5c (Fig. 3.1.4.1-A, picture 5c) were distributed within the cytoplasm in a granular-reticular pattern that was imposable on the intracellular membranous structures, as revealed by double staining with the ER-markers PDI for Wt surface proteins (Fig. 3.1.4.1-C, picture Wt). Colocalization experiments were also performed with calnexin (another ER-marker) with similar results (data not shown). These results are consistent with the surface protein's entering the usual secretory pathway. In contrast, the intracellular variant 5a surface proteins were markedly different localized in discrete, granular areas near the nucleus, representing a small region of the cytoplasmic membranes with a rather speckled-distribution (Fig. 3.1.4.1-A and B, picture 5a). These results confirmed that synthesis of the variant 5a surface proteins resulted in intracellular retention of all forms of surface proteins in an abnormal region close to the nucleus.

According to colocalization studies (Fig. 3.1.4.1-C and D, picture 5a), variant 5a proteins were not retained in the Golgi apparatus, as revealed by double staining with 58-kDa Golgi-protein staining. Therefore, the immunofluorescence results indicate that retention of variant 5a surface proteins occurs in pre-Golgi compartments as previously suggested by others (Persing, Varmus, and Ganem, 1986; Xu and Yen, 1996).

3.2 Mutations responsible for the secretion block

Several non-aa-altering (synonymous) and aa-altering (nonsynonymous) nt-mutations (Fig. 3.2.1-B) were reported to be mutated all over the whole variant 5a genome (Sterneck et al., 1996; Sterneck et al., 1998).



								preS1										preS2										S								
Nucleotide	2351	2409	2416	1619	2645	2655	2749	2848	2873	2973	3008	3033	3036	3072	3098	3087	3159	3171	1	25	53	95	111	113	128	154	348	409	520	528	533	548	554	566
Wt	C	C	A	A	C	A	A		A	A	T	C	T	A	T	T	T		T	G	T	T	C	T	T		T	A	G	T	A	G	T	T
5c	G	A	C	C			G		G	G	C	A	G	C	C	A	C		C	A	C	C	A	A	A		C	C	A	C	C	.	C	.
5a	G	A	.	C	T	G	.		.	.	C	A	G	C	C	A	C		C	A	C	C	A	A	A		C	C	A	C	C	A	.	C
Amino acid									9		54				77	80					22	36	41	42	47					125	127	130	134	138
Wt									N		L				L	N					F	L	P	L	S					M	T	G	Y	C
5c									S		P				V	K					L	P	H	I	T					T	P	.	H	.
5a									.		P				V	K					L	P	H	I	T					T	P	E	.	R

Fig. 3.2.1: **Nucleotide mutations of the variant genomes**. HBV full-length genome (panel A) is represented by the preC/C-, preS1/preS2/S- P- and X-genes. Genomes 5c and 5a are presented below with single mutations represented by vertical bars, compared to Wt sequence genotype D. Table of nt- and aa-mutations (panel B) is shown for the surface protein region of the variants. Three starting points for HBV surface protein expression are depicted on the first line of the table by: preS1, preS2 and S. The numbers refer to the mutated nts-position in the HBV genome (above) or alternatively to the position of mutated aas in the envelope proteins (below), whereas the dots point to parts identical with the Wt genome.

According to the Southern blot analysis, these mutations together did not affect significantly the variant's replication ability compared to Wt (Fig. 3.1.2.1-A). However, variant 5a was shown to be defective in viral particle secretion, because neither the 5a S- nor the 5a L-proteins were detected in the medium of the corresponding transfected cells. For hepadnaviruses, overexpression of envelope proteins in absence of other HBV-genome regions lead to self-assembly and secretion of non-infectious SVPs (spheres and filaments). Therefore, mutations in the surface proteins might be mainly responsible for the block in secretion. The secretion defect of these particles and the reason for the secretion block of these HBV variant are subject of this chapter.

3.2.1 Association of the secretion block with mutations in 5aS-gene

Envelope proteins serve as transport vectors for viral particles through the secretory pathway, the emergence of mutations within the preS- or S-region of variant HBV genomes

can alter the capacity of viral particle formation and secretion. In order to investigate the envelope protein region responsible for the secretion block, the preS- or S-region of the variants was swapped into the Wt genome by molecular cloning. The HBV constructs were then named Wt/preS-5c and Wt/preS-5a for the preS-gene in Wt context or Wt/S-5c and Wt/S-5a for the S-gene in Wt context (Fig. 3.2.1.1-A). Two clones from each construct were amplified and sequenced and each construct contained the expected insert.

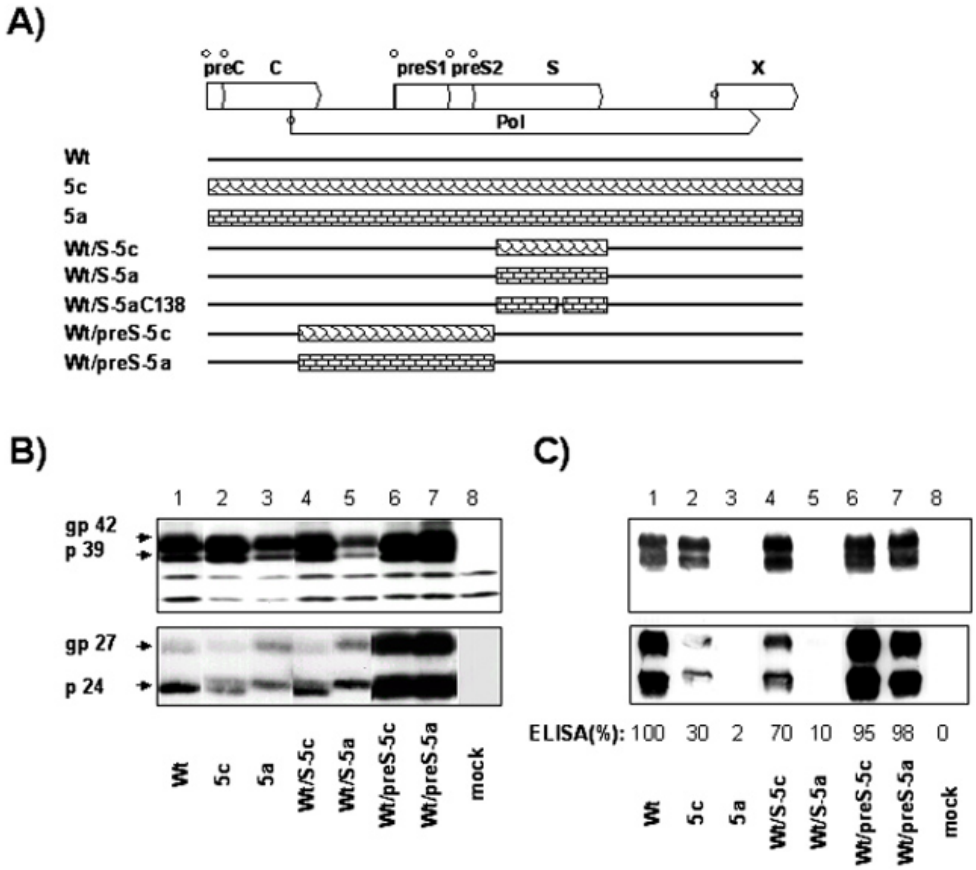


Fig. 3.2.1.1: **Envelope protein expression and secretion after transfection with pre-S and S-constructs**. HBV full-length genome (panel A, upper part) is represented by the corresponding genes. The constructs used for transfection are represented by lines (panel A, above): thin lines represent the Wt genome and patterned thick lines represent variant genomes. Huh-7 cells were transfected with preS- (Wt/preS-5a and Wt/preS-5c) or S-constructs (Wt/S-5a and Wt/S-5c) and envelope protein expression (panel B) and secretion (panel C) was studied by Western blot analysis of cell extracts and PEG-precipitated particles, respectively, and detected with anti-S and anti-preS1 MAbs. The envelope proteins detected were the L-protein glycosylated (gp42-kDa) and nonglycosylated (p39-kDa), and the S-protein glycosylated (gp27-kDa) and nonglycosylated (p24-kDa).

Functional analysis of Wt and HBV-hybrids were performed after transient transfection in Huh-7 cells. Transfection efficiency was ascertained by measuring secreted SEAP-activity and was found to be similar for all constructs. The HBV genome of all S- and preS-variant genes in Wt context replicated like Wt (data not shown).

The envelope secretion phenotype of these genomes was first determined by ELISA for HBsAg. Values were calculated as percentage of Wt secretion, set arbitrarily to 100%. While SVPs secretion from the genome Wt/S-5a was reduced to 10%, the genome Wt/S-5c

showed a relative secretion efficiency of only 70% (Fig. 3.2.1.1-C assigned as ELISA (%), lanes 5 and 4). In contrast, the preS-variant gene in Wt context showed an almost normal surface protein secretion phenotype, close to 100% in these ELISA measurements (Fig. 3.2.1.1-C assigned as ELISA (%), lanes 6 and 7). To continue this further, particles present in the medium were concentrated, and intracellular or secreted surface protein were also analysed by Western blot analysis with anti-S and anti-preS MAbs (Fig. 3.2.1.1-B and C, respectively). The S- and L-proteins were clearly detectable in cell lysates and supernatants for the secretion-competent HBV genomes Wt, variant 5c, and Wt/S-5c (Fig. 3.2.1.1-B and Fig. 3.2.1.1-C; lanes 1, 2 and 4, respectively). In contrast, 5a and Wt/S-5a (Fig. 3.2.1.1-C, lanes 3 and 5, respectively) protein secretion was dramatically reduced as shown by the lack of signal for this genomes. This result is consistent with results of the ELISA for HBsAg. Furthermore, the same antibodies detected on blots the 5a and Wt/S-5a surface proteins derived from cell extracts (Fig. 3.2.1.1-B, lanes 3 and 5, respectively).

No extraordinary change in secretion was detected for the preS-variant genes in Wt context (Fig. 3.2.1.1-C, lanes 6 and 7) compared to Wt (Fig. 3.2.1.1-C, lane 1), whereas the intracellular surface protein levels were increased for both constructs. The reason for the increase in protein expression might be connected to mutation/s in the variant preS-region, which are common to both variant 5a and 5c because the intracellular surface protein levels were similarly increased for both Wt/preS-5a and Wt/preS-5c genomes. Expression of the HBV S-protein under the control of a heterologous promoter has previously been reported for Wt, and leads to the secretion of SVPs (Aden et al., 1979; Prange, Nagel, and Streeck, 1992). Therefore, in order to confirm that only the S-region is responsible for the secretion block and no cooperation with an additional HBV region is required, Wt and variant S-domain was expressed under the foreign cytomegalovirus (CMV) early promoter. Wt and variant 5a S-genes were swapped by molecular cloning into a plasmid expressing the HBV S-protein: CMV-S plasmid (Xu, Jensen, and Yen, 1997). Two identical clones were obtained for each construct, which were designated CMV-S/Wt and CMV-S/5a. In addition, the preS-region of Wt and 5a constructs was also subcloned into a plasmid expressing the HBV L-protein (Xu, Jensen, and Yen, 1997). Two identical clones were obtained for each construct, and were designated CMV-L^+X^-/Wt and CMV-L^+X^-/5a, respectively.

Transient expression of the constructs CMV-S/5a, CMV-S/Wt, CMV-L^+X^-/5a, and CMV-L^+X^-/Wt was performed in Huh-7 cells. Transfection efficiency was not calculated, because the SEAP-plasmid expresses the SEAP-protein also under CMV-promoter, which might induce expression interferences. However, the secretion phenotype was analysed by

ELISA for HBsAg and calculated as a percentage related to the secretion for the CMV-S/Wt construct, which was set arbitrarily to 100%. SVPs were efficiently secreted by CMV-S/Wt, but were reduced to less than 3% for the construct CMV-S/5a. As expected, ELISA values were close to mock-control for the constructs CMV-L^+X^-/Wt and CMV-L^+X^-/5a, probably due to the L-protein retention signals (Gallina, Gazina, and Milanesi, 1995; Yu, 1991) or myristylation (Prange, Clemen, and Streeck, 1991).

These results strongly suggest that mutations of variants in the S-region might be mainly responsible for the secretion block, both when expressed under homologous and heterologous promoter control.

3.2.2 Mutant genomes bearing single or several mutations

HBV contains a small DNA genome with overlapping ORFs. The P-gene extends over more than two-thirds of the HBV genome; therefore, aa-changes affecting important regions of this gene often interfere with efficient HBV replication. Mutations or deletions in the preS-region are less critical, because they overlap with the spacer region of the P-protein. This is obviously the reason why only a few aa-exchanges are found within the variant 5a S-domain (Fig 3.2.1-B).

It was interesting to study whether a single or several aa-exchanges are synergistically responsible for the block of secretion. The variant 5a envelope proteins have four aa-exchanges Met125Thr, Thr127Pro, Gly130Glu, and Cys138Arg inside the S-domain. Additional three synonymous (non-aa-altering) mutations (T346C, A499C, and G520A) are present in the S-gene (Sterneck et al., 1996).

Several HBV genomes were made and used to investigate the secretion phenotype of each single and the combination of several mutations (Fig. 3.2.2.1). To test the effect of the important Cys residue in position 138 in the variant, the construct 5aC138 were investigated, which bears a single mutation Arg138 to Cys compared to the variant 5a genome. The counterpart WtR138 HBV genome, which contains the Wt genome expressing envelope proteins with the aa-exchange (Cys138Arg), was also examined. The aa-exchanges Met125Thr and Thr127Pro were simultaneously introduced into Wt-genome by site-directed mutagenesis (construct WtT125P127). Finally, the effect of the exchange Gly130Glu was analysed with the construct Wt/S-5aC138, which expresses surface proteins bearing three aa-exchanges Gly130Glu, Met125Thr, and Thr127Pro simultaneously.

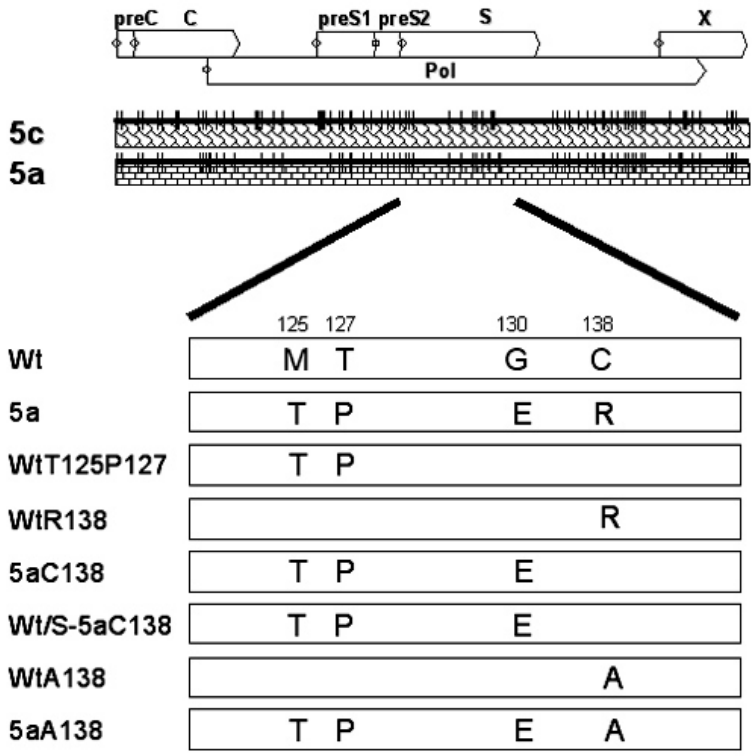


Fig. 3.2.2.1: **Amino acid changes in the a-determinant of the envelope proteins**. HBV full-length genome (panel A) is represented by the preC/C-, preS1/preS2/S- P- and X-genes. Genomes 5c and 5a are presented below with single mutations represented by vertical bars, compared to Wt sequence genotype D. The S-region of the HBV genome is represented in an extended graphic below, where the aa-exchanges (left) between Wt, variant 5a, and mutant genomes are depicted. In the first line, the aa-position where mutations were introduced into the constructs is given. Below, the constructs are represented. The appearance of a letter, different from Wt, indicates an aa-exchange in the construct. Aas nomenclature: M-Met, T-Thr, G-Gly, C-Cys, E-Glu, P-Pro, and R-Arg.

The construct Wt/S-5aC138 was created by swapping the 5aC138 S-gene in the Wt genome (Fig. 3.2.1.1-A). Constructs bearing mutants with the Cys138Ala mutations are described in chapter 3.2.6. Details of cloning of all mutant genomes are described in Material and Methods.

3.2.3 Functional analysis of HBV mutant genomes

The DNA of the hybrid genomes variant 5a and Wt were transiently transfected into Huh-7 cells. Transfection efficiency, as measured by SEAP-activity, was similar for all transfected constructs. All HBV genomes replicated well because the amount of intracellular encapsidated replicative intermediates (Fig. 3.2.3.1-A) as well as HBcAg expression (Fig. 3.2.3.1-B) were similar in Huh-7 transfected cells.

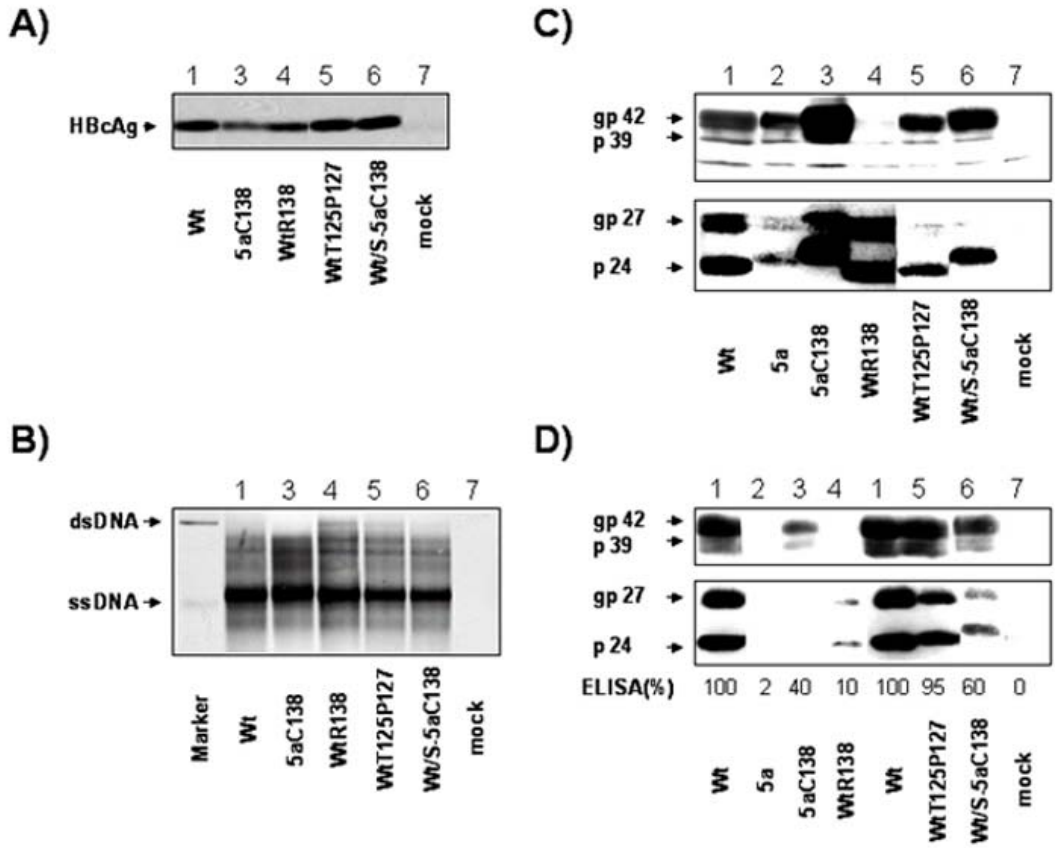


Fig. 3.2.3.1: **Functional analysis of the HBV genomes**. Huh-7 cells were transfected with the constructs named below each line (for description of the constructs see Fig. 3.2.2.1). In panel A, Western blot analysis of cell extracts detected with anti-C PAb, a band is detected corresponding to HBcAg. Replicative intermediates were detected by Southern blot analysis with a HBV full-length DNA probe (panel B): Single-stranded (ssDNA), and double-stranded (dsDNA). Western blot analysis of cell extracts (panel C) and PEG-precipitated particles (panel D) was performed with anti-S and anti-preS1 MAbs. Two forms of envelope proteins were detected for L in glycosylated (gp42-kDa) and nonglycosylated (p39-kDa), and for S in glycosylated (gp27-kDa) and nonglycosylated (p24-kDa) version.

In order to analyse the mutation/s responsible for the secretion block, secretion of SVPs was monitored first by ELISA for HBsAg. Wt secretion was standardized to 100% and the secretion phenotype of the HBV genomes was calculated as a percentage related to Wt value. Introduction of Arg in position 138 (WtR138) reduced the secretion to 10%, while reversion of the variant 5a Arg138 to Cys (5aC138) rescued secretion to 30-50%. The other constructs, WtT125P127 and Wt/S-5aC138, presented a secretion phenotype of 95% and 60%, respectively.

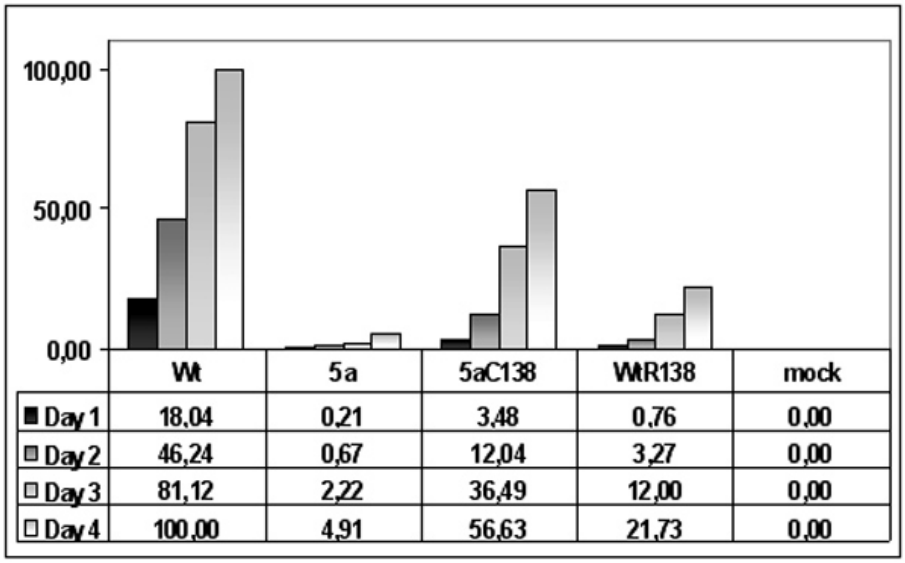
Surface protein expression (Fig. 3.2.3.1-C) and secretion (Fig. 3.2.3.1-D) were investigated by immunoblot analysis. Secreted particles were previously concentrated by PEG-precipitation. Additionally, cells were lysed and the cytoplasmic fraction was separated by a three time freeze-thawing procedure. Proteins were separated by SDS-PAGE and detected with anti-S and anti-preS MAbs. Representative signals were detected for Wt S- and L-proteins in cell lysates and from the medium (Fig. 3.2.3.1-C and D, lane 1). Both, the

signals for L- and S-containing particles were low for the construct WtR138, despite strong signals for intracellular S-proteins (Fig. 3.2.3.1-C and D, lane 4, respectively). Interestingly, the intracellular WtR138 L-protein level was reduced by about four-fold compared to the Wt-counterpart (Fig. 3.2.3.1-C, lane 4 compared to lane 1, gp42 and p39). In contrast, secreted viral particles containing 5aC138 L-protein were detected by immunoblot analysis, whereas 5aC138 S-containing particles were hardly detected with the anti-S MAb (Fig. 3.2.3.1-D, lane 3, gp27 and p24). For unknown reasons, this result dispairs to the ELISA measurement for HBsAg, where 5aC138 genome secreted SVPs with 30-50% efficiency compared to 100% of Wt. The other constructs, WtT125P127 and Wt/S-5aC138 induced secretion of significant amounts of S- and L-containing particles into the medium of transfected cells by detection with MAbs (Fig. 3.2.3.1-D, lanes 5 and 6, respectively).

In summary, these results demonstrate that aa-exchanges in the S-protein at positions 125, 127, and 130 are compatible with assembly and secretion of HBV-SVPs. In contrast, exchange of the Cys138Arg reduces the secretion of these particles by about ten-fold and reduced significantly the level of L-protein intracellularly compared to its counterpart S-protein. The reason for the lack of detection of the 5aC138 S-protein was further investigated.

3.2.4 Detection of the 5aC138 S-protein in supernatants

To confirm that the ELISA signal for HBsAg observed for the construct 5aC138 is specific for secretion of SVPs, kinetic studies for secreted particles were performed.



	Wt	5a	5aC138	WtR138	mock
Day 1	18,04	0,21	3,48	0,76	0,00
Day 2	46,24	0,67	12,04	3,27	0,00
Day 3	81,12	2,22	36,49	12,00	0,00
Day 4	100,00	4,91	56,63	21,73	0,00

Fig. 3.2.4.1: **Kinetic of HBsAg secretion**. ELISA for HBsAg was performed with cellular supernatants in duplicates in dilutions of 1:10 using the constructs listed under the columns. Measurements were performed every 24-hours (from day-1 to day-4) and represented as a percentage related to Wt value set to 100% (table below). ELISA values are visualized above in the graphic of columns; the values are given as a percentage compared to Wt ELISA value set to 100%.

