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Characterization of the hepatitis B virus matrix domain function in virion assembly

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CHARACTERIZATION OF THE HEPATITIS B VIRUS MATRIX DOMAIN FUNCTION IN VIRION ASSEMBLY

1 Introduction

1.1 Hepatitis B virus

Hepatitis is a disease probably as old as mankind since reports of jaundice being the most prominent symptom date back more than 2000 years. Already in the beginning of the 19th century the hypothesis of a virus causing liver inflammation had been postulated and was substantiated in 1938 by F. O. Maccallum who investigated the cause of 89 cases of jaundice after immunization of 3100 humans against yellow fever using vaccines based on human (immune) serum (Findlay et al., 1938). Nine years later Maccallum coined the two terms hepatitis A and hepatitis B to differentiate between two forms of the disease, one spreading through water and food and the other one being transmitted by blood. Despite the efforts of many scientists, it took nearly 20 years until the next important step in hepatitis B research happened. B. S. Blumberg discovered an antigen in the blood of an Australian aborigine which was recognized by antibodies in the serum of a haemophilia patient having received multiple blood transfusions. This antigen was linked to the development of hepatitis B by different researchers (Blumberg et al., 1967; Prince et al., 1968). In 1970, D. S. Dane finally resolved the last part of the puzzle. He managed for the first time to visualize 42 nm large virus particles (so-called Dane particles) in the serum of three Australia-antigen-positive hepatitis patients by electron microscopy. Next to whole virus he also discovered 22 nm spherical particles and filamentous forms which became known as subviral particles (Dane et al., 1970). The Australia antigen was identified to be the hepatitis B virus surface antigen of subviral particles (and virions).

1.1.1 Classification

Taxonomically, the hepatitis B virus belongs to the *Hepadnaviridae*, a family of viruses which is characterized by their hepatotropy and their DNA genome which is transcribed from a pregenomic RNA by reverse transcription. The term *Hepadnaviridae* was deduced from *hepar*, the Greek word for liver, and *DNA*, specifying the genetic material. The virus family can be divided into two genera: the Orthohepadnaviruses infecting mammals, and the Avihepadnaviruses using birds as hosts. Both genera are further divided into species, hepatitis B virus being the most prominent member of the Orthohepadnaviruses. It can be further subdivided into 8 genotypes (A – H) and 24 sub-genotypes. Besides HBV, woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) belong to the

Orthohepadnaviruses. Notable Avihepadnavirus members are duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV).

1.1.2 Epidemiology and pathogenesis

HBV is one of the most important human pathogens of our times. At present, an estimated 360 million people worldwide are chronically infected with HBV (Previsani and Lavanchy, 2002). These patients are lifelong carriers of the virus. They are at high risk of developing hepatocellular carcinoma (HCC) since chronic HBV infection accounts for around half of the cases of this tumour. The incidence of HCC among chronic HBV carriers HBV is elevated more than 200-fold compared to non-carriers (Beasley et al., 1981).

Furthermore there is a striking correlation between HBV endemic regions and regions with a high incidence of HCC (Beasley, 1988). Up to 90 % of HCC cases in these parts of the world can be ascribed to persistent HBV infection. HBV is highly endemic in South-East and central Asia, sub-Saharan Africa, parts of Southern America, Greenland and Northern Canada, and the Eastern Mediterranean region. In these areas more than 8 % and up to 20 % of the population are chronic HBV carriers. Low endemic regions include Northern and Western Europe, North America and Australia with less than 2 % chronically infected inhabitants (see Fig. 1). Transmission occurs horizontally through direct contact with infectious blood or other body fluids, e.g. through blood transfusion or sexual contact, or peri- or postnatally from mother to child during or after birth.