If the construct is able to induce secretion of viral particles into the medium of transfected cells, there should be a specific HBsAg expression kinetics. Experimentally, Huh-7 cells were transfected with different HBV genomes (Wt, 5a, 5aC138, and WtR138) and small aliquots of the medium were collected every 24-hours. ELISA for HBsAg was performed with all aliquots. The study revealed a similar kinetic of particle secretion for Wt and 5aC138, which was characterized by an accumulation of particles in the supernatant of transfected cells over the time, as indicated by an almost linear increase of the HBsAg signal (Fig. 3.2.4.1). The progressive increase of an ELISA-signal for HBsAg 5aC138 with the time after transfection suggests that this construct produced particles, which might be selectively secreted into the supernatant of Huh-7 cells.

To further analyse this point, the 5aC138 S-gene was cloned under a heterologous CMV-promoter (CMV-S/5aC138). As a control, the constructs expressing the 5aC138 L- and the corresponding WtR138 S- and L-proteins (CMV-L^+X^-/5aC138, CMV-S/WtR138, and CMV-L^+X^-/WtR138 constructs, respectively) were also established by molecular cloning. Transient transfection was performed in Huh-7 cells, but cotransfection experiments monitoring the transfection efficiency were not performed to avoid a possible interference between the CMV-promoter of the SEAP-plasmid and the CMV-protein expression constructs studied. Three days after transfection, the expression of the 5aC138 S-protein from construct CMV-S/5aC138 led to secretion of SVPs of about 50% the amount compared to those expressing Wt protein as determined by ELISA for HBsAg, which was standardized to 100%. In contrast, expression of the WtR138 S-protein from construct CMV-S/WtR138 was reduced about 10-fold (to 10%). These results are similar to those obtained with the proteins expressed under homologous HBV promoter control (chapter 3.2.3). The CMV-L^+X^-/5aC138, and CMV-L^+X^-/WtR138 constructs didn't result in secretion of detectable amounts of SVPs.

Subsequently, the secretion phenotype of SVPs produced from these CMV-constructs or using full-length HBV-DNAs was investigated by Western blot analysis (Fig. 3.2.4.2, lanes 5-8 or lanes 1-4 and 9-12, respectively). Proteins of particles concentrated by immunoprecipitation with an anti-S PAb or by ultracentrifugation were separated by SDS-PAGE and the S-protein was detected with anti-S MAb. Surface proteins expressed by 5aC138 full-length HBV genome or CMV-S/5aC138 and concentrated by anti-HBs PAb immunoprecipitation (Fig. 3.2.4.2, lanes 3 and 11 or lane 8, respectively) were almost as reduced compared to Wt-signals (Fig. 3.2.4.2, lanes 1 and 9 or lanes 5 and 7) as in former experiments (Fig. 3.2.3.1, lane 3). In contrast, particles secreted by CMV-S/5aC138 were detectable after ultracentrifugation with similar amounts as for Wt CMV-construct (Fig.

3.2.4.2, lane 6 compared to lane 5). Taking into account that transfection efficiency was not measured for the CMV-driven constructs, these results suggest that 5aC138 S-gene has the capacity to produce and secrete SVPs efficiently.

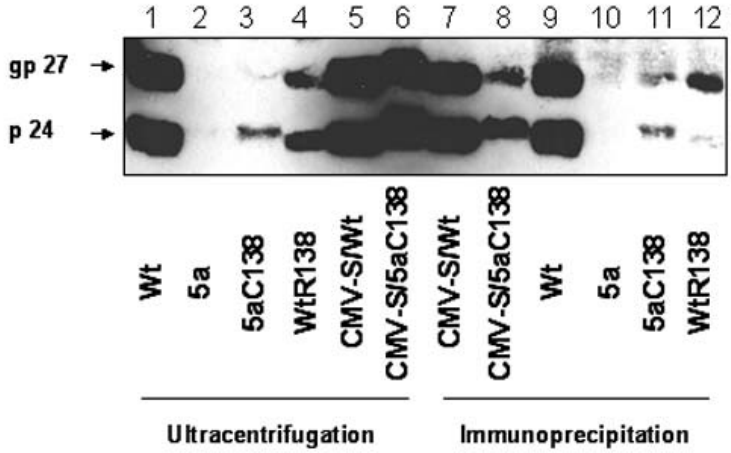


Fig. 3.2.4.2: **Detection of secreted 5aC138 S-containing particles**. Huh-7 cells were transfected with constructs named in the figure below each line. Secreted particles were concentrated by ultracentrifugation (lanes 1-6) or by immunoprecipitation (lanes 7-12). Proteins were separated by SDS-PAGE, and detected with monoclonal anti-S antibody. The envelope proteins detected were the S-protein glycosylated (gp27-kDa) and nonglycosylated (p24-kDa).

3.2.5 Comparison of the secretion phenotype in two cell lines

Due to the lack of cells permissive for HBV infection, HBV studies are restricted to investigations with hepatoma cell lines transfected with HBV-DNA. Different results obtained with various cell lines can be due to many factors: different susceptibility in transfection, differences in transcription and protein stability, efficiency in assembly and secretion of viral particles and others. In all studies performed so far, a Huh-7 cell line was used. The question was whether the block of particle secretion was characteristic only for this cell line or common also to other hepatoma HBV-replication competent cell lines. For that reason, the mutant phenotypes were also studied in the HepG2-C3A cells.

Wt, variant 5a, mutant WtR138 and mutant 5aC138 HBV genomes were transfected into HepG2-C3A or Huh-7 cells in parallel. Although transfection efficiency was about two-fold higher in HepG2-C3A cells than in Huh-7 cells, transfection of the different constructs within a certain cell line was similarly efficient. In addition, the mutant envelope protein expression and secretion were always compared to Wt in each cell line and only the phenotype of secretion was compared between different cell lines.

Secretion of SVPs during 4-days was determined by ELISA in duplicate for HBsAg and data were calculated as a percentage related to Wt set arbitrarily to 100% for each cell line. In HepG2-C3A cells transfected with 5a, secretion of SVPs appeared to be less than 5%.

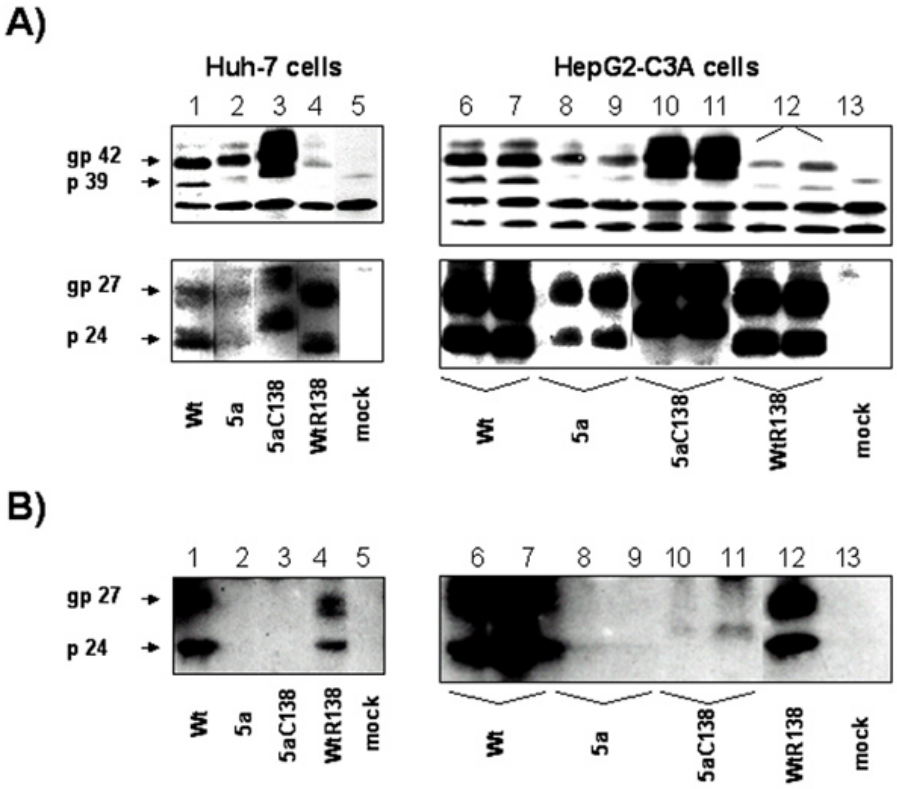


Fig. 3.2.5.1: **Functional analysis of the variants in hepatoma cell lines**. The HBV constructs named below each line were used for transfection of Huh-7 cells (lanes 1-5) or HepG2-C3A (lanes 6-13). The envelope protein expression (panel A) was investigated by Western blot analysis with anti-preS1 or anti-S MAbs. In panel B, viral secretion was investigated by immunoblot analysis of PEG-precipitated particles and HBs detected with anti-S MAb. The envelope proteins detected were the L-protein glycosylated (gp42-kDa) and nonglycosylated (p39-kDa), and the S-protein glycosylated (gp27-kDa) and nonglycosylated (p24-kDa).

For the construct WtR138 and for 5aC138, it was reduced to 10% and to 50%, respectively. These results were similar to those observed for ELISA measurements of HBsAg in Huh-7 transfected cells in former experiments (chapter 3.2.3). In addition, surface protein expression and secretion was investigated by Western blot analysis. Cells were lysed, and cytoplasmic fractions were obtained after application of a three-times freeze-thawing procedure (Fig. 3.2.5.1-A). Particles in supernatant of transfected cells were precipitated by addition of PEG (Fig. 3.2.5.1-B). Proteins were separated by SDS-PAGE, blotted to a nitrocellulose membrane, and detected by anti-S and anti-preS1 MAbs. A block of secretion was observed for the variant 5a in the HepG2-C3A cell line, while proteins were detected intracellularly (Fig. 3.2.5.1-B and A, lanes 8-9, respectively). However, intracellular levels were reduced to about 50% for the 5a S-protein (Fig. 3.2.5.1-A, lanes 8-9, gp27 and p24). The phenotype of expression and secretion of the mutant genomes in HepG2-C3A cells were similar to those in Huh-7 cells. These results suggest that the secretion phenotype of the HBV constructs were not restricted to Huh-7 cells only and that factors inducing abrogation of 5a surface protein secretion might be present in both hepatoma cell lines.

3.2.6 Significance of the Cys138 amino acid exchange

To investigate the significance of the Cys 138 mutations, plasmids with HBV genomes expressing Ala in position 138 of the S-gene were constructed by site-directed mutagenesis in the sequence context of Wt or 5a, WtA138 and 5aA138 genes, respectively. The expression and secretion of the WtA138 S-protein was previously studied by Mangold and colleagues, and Cys138 was found to be indispensable for efficient secretion of SVPs in a heterologous protein expression system (Mangold and Streeck, 1993). However, analysis of the effect of exchange Cys138Ala has never been investigated by expression of the surface proteins in a human liver derived cell line under homologous HBV promoters.

For functional analysis, hepatoma Huh-7 cells were transfected with HBV constructs expressing Cys, Arg, or Ala in position 138 of the S-protein. Transfection efficiencies were calculated by SEAP-activity in the supernatants, and found similar for all transfected constructs. HBcAg expression (Fig.3.2.6.1-A) was slightly reduced for the constructs 5aC138 and 5aA138 compared to Wt HBcAg levels. The other constructs expressed HBcAg to similar levels as Wt. Intracellular *de novo* DNA replicative intermediates were analysed by Southern blot analysis (Fig.3.2.6.1-B) and the HBV genomes of all constructs studied were replication competent. The replicative intermediate levels of the construct WtA138 (Fig.3.2.6.1-B, lane WtA138) were slightly reduced two-fold compared to those from Wt. This reduction could be caused by the double S-gene mutation introduced when the construct WtA138 was created by site-directed mutagenesis: codon TGC was mutated to GCC in the S-ORF and codon TTG was mutated to TGC in the P-ORF. These mutations are nonsynonymous (aa-altering) mutations in the P-protein resulting in a Leu481Cys exchange. Mutations in the S-domain affecting the P-protein are rarely neutral because they can affect the reverse-transcriptase DNA-polymerase activity of these HBV genomes. Note that a small cluster of mutations in the P-protein is present in the S-gene of the variant 5a between aa 460 and 490 inducing in the variant 5a an aa-exchange Leu481Ser in the P-protein.

The significance of the Cys in position 138 of the S-protein for secretion of SVPs was next investigated by ELISA for HBsAg (Wt secretion was set to 100%). The aa-exchange of Cys138 to Ala led to a decrease in secretion to 30% (construct WtA138), and to 10% with the Cys138Arg mutant (construct WtR138). This result showed that introduction of Arg in position 138 of the surface protein induces a stronger secretion block than introduction of Ala. On the other hand, secretion of SVPs was extremely low (1-2%) for the construct 5aA138 compared to Wt. These results suggest that mutations in the 5a context might play a secondary role in the secretion phenotype.

In order to differentiate which type of SVPs (spheres or filaments) had a reduced secretion phenotype, particles in supernatants of cells were concentrated by PEG-precipitation, and proteins were investigated by Western blot analysis with anti-preS1 and anti-S MAbs (Fig. 3.2.6.1-D). Spherical SVPs mainly contain S-protein and they are here designated as S-containing particles; in contrast, filamentous SVPs bear a relatively high percentage of L-protein and they are here designated as L-containing particles. For the construct WtA138, the secretion of S-containing particles (Fig. 3.2.6.1-D, lane 5, p24 and gp27) was reduced to 30% in accordance with ELISA measurement for HBsAg. However, a stronger block of secretion was detected for WtA138 L-containing particles compared to Wt particles (Fig. 3.2.6.1-D, compare lane 5 to lanes named as 1, p39 and gp42). Secreted surface proteins were undetectable by Western blot analysis for the construct 5aA138 (Fig. 3.2.6.1-D, lanes 6) in accordance with the low values of 1% observed by ELISA when measuring SVP secretion.

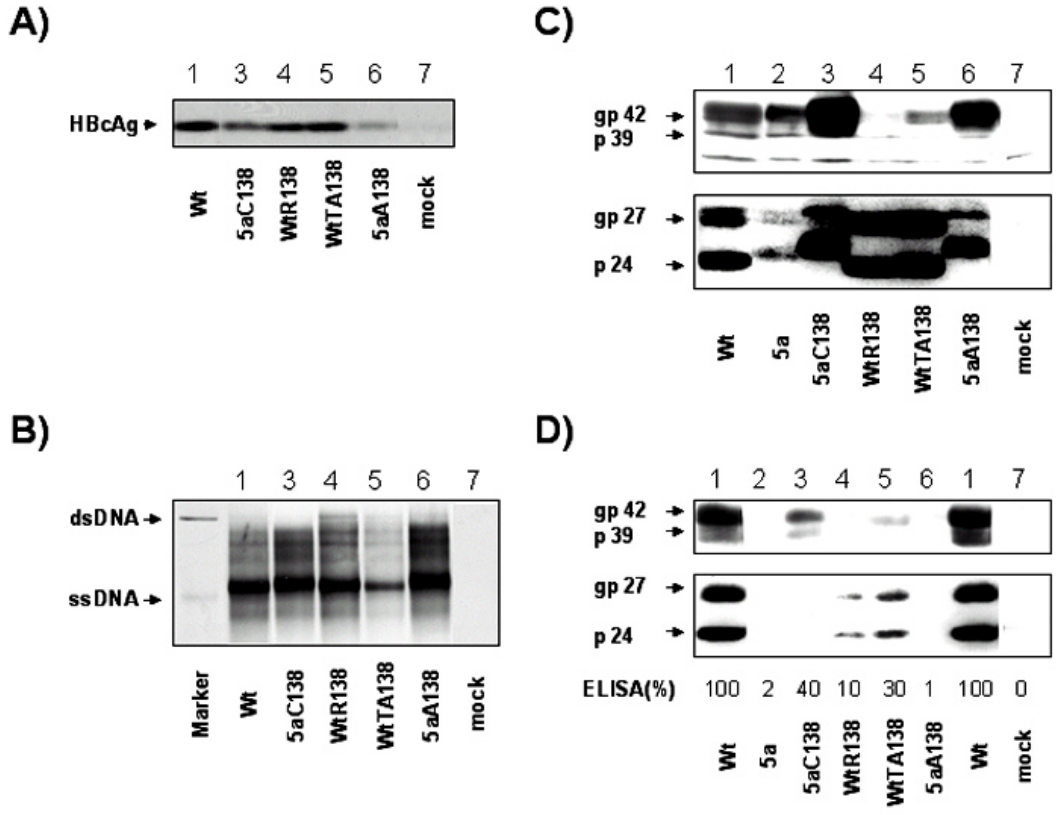


Fig. 3.2.6.1: **Functional analysis of HBV constructs**. Replication ability of the constructs was tested by the capacity of the HBV genomes to express capsid protein (panel A) and encapsidated viral DNA (panel B) in Huh-7 transfected cells. The HBcAg was detected by Western blot analysis with anti-HBc PAb and a single band corresponded to the HBcAg. Several replicative intermediates were detected by Southern blot analysis with an HBV-DNA full-length probe, double-stranded (dsDNA) and relaxed-circular DNA (rcDNA). The envelope protein expression (panel C) in Huh-7 cells was investigated by Western blot analysis. Viral particle secretion was investigated by immunoblot analysis of PEG-precipitated particles from Huh-7 cells (panel D). The envelope proteins detected were the L-protein glycosylated (gp42-kDa) and nonglycosylated (p39-kDa), and the S-protein glycosylated (gp27-kDa) and nonglycosylated (p24-kDa).

An obvious reason for the selective reduction of L-containing SVPs could be a reduction in the L-protein levels. Cells transfected with the HBV constructs were lysed and subjected to Western blot analysis with anti-preS1 and anti-S MAbs. The expression of the WtA138 L-protein was reduced about four-fold compared to Wt (Fig. 3.2.6.1-C, lanes 5 and lanes named as 1, respectively) while the WtA138 S-protein was slightly increased (Fig. 3.2.6.1-C, lane 5). Protein expression levels for the construct 5aA138 were similar to those for Wt (Fig. 3.2.6.1-C, compare lane 6 and lanes named as 1). Surprisingly, the shift in the 5aA138 S-protein mobility and the ratio p24-/gp27-kDa was similar to those observed for the S-protein expressed by construct 5aC138. The reason for this phenomenon is unknown.

3.2.7 Subcellular localization of mutant surface proteins

Secretion deficient proteins for the variant 5a showed an aberrant localization. In addition, the mutation Cys to Arg in position 138 of the S-protein was mainly responsible for the secretion block. To assess the subcellular localization of the surface proteins bearing this mutation in Wt or variant context, immunofluorescence staining and immunofluorescence microscopy were performed. Huh-7 cells were transfected with plasmids WtR138 or 5aC138. Four-days after transfection, the cells were split and seeded onto coverslips for 24-hours. After fixation, surface proteins were detected with anti-S PAb and anti-preS1 MAb. Specific cellular markers for the secretion pathway compartment were also used: anti-PDI (ER-marker) and anti-58kDa protein (Golgi-marker) Abs (Fig. 3.2.7.1).

Secretion deficient envelope proteins expressed by constructs WtR138 and for WtA138 had a cytoplasmic distribution (Fig. 3.2.7.1-D). In contrast, HBV mutant 5aC138 envelope proteins showed a more limited distribution adjacent to the nuclear membrane (Fig. 3.2.7.1-A). This result suggests that the aberrant intracellular localization might be associated with but not sufficient for a secretion defect.

3.2.8 Lack of accumulation of the variant 5a envelope proteins

So far, S-protein steady-state levels from the variant 5a genome was shown to be slightly reduced compared to the Wt-counterpart (Fig. 3.1.2.1-D, lane 3 and Fig. 3.1.2.2, lanes 3 and 4); in addition, exchange of a single Cys138 to Arg or Ala in the Wt context (construct WtR138 and WtAla138) led to representative decreased amounts of detectable L-protein (Fig. 3.2.3.1-C, lane 4 and 1 and Fig. 3.2.6.1, lanes 4, 5 and 1, respectively). In contrast, Northern blot analysis showed that the 5a mRNAs are transcribed in Huh-7 transfected cells almost as

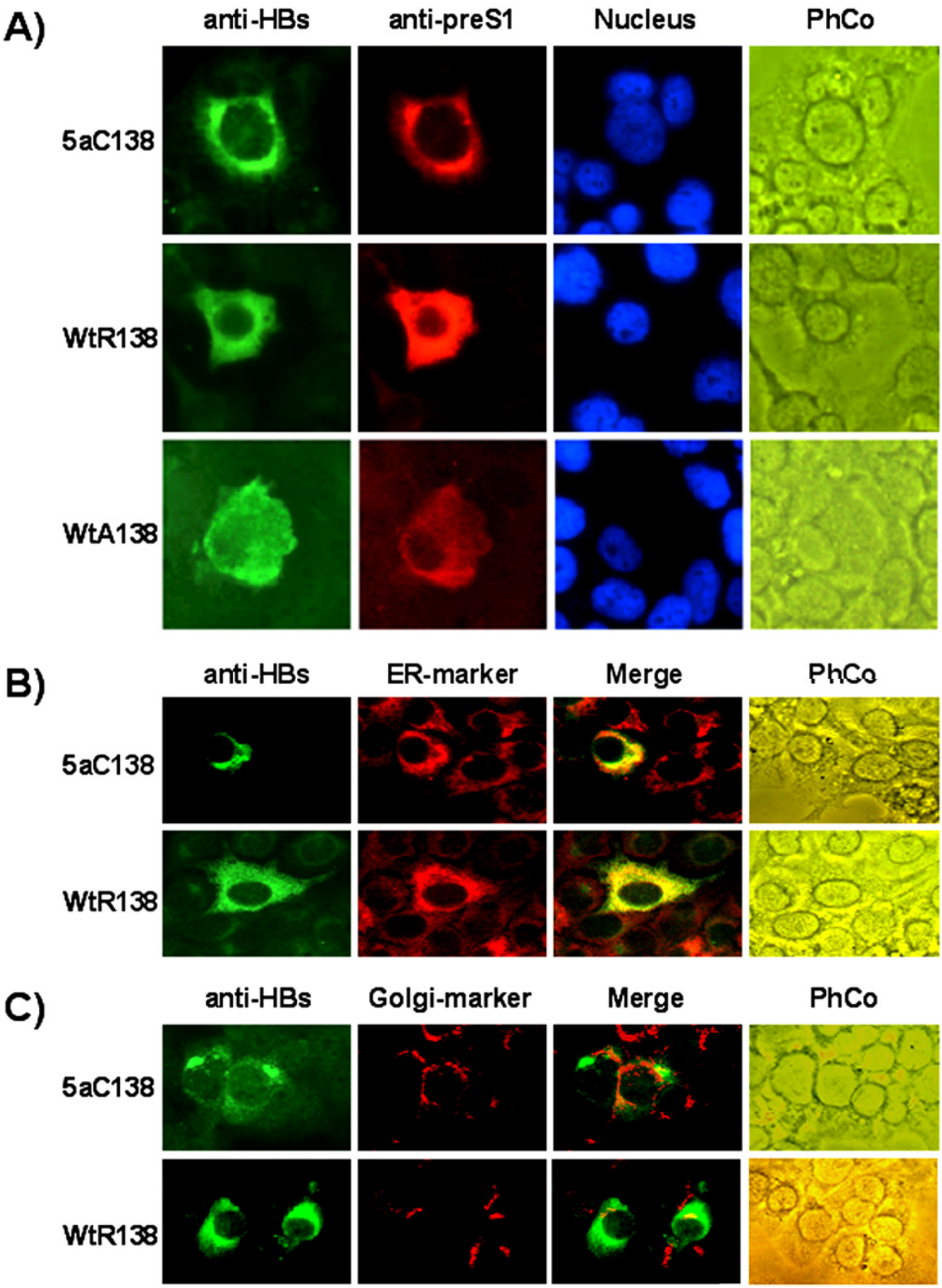


Fig. 3.2.7.1: **Subcellular localization of the mutant surface proteins**. Huh-7 cells were transfected with the HBV construct 5aC138, WtR138 and WtA138. Four days posttransfection, cells were splitted and seeded onto coverslips. In panel A, surface proteins were co-stained with goat anti-S (green) and anti-preS1 MAbs (red), and DNA-binding-dye (blue) was added for 10 minutes in the last washing procedure. In panel B and C, co-staining of surface proteins (anti-S PAb) was performed with PDI the cellular marker for the ER (ER-marker, red) and the 58kDa Golgi protein (Golgi-marker, red), respectively. The merge, demonstrating possible colocalization, is also shown (Merge) and the cells are also visualized by phase contrast microscopy (PhCo).

efficiently as the Wt counterparts (Fig. 3.1.2.1-C, lane 3 and 1, respectively). If RNA levels of HBV variant 5a genome are similar to HBV genomes expressing Cys in the S-protein, several other reasons could lead to the reduction in these protein levels: reduction in translation efficiency, decreased stability of primary translation products, or increase in the protein degradation. It is known that misfolded and intracellularly retained mutant proteins might be less stable and thus more accessible to proteolytic degradation, introduction of Arg in proteins might as well decrease protein stability.

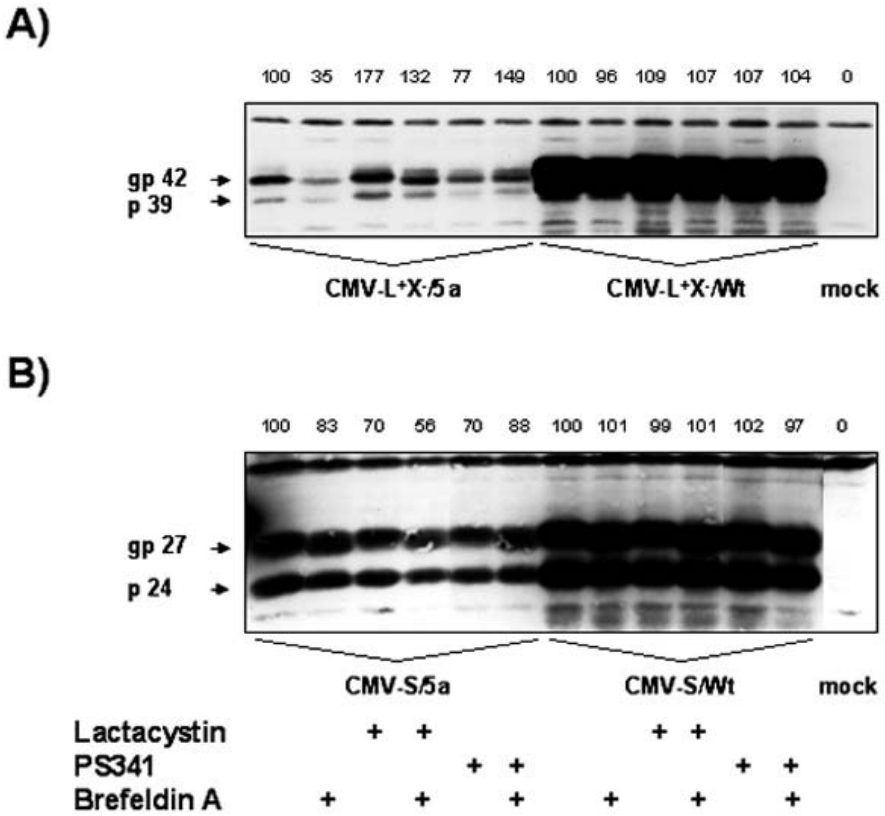


Fig. 3.2.8.1: **Surface protein levels under proteasome inhibition**. Huh-7 cells were transfected with CMV-constructs expressing the L- (CMV-L^+X^-/5a or CMV-L^+X^-/Wt, panel A) or the S-protein (CMV-S/5a or CMV-S/Wt, panel B). Three days posttransfection, cells were not treated or treated with the combination of drugs depicted below each line: cells were either not or treated with the secretion pathway-blocking drug BfA, the irreversible (Lactacystin) or the reversible (PS341) proteasome inhibitor. Intracellular L-protein (glycosylated gp42-kDa and nonglycosylated p39-kDa) or S-protein (glycosylated gp27-kDa or nonglycosylated p24-kDa) levels after treatment were investigated by Western blot analysis. For detection, anti-preS1 (panel A) or anti-S (panel B) MAbs were used, respectively.

Several proteolytic degradation pathways are present in mammalian cells, but the most important system is the proteasome (Garber, 2002). Proteins targeted to degradation by proteasomes can be partially stabilized by inhibition of the proteasome activity. Lactacystin is an irreversible inhibitor of the proteasome activity (Fenteany and Schreiber, 1998), while PS341 is a reversible inhibitor (Garber, 2002). To investigate if the reduction of envelope protein levels was due to an increase of their degradation by the proteasome system, Huh-7 cells were transfected with plasmids CMV-L^+X^-/5a (Fig 3.2.8.1-A) and CMV-S/5a (Fig

3.2.8.1-B) to express the 5a-genome specific L- or S-proteins, respectively, and treated with Lactacystin or PS341. As an additional control, cells treated or non-treated with proteasome inhibitors were also treated in addition with a drug that disrupt the Golgi apparatus (BfA) to block the constitutive secretion pathway (Kano, 2000). Wt envelope protein expression was also performed under the same conditions by transfection with the plasmids: CMV-$L^{+}X^{-}$/Wt (Fig 3.2.8.1-A) and CMV-S/Wt (Fig 3.2.8.1-B).

No stabilization of the 5a S-protein was observed after proteasome inhibition (Fig. 3.2.8.1-B). In contrast, levels of 5a L-protein increased two-fold indicating a minor stabilisation by the treatment with lactacystin (Fig. 3.2.8.1-A). Besides, expression of the Wt L-protein appeared not to be affected by treatment of the cells with the drugs (Fig. 3.2.8.1-A). This result seems to be in accordance with the fact that the mutated L-protein interacts with the chaperone Hsc70 and in case of retention the protein might be target to degradation in the proteasome after translocation to the cell cytoplasm. Treatment with BfA decreased the 5a L-protein levels, probably by induction of the unfolded protein response and through a decrease in protein synthesis (Patil and Walter, 2001).

3.3 Virion secretion and viral propagation

Hepadnaviruses require nucleocapsids for encapsidation of viral pgRNA and envelope protein for transport out of the cell. In addition, secretion is a prerequisite for the establishment of new infections.

The presence of high mannose in glycosylated complexes of surface proteins in HBV viral particles indicates that the constitutive secretory pathway might be the main viral secretion pathway. Cell lysis as a consequence of viral infection with Wt HBV is not observed. Three forms of virus-associated particles are normally found in the serum of HBV infected patients: virions (Dane particles of about 42nm diameter) as well as spherical and filamentous particles (SVPs about 20-22nm in diameter, see introduction section). Additionally, naked capsids without envelope can be secreted or released as such by infected hepatocytes cultures or by transfected cell lines (Lenhoff and Summers, 1994). These particles were never found in patient's serum or in immunosuppressed patients.

The HBV constructs Wt, variant 5a, and mutants are replication competent, produce nucleocapsids and envelope proteins. As a consequence, the presence of secreted virions depends only on the ability of the surface proteins to transport these particles through the secretion pathway. In the present chapter, the capacity of the HBV constructs to secrete virions and their physical properties will be described.

3.3.1 Secretion of virions for Wt and mutant genomes

Since cells transfected with variant 5a HBV-DNA did not secrete detectable amounts of SVPs, it was anticipated that no virion particles would be secreted either. However, variant 5a HBV-DNA particles were reported to be found in the medium of transfected cells by a highly sensitive Southern blot analysis (Sterneck et al., 1998), but in that study it was not discriminated whether the 5a HBV-DNA signal belonged to virion or naked nucleocapsids.

Discrimination between virion and naked nucleocapsid secretion can be achieved by their electrophoresis separation throught a native agarose gel. In former reports (Xu and Yen, 1996), virions from Wt-transfected cells ran in native gels with the same mobility as authentic virions isolated from the serum of an infected person, while naked nucleocapsids had a higher electrophoretic mobility. In this thesis, further evidence for the different position of specific type of viral particles in native agarose gels was obtained by using culture medium from transfected Huh-7 cells with Wt, variant 5c, and variant 5a HBV-genomes, or from mock-treated Huh-7 cells. Particles present in cell culture supernatants were concentrated by

addition of PEG and separated by native agarose gels. Three identical gels were run in parallel, the particles in the gel were transferred to a nylon membrane and the viral-DNA detected by hybridisation with DIG-labelled HBV-DNA (Gel-1, Fig. 3.3.1.1-A). The surface proteins were detected by incubation with MAbs: anti-preS1 (Gel-2, Fig. 3.3.1.1-B), or anti-S (Gel-3, Fig. 3.3.1.1-C), respectively.

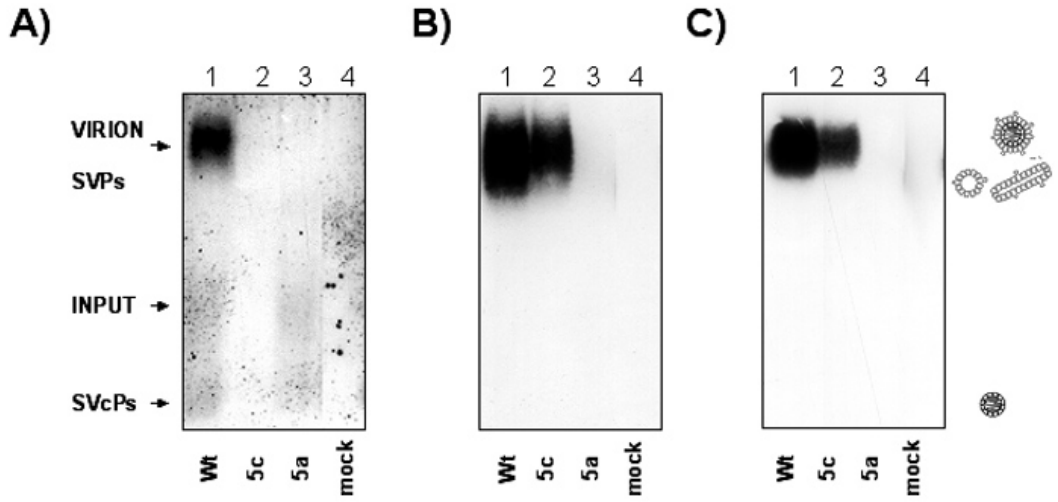


Fig. 3.3.1.1: **Viral particles from Wt and variant genomes separated by electrophoresis through a native agarose gel**. PEG-precipitated particles from cells transfected with the constructs indicated below each lane were divided into three parts, separated through three agarose gels in parallel and transferred to three nylon membranes. Viral-DNA and envelope proteins were detected by incubation of one membrane with full length digoxigenin-labeled HBV-DNA probe (panel A), with anti-preS1 MAb (panel B), or with anti-S MAb (panel C). The area on the upper part of the gel is designated VIRION/SVPs, while the area below to naked nucleocapsids (here depictured as SVcPs), respectively. In addition, traces of linear HBV-DNA (3,2 Kb) used for transfection was detected in the gels by DNA hybridisation, this signal was designated INPUT.

Two HBV-DNA species were detected for secreted Wt virus particles in gel 1 (Fig. 3.3.1.1-A): a slow-migrating band (designated VIRION) and a quicker-migrating band (designated SVcPs). The upper-band corresponds to virions as also the envelope proteins run in the same position in gel-2 (Fig. 3.3.1.1-B, lane 1). Particles in the lower part of the gel correspond to naked nucleocapsids, consistent with the fact that no envelope proteins were detected in the same position in gel-2 or gel-3. The HBcAg levels in secreted HBV particles are usually too low to be detected by blotting with an anti-HBc PAb in this type of experiments.

Viral particles produced by variant 5c were also investigated in native gels. This variant is competent in secretion of SVPs but defective in formation of virions, as shown by Southern blot analysis (Fig. 3.1.2.1-A, lane 2). For variant 5c neither virion nor non-enveloped core-capsids were detected in gel 1 (Fig. 3.3.1.1-A, lane 2). However, in gel 2 and gel 3, S- and L-containing particles (Fig. 3.3.1.1-B and C, lane 2, respectively) were detected in the same position as those for Wt-virions. These particles might correspond to SVPs, but

run in the same position as virions. Cells transfected with HBV-DNA of the variant 5a genome showed neither secretion of virions nor SVPs, as no particles were observed in gel 1 and in gel 3 (Fig. 3.3.1.1-A and C, lane 3). Only a weak hybridisation signal in the position of naked nucleocapsids could be appreciated using an HBV-DNA probe (Fig. 3.3.1.1-A, lane 3).

In order to study the influence of mutation Cys138 in the S-domain of the surface proteins on the secretion of virions, Huh-7 cells were transfected with Wt or mutant HBV-genomes named in the figure below each lane (Fig. 3.3.1.2-A) and secreted particles were separated by native agarose gel. As expected, introduction of Cys in position 138 of the 5aS-proteins (construct 5aC138) led to rescue of virion secretion (Fig. 3.3.1.2-A, lane 3). However, 5aC138 secreted virion levels did not reach Wt levels (Fig. 3.3.1.2-A, lane 1).

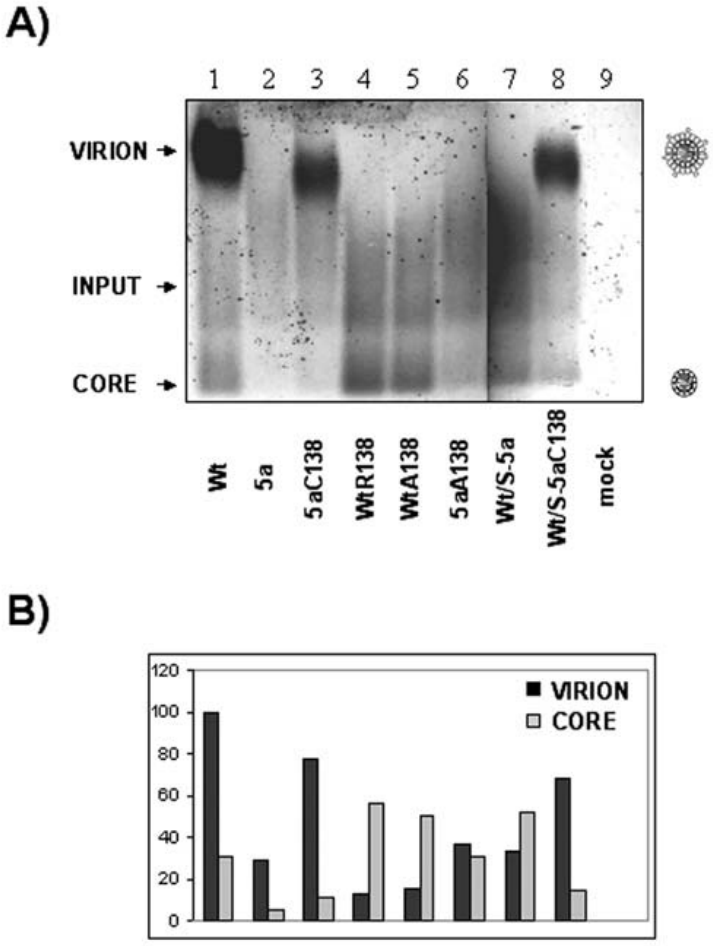


Fig. 3.3.1.2: **Viral particles secreted after transfection with HBV genomes**. PEG-precipitated particles of the HBV-constructs indicated were separated through an agarose gel and transferred to a nylon membrane. Detection was performed with full-length digoxigenin-labeled HBV-DNA probe. Separated particles were found in two areas: Virions/SVPs (upper part of the figure, designated here as VIRION), and naked nucleocapsids (lower part of the figure, designated here as CORE). Traces of linear HBV-DNA (3,2 Kb) used for transfection was also detected in the gels by DNA hybridisation, this signal was designated here IMPUT. Quantification of the HBV-DNA signals intensities are shown in panel B for virions (black columns) and naked nucleocapsids (grey columns). Signals from the upper blot were scanned and dot-intensities were measured with the computer program TINA.

As shown above, absence of Cys in position 138 of the S-protein in the variant genome context -variant 5a (Fig. 3.3.1.2-A, lane 2) or construct 5aA138 (Fig. 3.3.1.2-A, lane 6)- led to no detectable virions secretion.

HBV genomes containing the single aa-substitution in the S-region both Cys138Arg and Cys138Ala were compared to Wt. Although these HBV genomes presented a partial reduction in SVPs secretion shown by HBsAg-ELISA and Western blot (chapter 3.2.6) of about 10% and 30%, respectively; no HBV-DNA signals were observed after running native agarose gels (construct WtR138, Fig. 3.3.1.2-A, lane 4; and construct WtA138, Fig. 3.3.1.2-A, lane 5). These results suggests that aa-exchanges in Cys138 might have a stronger effect on secretion of virions than on that of SVPs. Therefore, this aa-exchanges seems to be essential for virion secretion. Surprisingly, cells transfected with these constructs (WtR138 and WtA138) released a higher amount of naked nucleocapsids than the corresponding Wt-ones. This was not due to an increase of cell death as evident by light microscopy and secretion of these particles might be specifically increased by the block of virion secretion.

In order to assure that this effect was only promoted by aa-exchange 138 in the S-domain, the virion secretion phenotype of two S-constructs was analysed: first, a construct that contained the mutant 5aC138 S-gene in Wt sequence context (Wt/S-5aC138) and second, a construct bearing the 5aS-gene in Wt sequence context (Wt/S-5a). Secretion of virions was again abolished in cells transfected with the construct expressing Arg in position 138 of the S-gene (construct Wt/S-5a, Fig. 3.3.1.2-A, lane 7); in contrast, transfection with the construct expressing Cys in position 138 (construct Wt/S-5aC138, Fig. 3.3.1.2-A, lane 8) led to secretion of virions.

3.3.2 Characterization of virion particles

Since the 5aC138 HBV-genome is competent for virion secretion, the physical properties of virions produced from this construct were therefore analysed by isopycnic CsCl gradient centrifugation, and compared to Wt-virions.

Ultracentrifugation in CsCl-gradient allowed banding of different particles present in the supernatant of transfected cells: SVPs, virions, and HBV-DNA nucleocapsids. Huh-7 cells were transfected with 5aC138 or Wt genome, the medium was collected 5-days posttransfection and directly used for ultracentrifugation. Separation of Wt virions (designated here as Virion) from HBcAg particles (designated here as CORE) was successful as judged by the appearance of two regions presenting HBV-DNA bands at about 1.37g/ml and 1.22g/ml CsCl density, respectively, after dot blot analysis in Fig. 3.3.2.1-A. Similar physical properties

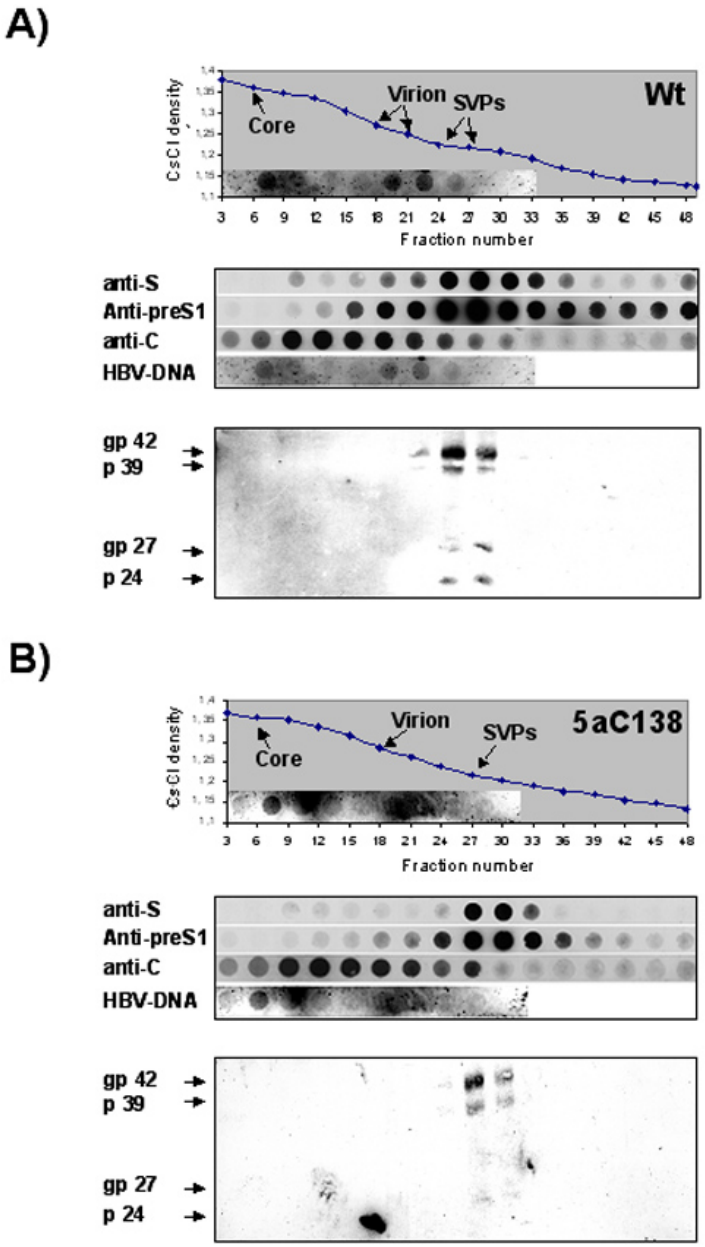


Fig. 3.3.2.1: **Separation of secreted HBV particles by ultracentrifugation in CsCl gradients**. Particles released into the medium of cells transfected with Wt (panel A) or 5aC138 viral genomes (panel B) were banded by ultracentrifugation in CsCl. About 50 fractions (fraction number) were collected and the densities values were calculated by measuring the refractory indices in a Zeiss refractometer (CsCl density). HBV-DNA Dot blot is shown in the left corner of the graph (above left). Protein dot blot was performed with anti-S, anti-preS1 MAbs, and anti-C PAb (panel B and D, upper parts). Western blot analysis was also performed directly with aliquots of fractions (panels on the lower part) using a mixture of anti-preS1 (L-protein glycosylated gp42-kDa and nonglycosylated p39-kDa) and anti-S (S-protein glycosylated gp27-kDa and nonglycosylated p24-kDa) MAbs for detection.

were observed for HBV-DNA particles in the medium of 5aC138 genome DNA-transfected cells (Fig. 3.3.2.1-C), but the amount of virion and naked nucleocapsids were lower than those estimated for Wt. Notably, the ratio of virion and naked nucleocapsids both for Wt- and 5aC138-secreted particles was decreased in comparison to the former experiment (chapter 3.3.1). The increase in core-free-capsids could be caused by the destruction of the virion envelope by the centrifugation process.

The presence of S- and L-containing particles was investigated by protein dot-blot (Fig. 3.3.2.1-B, upper part) and Western blot analysis (Fig. 3.3.2.1-B, lower part). The S- and L-signals were very strong in the banded material at a density of 1.19 g/ml. This band might correspond to the position of SVPs being similar to that of 22-nm particles from a patient (Kaplan, 1976). The SVPs secreted by construct 5aC138 were also detected in the supernatants, however as reported before, 5aC138 S-protein, gp27- and p24-kDa expressed less according to the Western blot than the corresponding Wt S-protein (Fig. 3.3.2.1, compare the lower parts of C and D). In conclusion, cells transfected with the 5aC138 HBV-construct secrete virions, which have similar properties than those secreted after transfection with the Wt-genome in the transient transfection system used.

3.3.3 Transcomplementation of Wt and mutant surface proteins

Analysing the variants present in liver biopsy, a mixture of HBV variant population was detected; at least 30% of the population exhibited Arg in position 138 of the S-domain of the surface proteins, while the rest expressed Cys in the same position of the surface proteins (chapter 3.1.1). However, the variant with mutation Cys138Arg seems to be a minor population. Besides that, this mutation was shown above to be responsible for a complete virion secretion block (chapter 3.3.1 and Fig. 3.3.1.2). These results make it difficult to understand how a secretion deficient variant can spread and constitute 30% of the viral population, when HBV-variants requires virion exit for proper propagation (Funk, 2004). Certain deficient viruses can be complemented by other partially deficient viruses or by Wt and thus lead to efficient virus propagation (Melegari, Scaglioni, and Wands, 1997). Whether variant 5a can be complemented by Wt or not, was examined next.

For this analysis, constructs were built expressing only S-protein (WtL^-M^-, that means deficient in L- and M-protein translation) or expressing M- and L-proteins (WtS^- and $5aS^-$, that means deficient in S-protein translation). Briefly, the construct WtL^-M^- is a HBV full-length genome in which the preS1-initiation codon was mutated to ACG and in which the third codon in the preS2-region was switched to the stop codon TGA. The constructs WtS^- and $5aS^-$ are also HBV full-length genomes in which the S-initiation codon was converted into stop-codon TGA. Detailed description of the construction of the plasmids is in the Method section and for a brief overview of the constructs see Fig. 3.3.3.1-A. Protein expression and viral particle secretion was tested by double transfection of each single constructs and two independent clones from each type of construct were isolated and tested.

Huh-7 cells were transfected with Wt or mutant HBV-constructs (Fig. 3.3.3.1-A), and surface protein expression was studied by Western blot analysis of cell lysates (Fig. 3.3.3.1-B). After transfection with the Wt-genome, both the S- and L-protein were detectable (Fig. 3.3.3.1-B, lanes 1-2). S-protein, but not L-protein, was detected in lysates from cells transfected with construct WtL^-M^- (Fig. 3.3.3.1-B, lanes 3-4), as expected, indicating an efficient block of L-protein expression. Notably, the mutant WtL^-M^- S-protein levels were intracellularly increased compared to Wt, in absence of L-protein expression of the mutant HBV-genome. The L-protein, but not the S-protein, was detectable for both constructs: WtS^- and $5aS^-$ (Fig. 3.3.3.1-B, lanes 5-6 and 7, respectively). However, the steady-state level of the L-protein produced from the construct WtS^- was reduced, and dramatically reduction was observed with the construct $5aS^-$.

In addition to the intracellular viral expression, secretion of viral particles into the cell culture medium was investigated by immunoblot analysis (Fig. 3.3.3.1-C). The viral particles in the medium of cells transfected with the constructs mentioned above was concentrated by addition of PEG, separated by SDS-PAGE, and the viral envelope proteins were detected with anti-S or anti-preS1 MAbs. While Wt and WtL^-M^- secreted particles as expected (Fig. 3.3.3.1-C, lane 1-2 and 3-4, respectively), cells transfected with the construct deficient in S-protein expression (WtS^- and $5aS^-$) secreted neither S- nor L-containing viral particles (Fig. 3.3.3.1-C, lane 5-6 and 7, respectively). This result was consistent with previous reports in the literature describing that the L-protein is retained intracellularly when expressed without its S-counterpart (Bruss et al., 1996). Furthermore, no HBsAg secretion in the medium of transfected cells could be detected by ELISA, although the construct WtS^- should express L- and M-protein, which both contain at their carboxy-termini the sequences of the S-protein. This result suggests that M-protein does not act as a transport vector for viral particles in the absence of S-protein.