Approximately 2 billion people worldwide have been infected with HBV once in their lives. Generally, the course of infection can either be transient or persistent. In adults, 90 % of infections are transient while in newborns it is only 10 %. The symptoms are often subclinical and rarely, HBV infection can result in a fulminant hepatitis leading to death. Patients having experienced a transient infection gain lifelong immunity. HBV is a noncytopathic virus. In fact it is the immune response of the host which is responsible for pathogenesis (Moriyama et al., 1990; Chisari and Ferrari, 1995). Furthermore, it is responsible for clearance or persistence of the virus. Strong, polyclonal and multispecific T cell responses result in successful clearance while weak and oligoclonal responses are characteristic for chronic HBV infection (reviewed in Chisari et al., 2010).

There is no treatment available for acute hepatitis B. Chronic carriers are treated with the immune modulator interferon alpha and nucleoside or nucleotide analogues like Lamivudine or Adefovir which act as antivirals inhibiting viral replication. They can be administered as

monotherapy or combination therapy. Vaccines are available since the early 1980s (McAleer et al., 1984).

1.1.3 Structure of the virion

Hepatitis B virions consist of a nucleocapsid containing the viral genome and a surrounding envelope (Fig. 2). They measure 42 nm in diameter (Dane et al., 1970) and under the microscope they can therefore easily be distinguished from the filamentous or spherical subviral particles (SVP) exhibiting a diameter of 22 nm. Consisting of only the envelope and lacking the nucleocapsid, subviral particles are produced by infected cells in great excess over Dane particles and may possibly act as immunological decoys. The envelope of both Dane particles and SVP consists of lipids and the three virus encoded surface proteins (small, middle and large). In comparison to infectious virions spherical subviral particles contain less large and proportionally more small and medium surface proteins (Heermann et al., 1984). The lipid composition of the envelope of spherical SVP has been determined by different groups who could show that it is very characteristic and clearly distinct from the lipid composition of the host cell and ER membranes (Gavilanes et al., 1982; Satoh et al., 1990; Satoh et al. 2000) which led to the assumption that subviral particles do not contain ordered lipid bilayers. In contrast, 3D image reconstructions of cryo-electronmicroscopical images of Dane particles clearly showed that the envelope of virions contains a classical lipid bilayer (Seitz et al., 2007). The nucleocapsid harbouring the viral genome shows an icosahedral shape (Almeida et al., 1970) of around 30 nm diameter and can either be built of 180 or 240 core proteins which leads to a symmetry of T=3 and T=4, respectively. The crystal structure of the T=4 capsid could be solved by Wynne et al., 1999. Inside the capsid the partially double stranded and 3.2 kb large HBV genome can be found along with the virally encoded polymerase (Kaplan et al., 1973) which is attached to the 5’ end of the minus strand.

1.1.4 Genome organization and transcripts

The HBV genome (Fig. 3) consists of circular DNA of around 3.2 kb (Summers et al., 1975; Landers et al., 1977) and is partially double stranded. It is called rcDNA (relaxed circular DNA). The negative strand is the coding strand and contains the full length of the genome while the positively sensed strand has a variable length and is mostly shorter than unit length (Summers et al., 1975). In the viral life cycle, the genome is produced by reverse transcription of a pregenomic RNA by the viral polymerase. This enzyme is covalently bound to the 5’ end of the negative strand by its aminoterminal domain (terminal protein; TP) (Wang and Seeger,

1992). The 5’ end of the positive strand contains a short capped RNA oligomer which serves as primer for the positive strand DNA synthesis. Other important features for the genomic replication are two short direct repeats (DR1 and DR2) which are located at the 3’ end of the negative and the 5’ end of the positive strand, respectively. They have a length of 11 nucleotides and are also implicated in integration of the viral genome into the host genome as observed in hepatocellular carcinoma DNA (Dejean et al., 1984). The circularization of the genome is achieved by overlapping cohesive ends since heating of the circular form leads to conversion to the linear form (Sattler and Robinson, 1979). The start of the numbering of the HBV sequence was fixed by convention to be directly after the single EcoRI restriction site due to the fact that restriction analysis were the first analyses which were made of the HBV genome.