On the other hand, lack of the expression of preS-proteins (WtL^-M^-), increased the efficiency of secretion for viral particles containing only S-protein (Fig. 3.3.3.1-C, lanes 3-4). This result was confirmed by ELISA for HBsAg where WtL^-M^- showed 133% secretion efficiency compared to Wt, set arbitrarily to 100%. The increase in secretion of particles by L-expression deficient construct (WtL^-M^-) could be caused by the lack of intracellular retention induced by the L-protein.

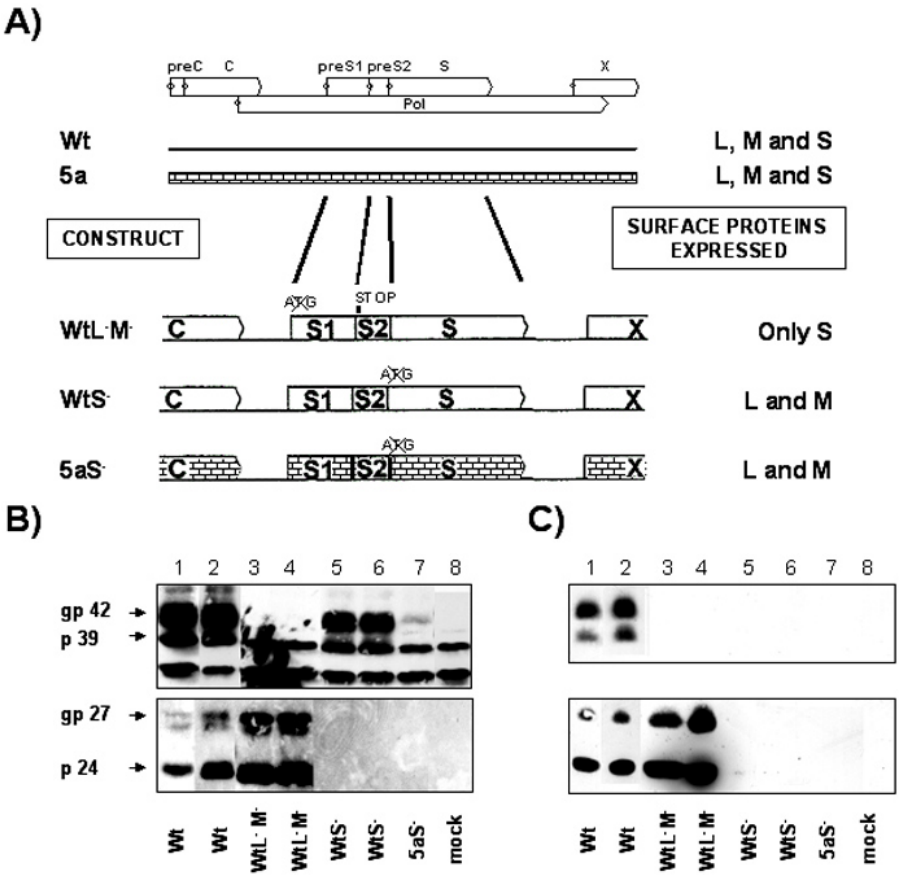


Fig. 3.3.3.1: **Protein expression and secretion of the HBV expression-deficient constructs**. Panel A, schematic representation of the HBV-genome with its four ORFs: preC/C, S1/S2/S (preS1/preS2/S), X and P. Below that, representation of Wt and 5a variant genomes that express all HBV surface proteins (L, M and S), right. In the lower panel, the region S1/S2/S region was amplified. The construct WtL$^-$M$^-$ presents a mutation of the initiation codon of the preS1-region (S1) and a stop codon (STOP) at the beginning of the preS2-region (S2), expressing only S-protein. On the other hand, expression of S-protein in Wt genome was blocked by mutation of its start codon in the construct WtS$^-$. Similarly, in the variant 5a the translation initiation codon was also mutated to form the construct 5aS$^-$. The constructs, WtS$^-$.and 5aS$^-$, expressed only its corresponding L- and M-proteins. Western blot analysis was performed for cell lysates (panel B) and PEG-precipitated particles from the medium of transfected cells (panel C) with the constructs shown below. Immunoblot were developed with the anti-preS1 MAbs (L-protein glycosylated gp42-kDa) and nonglycosylated p39-kDa) or anti-S MAbs (S-protein glycosylated gp27-kDa and nonglycosylated p24-kDa). As control, Wt protein expression (lanes 1-2) was compared to expression of the constructs with variant genomes, as well as mock control (lane 8).

To determine whether the Wt S-protein can complement variant 5a, which would be essential to explain spreading within the liver of the patient 5, cotransfection experiments were performed by transfecting into Huh-7 cells equal total amounts of HBV-DNA but different ratios of WtL$^-$M$^-$ and 5a viral genomes. As a control, Wt, 5a and WtL$^-$M$^-$ constructs were transfected alone.

3.3.3.1 Coexpression of WtL$^-$M$^-$ and mutant genomes

As described in chapter 3.3.3, expression of an HBV-SapI construct defective for S-protein expression (WtS$^-$) showed reduced L-protein levels (Fig. 3.3.3.1-B, lanes 5-6). This result suggested that the S-protein might have a chaperone role for the L-protein both in

variant and in Wt-sequence context. On the other hand, functional analysis of a natural occurring variant, designated 5a, isolated from a patient suffering from FHB showed a block of virus and SVPs secretion. Surprisingly, compared to Wt no intracellular accumulation of secretion deficient surface proteins was detected with 5a genome in cells 5-days posttransfection. To determine whether variant 5a surface proteins could be stabilized to a certain extent by the Wt S-protein, construct WtL$^-$M$^-$, trans-complementation studies were performed next.

Strategically, Huh-7 cells were transfected with equal total amounts of HBV-DNA containing plasmids but changing the ratios of WtL$^-$M$^-$ to variant 5a or to mutant WtR138 genomes. As control, 5a or WtR138, Wt, WtL$^-$M$^-$ constructs, and mock were transfected alone. Additionally, SEAP-plasmid was cotransfected for calculation of transfection and secretion efficiency. Transfection efficiency as determined by SEAP-activity measurements was similar for all cotransfections studied. Intracellular proteins levels were investigated by immunoblot analysis (Fig 3.3.3.1.1).

Expression *in trans* of Wt S-protein with the variant 5a 1:1 increased the steady-state level of the variant 5a and WtR138 L-protein about two-fold (Fig 3.3.3.1.1, lanes 4 and 8, respectively). A more drastic stabilization was detected when the signals were compared to the L-protein expressed by the construct 5aS$^-$ (Fig 3.3.3.1-B). This result suggests that the HBV S-envelope protein could play a role in protein stabilization of intracellular L-protein both for Wt and mutant surface proteins.

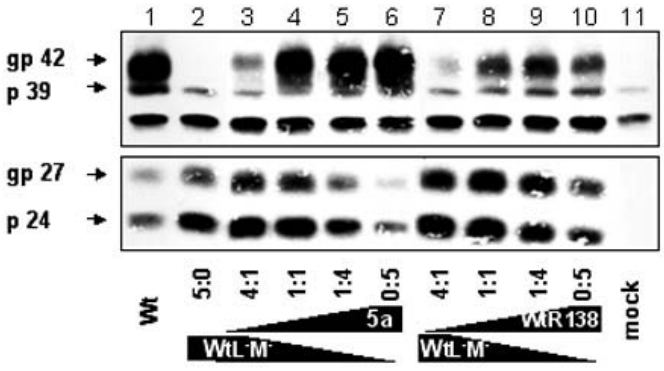


Fig. 3.3.3.1.1: **Intracellular surface protein levels after cotransfection of plasmids expressing Wt S-protein with 5a and WtR138 genomes**. Cotransfection experiments were performed with the constructs WtL$^-$M$^-$ and variant 5a (3-5) or the mutant WtR138 (7-9). Transfection experiments were always performed with equal amounts of total HBV-DNA in Huh-7 cells with changing ratios between WtL$^-$M$^-$ and mutant genomes from 4:1, 1:1, to 1:4. As controls, Wt (1), 5a (6), WtR138 (10), WtL$^-$M$^-$ (2) full-length HBV-DNAs, and mock (11) were transfected alone. In the figure, Western blot analysis of intracellular cell lysates was done with anti-preS1 (gp42- and p39-kDa) or anti-S (gp27- and p24-kDa) MAbs.

3.3.3.2 Mutant L-protein secreted by coexpression of Wt S-protein

Particles secreted into the medium of cells co-transfected with WtL$^-$M$^-$ and 5a plasmids were studied in order to determine whether the secretion deficient variant 5a genome could be rescued by the Wt S-protein, a prerequisite for spreading of virus genome 5a in the liver of patient 5. Additionally, viral particle secretion in a complementation experiment with transfected mutant WtR138 with WtL$^-$M$^-$ genomes was also studied.

Cotransfection experiments were performed as described in chapter 3.3.3.1 by changing the ratio of WtL$^-$M$^-$ and mutant genomes from 5:0, 4:1, 1:1, 1:4, and 0:5, and keeping the total amount of transfected pHBV-SapI DNA constant. Particles accumulating in cell supernatant during 4-days were collected, cleared, and subsequently investigated. WtL$^-$M$^-$ and variant 5a genomes containing plasmids were co-transfected and the phenotype of secretion was monitored by ELISA for HBsAg (Fig. 3.3.3.2.1-A). There was a dose-dependent increase of the viral particle secretion after increasing the amount of WtL$^-$M$^-$ compared to secretion deficient HBV-DNA in cotransfection experiment. By Western blot analysis of PEG-precipitated particles from cells transfected with 5a or with WtR138 genomes, both the 5a and the WtR138 L-proteins (Fig. 3.3.3.2.1-B, lanes 3-5 and 7-9, respectively) were detected in the medium of transfected cells after complementation *in-trans* with the Wt S-protein. In contrast, a block of L-protein secretion was observed after transfection of the secretion deficient HBV construct alone (Fig. 3.3.3.2.1-B, lanes 6 and 10, respectively).

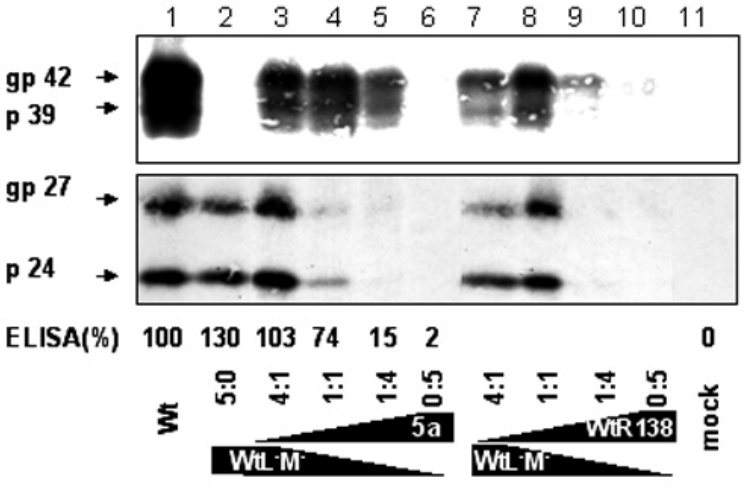


Fig. 3.3.3.2.1: **Extracellular HBV surface protein levels after cotransfection**. The supernatants derived from cells transfected with different ratios of plasmids containing WtL$^-$M$^-$ and 5a were analysed by ELISA for HBsAg (panel A). In panel B, cells were transfected with WtL$^-$M$^-$ alone (lane 2); this mutant genome was cotransfected with variant 5a (lanes 3-6) or with the mutant WtR138 (lanes 7-10). Particles were precipitated by addition of PEG, proteins were analysed by Western blot analysis using anti-preS1 (gp42- and p39-kDa) or anti-S MAbs (gp27- and p24-kDa). The supernatants of Wt- (lane 1) and mock- (lane 11) transfected cells were introduced as controls.

In summary, the intracellular L-protein steady-state levels produced by the variant 5a genome could be stabilized to a certain extent by co-expression of Wt S-protein and this results in the formation of mixed particles containing both Wt and variant 5a L-proteins, which were secreted into the medium of transfected cells. This implies that the 5a S-protein is causative for the secretion block of the variant 5a genome. This HBV quasi-species complementation experiment offers an explanation for the potential mechanism involved in spreading of secretion-deficient variants within the liver of a patient. However the co-secretion of the variant 5a L-protein does not prove that nucleocapsid containing variant 5a HBV genomes are also secreted; such an experiment remains to be done.

4. Discussion

The focus of this thesis was to study a naturally occurring HBV variant, designated variant 5a, present in the serum and the liver of the patient, who was suffering from FHB with liver failure in order to increase the scarce knowledge on the potential role of variants in development of hepatitis with fulminant stage (Liang et al., 1991; Petrosillo et al., 2000; Tanaka et al., 1995).

Although the hepatopathogenicity of the variant was not evident in cell culture, it was possible to establish a causal relationship between the genetic changes specific for the naturally occurring variant 5a and their biological effects. These included a perinuclear accumulation of envelope proteins and a partial proteolysis of mutant envelope proteins mediated by the proteasome. Intracellular accumulation of mutant hepadnaviral envelope proteins is known to be able to cause cellular stress and cell death (Chisari, 1996; Xu, Bruss, and Yen, 1997). In addition, the generation of 5a envelope peptides by the proteasome and their recognition by the immune system may play a role in the fulminant course of the infection seen in the corresponding patient.

The data obtained in the current study has also provided important information on the survival and persistence of HBV variants with an almost complete deficit in viral secretion when occurring as a minor virus population and their selection during the course of infection. In particular, it was shown for the first time that secretion-deficient variants can survive in vivo through complementation by Wt surface proteins and thus can be successfully secreted and spread from cell to cell.

4.1 Variant 5a mutations occur naturally in fulminant hepatitis

Variant HBV genomes belonging to the genotype D are characterized by the lack of 33-nt in a specific domain of preS1-region and by definition differ by less than 4.2% nt sequence homology from the first described prototype (Galibert et al., 1979). The variants 5a and 5c investigated in this thesis differ from this defined genotype D prototype virus by 59- and 64-point mutations in the complete HBV genome, respectively, but do not exhibit any deletions or insertions (Sterneck et al., 1998). This corresponds to a sequence homology of 98,2% (1.8% differences) and 97.9% (2.1% differences), respectively; which is in the range of the known intratype differences for the genotype ranging between 1,5% and 4,2%. In contrast, nt-sequences differences of complete genomes between different genotypes range from 8,8% to 14,5% (Gunther et al., 1999; Norder, Courouce, and Magnius, 1994). In accordance with the fact that there is frequently a very high homology between HBV-genomes of quasi-species from a specific patient, variants 5a and 5c differed by only 12-nt corresponding to a sequence homology of 99,6%.

The analysis of HBV variants present during acute and fulminant hepatitis is often very difficult due to the extremely low HBV-DNA concentrations present in the serum of the patients, which can result from the increased activation of the host immune system inhibiting viral propagation. In these cases DNA amplification methods or PCR have to be used, which might introduce artificial mutations. Amplification and sequencing of the viral DNA derived from at least two independent reactions as well as the analysis of the viral genome populations both in serum and in the liver of the patients is one way to eliminate or reduce potential sequence artefacts caused by PCRs. Therefore in this study, HBV-variants present in a paraffin-embedded liver tissue obtained from patient 5 during the fulminant stage of hepatitis B were also investigated and showed almost the same specific mutations as the variants (variant 5a and 5c) isolated from the serum of the same patient studied previously (Sterneck et al., 1998). Both in the serum (Sterneck et al., 1998) and in the liver of this patient, about 30-40% of the HBV viral population presented an Arg in position 138 of the S-domain of the envelope proteins, while the remaining viral population presented a Cys in the same position as the Wt genomes of all genotypes. Interestingly, all other mutations in the S-gene of the variant 5a genome isolated from the serum (nt position 499, 520, 528, 533, 543) were always present in all viral genomes derived from the liver. In contrast, variant 5c mutations leading to an exchange Thr134His in the a-determinant could not be detected in the liver population and

therefore may be not naturally occurring, represent a PCR artefact or be present in other parts of the liver of patient 5.

The data obtained proves that Cys138Arg mutation is not the result of a technical artefact of the amplification methods, but constitutes definitively a naturally occurring mutation. In addition, these data strongly indicate that mutations in variant 5a compared to the genotype D prototype represent a characteristic feature of the viral population, which was present at this specific period during the course of FHB. However, it is not possible to rule out completely that viral genomes isolated and amplified from the liver specimen actually derived from the extrahepatic spaces such as the serum derived from the blood vessels located between hepatocytes and not actually from the hepatocytes.

HBV variants with mutations in the a-determinant of the small envelope protein seems to be uncommon in FHB (Gunther et al., 1999), such mutants were observed only in few cases (Hsu et al., 1997). HBV with mutations in the a-determinant are frequently occurring in patients with anti-HBe, and are most prevalent in patients negative for HBsAg or positive for anti-HBs. This was taken as an evidence for the accumulation of a-determinant variants during the course of the chronic hepatitis, and in particular for their emergence in patients during or after seroconversion to anti-HBs. This might also apply to patient 5, a chronic HBeAg negative patient who developed an acute stage with a fulminant course of hepatitis B. A similar scenario has been reported for children infected with HBV by their anti-HBe-negative mothers despite vaccination (Bah et al., 1995). These children developed mostly fulminant and acute self-limited hepatitis. In contrast, children infected with HBV by their anti-HBe-positive mothers developed chronic infection.

The consequences of the presence of mutations in the a-determinant of the S-protein of HBV in patients with active hepatitis B has never been investigated in detail, and therefore the functional importance of these mutations remains unproven.

4.2 Functional analysis of the variants

The selection and thus accumulation of certain mutants depend not only on the replication activity of the virus, but also on the rate of elimination of Wt viruses and the resistance towards antiviral mechanism, amongst many other factors. If a particular mutation does not result in a biological disadvantage for the virus, the variant will be propagated. Mutations will be accumulated if they confer a functional gain for the virus, but the appearance of certain mutations can also induce viral phenotypes with a strong immune

response. Therefore, I characterized the biological properties of HBV variants, which were isolated during FHB stage, by transfection of cloned HBV genomes in hepatocarcinoma cells.

A difference in replication ability was observed between both variants studied: the variant 5a was replication competent, but variant 5c was not. In contrast, viral particle secretion of the variant 5a was 50-fold reduced compared to Wt genome, as judged by highly sensitive ELISA as well as by specific anti-S and anti-preS1 Western blot. The ELISA data was in agreement with data reported previously (Sterneck et al., 1998). In addition, the variant 5a envelope proteins localized predominantly in a perinuclear rim, a pattern different from Wt envelope proteins, which exhibit an almost homogeneous cytoplasmic staining. This aberrant localization was reminiscent of other mutant HBV envelope proteins with an intracellular retention phenotype as described by others (Melegari, Scaglioni, and Wands, 1997; Xu, Bruss, and Yen, 1997). Therefore, the consequences of single or the combination of several mutations in the variant 5a envelope proteins are further discussed. Variant 5c was used only as an internal control because this HBV genome secreted SVPs efficiently and was replicative incompetent.

4.3 Functional significance of the 5a S-gene mutations

After having reached a certain intracellular concentration, transmembrane HBV surface proteins dimerize in the ER, protein oligomerize and this induces viral particle formation by the budding from the ER membranes (Huovila, Eder, and Fuller, 1992). Even after S-protein expression with hetererologous systems SVPs are successfully secreted. These particles are designated as subviral because they lack nucleocapsids and therefore they do not contain genomic HBV-DNA. Therefore, we suspected that the variant 5a genome specific defect in secretion ability was attributable to mutations in the envelope ORF.

The three dimensional structure of the envelope proteins of HBV is not known, but an intensive biochemical and immunological study of HBV S-gene mutants has led to structural prediction (Stirk, Thornton, and Howard, 1992). Knowing the precise surface protein structure in the future might allow a better and faster characterization of the causes of viral particle retention. Currently conformational changes can be detected with biochemical analyses, which are difficult to be perform. However, not only one but several protein conformations might be compatible with their function in viral particle secretion, as mutant Cys121/124Ala S-protein appears to have a different conformation than Wt and this mutant genome is able to secrete SVPs (Mangold and Streeck, 1993). The phenotype of mutants bearing several or single aa-changes were progressively investigated in this thesis. The functional analysis of

mutant and Wt genomes included the subcellular localization and degradation of the surface proteins. The subcellular localization of those proteins may lead to the elucidation of the fate of the proteins within the cells, which are strictly controlled by the ER quality control. In this system, molecular chaperones and energy-dependent proteases play a dual role: the first ones promote proper protein folding and prevent protein aggregation, and the latter ones eliminate irreversibly damaged proteins.

4.3.1 Secretion phenotype of HBV S-mutants

As expected, mutations in the S-region and not in the preS-region (preS2/preS1 and upstream) of the variant 5a were mainly responsible for the secretion defect. This result was strongly corroborated by expression of variant S-proteins under a foreign promoter without the HBV context. On the one hand, mutations in the preS-gene increased the intracellular steady-state levels of both surface proteins and this was not due to a defect in secretion. On the other hand, reduced intracellular L-protein levels were detected for the HBV Wt construct with a single Cys in position 138 of the a-determinant, although an accumulation of this protein due to the block in secretion would have been expected. These results suggest a compensatory effect between S- and preS-mutations in the variant 5a, so that the surface proteins might be retained and their intracellular levels might be partially increased at the same time by mutations in these preS-constructs. To achieve the increase in intracellular surface protein levels, promoter mutations upstream of preS-region could be responsible for the L-protein increase, which, at the same time, might affect and upregulate the S-promoter activity (Xu, Jensen, and Yen, 1997). In this way, a proper surface protein ratio might favour viral particle secretion in the absence of secretion blocking S-mutations, and this would allow deficient variants to prevail. Nevertheless, the mutations in preS-region responsible for increase in surface protein levels remains to be investigated experimentally.

Concerning the contribution of specific aa-changes to the secretion phenotype (see column diagram in Fig 4.3.1.2), the double mutations Met125Thr and Thr127Pro does not contribute to the secretion phenotype, because both mutations in HBV Wt sequence context were compatible with efficient secretion. This result is in accordance with the idea that the double mutant is occurring naturally in HBV-isolates from several genotypes and subtypes (Norder et al., 1994). In contrast, the lack of the essential Cys in position 138 and its substitution with an Arg in the variant genome have strong consequences for the secretion of viral particles. It is noteworthy that in case of the variant 5a genome there is another aa-substitution Glu instead of Gly in position 130.

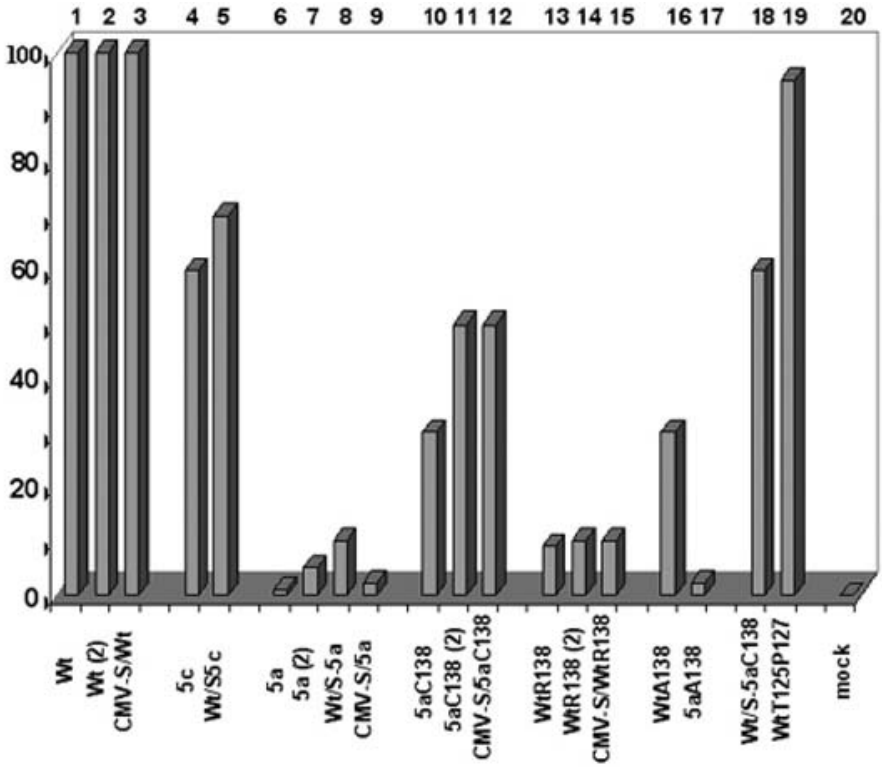


Fig. 4.3.1.2: **Summary of the ELISA-data**. ELISA measurements for HBsAg are presented in a table as a summary. The secretion phenotype is represented as a percentage of secretion compared to Wt, which was standardised to 100% in supernatants of Huh-7 transfected cells (without number), or in supernatant of HepG2-C3A transfected cells (designated as (2)). The data was derived from the result chapter: Columns 1, 4, and 6 from chapter 3.1.2; columns 5 and 8 from chapter 3.2.1; columns 3, 9, 10, 13, 18 and 19 from chapter 3.2.3; columns 12 and 15 from chapter 3.2.4; columns 2, 7, 11, and 14 from chapter 3.2.5; and columns 16 and 17 from chapter 3.2.6.

These exchanges might compensate the overall protein charge, as two closely located neutral Gly and Cys are simultaneously exchanged in the variant genome to opposing charged aas (see a-determinant postulated structure Fig 4.3.1.1). However, it cannot be excluded that 5a S-protein has a distorted or a twisted conformation compared to Wt S-protein. Exchanges in Gly130 are also naturally occurring in other HBV isolates (Asahina et al., 1996; Hou et al., 2001; Zhang, Nordenfelt, and Hansson, 1996); however, an aa-exchange of Gly130 to Glu has not been found in the literature. Aa-exchanges in Cys 138 were already reported in HBV population in other patients suffering chronic hepatitis, Cys138Ser (Gunther et al., 1999); chronic active hepatitis, Cys138Arg (Mathet et al., 2003); or as an escape mutant from a vaccinated carrier, Cys138Thr (Hou et al., 2001). These HBV variants, however, had other mutations in their HBV genomes, we cannot therefore exclude that exchange of Cys138 in the S-domain of the envelope protein was exclusively responsible for the outcome of the infection. The prevalence and consequences of the presence of this mutation in chronic HBV carriers and in patients with active hepatitis patients has never been investigated thoroughly and the functional importance of this mutation remains unproven.

A precise three-dimensional structure of the viral envelope proteins, which is so far not available, would very much help to understand the consequences of each single exchange, their possible functions, and potential contribution to hepatopathogenesis.

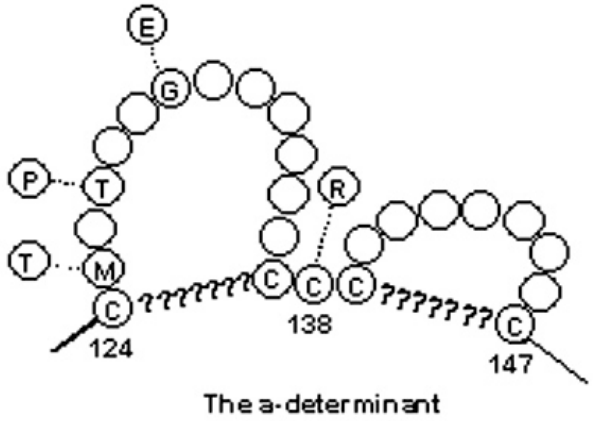


Fig. 4.3.1.1: Predicted structure of the a-determinant. The aa-positions are represented as circles from Cys124 until Cys147. Aa-exchanges in the variants 5a proteins are shown as circles (Thr, Pro, Gln, Arg designated here as T, P, E and R) above their corresponding aas from Wt (Cys, Met, Thr, Gly designated here as C, M, T and G).

Structurally, the a-determinant is located in the major antigenic region (HBsAg, aas 100-160) spanning aas 122 and 147. Its antigenic structure might be formed by at least two loops of aas 124-137 and 139-147 (Stirk, Thornton, and Howard, 1992). Eight Cys residues, highly conserved among genotypes and subtypes (Norder et al., 1994), help to maintain the conformational structure of the loop through disulfide-bonds (Fig 4.3.1.1). A single Cys can fill in the structural space with its positively polarized residue, can form intramolecular disulfide-bridges, or can influence the quaternary structure by intermolecular disulfide-bridges. It seems to be well established that Cys48, Cys65, Cys69, Cys121 and Cys147 are important for conformation of the S-protein quaternary structure (Mangold et al., 1997) and consequently they might form intermolecular disulfide-bridges. In contrast, Cys107 and Cys149 form intramolecular disulfide-bridges to give the a-determinant its ternary protein structure (Mangold et al., 1995; Mangold et al., 1997), which is recognized by MAbs.

The function of Cys138 for the conformation of the envelope proteins is not completely understood. Cys138 might not fill-in structural space as alkylation studies showed that this residue might form disulfide-bridges both in intracellular S-protein and in secreted particles (Mangold et al., 1997). Additionally, Cys138 might have little or no importance for the structure of the a-determinant, because exchange of the Cys138 to Ser still allowed the mutated protein to react with a set of a-determinant MAbs; in contrast, exchange of adjacent Cys137 and Cys139 considerably reduced recognition by MAbs (Bruce and Murray, 1995). Despite of the availability of these data, it remains an open question whether Cys138 is involved in intermolecular disulfide-bridge formation and whether it has an important

structural function on the quaternary structure of the second-loop of the surface proteins. In order to address these questions the mutants were analysed functionally by the use of corresponding HBV constructs created by site-directed mutagenesis.

In a first approach, we analysed the secretion phenotype of HBV full-length constructs where Wt had a single aa-exchange: WtR138 or WtA138 in the a-determinant. Both mutant surface proteins with a single aa-exchange led to a significant block in viral particle secretion. This is consistent with previous observations by others (Mangold et al., 1995), who found that Cys138Ala mutant S-protein was retained and formed aberrant oligomers. The secretion defect with the HBV full-length genome containing only one aa-exchange Cys138Arg in the S-protein was more pronounced than the defect with the aa-exchange Cys138Ala. This may be due to the positive charge of the aa-Arg, which may further affect interaction of the mutant envelope proteins with the ER quality control. From these results, one could conclude when Cys138 is changed to Ala or Arg there is a small difference in secretion phenotype. However, both mutant genomes presented a significant reduction in viral particle compared to Wt, therefore the cause of viral particle secretion block was not established.

More information on the significance of aa-position 138 may be gained from investigations of the secretion phenotype of an HBV construct bearing Ser in position 138 of the S-domain. Ser is structurally similar to Cys, but lacks the capacity to form disulfide-bridges. If the HBV genome bearing Ser in position 138 would present a block in viral particle secretion, we could speculate that it is due to a failure of the S-protein to form an appropriate quaternary structure (oligomerization). In case the Ser-mutant would be secreted normally, we could speculate that residual chains in the Ala or Arg aas disturb the ternary protein structure, and this would support the hypothesis that Cys138 is involved in the ternary structure interactions. In addition, characterization of the structural distortion eventually induced by mutations of Cys138 could be tested with a panel of MAbs by expression of Wt or mutated S-proteins.

By exchange of the Arg in position 138 of the variant 5a S-protein to Cys, about 50% of reversion of the secretion phenotype was observed compared to secretion competent Wt HBV genome. The partial reversion of the phenotype could have been caused by steric disturbances due to the presence of a single negative charge, a Glu, in the a-determinant compared to variant 5a. Although protein secretion is strongly regulated by the ER quality control and chaperones (Ellgaard and Helenius, 2003; Patil and Walter, 2001; Ellgaard, Molinari, and Helenius, 1999), I speculate that 5aC138 S-protein, which may have a different conformation than Wt S-protein, might also be competent for SVPs secretion. For example,

Cys121/124Ala presented a reduced antigenicity, and therefore a disturbed a-determinant, but were properly secreted (Mangold et al., 1995). An explanation for the partial re-establishment of the secretion phenotype by 5aC138 genome could be that some secretion-competent conformations might be faster in passing the quality control than others, and hence 5aC138 S-proteins might pass the quality control slowly. Another scenario possible to investigate in future meaningful experiments would be, whether nonglycosylated aberrant M could act in a dominant negative manner, thereby partially hindering secretion of competent proteins, as proposed by other authors (Mehta et al., 1997).

However, there was a discrepancy between the amounts of secreted HBsAg detected by ELISA and by Western blot analysis. Compared to the ELISA data, only weak signals were detected for the secreted 5aC138 S-protein in Western blots, while these proteins were clearly detected intracellularly with the same MAbs. This result was unexpected because secretion of the 5aC138 L-protein was observed, although L-protein should not be secreted without the support of its counterpart S-protein, as shown in this work (chapter 3.3.3) and by others (Bruss et al., 1996). At present, we have no good explanation for this discrepancy. Perhaps our techniques used for the analysis of secreted proteins did not allow us to visualize the 5aC138 HBsAg, which were clearly detected by the highly sensitive ELISA. The possibility that secreted 5aC138 S-protein bears an additional protein modification in late stages of the secretion pathway, which may impair detection by the MAb, cannot be excluded. This theory is well conceivable as this protein showed a decrease in the ratio of glycosylated to non-glycosylated S-protein and a slower mobility in SDS-PAGE than the variant 5a or the Wt S-proteins. As reported before, the degree of glycosylation of envelope proteins might be influenced by steric impediments, for instance by mutation of Cys 147/149 to Ala a partially increase in the glycosylation of the mutant S-protein was observed as described by others (Mangold et al., 1995). Insertion of an internal epitope into the S-domain, like the polio-epitope as established by Mangold and colleagues (Mangold et al., 1995), could help to detect these proteins.

Nevertheless, other mutations outside the S-region in the variant 5a genome may also have contributed to the secretion phenotype. First, the variant 5a secretion was severely reduced compared to the WtR138; and secondly, the mutant 5aA138 showed a strongly reduced secretion phenotype compared to the mutant WtA138. In addition, it is noteworthy that only a partial reversion of the secretion block was observed by reversion of the Arg138 to Cys mutation in the variant 5a context (construct 5aC138) and block of secretion by the construct bearing the variant 5a S-gene in the Wt-context was less efficient than the original

variant 5a. These additional mutations outside the S-region and their effects were not further characterized in this thesis.

Although having an identical carboxy-terminal aa-sequence, variant 5a mutations in the S-domain (T125, P127, Glu130 and Cys138), which causes a strong block of the 5a S-protein secretion, seem not to affect the 5a L-protein secretion. In fact, the 5a L-protein was efficiently secreted after cotransfection with a HBV construct expressing secretion competent Wt S-protein and deficient in Wt L-protein expression. At least two hypotheses could explain this fact: mutations in the S-region of the L-protein might not influence secretion by adoption of an alternative S-region conformation, and/or interaction between aas within the preS-region might drag the 5a L-protein into Wt S-protein particles. Additionally, Wt S-protein might form mixed particles with the variant 5a L-protein without preference for the Wt S-protein for itself as the 5a L-protein is secreted neither alone nor with its counterpart 5a S-protein. This fact occurs despite of the S-domain mutations in the variant 5a L-protein, the same mutations in the variant 5a S-protein lead to reduce dramatically its secretion. Although the 5a L-protein lacks Cys138, phenotype mixing could be supported by other essential aa for secretion, like Cys107 and/or Cys149 in the S-protein, as suggested elsewhere (Gerhardt and Bruss, 1995).

It is by no means self-evident that a protein that folds properly in one cell type will do so in another. Incompatibility is most often observed between cells of distantly related species such as yeast and mammals, but it also occurs between mammalian cell types. Two types of hepatoma cell line were used in this thesis for the analysis of viral particle secretion of Wt and mutant genomes. Although these cell lines differ certainly in many cellular factors, viral particle secretion and protein expression of Wt and variant HBV genomes were similar, suggesting that cellular factors leading to surface protein retention might act in both cell lines in a similar way. These factors are discussed in the following chapter.

4.3.2 Cellular localization of mutant surface proteins

Assembly and secretion of big viruses often occurs in so called ‘assembly compartments’ which can be created by reorganization of the host cell secretory compartment (Seo and Britt, 2006). HBV has a small and compact genome and therefore needs to use the cellular machinery for several steps in replication, assembly and secretion. In order to exit the host cell, HBV viral particles bud by the action of the ER inserted envelope proteins, and viral particle secretion might follow the constitutive cellular secretion pathway (Patzer, Nakamura, and Yaffe, 1984) as surface proteins are posttranslationaly modified by glycosylation.

However, the budding and secretion pathway for hepadnaviruses is still not well characterized.

In an effort to view the subcellular compartments, which are used by hepadnaviruses for viral particle secretion, we investigated the localization of the envelope proteins and their possible colocalization with cellular ER proteins responsible for the quality control: calnexin, calreticulin, and PDI. Studies with thiol-oxidoreductases of the PDI family were interesting because they catalyse the process of disulfide-bond oxidation and isomerization through the transient formation of mixed disulfides-bridges with substrate proteins (Bottomley et al., 2001; Puig et al., 1994). Therefore, cellular compartments belonging to ER with active chaperone-quality-control are here designated as PDI-compartments.

Some differences were found in colocalization of the different ER markers calnexin (data not shown), calreticulin (data not shown) and PDI suggesting that the ER is subcompartmentalized as already proposed by others (Huovila, Eder, and Fuller, 1992). In agreement with previous work performed in other cell lines (Huovila, Eder, and Fuller, 1992; Patzer, Nakamura, and Yaffe, 1984), HBV Wt surface proteins localized in a larger area than the one highlighted by the ER-markers (cytoplasmic-like localization), suggesting that these proteins pass the ER quality control (here designated as PDI compartments) and proceed into non-PDI compartments for oligomerization and budding of viral particles to the ER-lumen (Huovila, Eder, and Fuller, 1992). Consequently, HBsAg particles became resistant to protease digestion from the cytosolic side, indicating they might have budded into the lumen of intracellular membrane structures and they were thereafter secreted. In contrast, variant 5a surface proteins were localized within a small section of the perinuclear space. These surface proteins co-localized perfectly with the ER resident protein PDI, and therefore seem not to move to non-PDI compartments. It would have been interesting to determine whether this perinuclear localization of variant 5a envelope proteins corresponds to the ER-Golgi intermediate compartments (ERGIC). Unfortunately, colocalization studies with the ERGIC rab2 MAbs were not successful (data not shown). Therefore, other recycling proteins of this compartment could be used as markers in future studies, like ERGIC53 or p115. Since there was no colocalization of the 5a HBsAg with a well-known 58-kDa Golgi-marker protein, one can conclude that this protein is definitely not retained within the Golgi compartment.

Another approach to determine precisely the localization of the retained envelope protein could be to analyse the localization of envelope proteins with Wt and 5a S-protein tagged with secretion pathway retention signals, such as the well-known peptide sequences KDEL or RDEL. A change in the localization of 5a S-protein with retention signals could

give more insight into the fate of these proteins. In any case, introduction of the retention motif should be done in different parts of the S-domain: at the amino-terminus (supposed to be luminal), between Cys121 and Cys124 (where insertion with few aas is relatively well accepted) or at the C-terminus between transmembrane domain 4 and 5 (supposed to be a small luminal loop, see figure 2.2.2-B). HBV S-proteins bearing retaining signal sequences should be expressed alone under strong promoters (CMV), or in context of a complete HBV genome, and to be functionally well characterized in order to show the proper insertion of mutant proteins in ER membranes, their correct localization and retention. Such mutated S-proteins with retention signals, in case of being stable, could probably provide more information about the S-protein structure and dual topology of the S-domain of the envelope proteins, because partial or no intracellular retention of these proteins could indicate lack of luminal topology of the domain where the retention signal was inserted. Positive controls could be viral proteins with KDEL sequences (Hnatiuk et al., 1999) or cellular ER resident proteins, which also contain these retention sequences. Antibodies against these signal sequences are already available.

Another possible approach could be to assess the localization of variant surface proteins under BfA treatment, and compare it to the Wt envelope proteins localization under the same conditions. In case of a redistribution of 5a envelope proteins to an extended ER, Wt-like distribution could mean retrieval of 5a envelope proteins from ERGIC to the ER. In addition, subcellular fractionation could also bring valuable information in this respect.

Another biochemical approach to assess the localization of these proteins was performed in this thesis: the analysis of the glycosylation of the envelope proteins. In agreement with the colocalization studies, variant 5a HBsAg was endoH sensitive and hence remained within or before pre-medial Golgi compartments. In contrast, these proteins became partially endo-H resistant after disruption of the Golgi apparatus by treatment of cells with BfA. To achieve this, Golgi enzymes probably recycled to ER compartments by disruption of the Golgi tracks and fusion of Golgi vesicles back to ER (Kano, 2000; Sciaky et al., 1997).

It has been shown that the envelope proteins of secreted Wt HBV viral particles are endo-H resistant, suggesting that they pass through the Golgi compartments and are modified by Golgi enzymes to acquire this resistance. However, in contrast to reported by others (Hartmann-Stuhler and Prange, 2001), intracellular Wt surface proteins were neither colocalised with the Golgi-marker protein nor partially endoH resistant in my experiments. Therefore, we speculate that folded surface proteins in secretion-competent particles may

leave the cells rapidly in our experimental working conditions, and intracellular detection levels might therefore be limited to proteins in the folding process.

Due to the complete colocalization of PDI and the retained envelope proteins with mutations in essential Cys, we expected a possible functional interaction and retention of mutant envelope proteins by this chaperone and formation of aberrant oligomers as proposed by Mangold and colleagues (Mangold et al., 1995). In an effort to further characterize the nature of the retention, we searched a direct or a prolonged interaction between those proteins by immunoprecipitation assays (data not shown), without having success. Possible reasons may be that the protein interactions are too short-lived, retention might occur with other chaperones, or other experimental methods are necessary to detect this interaction, as it was shown for the Hsc70 interacting with the L-protein (Loffler-Mary, Werr, and Prange, 1997) or calnexin with the M-protein (Prange, Werr, and Loffler-Mary, 1999). The use of other experimental methods such as a novel ER like two-hybrid system (Nyfeler, Michnick, and Hauri, 2005) or protein cross-linking combined with immunoprecipitation assays could provide more information. An interaction of retained mutant envelope proteins with other ER chaperones remains to be studied; this applies for BiP binding to hydrophobic determinants exposed on the protein's surface and the GRP94 protein which has peptide binding activity (Ellgaard, Molinari, and Helenius, 1999).

The change in subcellular localization of variant 5a proteins could not be related to the mutation that mainly causes the secretion block, as secretion deficient WtR138 surface proteins showed the same localization pattern as Wt proteins. This result is in accordance with the idea that several protein conformations of the S-protein can pass the primary ER quality control and localize in PDI and non-PDI compartments (Huovila, Eder, and Fuller, 1992). In any case, WtR138 surface proteins could not leave late ER compartment, because these proteins were neither secreted nor localized in Golgi compartments. These results beg the question of how these proteins could be retained? Since Cys residues are responsible for disulfide-bridge formation, lack of Cys138 could block the protein oligomerization and viral particle aggregation necessary for the budding procedure, which might occur in late ER compartments. Houvila and colleagues (Huovila, Eder, and Fuller, 1992) have intensively studied this oligomerization process of HBV surface proteins in cell lines and could show that retention of Wt proteins with the Golgi disrupting drug BfA led to an accumulation of Wt envelope proteins in dimeric form. In contrast, the mutant Cys137/138/139Ala formed aberrant oligomers in the ER under the same conditions (Mangold et al., 1995). The authors suggested shuffling of disulfide-bonds as a cause for the aberrant oligomerization. This

implies that aberrant oligomers might have activated the ER quality control that recognize incorrect quaternary structure and promote retention (Ellgaard, Molinari, and Helenius, 1999). Whether the variant 5a surface proteins bearing the aa-exchange Cys138Arg form aberrant oligomers remains to be investigated, but it is probable.

Bock and colleagues suggested that in the absence of S-protein the L-protein localization could be determined by preS-sequences that urge particles to stay in an aberrant localization and be retained (Bock et al., 1999). In addition, L-protein retention could be related to prolonged folding processes mediated by the calnexin chaperone (Xu, Jensen, and Yen, 1997) and to pathogenesis. Indeed, after transfection with the HBV genome 5aC138, in which aa 138 of the a-determinant was reversed to Cys, the surface proteins showed a perinuclear-like distribution, although they were partially secreted. These proteins might still have difficulties, but once they have passed the ER quality control, they might form oligomers, and be secreted.

HBV particles containing altered a-determinant epitopes might be secreted to a certain extend as these mutants are found in the serum of patients and do not react with anti-HBs antibodies, therefore such variants constitute a risk of viral persistence.

4.3.3 Intracellular protein stability and degradation

Several hints point to the fact that surface proteins bearing a single aa-exchange in Cys 138 were not as stable as Wt S-proteins. First, functional analysis of these HBV constructs (WtR138 and WtA138) showed a reduction in intracellular L-protein levels. Surprisingly, the intracellular S-protein levels were slightly increased despite having 226 carboxy-terminal identical aas to the L-proteins. Secondly, reversion of the aa-exchange in the variant 5a Arg back to Cys (5aC138) re-established the L-protein levels intracellularly. Interestingly, the variant 5a L- protein levels were comparable to Wt, probably due to the effect of mutations in the promoter region before the preS-region in the variant genome, which might increase L-protein levels, as discussed in chapter 4.3.1. Briefly, mutations in the preS-constructs in Wt context induced an increase in intracellular steady-state levels of the expressed surface proteins. Thirdly, expression of the 5a L-protein without an S-counterpart led to extremely reduced 5a L-protein levels. This reduction was also observed by expression of the Wt L-protein without the Wt S-protein, but the reduction was not as pronounced as for the variant 5a L-protein expression. Vice versa, the expression of the S-protein alone showed no difference in intracellular levels compared to that of the full-length competent Wt HBV genome. After complementation of constructs expressing only L-protein with a HBV genome

expressing only the Wt S-surface protein and not Wt L-protein, both Wt and variant 5a L-protein levels were recovered. Taken together, these results suggest that the mutant L-protein is less stable than the mutant S-protein and compared to Wt envelope proteins. The S-protein may have a chaperone-like role for the L-protein.

In the eukaryotic cytosol, the 26S proteasome is responsible for degradation of 20% of newly synthesized cellular polypeptides (Garber, 2002). Proteasomes can be successfully inhibited irreversible or reversible by drugs, like lactacystin (Fenteany and Schreiber, 1998) or PS341 (Garber, 2002), respectively. Blocking of the proteasome with a reversible inhibitor resulted in an accumulation of variant 5a L-protein, suggesting that this protein is partially degraded by this machinery. A stronger 5a L-protein accumulation was observed by blocking the proteasome with the irreversible inhibitor lactacystin. These results could be explained by the fact that the inhibitors used target different subunits, and thus have different effects on the degradation. It could also be interesting to investigate the effect of overexpression of the HBx-protein on the degradation of the variant 5a L-protein, as the proteasome complex is reported to be a potential cellular target of this functionally undefined viral protein (Huang et al., 1996).

In contrast to the L-protein, the S-protein seems to be more stable as evident from the determination of the half-life of Wt and mutant S-proteins by pulse-chase experiments done during this thesis (data not shown). For comparison of the stability of secretion-competent and secretion-deficient envelope proteins, I blocked the constitutive secretion pathway with the Golgi-disrupting drug BfA. Therefore, all envelope proteins were thus retained. Under these conditions, the half-life of both variant 5a S-protein and Wt S-protein were too long (more than 12-hours) to be evaluated experimentally, because hepatoma cells did not tolerate prolonged treatment with BfA, and died probably by apoptosis (Guo et al., 1998). By using glycosidase transport inhibitors, Lu and colleagues found that HBV envelope proteins have a long half-life when retained intracellularly (Lu et al., 1997). The stability of envelope proteins might depend on the localization of the proteins and the stress status of the treated cells. Therefore, our preliminary experiments were not designed to get the ‘real’ half-life of the 5a S-protein located in this perinuclear compartment. Other experimental strategies, which might provide more information in future experiments, should include the use of HBV-Wt genomes expressing S-proteins with an ER or ERGIC retention signal. This would allow measuring the half-life of intracellularly retained HBV-Wt surface proteins without the need of secretion pathway-blocking drugs.

The lack of intracellular accumulation of the 5a S-protein despite the block in secretion as well as their long half-life raises the question whether translation or elongation of this protein may be slower than that of Wt proteins. These mechanisms are well conceivable and remain to be studied experimentally.

4.3.4 Secretion of HBV-DNA particles

Budding and secretion of HBV enveloped viral particles is mainly directed by the action of the S-protein. In addition, the release of virions needs specific interaction between HBcAg and surface proteins, mainly via the matrix function of the L-protein. These interactions have been intensively studied (Bruss and Ganem, 1991; Ostapchuk, Hearing, and Ganem, 1994; Prange and Streeck, 1995), but are still not well understood. The forces, that drive virion secretion, are supposed to be the same as those for SVPs, but there are still discrepancies to be sorted out (Xu and Yen, 1996).

Methodologically, difficulties occur when studying virion secretion in hepatocarcinoma cell lines due to the additional secretion or release of nucleocapsids without envelope (Lenhoff and Summers, 1994). The reason for this phenomenon is not known, but these particles are detected neither in patients' blood, not even in immunosuppressed patients, nor in cultured primary hepatocytes. Therefore, discrimination between both types of particles cannot be made by direct HBV-DNA detection methods, and requires separation methods prior to DNA analysis. In this thesis, two methods were used: native agarose gel electrophoresis and isopycnic CsCl gradient centrifugation. Both methods gave good resolution in the separation of virion from released nucleocapsids.

It is speculated that free capsids might be released by dead or lysed hepatocarcinoma cultured cells (Lenhoff and Summers, 1994), which may be enhanced under stress conditions (Xu, Bruss, and Yen, 1997). In our experiments, the ratio of Wt virions versus naked particles was about 5 to 1. However, release of naked nucleocapsids was increased at least three-fold after transfection of two HBV mutants bearing single mutations (WtR138 and WtA138), in which virion secretion was blocked because of a single mutation in the a-determinant. In these conditions, increased cell death in hepatoma cells transfected with this constructs was not observed. These data argue that hepatoma cells in addition to the unspecific release caused by cell death could specifically release naked particles, but further experiments in this direction remain to be done to clarify this point.

By investigating the variant 5a genome isolated from a patient suffering FHB, aa-exchanges in the a-determinant were associated with a strong decrease in SVPs secretion and

incompetence in secretion of virions. However, these aa-exchanges of the variant 5a surface proteins still allowed proper nucleocapsids formation, as variant 5a naked nucleocapsids presented similar properties as those released by Wt-transfected hepatoma cells. This is in accordance with the observation that nucleocapsid formation is not affected by mutations in the surface proteins. These intracellularly formed nucleocapsids were successfully enveloped and partially secreted only by the reversion of the Arg to Cys in position 138 of the variant envelope proteins pointing to an important function of this aa for the virion assembly process. Moreover, the physical properties of 5aC138 secreted virions were also similar to those secreted by Wt-transfected cells. Electron microscopy studies could give more information about the morphology and ratio of virions and their associated SVPs for these mutants. This experiments may provide interesting data because viral genome 5aC138 secretes more L- than S-protein, which may influence the shape of SVPs and virion compared to Wt, similar as reported by Xu and colleagues who have analysed another mutant HBV genome (Xu and Yen, 1996). Such studies may also reveal whether variant 5a envelope proteins are able to bud and form particles in the ER or ERGIC.