Four open reading frames (ORF) coding for in total 7 proteins are located on the HBV genome. They are to a great extent overlapping. The polymerase (P) frame is the longest one coding for a single protein while other frames like the surface or core (C) frames code for up to three proteins. The surface ORF consists of the preS1, preS2 and S domains and codes for the large, middle and small envelope proteins, the core frame of the precore and core domain and serves as template for expression of the E and the capsid proteins. The shortest ORF encodes the X protein.

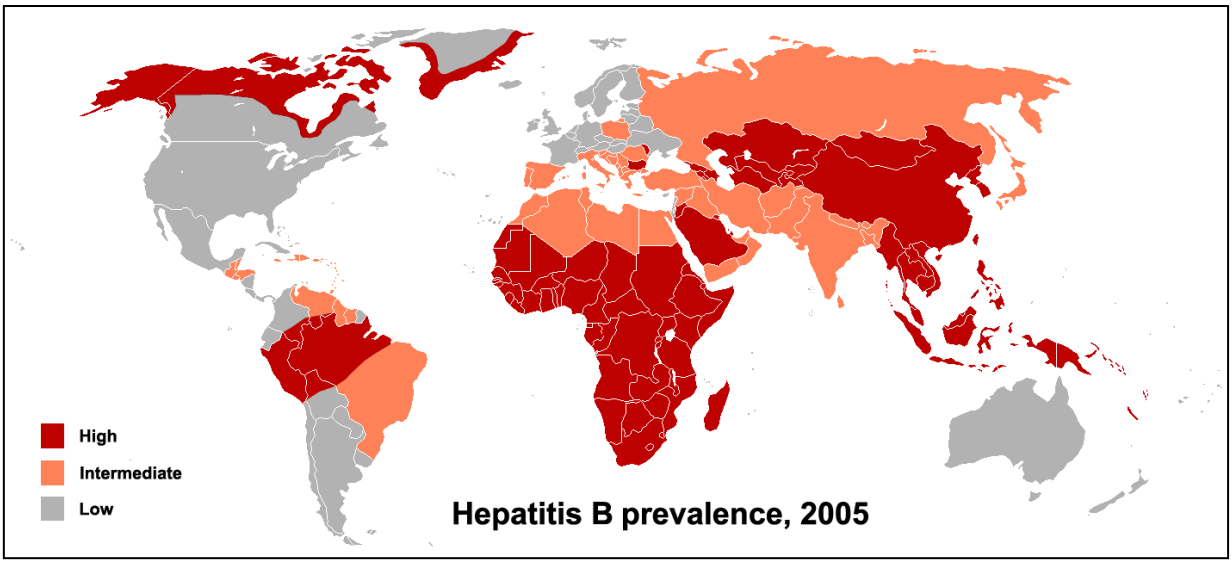

Fig. 1. Geographic distribution of chronic HBV (2005). Countries with high (intermediate, low) prevalence are shown in dark red (light red, grey) and more than 8 % (2 – 8 %, less than 2 %) of the population are chronic carriers of hepatitis B virus (from CDC Travelers' Health: Yellow Book).