A complete virion secretion block was observed for the HBV mutants bearing an exchange of Cys138 with either Arg or Ala (WtR138 and WtA138, respectively), although the reduction of SVPs secretion was only of 10% and 30%, respectively. This fact suggests that the block in virion secretion might not only be caused by retention of the envelope protein, but also by a specific deficit in virion assembly. Originally, preS1-peptides were found to directly interact with HBcAg and promote virion release, but other evidences argue that virion formation might be also influenced by S-peptides sequences (Poisson et al., 1997; Tan, Dyson, and Murray, 1999). A single aa (Arg92) in the first hydrophilic loop of the S-protein is essential for virion nucleocapsid interaction and secretion. This loop is postulated to face the cytoplasm after insertion of envelope proteins into ER membranes and therefore may be adequate for mediating this kind of interactions. Could also Cys138 be implicated in nucleocapsid interactions? Elegant experiments have suggested that surface protein topology might be more complex than expected for the second hydrophilic loop of the S-protein where Cys138 is located: the partial glycosylation of residue Asn146 and the partial protease digestion of the corresponding hydrophilic loop (Prange and Streeck, 1995) strongly suggest a dual topology of this loop. In fact, reversion of aa 138 to Cys in the variant 5a genome (5aC138) led to a change in the ratio of the glycosylation pattern of its surface proteins suggesting an extended arrest of the second hydrophilic loop in a cytoplasmic conformation. A possible reason for reduction of the glycosylation rate of the surface proteins of this

genome could be to promote surface protein interactions for virion formation. Whether Cys138 is implicated in this process remains to be proved and additional protease assays should be done to confirm the differences in the S-domain topology. In addition, HBV mutants with specific virion retention signals could be the targets for the design of antiviral peptides for therapy.

Taken everything together, a single aa-exchange Cys138Arg in the S-region of the HBV variant 5a is the major cause of viral particle retention. Phenotypically, this mutation did not lead to an intracellular envelope protein accumulation, but it caused a localization of these proteins in abnormal cellular compartments, and in addition led to increased sensitivity of the variant 5a L-protein towards proteasome degradation.

4.4 Variant phenotypes related to pathogenesis and prevalence

The pathology of hepatitis with fulminant-stage is defined by the sudden onset of severe liver injury accompanied by hepatic encephalopathy in an individual who previously had no evidence of liver disease. Disturbances in cerebral consciousness vary from mild decreases (in grades I and II) to severe stadium characterized by stupor, dementia or coma. However, a major complication of fulminant hepatitis failure is a cerebral oedema, which is the leading cause of death. Other major complications related to this failure include: hypoglycaemia (impaired gluconeogenesis), disturbances in acid-base balance (respiratory alkalosis and metabolic acidosis, as a result of increased lactate production), severe coagulopathy (decreased synthesis of clotting factors II, V, VII and IX and disseminated intravascular coagulation arising from liver cell necrosis) compromised immunological competence (decreased complement synthesis, leukocyte dysfunction) and development of bacterial and fungal infections which can cause as well mortality. In addition, other organ systems are affected with high incidence of renal, pulmonary and cardiac dysfunction and often succumb to renal failure secondary to the hepatorenal syndrome. Therefore, most of the patients with fulminant hepatitis failure require liver transplantation. This was the case for patient 5 who suffered hepatitis because of HBV infection. After seroconversion to anti-HBe, the patient suffered a HBV remission and FHB (Sterneck et al., 1996). During the course of the FHB, HBV variants 5a and 5c were isolated from the serum (Sterneck et al., 1998). The patient survived thanks to a liver transplantation.

The most frequent cause of fulminant hepatitis is the hepatitis B virus but other hepatic and extrahepatic viruses can induce this disease. Since HBV is a noncytopatic virus (Guidotti et al., 1999), it is unclear how it can induce such a severe stage. In general, HBV S-gene

mutations are rarely associated with FHB (Gunther et al., 1999), but studies with animal models and some clinical cases support the idea that variants with similar phenotype as variant 5a could play a role.

First, an indirect evidence is that some patients infected with HBV variants bearing an exchange in Cys138 or Gly130 of the small HBV envelope proteins were reported to suffer severe hepatitis, but these variant genomes contained in addition other mutations elsewhere in their genomes. These exchanges could so far not be related to outbreak of FHB, unlike it has been described for other HBV variants related to the same pathology (Hasegawa et al., 1994; Liang et al., 1991; Petrosillo et al., 2000; Tanaka et al., 1995).

Secondly, variant genomes isolated from patient 5 in the fulminant-stage could not express and secrete HBeAg, due to a preC-stop codon (T1896A), and two mutations in the C-promoter (A1762T/G1764A). These mutations were related to variants isolated from patients suffering from FHB (Gunther et al., 1999). However, these mutations seem to be insufficient to have caused the fulminant course of the infection (Sterneck et al., 1996). Nevertheless, HBeAg seems to be a modulator of the host response to viral infection and its absence may play a role. HBeAg depletion might increase the aggressive character of CTLs (Blum, 1993; Miska and Will, 1993), and seems to be associated with an increased viral persistence, possibly mediated by escape of the virus from host immune recognition.

Third, aberrantly localized envelope proteins have been related to pathogenesis. In transgenic mice, intracellular retention of L-protein was found to render hepatocytes more susceptible to cell death induced by cytokines, such as interferon-gamma and tumour necrosis-alpha, and to represent a factor that promotes development of severe or FHB in these animals (Chisari, 1996). In this context, it is interesting to note that the intracellularly retained envelope proteins of variant 5a presented a similar localization than particles formed by expression of Wt L-protein without its S-counterpart analysed in previous studies (Xu, Bruss, and Yen, 1997). Such particles are similar to those found within ground glass cells in the liver of human and transgenic mice (Xu, Bruss, and Yen, 1997; Chisari, 1996). However, the cell morphology of hepatoma cells transfected with HBV Wt and variants genomes was followed both by light microscopy and immunofluorescence staining. Neither Wt- nor variant-transfected cells showed ground glass cell formation, apoptotic signs (like nuclear DNA condensation or preapoptotic body formation) or excessive vacuole formation. However, the transfected cells were only investigated for short periods of time with a maximum of eight days and pathogenic effects may become obvious only after longer observation periods.

Additional hints towards pathogenic effects of variants derive from the functional study of a HBV variant related to an outbreak of FHB in tupaia primary hepatocytes (Baumert et al., 2005). This variant presented in this animal model reduced amounts of viral particle secretion, no intracellular accumulation of surface proteins and induction of apoptosis. Interestingly, the viral phenotype related to FHB in these studies was dependent on the variant envelope proteins and not related to enhanced viral replication (Baumert et al., 2005). Reduction in viral particle secretion and apoptosis are phenotypes that could lead to fatal fulminant-stage. This animal model could be therefore suitable for further functional studies that relate variant 5a phenotype with outcome of the infection.

An increased intracellular accumulation of the variant 5a L-protein was observed after proteasome-inhibition, which argues for an increased degradation of the 5a L-protein. Evidences have point to a critical role of this complex in the protein degradation and delivery of peptides to the major histocompatibility complex class I molecules (York and Rock, 1996). This observation might be relevant as a strong activation of the immune system might be the major cause of liver destruction in FHB. The highly immunogenic a-determinant in the S-region of all HBV surface proteins is the main target for neutralizing antibodies (Peterson, 1987). But the L-protein has highly immunogenic preS1- and preS2-sequences (Maeng et al., 2000; Milich et al., 1986), which seem to be masked by glycosylation in the M-protein. Whether the increase in 5a L-protein degradation really leads to an increase in peptide presentation and hepatopathology could be investigated by genetic immunization of mice with plasmids expressing Wt or variant 5a envelope proteins.

Transcomplementation studies performed with secretion deficient variant 5a and HBV genomes expressing all viral proteins except the L- and M-protein (chapter 3.3.3) contribute interestingly to the dynamics of HBV-infection with a mixture in viral population and hints to a mechanism how secretion incompetent variants can prevail in HBV infected patients. Secretion rescue of the incompetent variant genomes 5a was estimated by the presence of mutant 5a L-protein in the serum of transfected cells; however it remain to be investigated whether the variant 5a viral genome was also secreted with the same efficiency. Interestingly, secretion of the variant 5a L-protein was already possible with a low ratio of variant 5a genome versus the HBV genome with the deficit in L- and M-proteins, therefore expressing only the S-envelope protein. This suggests that small amounts of secretion-efficient Wt S-protein may be enough to drag the variant 5a L-protein into mixed particles for secretion.

In contrast to the S-protein, which is mainly important for immunity and secretion of viral particles, L-protein is known to be important in several steps of the HBV viral life cycle.

This protein acts as a matrix-like protein for virion formation (Tan, Dyson, and Murray, 1999) and has at least two determinants and a protein modification that are involved in virion attachment to the host cell and internalisation. Therefore, efficient secretion of the 5a L-protein might favour persistence of this HBV variant genome in case HBV-DNA containing viral particles for this genome are secreted an as efficient as the variant 5a L-envelope protein. This fact remains still to be investigated but it is probable. The lack of enhanced replication of the variant 5a genome could be one explanation why this genome is only a minor population (about 30%) in the serum of the patient. However, the presence of HBV variant genomes in the liver of the specific patient with a Cys in position 138, and therefore secretion competent HBV genomes, supports the transcomplementation mechanism that might allow persistence of secretion-incompetent variant 5a genomes. Transcomplementation experiments with secretion competent variants (variant 5c) should be considered for further meaningful experiments. Unfortunately, infectivity of variant 5a could not be tested in the time-frame of this work.

To summarize, transient transfection of full-length HBV genomes allowed the functional characterization of several viral phenotypes that may play a role in FHB. Due to technical limitations of the *in vitro* system used, the direct cytopathic effects of the variant 5a could be further investigated with the help of animal models such as transgenic mice (Chisari, 1996), mice transplanted with human hepatocytes (Dandri et al., 2001) or with the help of the tupaia model (Kock et al., 2001). In addition, the variant L-protein could be secreted by transcomplementation with a secretion efficient S-protein. This results hints to a mechanism for the persistence of secretion incompetent variant genomes in HBV infected patients.

4.6 Perspectives

Effective vaccines against HBV infection have been in use for some years, and more recently nucleoside analogues able to inhibit HBV replication have become available. Despite these advances, the global control of the infection is still a future goal for the future and the appearance of cytotoxic variants complicate new trials. In this respect, it is important to increase the understanding of the biology and the clinical impact of mutations in the HBV genome.

Although the liver damage caused by most HBV mutants is believed to depend primarily on the level of HBV replication, the role of mutant surface proteins in hepatopathogenesis is well documented both for HBV and the duck hepatitis B virus. Whether the block of viral particle secretion by specific drugs or the use of dominant negative proteins are potential strategies for novel antiviral therapies remains to be investigated. Such studies

need to take into account the consequences of accumulation and further processing of intracellularly retained viral surface proteins, which may enhance liver inflammation and even enhance development of liver cancer. One challenge for the future in understanding the role of mutant envelope proteins is to define further the quality control of the HBV surface proteins, to pursue the analysis of alternative, proteasome-independent mechanisms of ER-mediated degradation system targeting these proteins. The capacity to modulate folding, quality control and protein degradation in HBV infected cells may lead to novel treatments, which hopefully prevent the frequent lethal outcome of FHB or other severe courses of hepatitis infection.

5. Material and Methods

5.1 Material

5.1.1 Patient

Virus from a patient, designated patient 5, who suffered from FHB was previously investigated by Sterneck et al (Sterneck et al., 1996). Briefly, infection of the patient 5 with HBV led to a severe form of hepatitis, so-called FHB; this patient was negative both for HCV and hepatitis delta virus. HBsAg and anti-HBc IgM were positive during the fulminant course of the illness. On the other hand, the serum was negative for HBe as well as anti-HBe. Histology of the tissues showed low or no presence of HBcAg. The patient underwent liver transplantation surviving with conservative management.

The sequence of the dominant HBV population of the serum during the course of the FHB was analysed after DNA isolation and PCR amplification by direct sequencing (Sterneck et al., 1996). Sequences were always compared to that of Wt. In the dominant HBV population of the serum of the patient a stop codon was observed, being probably the cause of the absence of HBe antigen in the blood. In addition an A-to-T substitution in position 1762 and a G-to-A substitution in the position 1764 were observed. In the a-determinant the aa-exchanges T125, P127 and, E138 were observed.

Sequences of three cloned HBV genomes, designated 5a, 5b and 5c, selected from DNA purified and cloned from the serum of patient 5 were also previously analysed (Sterneck et al., 1998). Clones 5a and 5b bear additional mutations in the a-determinant of the S-protein in position T125, P127 and E130, as well as R138 was observed.

5.1.2 Liver and serum samples

- Paraffin embedded liver biopsies from patient 5 suffering FHB (Sterneck et al., 1996), from second patient infected with HBV (used as a positive control), and from a non-infected patient with HBV (used as a negative control) were kindly provided by Dr. M. Sterneck (Medizinische und chirurgische Abteilung am Universitätskrankenhaus Eppendorf, Hamburg).

- Serum from a third HBV-infected patient with high titer (used as a HBV PCR positive control) was also provided from Dr. M. Sterneck.

5.1.3 Enzymes

Restriction endonucleases

Digestions with restriction enzymes were performed according to the protocols of the supplier:

- AvrII, BsrGI, BspEI, RsrII, BbsI, Sap I and SstI — New England Biolabs
- Buffer II and buffer III — New England Biolabs.

Other enzymes

Enzymatic treatments were performed according to the protocols of the supplier:

- Endoglycosidase-H (endoH) — New England Biolabs

  Briefly, proteins were treated with denaturing buffer (0.5% SDS, 1% ß-mercaptoethanol) for 10 minutes at 100°C. Then, 0.05M sodium citrate and 1U (enzymatic units of reaction) of endoH were added to the sample and incubated overnight at 37°C (Robbins et al., 1984). Mock reactions were run in parallel without addition of enzyme.
- PeptidoglycanaseF (PNGaseF) — New England Biolabs

  Briefly, proteins were treated with denaturing buffer (0.5% SDS, 1% ß-mercaptoethanol) for 10 minutes at 100°C. Then, 0.05 sodium phosphate, 1% NONIDET P-40 and 1U of PNGaseF were

added overnight at 37°C. Mock reactions were run in parallel without added enzyme (Maley et al., 1989).

- Expand™ High Fidelity — Boehringer Mannheim
  For PCR amplification with Hifi-DNA polymerase (*Taq* and *Pwo* DNA polymerase mixture), elongation steps were performed at 68°C. The other steps of the PCR were performed as described in the manufacture's protocols.
- *Taq*-DNA polymerase — Boehringer Mannheim
  For PCR amplification with Taq-DNA polymerase, elongation steps were performed at 72°C. The other steps of the PCR were performed according to the protocol described above.
- Proteinase K (20mg/ml) — Boehringer Mannheim
- Dnase I (10ng/ml) — Roche Biochemicals
- DNase I, RNase free (10ng/ml) — Roche Biochemicals.

5.1.4 Antibodies

Antibody dilutions were used as recommended by the supplier:

- Abbott AxSYM HBsAg (for ELISA) — Abbott Laboratories
- Abbott AxSYM HbeAg (for ELISA) — Abbott Laboratories
- Polyclonal goat anti-S antiserum — DAKO Corporation
  This antiserum was used for immunoprecipitation (dilution 1:100) and for immunofluorescence analysis (dilution 1:250).
- Polyclonal anti-HBc antiserum — DAKO Corporation
  This antiserum was used for detection of HBcAg and HBeAg in Western blot analysis (dilution 1:1000).
- MAb 18/07 (kindly provided by Dr. Heermann and Gerlich) — Göttingen University
  For the anti-pre-S1 antibody MAb 18/7, the minimal epitope proved to be the hexamer LDPAFR (aa30-35 of the pre-S region) (Kuttner et al., 1999). It was used for Western blot analysis (dilution 1:8000) and immunofluorescence staining (dilution 1:300).
- MAb H166 (not commercially available, but kindly provided) — Abbott Laboratories
  The MAb recognizes C121-C124 of all HBV surface proteins (Mimms et al., 1990), but the S-protein is detected with higher efficiency in Western blot (dilution 1:2000-1:16000), than when used for immunofluorescence staining (dilution 1:250).
- Peroxidase-conjugated antibodies — Dianova GmbH
  The secondary antibody was anti-rabbit and anti-mouse (dilution 1:50000).
- Mouse anti-PDI monoclonal antibody — StressGen
  This antibody recognizes the human PDI protein.
- Monoclonal anti-Golgi (58 kDa protein) — StressGen
  This is a primary antibody that recognizes a Golgi-resident protein, which has a molecular weight of 58 kDa.
- Alexa Fluor® 594 goat anti-mouse IgG — Molecular Probes, Inc
  This is a secondary antibody for immunofluorescence staining coupled to a fluorochrom, emitting green fluorescence.
- Rhodamine Red™-X-conjugated Rabbit anti-goat — Dionova GmbH
  This is a secondary antibody for immunofluorescence staining coupled to a fluorochrom, emitting red fluorescence.
- Anti-Digoxigenin-AP (alkaline phosphatase), Fab-Fragments — Roche Biochemicals
  This antibody recognizes digoxigenin-labeled nts, and was used in Southern blot and Northern blot analyses.

5.1.5 Kits

Kits were used according to the protocols of the supplier:

- ABI PRISM Dye Terminator Cycle Sequencing Kit — Perkin Elmer

- DNA ligation kit — Roche Biochemicals
- QIAamp^{R}DNA mini kit — Qiagen GmbH
- Qiagen gel extraction kit — Qiagen GmbH
- Qiagen Plasmid Maxi Kit — Qiagen GmbH
- Gel drying kit — Promega
- Qiashredder^{R} spin column — Qiagen GmbH
- RNeasy Mini kit — Qiagen GmbH
- Dig RNA Labeling Mix — Roche Biochemicals.

5.1.6 Oligonucleotides

- PCR amplification and sequencing:

Name	HBV nt positions	Sequence
+67	67 to 90$_{(2)\,(3)}$	5'-CTCCAGTTCAGGAACAGTAAACCC-3'
+191	191 to 216$_{(4)}$	5'-CTCGTGGTTACAGGCGGGGTTTTTCTT-3'
+301	301-326$_{(4)}$	5'-TGGCCAAAATTCGCAGTCCCCAACCT-3'
+368	368-390$_{(4)}$	5'-TATCGCTGGATGTGTCTGCGGCG-3'
+408	408 to 432$_{(2)}$	5'-CATCCTGCTGCTATGCCTCATCTT-3'
+2090	2090 to 2118$_{(1)}$	5'-TCTAGCTACCTGGGTGGG-3'
+2812	2802 to 2832$_{(1)\,(3)}$	5'-GCCTCATTTTGTGGGTCACCATATTCTTGGG-3'
-477	477 to 454 $_{(2)}$	5'-GTTCCATACAACGGGCAAACAGGAGA-3'
-700	698 to 668$_{(4)}$	5'-CCGAGTCAAATGATCACGGTAAACAAGTCA-3'
-1164	1164 to 1137$_{(2)\,(3)}$	5'-GCCAGGTCTGTGCCAAGTGTTTGCT-3'
+1822	1821-1841$_{(5)}$	5'-TTTTTCACCTCTGCCTAATCA-3'
-1822	1825 to 1806$_{(5)}$	5'-AAAAAGTTGCATGGTGCTGG-3'

$_{(1)}$Sequencing of the C/PreS region, $_{(2)}$Sequencing of the S region, $_{(3)}$Mutagenesis, $_{(4)}$Primers for PCR $_{(5)}$Primers for full-length PCR amplification

- Site-directed mutagenesis:

Name	Mutations	Sequence
+528*	+528C/533C*	5'-CACACAGAAGACGCACGACTCAAGGAACCT-'3
-528*	-528C/533C*	5'-CACACAGAAGACAGCGTGCAGGTCCGGCATGGTCCCGT-'3
+566*	+566G/567C*	5'-CACACAGAAGACTGTGCCTGTACCAAACCTTCGGACGG-'3
-566*	-566G/567C*	5'-CACACAGAAGACCAGGCACAGGAGGGATACATAGAGGT-'3
+156*	+156C/156A*	5'-CACACAGAAGACAACCAGGAGAACATCACATCAGGATT-3'
-156*	-156C/156A*	5'-CACACAGAAGACTCCTGGTTCAGCGCAGGGTCCCCATCCT-3'

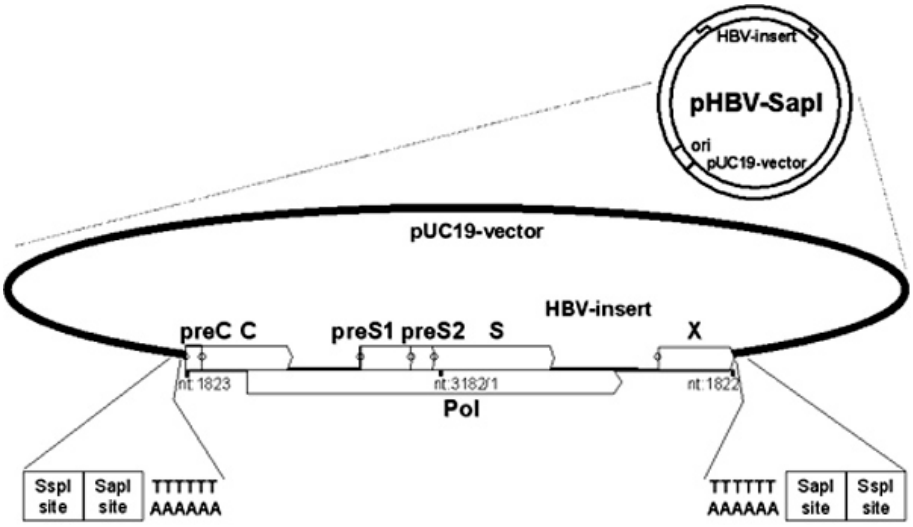
* BbsI restriction site present in the oligonucleotides is underlined.

5.1.7 Plasmids

- Plasmids pUC19-HBV-SapI monomers:

A set of pHBV-SapI plasmids (5.8-kb) (Gunther et al., 1995b) is represented in the figure above, which contain a HBV full-length genome and two identical flanking non-HBV homologous regions (depicted below the plasmid figure) cloned into the SstI restriction site of the polylinker region of the carrier plasmid pUC19 (Yanisch-Perron, Vieira, and Messing, 1985). The HBV-genome is cloned

starting from the preC-region (nt1822) until the X-gene (nt1822) between the T:A repeat sequences. The cloning vector contains also Ampicillin resistance gene (Amp^R, ß-Lactamase) as a selection marker for transformed cells.



1) The plasmid pWt-SapI, designated as Wild-type (Wt), contains the HBV full-length genotype D, ayw3, genome amplified from pSM2 plasmid. Wt is here defined as virus, which is characteristic for the immune tolerance phase and lack mutations emerging during the course of acute or chronic infection.
2) The plasmids p5a-SapI and p5c-SapI, designated as 5a and 5c respectively, contain the HBV full-length genome of the variants isolated from the serum of a patient, designated as 5, suffering from FHB (Sterneck et al., 1998).
3) The plasmid p5aC138-SapI, designated as 5aC138, contains the HBV full-length variant 5a with a reverse mutation that leads to Cys in position 138 of the S-gene. The plasmid pWtR138-SapI, designated as WtR138, containing a mutation in the S-gene of the HBV Wt full-length genome, leads to expression of Arg instead of Cys in aa position 138. These plasmids were kindly provided from Dr. Kalinina (Heinrich-Pette Instituts, Hamburg).

- Plasmid SEAP (Cullen and Malim, 1992):

The vector contains a gene that codes for an alkaline-phosphatase under the control of a CMV-promoter, which is secreted into the medium of transfected cells. The plasmid also contains the Ampicillin resistance gene (Amp^R, ß-Lactamase) as a selection marker for transformed bacterial cells.

- Plasmid pSM2:

A vector pMa5-8 containing a head-to-tail HBV dimer of subtype ayw3 (Galibert et al., 1979; Will et al., 1985).

- Plasmid pBSK-c/x:

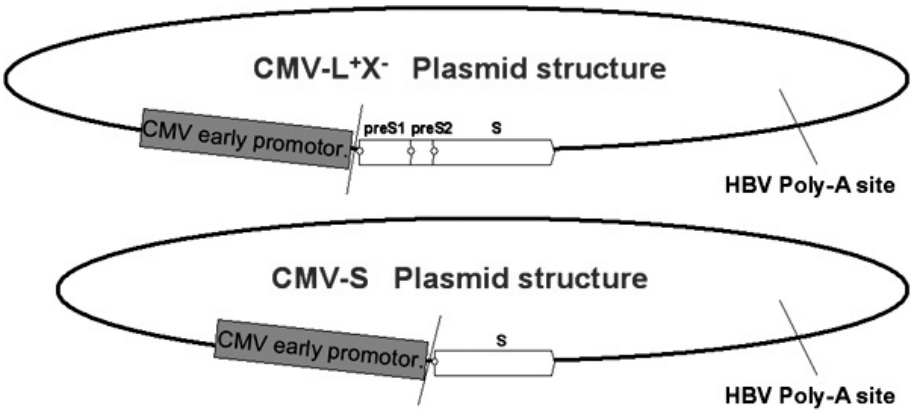
A plasmid containing the HBV sequence encompassing the C- and X-ORF under the control of the T3 promoter (Sommer, 1997). This vector was used for transcription of the RNA-probe.

- Plasmid p14:

A Wt HBV construct (6,338 kb) in which the preS1 initiation codon was mutated to ACG, and in which the third codon in the preS2 region was switched to the stop-codon TGA. This plasmid was kindly provided from Dr. Kalinina (Heinrich-Pette Instituts, Hamburg)

- CMV-plasmids:

Two sets of plasmids that express the HBV surface proteins under the early CMV-promoter. First, CMV-L^+X^-/constructs were build from the pCMV-$LM^-S^-X^-$ (Xu, Jensen, and Yen, 1997). The CMV-S/constructs were made from the pCMV-S construct (Xu, Jensen, and Yen, 1997).



5.1.8 Cell culture media and antibiotics

Medium for bacteria (LB-medium)

10g	Bactotryptone	Gibco BRL
5g	Yeast extract	Gibco BRL
10g	NaCl	Sigma Chemie GmbH

H_2O to 1000ml, pH7.0, autoclave

Ampicillin (100mg/l end concentration) Gibco BRL.

Medium for hepatoma cell lines

Dulbecco's Modified Eagle Medium Gibco BRL

Additionally, the medium was supplemented with:

10%	fetal calf serum	Gibco BRL
2mM	L-glutamine	Gibco BRL
3.5mM	L-arginine	Sigma Chemie GmbH
1mM	pyruvate	Gibco BRL
100IU	penicillin	Gibco BRL
100IU	streptomycin	Gibco BRL
10ml/l	nonessential aas	Gibco BRL.

Media for hepatoma cell lines (pulse experiments)

Met-free Dulbecco's Modified Eagle Medium Gibco BRL

Additionally, the medium was supplemented with:

2mM	L-glutamine	Gibco BRL
3.5mM	L-arginine	Sigma Chemie GmbH
1mM	pyruvate	Gibco BRL
100IU	penicillin	Gibco BRL
100IU	streptomycin	Gibco BRL.

5.1.9 Bacterial strain and cell lines

- *E. coli* (*Escherichia coli*) DH5α (Hanahan, 1983):
  *Sup*E44, Δ(*lac*ZYA-*arg*F)U169, Φ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1, *hsd*R17(r_k^-, m_k^+), *deo*R

- Huh-7 cell line (Nakabayashi et al., 1982):
  This cell line originates from a hepatoblastoma of a 57 year-old man, and was established in 1981.

5.1.10 Buffers and solutions

Buffer for cell extracts

- **DNA lysis buffer** (Southern blot) 50mM Tris pH=7.8, 1mM EDTA ph=8.0 and 1% NONIDET^{R} P-40
- **5x loading buffer** (agarose gel electrophoresis) 1mg/ml bromphenol blue, 1mg/ml xylencyanol, 2mg/ml orange G and 80% glycerol)
- **Lysis buffer for immunoprecipitation** (pulse experiment) 50mM Tris-Cl (ph=7.5), 150mM NaCl, 1% Nonidet^{R}P-40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete^{TM} protease inhibitor
- **Protein lysis buffer** (Western blot) 100mM Tris-Cl pH=7.8, 100mM NaCl, 10mM EDTA acid, 0.5% Nonidet^{R}P-40 and Complete^{TM} protease inhibitor
- **2x SDS-loading buffer** (SDS-PAGE) 100mM Tris-Cl (pH=6.8), 0.2M dithiothreitol, 4% SDS, 0.2% bromphenol-blue, and 20% glycerol.

Solutions and buffers

- **EDTA** (block of enzymatic reactions by arresting binary ions in complexes) 0.5M disodium ethylenediaminetetraacetic (pH=8 with NaOH)
- **PBS buffer** 140mM NaCl, 8mM Na_2HPO_4, and 2mM NaH_2PO_4
- **PBS-EDTA buffer** PBS buffer and 10mM EDTA
- **TE buffer** 10mM Tris-Cl and 1mM EDTA, pH=7.4
- **SEAP-buffer** 1mM $MgCl_2$, 2M diethanolamin, and 20mM L-homoarginin
- **Cracking buffer** 50mM Tris-Cl (pH=6.8), 1% SDS, 2mM EDTA, 400mM sucrose, 0.01% bromophenol blue, 0.01% xylencyanol FF, 0.01% orange G, and 100µg/ml RNAse A
- **TAE buffer** 40mM Tris-acetate, and 1mM EDTA
- **10xTNE buffer** 10mM Tris-Cl, 1mM EDTA and 1.5M NaCl
- **TBS buffer** 50mM Tris-Cl and 150mM NaCl (pH=7.5 with HCl)
- **TG buffer** 25mM Tris, 250 mM Glycin (pH=8.3) and 0.1% SDS
- **TGE buffer** 25mM Tris, 192mM Glycin (pH=8.4) and 20% ethanol
- **20x SSC** 3M NaCl and 0.3M Na-Citrate (pH=7.0) autoclave.

5.1.11 Chemicals and other general materials

• Acrylamid 30% solution	ICN
• Aqua-Phenol	Appligene-Oncor
• Aqua ad in iectabilia (H_2O-bidestillated)	Braun
• Amplify solution	Amersham Biotech
• Bacto-agar	ICN
• Agarose	Sigma Chemie GmbH
• Agarose Type I Low EEO	Sigma Chemie GmbH
• Ammonium persulfate	Merck
• Blocking reagent	Boehringer Mannheim
• Bisacrylamide	ICN
• Brefeldin A	Sigma Chemie GmbH
• BenchMark^{TM} Prestained Protein Lader Protein molecular weights kDa: 187, 118, 85, 61, 50, 38, 26, 20, 15, and 9.	Life Technologies
• Bromphenol blue	Merck
• Cell culture plates and flasks	Falcon
• Cell culture well-multiplates	Greiner
• Chloroform	Merck
• Complete^{TM} protease inhibitor	Roche Biochemicals
• CDP-Star	Roche Biochemicals
• 2'-Deoxyribonucleotide triphosphate, Lithium-salt	Roche Biochemicals
• 4',6'-diamidino-2-phenylindole (DAPI)	Roche Biochemicals
• Dig Easy Hyb (Northern blot)	Roche Biochemicals
• Diethylpolycarbonat	Sigma Chemie GmbH
• Dimethylsulfoxid	Merck

- DNA molecular weight marker **III**: Roche Biochemicals
  Number of bps of the bands: 21226, 5148, 4268, 3530, 2027, 1907, 1884, 1375, 947, 831, and 567.
- Ethanol Merck
- Ethidium bromide Roche Biochemicals
- Ethylendiaminotetraacetic acid (EDTA) Merck
- Formaldehyde (37%, acid free) Merck
- Formamide Merck
- FuGENE^{TM} 6 Transfection Reagent Roche Biochemicals
- Glicerol Serva GmbH & Co
- Goat serum Sigma Chemie GmbH
- Glycerol Serva GmbH & Co
- Herring sperm-DNA Promega
- Hybond-N Transfer Membran Amersham Biotech
- Low Molecular Calibration kit for SDS electrophoresis Amersham Biotech
  Protein molecular weights in kDa: 95, 65 (albumin), 45, 30, 20, and 12.
- Morphdinopropansulfonsäure (MOPS) Sigma Chemie GmbH
- Nonidet^{R}P-40 Sigma Chemie GmbH
- N,N,N,N'-Tetraethylendiamine (TEMED) Serva GmbH & Co
- Nitrocellulose membrane Schleicher&Schuell
- Ponceau S solution Serva GmbH & Co
- Polyethylenglycol 8000MG (PEG) Sigma Chemie GmbH
- Protein G-Sepharose Pharmacia Biotech
- P-Nitrophenol-Phosphate (x $6H_2O$) Roche Biochemicals
- Robocycler Stratagene
- (^{35}S)Met-label (specific activity >37 TBq/mmol) Hartmann Analytic
- Sodium dodecylsulfate (SDS) Serva GmbH & Co
- SuperSignal^{R} West Dura Chemiluminescent Substrate Pierce
- RNA molecular weight marker I Roche Biochemicals
- tRNA Roche Biochemicals
- Tween 20 Serva GmbH & Co
- Tris-(hydroxylmethyl)-aminomethane (Tris) ICN
- Whatmann chromatography paper Whatmann Ltd.
- X-ray film Fuji RX GCU FUJI

All other chemicals were obtained from the following suppliers:

- Merck
- Riedel de Haen
- Roche Biochemicals
- Serva Feinchemikalien GmbH & Co
- Sigma Chemie GmbH

5.1.12 Computer programs

- Corel Photopaint 5.0 Corel Corporation
- Corel Draw 6.0. Corel Corporation
- Photoshop 7.0 Adove
- Word 6.0 Microsoft
- Excel 4.0 Microsoft
- Endnote 4 ISI ResearchSoft
- Mac Vector 6.0.1 Oxford-Mol-Group
- TINA 2.09 Raytest
- Bas reader 2.0 Raytest

5.2 Methods

5.2.1 DNA-preparation from liver tissue (Chen 1991, modified)

Extraction of DNA from paraffin embedded tissue

Three paraffin embedded tissues were available: A single biopsy of a patient, patient 5 (Sterneck et al., 1996), which was obtained during the course of the FHB stage, was investigated. Additionally, a biopsy section HBV-positive and an HBV-negative tissues were studied.

Tissues were dewaxed and total-DNA was extracted and purified with a tissue DNA purification kit. Briefly, approximately 25mg was dewaxed with 1200µl xylene, and cleaned with 1200µl ethanol absolute at least three-times. These conditions were used for optimal DNA extraction with a tissue DNA purification kit. The pellet containing the tissue was dried 15 minutes at 37°C until ethanol evaporated. The tissue was further solved in 180µl of buffer from the tissue DNA purification kit and digested with proteinase K over night at 37°C. The DNA was isolated with the mini column of the kit and solved in 20µl of elution buffer, which were used directly for PCR amplification.

5.2.2 DNA-plasmid preparation from *E.coli* (Hannahan 1983, modified)

Production of competent bacteria

E.coli DH5α bacteria were treated to obtain competent bacteria for transformation of plasmid DNA. A tip-point from a glycerol-stock of non-competent bacteria was grown overnight with 5ml LB medium; an aliquot of 1ml was passaged in 100ml new fresh LB medium with 4ml $MgCl_2$, grown for 2-3 hours at 37°C and 300rpm until OD_{550} reached 0.45-0.55. The bacteria were incubated in ice for 10 minutes, peleted by centrifugation with 3000rpm in a SS34 rotor at 4°C for 10 minutes. The cells were equilibrated in 30-40ml ice-cold FSB buffer (10mM KAc, 45mM $MnCl_2$ x $4H_2O$, 100mM KCl, 10mM $CaCl_2$ x $2H_2O$, 3mM hexamine cobalt III chloride and 10% glycerol). Cells were centrifuged with 3000rpm in a SS34 rotor at 4°C for 10 minutes and resuspended in 8ml of FSB buffer; 560µl DMSO were then added and incubated in ice for further 15 minutes. Again 560µl of DMSO were added, incubated in ice for 15 minutes, and aliquots were frozen in liquid nitrogen. The competence of the cells prepared according to this protocol was about $5x10^7$ clones/µg supercoiled DNA as tested by transformation.

Bacteria agar dishes and cultures

Bacteria were grown both in agar-dishes and glass bacteria cultures overnight in an incubator at 37°C. The agar dishes were prepared by boiling 2.5% LB medium with 1.5% bacto-agar (eventually Ampicillin was added at about 40°C), cooled down over night at room temperature, and stored at 4°C.

Selection of transformed bacteria was achieved by expression of the ß-lactamase gene resulting in Ampicillin resistance.

Transformation

This technique allows the introduction of plasmid-DNA into competent bacteria for isolation of single clones and plasmid amplification.

Briefly, an aliquot of *E. coli* DH5α competent bacteria (about 100µl) was thaw for 15 minutes on ice. Plasmid-DNA or ligation products were then added and the mixture was incubated on ice for further 30 minutes for absorption of the plasmid-DNA to the bacteria. Internalisation of the plasmid-DNA into the bacteria was achieved by passing the cells from ice to 42°C for 90 minutes, after this heat shock the bacteria were cooled down again on ice for 2 minutes. Then 0.9ml LB-medium (without antibiotics) was added, cells were grown for 1 hour at 37°C with 200 rpm. Finally, the culture was passed to a big culture flask or split onto agar-dishes (with antibiotics for colony-selection) and grown overnight.

The transformation efficiency for competent bacteria was always calculated. Briefly, 1µg of pUC19 vector-DNA was transformed into 100µl *E.coli* DH5α competent bacteria, and the number of bacterial clones grown in an agar-dish was counted. As a negative control, bacteria were mock-transformed. Transformation efficiency was always around $5x10^6$ bacterial clones per µg of vector-DNA transformed.

Cracking method

Cracking was performed for rapid isolation of plasmid DNA to determine size and amount within transformed bacteria. Additionally to cracking, screening PCR was performed for detection of insert introduction by molecular cloning or site-directed mutagenesis (see above).

About 300µl of bacteria culture were pelleted for 30'' at 13000rpm in a tabletop centrifuge. Lysis was achieved with 100µl of cracking buffer (50mM Tris-Cl (pH=6.8), 1% SDS, 2mM EDTA, 400mM sucrose, 0.01% bromophenol blue, 0.01% xylencyanol FF, 0.01% orange G, and 100µg/ml RNAse A), mixed thoroughly by vortexing and incubated for 10 minutes at room temperature. Cell debris was pelleted for 20 minutes at 13000rpm, and 25µl of the solution was loaded onto an 1% agarose gel. As a control, DNA marker III and plasmid (purified WT-pUC19) were used.

Maxi-preparation

Bacteria were grown in glass-flask with 200ml LB medium overnight, the cells were pelleted and plasmid was purified with Qiagen Maxi-preparation kit according to the QIAGEN Handbook protocol. Plasmid DNA was dissolved in 0.5-1ml TE buffer and the concentration was calculated at $OD_{240/260}$. This purified plasmid DNA was then used for sequencing, transfection, PCR, or other biochemical techniques.

5.2.3 DNA-Plasmid construction

The constructs created during this work are listed in the two tables below with the parameters used for construction. Two methods were used for construction, molecular cloning and site-directed mutagenesis.

Constructs and construction parameters

Construct	Restriction	Vector (size)	Insert (size)	Ligation (vector:insert)	Primer for Screening	Primer for Sequencing
Wt/S-5a	AvrII and BsrGI buffer II	Wt (4773bp)	5a (1097bp)	1:4	+67 and –1164	+67, +408
Wt/S-5c	AvrII and BsrGI buffer II	Wt (4773bp)	5c (1097bp)	1:4	+67 and –1164	+67, +408
Wt/S-5aC138	AvrII and BsrGI buffer II	Wt (4773bp)	5aC138 (1097bp)	1:4	+67 and –1164	+67, +408
Wt/preS-5a	BspEI and AvrII buffer II	Wt (4840bp)	5a (1030bp)	1:4	+67 and –1164	+2090, +2812
Wt/preS-5c	BspEI and AvrII buffer II	Wt (4840kb)	5c (1030kb)	1:4	+67 and –1164	+2090, +2812
WtL^-M^-	BspEI and AvrII buffer II	Wt (4840bp)	P14 (1030bp)	1:3	+67 and –1164	+2812, +67
CMV-WtL	AvrII and RsrII Buffer II	$CMVL^+X^-$	Wt (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-5aL	AvrII and RsrII Buffer II	$CMVL^+X^-$	5a (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-5aC138L	BspEI and AvrII Buffer II	$CMVL^+X^-$	5aC138 (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-WtR138L	BspEI and AvrII Buffer II	$CMVL^+X^-$	WtR138 (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-WtS	AvrII and RsrII Buffer II	CMVS	Wt (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-5aS	AvrII and RsrII Buffer II	CMVS	5a (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-5aC138S	BspEI and AvrII Buffer II	CMVS	5aC138 (1416bp)	1:3	+2812, +67	+2090, +2812
CMV-WtR138S	BspEI and AvrII Buffer II	CMVS	WtR138 (1416bp)	1:3	+2812, +67	+2090, +2812

Constructs created by molecular cloning are listed in the table above: the restriction enzymes used for cloning (Restriction), the size of vector (Vector) and insert (Insert), the proportion of vector and insert for cloning (ligation), and the primers used for screening and sequencing are listed for each construct.

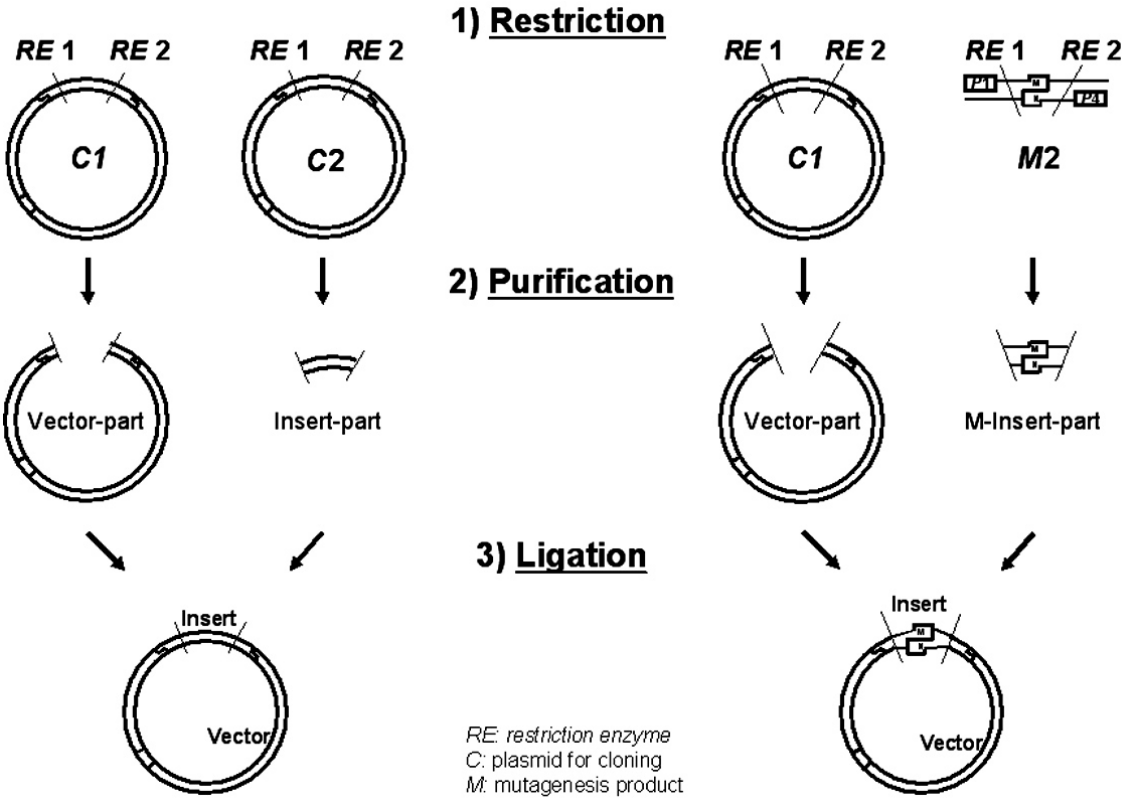
Construct	Vector plasmid	Primer-Mutagenesis (P1-P4)	Inserts (size)	Restriction enzymes	Primer-Sequencing
WtT125P127	Wt	PCR 1: +67, -528* PCR 2:+528*, -1164	PCR1: 461bp PCR2:639bp	AvrII, BsrGI, BbsI buffer II	+67, +408, -477
WtA138	Wt	PCR 1: +67, -566* PCR 2: +566*, -1164	PCR1:499bp PCR2:601bp	AvrII, BsrGI, BbsI buffer II	+67, +408
5aA138	5a	PCR 1: +67, -566* PCR 2: +566*, -1164	PCR1:499bp PCR2:601bp	AvrII, BsrGI, BbsI buffer II	+67, +408
WtS^-	Wt	PCR 1: +2812, -156* PCR 2: +156*, -1164	PCR 1: 538bp PCR 2: 1011bp	BspEI, BsrGI, BbsI buffer II	+67, -477
5aS-	5a	PCR 1: +2812, -156* PCR 2: +156*, -1164	PCR 1: 538bp PCR 2: 1011bp	BspEI, BsrGI, BbsI buffer II	+67, -477

The constructs built by site-directed mutagenesis are listed in the table above, the plasmid used and indicated as vector (Vector), the primers used for amplification of the inserts (Primer-Mutagenesis), the size

of the inserts (Inserts), the restriction enzymes used for molecular cloning (Restriction), and the primers used for sequencing are listed for each construct. The proportion of the vector and insert for cloning was always 1:4.

Molecular cloning

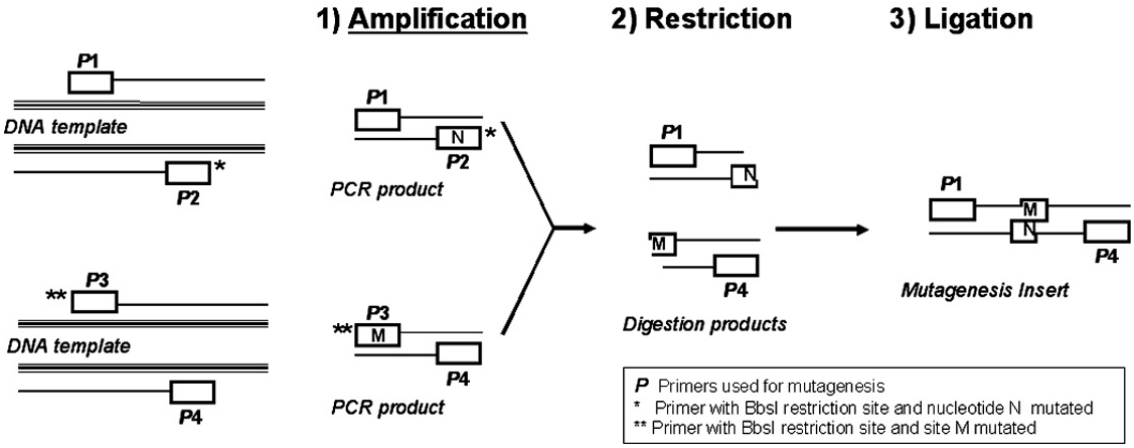
Generally, molecular cloning was performed by swapping the insert region in a vector-plasmid as shown into the figure below. Two different restriction enzymes were used, and allowed the introduction of the insert in the correct position and orientation into the vector plasmid. Methodically, plasmids (C1 and C2) or PCR-fragments (M2) containing insert or vector were digested (1 Digestion) with the corresponding restriction enzymes (RE1 and RE2) in the appropriate buffer (as named in the table for each construct). Complete restriction was checked by loading a small sample aliquot in an agarose gel, and DNA was detected by staining with EthBr. Subsequently, the rest of the digestion material was separated by agarose gel electrophoresis (without EthBr) and the fragments, vector, and insert, were cut and purified from the gel with Qiagen gel extraction kit (2 Fragment purification). Vector and insert were combined in appropriate amounts (as described in the table above for each construct), and used directly for ligation reaction 30 minutes at room temperature with the help of a DNA ligation kit (3 Ligation). Resulting plasmids were transformed in bacteria and purified with Qiagen Plasmid Maxi kit for functional analysis. The origin of replication of the vector plasmid is depicted as 'ori'.



Site-directed mutagenesis

Mutations leading to a single aa-exchange were introduced by site-directed mutagenesis (figure below). The nts to be mutated are here represented by the letters -complementary generic nt- M and N, which were introduced with the primers P2 and P3, respectively. Briefly, a double PCR reaction (PCR-amplification) was performed with the plasmid, in which the mutation had to be introduced (DNA template) after the protocol for 'subgenomic-PCR'. The first reaction (tube 1) was performed with an upstream HBV-primer (P1) and an anti-sense mutagenesis oligonucleotide including the N-mutation (P2). The second PCR (tube 2) was performed with the sense mutagenesis primer including the M-mutation (P3) and a downstream HBV-primer (P4). The mutagenesis primers contain an HBV-homologous region and a non-HBV region, the non-HBV region includes a BbsI attachment- and restriction-site (designated as * and **). The size of the PCR-fragments was between 0.4-1.2 kb, amount and size were checked by agarose gel electrophoresis. Fragments were separated in an agarose gel, and purified with Qiagen gel extraction kit. After purification, the same amounts of PCR-fragments (tube 1 and tube 2) were mixed, digested with the BbsI restriction enzyme, and

ligated. Ligation of these two PCR-products reconstituted the mutagenesis-insert, in which the mutation M:N is introduced. The mutagenesis-insert was cloned into the corresponding plasmid by molecular cloning.



5.2.4 Polymerase Chain Reaction (Saiki et al 1988, modified)

For PCR-reaction, two PCR-mixes were prepared: the PCR reaction-mix contained deoxyribonucleotide triphosphate, a sense and an anti-sense primer as well as DNA-template; the PCR enzyme-mix contained the DNA-polymerase. Amplification was performed with Taq- or Hifi-DNA polymerases, which required different enzyme concentrations of 5U or 2.6U and also different annealing temperatures at 68° or 72°C, respectively. Hifi-DNA polymerase was used for amplifications where a minimal error rate was essential.

Amplification of full-length HBV-genomes

Amplification of complete HBV genomes was carried out as described in Günther et al (Gunther et al., 1995a) for detection of variants in paraffin-embedded tissues and for synthesis of the DNA-probe for Southern blot analysis.