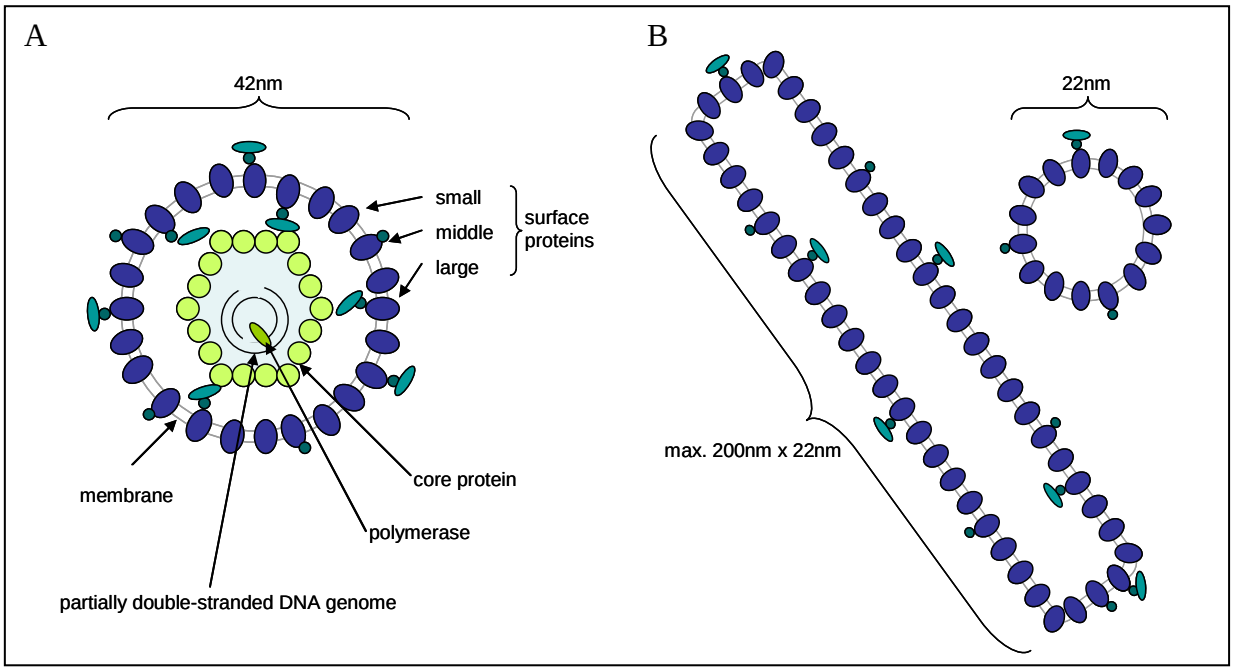

Fig. 2. Structure of the infectious hepatitis B virion (A) and HBV subviral particles (B). Virions (A) consist of a capsid made up of core protein (green circles) containing the partially double stranded viral DNA genome (black incomplete circle) and the viral polymerase (light green oval) and the surrounding envelope containing the small, middle and large surface proteins (blue oval: S domain; small green circle: preS2; dark green oval: preS1). Subviral particles (B) lack the nucleocapsid and can appear either as filaments or spheres.