The reaction-mix (total volume of 45µl) was composed of 1x PCR-buffer, 200µM deoxyribonucleotide triphosphate, 0,5µM HBV-primer (-1822), 0,5µM HBV-primer (+1822), and 1-10ng/µl of template. The enzyme-mix (total volume of 5µl) was composed of 1x PCR buffer and DNA polymerase.
PCR conditions were:

Pre-denaturation:	'hot start'	94°C for 1 minute.
Addition of the enzyme mix.		
Amplification (40 cycles)	denaturation	94°C for 40 seconds
	polymerisation	60°C for 90 seconds
	annealing	68°C for 3, 5, 7 and 9 minutes.

The addition of the enzyme-mix was done during the polymerisation step within the first cycle. The annealing time was increased by 2 minutes after 10 PCR cycles.

Screening-PCR

PCR was performed for identification of the correct insertion of the clones (site-directed mutagenesis and cloning procedure).

The reaction-mix (total volume of 20µl) was composed of 1x PCR buffer, 200µM dNTP (deoxyribonucleotide triphosphate), 0,5µM primer 1, 0,5µM primer 2, and variable amounts of template in 1µl solution. The enzyme-mix (total volume of 5µl) was composed of 1x PCR buffer and DNA polymerase.
The PCR conditions were:

Pre-denaturation:	'hot start'	94°C for 1 minute.
Amplification (40 cycles):	denaturation	94°C for 40 seconds
	polymerisation	60°C for 90 seconds
	annealing	72°C for 1-3 minutes.

The annealing temperature depended on the size of the DNA-fragment being amplified, for small fragments 1 minute was used, and for fragments bigger than 1-kb 2-3 minutes were used.

Subgenomic- and nested-PCR

Both PCR-reactions were used for amplification of HBV DNA from the paraffin-embedded tissues. Subgenomic-PCR was carried out using specific sets of primers, encompassing the a-determinant: Combinations of sense (+67 and +191) and antisense (-700 and -1164) primers were used. For nested-PCR,

subgenomic-PCR product (using the primers +67 and –1164) was used as a template in combination with the primers +191 and -700. The PCR-mixes and the PCR conditions were the same for both amplifications.

The reaction-mix (total volume of 20µl) was composed of 1xPCR buffer, 200µM deoxyribonucleotide triphosphate, 0.5µM of the corresponding primers, and template contained in 1µl of solution. In addition, the enzyme-mix (total volume of 5µl) was composed of 1xPCR buffer and DNA polymerase.
The PCR conditions used for both reactions were:

Amplification (25-40 cycles):	denaturation	95°C for 40 seconds
	polymerisation	60°C for 1 minute
	annealing	68°C for 60-90 seconds.

DNA labelling amplification for sequencing

For sequencing, the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit was used according to the protocol recommended by the supplier. Briefly, a unique PCR reaction mix (total volume of 20µl) was composed of 8µl of kit-mixture, 1µl of HBV-primer (100nM), 0.5-1µg of DNA-fragment or plasmid to be sequenced (in 1µl of volume), and 10µl H_2O.

The PCR conditions were:

Amplification (25 cycles):	denaturation	94°C for 40 seconds
	polymerisation	60°C for 90 seconds
	annealing	68°C for 3 minutes.

5.2.5 DNA sequence analysis

Sequence analysis was performed for plasmid- and PCR-fragments to confirm oligonucleotide sequences. Methodically, DNA was labelled with the protocol 'PCR for sequencing' containing a dye-terminator. For sequencing of the HBV S-region, the primers +67, +408, and –477 were used, and the primers +2090 and +2812 were available for sequencing of the HBV preS-region.

PCR products were then incubated with 0.1 volume sodium acetate (3M) and 2.5 volumes 97-100% ethanol over night. Nucleic acids were pelleted for 20 minutes at 13000 rpm on a table centrifuge. The bands corresponding to the different DNA-fragments were separated by a Sequencing Service (Hamburg University). Alignments of the sequences were performed with MacVector software.

5.2.6 DNA and RNA gel electrophoresis

Agarose gel electrophoresis

This technique was used to separate DNA-fragments for Southern blot, in molecular cloning, and site-directed mutagenesis, as well as to separate DNA-plasmids for cracking procedure and other techniques.

Agarose was used in concentration ranging between 1-2% in TAE buffer: High concentrations (about 2%) were used for separation of small DNA fragments (100-800kb), and 1% agarose concentration was used for plasmids or big DNA-fragments. TAE 1x buffer served as a running buffer, each sample was solved in 5x loading buffer (1mg/ml bromphenol blue, 1mg/ml xylencyanol, 2mg/ml orange G and 80% glycerol). The gel was run 100-120 volts until the dye reached three-quarters of the gel length.

Formaldehyde agarose gel

This gel was used for RNA separation followed by Northern blot analysis.
A 1% agarose gel (low EEO) was prepared with 20xRB buffer (200mM MOPS, 50mM NaAcx3H_2O pH=7.0, 1mM EDTA pH=8.0 in diethylpolycarbonat-H_2O), and was cooled down to 60°C, then formaldehyde was added until an end concentration of 2.2M. RB 1x buffer was used as running buffer, and RNA was diluted in 5x RNA loading buffer (50% formamide, 2.2M formaldehyde, 1xRB buffer). The formaldehyde gel was run at 60V over night to avoid excessive warming.

Non-denaturing Agarose gel separation

Separation of viral particles was achieved by an agarose gel under non-denaturing conditions. The gel was prepared with Agarose (low EEO) to a final concentration of 1% in TNE buffer, which was also used as a running buffer. Particles were precipitated by addition of PEG, and input DNA was digested by DNase I (1µg/ml) and $MgCl_2$ (0,5 mM) for 30 minutes at 37°C, the reaction was stopped by addition of EDTA (1mM). Particles were solved in 5x loading buffer, and loaded onto the non-denaturing gel, which was run 100V for 30 minutes, and then about 35V overnight.

5.2.7 Transfection of cell lines

Hepatoma cell line cultures

Huh-7 or HepG2-C3A cells were maintained in media for hepatoma cell lines (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10mM glutamine, 5% non-essential aas and 100 U/ml penicillin-streptomycin).

For cell splitting, confluent cells were washed twice with PBS, incubated with Trypsin-EDTA solution for 5 minutes, whereupon the trypsin solution was discarded and cells were collected in 10ml medium. 1-2ml were passaged onto a new plate (dilution 1:10 or 1:5, respectively).

Preparation of the full-length HBV-DNA for transfection

When transfecting cells full-length pHBV-SapI constructs were used. HBV linear monomers were released from the plasmid by cleavage with 1-2U of SapI for 3-14 hours. These genomes contained SapI sticky-ends. Sap I enzyme was inactivated for 10 minutes at 65°C on a thermo-block before transfection.

Transfection of hepatoma cells

Hepatocarcinoma cell lines were plated the day before transfection at a density of $1.6x10^6$ cells per 60-mm-diameter petri-dish; alternatively, $0.9x10^6$cells were plated on 6-well plates. The medium was changed 2 hours before transfection and 2.5 or 5µg of plasmid HBV-DNA were used per 6-well plate or per 60-mm-diameter petri-dish, respectively. When needed, 0.25-0.5µg of reporter SEAP-plasmid was added to the total amount of plasmid DNA. Plasmid-DNA transfer into mammalian cells was always performed with the Fugene 6 transfection system (3µl reagent/µg of DNA) according to the protocol recommended by the supplier. Briefly, the FUGENE reagent was dissolved in medium (fetal calf serum-free) for mammalian hepatoma cells and incubated at room temperature for 5 min. The reagent was then dropped carefully to the plasmid-DNA mixture and incubated for 15 minutes at room temperature. Before transfection, cells were washed twice with PBS. The mixture was finally added to the cells, which were then incubated at 37°C and 5% CO_2 for 16-20 hours. After that, cells were washed twice and fresh medium was added. Untransfected cells were treated accordingly and used as a mock control.

Transfection efficiency

Transfection efficiency was estimated after transfection with 0.5-1 µg plasmid expressing the green-fluorescent protein. Percentage of transfected cells was roughly determined by cell counting. In Huh-7 cells, transfection efficiency was between 10-30%.

Transfection and secretion efficiency was precisely determined after co-transfection of each single sample with SEAP-plasmid, and measurement of the enzymatic activity of the alkaline-phosphatase enzyme in the medium (Cullen and Malim, 1992). For SEAP analysis, endogenous phosphatases from 100µl cell culture supernatant of each single transfection plate were inactivated at 65°C for 5 min, and cell debris were discarded after centrifugation 2 minutes at 13000 rpm in a table centrifuge. Cell culture supernatants were diluted 1:5 or 1:2 (10 or 50µl of supernatant in a total volume of 100µl, respectively) and mixed with 100µl SEAP-dilution buffer in a microtiter-plate. Both the ELISA reader and the microtiter-plate were warmed to 37°C for 10 minutes and the substrate (50µl of p-nitrophenol-phosphate concentration 31.6 mg/ml in water) was added. Measurements of the kinetic were done at a wavelength of 405 nm at 37°C with a run time of 30 minutes and read-interval of 1 minute. The transfection efficiency was similar for all the constructs tested.

5.2.8 Immunofluorescence staining

Cells were cultured on glass coverslips in 24-well culture plates. To visualize surface staining, the cells were fixed by ice-cold methanol for 5 minutes and incubated three seconds with ethanol for drying. After blocking with normal goat serum in PBS for 45 minutes, cells were reacted with primary antibodies diluted in blocking buffer for 1 hour, washed four-times with PBS, and incubated with secondary antibody diluted in blocking buffer for 1 hour. After washing twice with PBS, cells were incubated with DNA-binding dye in PBS for 10 minutes and washed three-times with PBS. Coverslips were mounted in glass slices and staining was visualized with a Zeiss Axioplan 2 fluorescence microscope (Zeiss). PreS1 and S-proteins were detected with M18/07 (1:1000) MAb and anti-S PAb (1:250), respectively.

5.2.9 Bradford test

One hundred µl sample and 100µl 40% Biorad solution were mixed together, whereupon the mixtures were measured by OD at 595 nm after 5-30 minutes.

5.2.10 Enzyme-linked immunosorbent assay

ELISA for HBsAg

Supernatant of transfected cells were assayed for HBsAg secretion. To discard cell debris medium was centrifuged with 3000 rpm for 20 minutes at 4°C. Dilutions of the cell culture medium were prepared 1:10 to 1:20 in PBS buffer. Samples were considered positive with a signal noise ratio (S/N) greater than 2. HBsAg absolute values for each HBV genome were equivalence by subtraction of mock absolute value, Wt was standardized to 100%, and relative values for HBV particle secretion were calculated compared to Wt values and given also as a percentage.

ELISA for HBeAg

Supernatant of transfected cells were assayed for HBeAg secretion. To discard cell debris the medium was centrifuged 3000rpm for 20 minutes at 4°C. Dilutions of the cell supernatant of (1:5) were always prepared in PBS buffer. Samples were considered positive with a signal noise ratio (S/N) greater than the mock control, and given as HBeAg positive or negative.

5.2.11 Pulse experiment

Metabolic-labelling

Two days post-transfection, cells were washed twice with PBS and starved for 2 hours in a specific medium for pulse experiment (Met-free dulbecco's modified eagle medium supplemented only with 2mM glutamine, 3.5mM arginine, and antibiotic). Then, in vivo labelling was performed with 200µCi (^{35}S)Met per well in Met-free medium. Cells were immediately washed twice with ice-cold PBS-buffer, and incubated on ice for 20 minutes in PBS+EDTA-buffer, and harvested. Lysis was performed with 500µl lysis buffer (50mM Tris-Cl (pH=7.5), 150mM NaCl, 1% Nonidet^{R} P40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete^{TM}protease inhibitor), and spun for 20 minutes in a table centrifuge at 4°C.

Immunoprecipitation

For immunoprecipitation, anti-S PAb (polyclonal goat anti-S anti-serum) was preincubated with 100µl of 10% protein G-Sepharose slurry in PBS-buffer, afterwards the cell extract was added to the slurry and mixture was incubated overnight at 4°C under 150 turns/minute rotation. Sepharose beads were then washed, boiled in loading buffer, and the supernatants were loaded onto SDS-PAGE gels. After electrophoresis, gels were stained with methylen-blue solution for 3 hours, incubated with Amplify solution for 30 minutes at room temperature and dried over night between cellophane papers. Exposition to a screen was prolonged between 6 hours to 1 week, and images were obtained with a phosphor imager.

5.2.12 Separation and precipitation of viral particles

Polyethylenglycol-precipitation of particles

Particles released into the medium were precipitated by addition of 100ng/ml of PEG. Supernatants were incubated for 90 minutes at 4°C in a table rotor with 150 turns/minute, thereafter for 30-50 minutes at 4°C without rotation. Afterwards precipitation was performed at 8000 rpm for 20 minutes at 4°C in a table centrifuge. Supernatants were discarded and pellets were solved in PBS.

Ultracentrifugation through a sucrose cushion

Supernatants from the medium of transfected cells (2 ml in volume) were carefully placed in a centrifugation tube on top of a 20% sucrose solution (0.5 ml in volume). Ultracentrifugation was performed with a SW60 rotor (Beckman) with 50000rpm speed for 4 hours at 4°C. Particles were pelleted through the sucrose-cushion and then collected with PBS-buffer or with SDS-loading buffer. Supernatant was saved for HBsAg measurement.

CsCl-centrifugation

Enveloped viral particles were separated from non-enveloped nucleocapsids by isopynic centrifugation in a CsCl gradient. CsCl was added directly into the medium of transfected cells (6 ml in volume) at a starting density of 1.22g/ml. Ultracentrifugation was performed in a SW41 rotor (Beckman) with 38000 rpm speed for 36 hours at 20°C. About 50 fractions were collected from the top to the bottom of the tube, and the CsCl densities of the fractions were measured using a refractometer. An aliquot of every two or three fractions of the gradient were used for the HBV-DNA and protein detection that follows.

5.2.13 Southern blot analysis

DNA probe

As probe full length HBV-DNA genome was used, which was created by 'full-length PCR', using 20ng pSM2 plasmid as PCR template, and additionally 10nM dig-labelled dUPT. Amplified HBV-DNA was semiquantified by gel electrophoresis, being of concentration about 15ng/µl. The introduction of labelled-dUTP was confirmed by the presence of a shift in the DNA-product mobility. Labelled capacity of the DNA probe was further tested by Dot-blot analysis, and dilution 1:8000 was found optimal for good specific signal detection and low background in Southern blot analysis.

Asymmetric marker

A marker was made consisting of double-strand-DNA and single-strand-DNA HBV-genomes. This HBV-DNA combination was achieved by 'full-length PCR' using asymmetric ratios of the amount of the primers: Primer +1822 was diluted 1/25 to 1/50, while primer -1822 was used with normal concentration (1mM). Template for the PCR was pSM2 plasmid, which was linearized by the restriction enzyme Sap I. The PCR-product was checked and semiquantified after electrophoresis in a gel, and was then diluted 1/10000 and used for Southern blot analysis.

DNA extraction

For Southern blot analysis, cells were transfected and collected 4 days posttransfection. Cells were washed twice with ice-cold PBS-buffer and pelleted in an Eppendorf-tube. Cell content was solved in 1ml DNA lysis-buffer (50 mM Tris pH=7.8, 1 mM EDTA pH=8.0 and 1% NONIDET^{R} P-40), and the cytoplasmic fraction was separated from insoluble material by centrifugation at 8000 rpm for 20 minutes. The cytoplasmic DNA not packed in nucleocapsids was digested with 1U DNAse I in the presence of 2.5 mM $MgCl_2$. Then cell extracts were digested with proteinase K in the presence of 2% SDS. The DNA was extracted with a phenol-chloroform mixture 1:1 and eluted in 100µl TE-buffer. Then, the nucleic acids were precipitated with high salt concentration buffer (0.1 volume 3M NaAc, 20mg tRNA and 1 volume isopropanol), washed twice with 75% ethanol, and resuspend in 20µl TE-buffer.

Southern blot analysis

The extracted DNA was loaded onto a 1.5% agarose gel, and run for 5 hours at 90 volts. DNA was incubated for 45 minutes in denaturing solution (0.5M NaOH and 1.5M NaCl) and further incubated twice for 10 minutes in the neutralizing solution (1M Tris pH 7.5 and 1.5M NaCl, pH=7.5). The transfer was performed by liquid capillarity in 20x SSC solution over night at room temperature. The membrane was finally washed briefly with 2xSSC solution, dried, and the DNA was then cross-linked by UV-light to the membrane.

Hybridisation and detection with Dig-method

The membrane was first equilibrated with 20ml hybridisation solution (5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent and 100µg/ml herring sperm-DNA) for 2h at 68°C and then hybridised over night at the same temperature with a digoxigenine-labelled HBV DNA probe (dilution 1:8000) in 20 ml hybridisation solution. Thereafter, the membrane was washed twice with WL1 (2x SSC, 1% SDS) for 5 minutes at room temperature and twice with WL2 (0.1x SSC and 0.1% SDS) for 20 minutes at 68°C. Detection started with equilibration to P1-buffer (0.1M malein acid pH=7.5 and 0.15M NaCl). Then, the membrane was blocked with P2 (2% blocking reagent in P1-buffer), and incubated with anti-digoxigenin coupled to alkaline phosphatase (dilution 1:20000) in P2-buffer. The membrane was equilibrated to P3-buffer (0.1M Tris-HCl pH=9.5, 0.1M NaCl, and 50mM $MgCl_2$) and incubated with CDP-Star substrate (dilution 1:100). Chemiluminescence signals were detected with X-ray films.

5.2.14 Northern blot analysis

RNA probe

The probe was prepared by *in vitro* transcription with a DIG-labelling system. The template for transcription was the plasmid pBSK-c/x, which contain a HBV region (C- and X-ORF) common to all HBV-RNAs (pgRNA, L mRNA, S mRNA and X mRNA) under the transcription of the T3-promoter. *In vitro* transcription was performed for 20 minutes at 37°C with the RNA transcription kit according to protocols of the supplier. The introduction of digoxigenin was tested by dot-blot analysis.

RNA extraction

Huh-7 cells were transfected with HBV-constructs. Three days posttransfection, cells were washed twice with ice-cold PBS, incubated in ice for 20 minutes with PBS-EDTA solution, and pelleted. Cell lysis

was performed with 1ml of lysis buffer from the RNA purification kit, lysis mixture was previously homogenized using a QIAshredder column, and RNA was extracted by a RNA mini kit. Elution was done with 2x 50µl buffer.

DNA impurity was digested by addition of DNase I (RNAse-free 10U/ml) with 0.5 mM $MgCl_2$ for 20 minutes at 37°C. Free-deoxynucleotides and enzyme were removed with the precleaning procedure with RNA mini kit.

For measurement of the RNA-quantity and purity, an aliquot of RNA preparation (dilution 1:10-1:50) was measured with the spectrophotometer for $OD_{260/280}$. In order to check RNA integrity, an aliquot of the RNA preparation was run in an 1% agarose gel (prepared with DEPC-(Diethylpolycarbonat) TAE-buffer) and detection was performed with EthBr.

Northern blot analysis

RNA was quantified with the spectrophotometer OD_{260}, 20µg of each RNA preparation was solved in RNA-loading buffer, and loaded onto 1.5% formaldehyde agarose gel for RNA-separation. The gel was run over night at 60 volts in order to avoid excessive warming. Transfer of the RNA to a Nytran-N was performed at room temperature for 5 hours. The RNA was then cross-linked to the membrane and was stained with methylenblue. The membrane was hybridised with dig-labelled RNA probe prepared before and developed with chemiluminiscence detection. Hybridisation with a dig-labelled probe was performed according to the method described for Southern blot analysis. The RNA-probe was used at a dilution of 1:1000.

5.2.15 Western blot analysis

Protein from cell extracts and particles

Cell culture medium was also collected and cleared by centrifugation 3000 rpm for 20 minutes. Particles in the medium of transfected cells were concentrated by PEG-precipitation or ultracentrifugation and solved with 1:1 proportion of SDS-loading buffer proteins were then separated by SDS-PAGE.

Western blot analysis

Protein marker for SDS electrophoresis contained the protein bands at 95-, 65-(albumin), 45-, 30-, 20- and 12-kDa.

Proteins could be separated by SDS-PAGE. Briefly, acrylamide gel was composed of a 15% running gel (15ml 30% acrylamide, 1.3ml 2% bis-acrylamide, 7.5ml 1.5M Tris-Cl (pH=8.8), 5.8ml H_2O, 300µl 10% SDS, 100µl 10% APS, and 10µl TEMED in a total volume of 30ml) and a 5% collecting gel (3.34ml 30%Acylamide, 1.3ml 2% Bis-acrylamide, 2.5ml 1.5M Tris-Cl (pH=6.8), 12.5ml H_2O, 200µl 10%SDS, 100µl 10% APS, and 10µl TEMED in a total volume of 20ml). Proteins were always run at 50 Volts overnight with TG-running buffer and then electrotransferred from the gel to a nitrocellulose sheet using a blotting apparatus with TGE-buffer at 4°C for 3 hours with constant voltage of 0.4 Volts.

Proteins were stained and fixed into the nitrocellulose membrane with Ponceau S solution. The membrane was then blocked with 5% (w/v) fat-free instant milk powder in TBS-buffer and incubated with primary antibodies diluted in 2% (w/v) fat-free milk in TBS. After washing with 1xTBS-0.1% Tween 20 buffer and with 1xTBS, each three-times for 10 minutes, the membrane was incubated with secondary peroxidase-conjugated antibodies in 2% fat-free milk in TBS buffer. After washing SuperSignal™ DURA chemiluminescence substrate was added for 1 minute at room temperature and the membrane was exposed to a X-ray film between 1-30 seconds. Signals intensities were semi quantified by TINA program.

5.2.16 Dot blot

Protein dot-blot

On a nitrocellulose membrane, 5µl of cell culture supernatant was dropped and dried. The filter was blocked with fat-free milk. Detection was performed as described for Western blot analysis with anti-S and anti-preS1 MAbs or anti-C PAb.

DNA dot-blot

On a Hybond-N membrane, 5µl of cell culture supernatant was dropped and dried. The DNA was denatured twice with basic solution (1M NaCl and 0.5M NaOH) for 5 minutes and with a neutralizing solution (3M NaCl and 0.5M Tris-Cl pH=8.0) for 10 minutes. The filter was dried and DNA was cross-linked to the membrane. Detection was performed as described for Southern blot analysis with a HBV full-length DNA-probe.

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Sep 88-Jun 89 ”PROA” graduation course in Barcelona

Jun 89: Graduation.

University Studies:

Oct 89-Sep 96 ”Barcelona University”, degree in Chemistry

Specialization in Biochemistry with the subjects: genetics, enzymology, pathology and metabolism

Sep 96: Graduation in Chemistry.

Nov 95-Jun 96 ”Politechnical University”

Jun 97: Certificado Pedagogico.

Oct 96-Dec 96 ”Politechnical University”, Technical Autoedition and Design

Project: General Conference news magazine.

Jul 97-Oct 97 ”Hamburg University, Chemistry Faculty”, WG. Prof. Dr. Francke

Co-operation in two projects: 1) extraction and analysis of fresh fish compounds producing bad smell, 2) synthesis and gas chromatography analysis of monosaccharides glycosides pheromones.

Nov 97-Dec 97 ”Heinrich-Pette Institut ”, WG. Prof. Dr. Deppert.

Practikum in basic techniques in biochemistry research: electrophoresis, blots, radioactive markers.

Jan 98-Jun 98 ”Hospital Clínico de Barcelona ”, WG. Prof. Dr. Sanchez-Tapias.

Research project: hepatitis B virus preS-region genotyping and hepatitis C virus NS5 region genotyping.

Oct 98-Jan 00 ”Heinrich-Pette Institut”, WG. Prof. Dr. Hans Will.

Preparation of the manuscript for the german Diplom. Practikum in basic techniques in molecular biology research: site-directed mutagenesis, DNA and RNA blots, cell culture, immunofluorescence microscopy.

Jan 00- Dec 04 ”Heinrich-Pette Institut”, WG. Prof. Dr. Hans Will.

In the group of Dr. Martina Sterneck with the research project for doctorate degree: functional analysis of a natural occurring HBV variants isolated from a patient suffering fulminant hepatitis with a severe block of viral particle secretion.

Publication:

Kalinina T, Riu A, Will H and Sterneck M. (2003) Selection of Secretion-incompetent Mutant in the Serum of a Patient with severe Hepatitis B. Gastroenterology. 125(4):1077-84

Kalinina T, Riu A, Fischer L, Will H. and Sterneck M. (2001) A dominant Hepatitis B Virus Population Defective in Virus Secretion Because of Several S-Gene Mutations From a Patient With Fulminant Hepatitis. Hepatology 2001; 34: 385-394.

Abstracts:

Sterneck M., Will H, Riu A, Fischer L. and T. Kalinina (2003). Evidence for a reduced fitness of the HBV immune escape mutant G145R. Hepatology Vol.38, No4, Suppl. 1.

Kalinina T, Riu A, Will H and Sterneck M. (2000) Individual and synergistic effects of naturally occurring S-gene mutations determine type and quantity of secreted viral particles. The molecular biology of hepatitis B viruses annual meeting. September 17-21.

Kalinina T, Riu A, Günther S, Will H and Sterneck M. (1999).Mutations in the small S-gene leading to a defect in viral particle secretion occur in patients with fulminant hepatitis B. The molecular biology of hepatitis B viruses annual meeting. July 18-20.

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