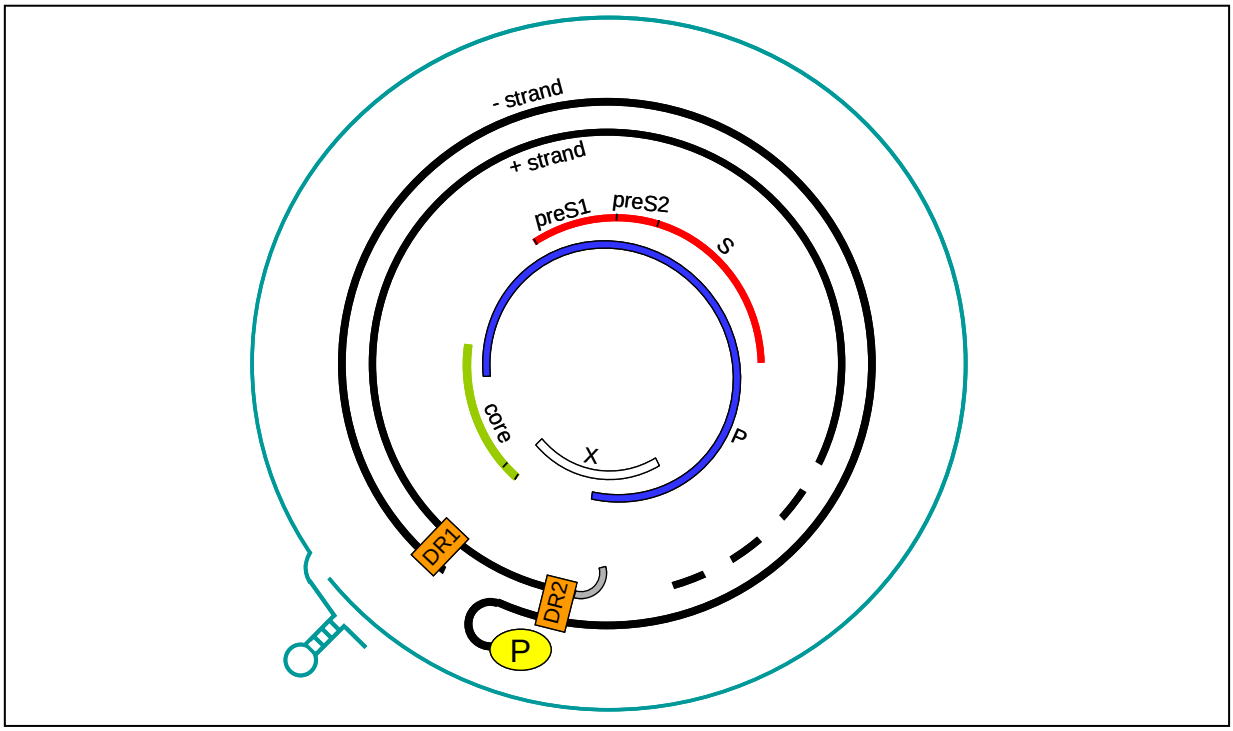

Fig. 3. Organization of the HBV genome. The partially double stranded DNA genome (thick black lines) contains four open reading frames which are depicted inside of the genome (green ORF: preC and C; red: preS1, preS2 and S; blue: polymerase; white: X). The polymerase (yellow oval) is covalently bound to the 5' end of the negative (coding) strand, the 5' end of the positive strand contains a small RNA primer (grey line). The orange boxes localize the direct repeats DR1 and DR2. Upon transcription a pregenomic RNA is made (cyan) which contains the epsilon signal (a stem loop structure) at its 5' end.

1.1.5 Transcripts

HBV transcripts are made from the so-called cccDNA (covalently closed circular DNA) which is formed upon repairing of the rcDNA by both viral and cellular enzymes. Pregenomic (pg) and subgenomic (sg) RNAs which are capped at the 5‘ end and contain a poly(A) tail at the 3’ end are produced by the host cell machinery. All transcripts share the same 3’ terminus which is due to the use of a common polyadenylation signal on the viral genome. The pgRNA of about 3.5 kb which is more than unit length is used for generation of new HBV genomic DNA by reverse transcription (Summers and Mason, 1982). Furthermore, the polymerase and the core proteins are translated from this RNA. The so-called epsilon structure, a stem loop structure located on its 5’ end, plays an important role in replication of the viral genome and encapsidation. Another RNA (preC RNA) which is slightly longer than the pgRNA and contains the start codon for the precore sequence serves for translation of the soluble E protein which is secreted from infected cells. It is neither used as pregenomic RNA nor for translation of P or C (Nassal et al., 1990). The sgRNAs of 2.4, 2.1 and 0.9 kb express the large (L), the middle (M) and small (S) surface proteins and the X protein, respectively.

1.1.6 Replication of the genome

After entering the nucleus, the viral genome is converted from its relaxed circular form to covalently closed circular DNA by both viral and cellular enzymes. Next, a pregenomic transcript is made by cellular RNA polymerase II using the negative strand of the genome as template. The pgRNA has more than unit length (see Fig. 3) and contains a special stem loop structure at its 5’ end, the so-called epsilon signal. The viral polymerase binds to this structure and a tyrosine of the aminoterminal domain serves as protein primer for copying 4 nucleotides from the bulge of epsilon by reverse transcription (Bartenschlager and Schaller, 1988; Zoulim and Seeger, 1994; Nassal and Rieger, 1996). In this way a short oligonucleotide is produced which is bound to the terminal protein of the polymerase. The complex is then translocated to the 3’ proximal DR1 sequence (Tavis et al., 1994) where the oligonucleotide binds to its complementary sequence and is elongated by the reverse transcription function of the viral polymerase until the 5’ end of the pgRNA. At the same time the RNA is degraded by the RNase H domain of P (Summers and Mason, 1982; Radziwill et al., 1990). Only the capped region at the 5’ end of the pgRNA including the 5’ DR1 is spared. It serves as primer for synthesis of the positive DNA strand (Lien et al., 1986). First, it is translocated to the 3’ proximal DR2 and then elongated until the 5’ end of the newly synthesized negative DNA strand (Staprans et al., 1991). Since there is a short terminal redundancy (r) on this strand, the

5’r is also copied. By circularization of the genome another template switch is achieved (Lien et al., 1986). The newly synthesized DNA is transferred to the 3’r on the negative strand and further elongated until synthesis stops for yet unknown reasons after around 1000-2000 bases.

1.1.7 Proteins

In total 7 proteins are expressed from the four open reading frames located on the minus strand of the viral genome. They are both transcribed and translated by the cellular machinery. The X protein and the surface proteins are translated from subgenomic RNAs while the core protein and the polymerase trace back to pgRNA. The E protein is derived from another RNA which is longer than the encapsidated one.

1.1.7.1 Polymerase

The open reading frame for the viral polymerase (P) covers more than 75% of the HBV genome. The protein is expressed from the pgRNA and is the largest protein the virus codes for (~ 90 kDa). It serves different functions and consists of several domains: the terminal protein (TP), a spacer domain, a reverse transcriptase (RT) and an RNase H domain. In virions, the TP domain is covalently bound to the 5’ end of the negative strand of the viral genome. Binding to the epsilon structure on pgRNA primes synthesis of the negative strand. So far this domain is unique in its sequence since no similarities to other proteins could be found. TP is separated from the RT domain by a very flexible and largely dispensable spacer (Radziwill et al., 1990). The RT domain performs both the generation of minus strand DNA from pgRNA by reverse transcription and synthesis of the plus strand by its DNA-dependent DNA polymerase function. Furthermore it is assumed to be involved in conversion of rcDNA to cccDNA by completing the positive strand of the genome (Köck et al., 2003). The RNase H domain is responsible for degrading the pgRNA during its reverse transcription into DNA.

1.1.7.2 X protein

The HBV X protein is the smallest of the 7 viral proteins with a molecular weight of 17 kDa. Translation occurs from the 0.9 kb subgenomic RNA produced upon transcription of the X ORF. In contrast to the orthohepadnaviruses, avihepadnaviruses lack this protein. The role of X is not fully elucidated yet. It plays a role in hepatocarcinogenesis (reviewed by Kew, 2011) since it promotes cell cycle progression and inactivates or downregulates tumour suppressor genes like p53. Furthermore it modulates cellular signal pathways such as the *Jak-Stat*

pathway. X also exerts regulatory functions in the viral life cycle. It is absolutely required to initiate and maintain viral replication upon infection (Lucifora et al., 2011).

1.1.7.3 Core protein

Translation of the core open reading frame from the pregenomic RNA yields a protein with a length of 185 amino acids and a molecular weight of 21 kDa in HBV genotype A. The arginine-rich C terminus of the protein is important for pregenome encapsidation and synthesis of the genomic positive DNA strand (Nassal, 1992) but not for assembly of the capsid (Gallina et al., 1989). For assembly of the capsid, the N-terminal 140 amino acids are sufficient (Birnbaum and Nassal, 1990). Structure and fold of the N-terminal 140 amino acids of the core protein have been resolved by cryo-electron microscopy (Conway et al., 1997; Böttcher et al., 1997) and crystallization (Wynne et al., 1999). It contains five α-helices two of which form a prominent hairpin structure (α3 and α4) (Fig. 4A). The loop connecting these two α-helices holds the major epitope of the capsid (Salfeld et al., 1989). Core proteins self-assemble into dimers linked by a disulfide bridge between the cysteines at position 61 (Nassal et al., 1992; Zheng et al., 1992) (Fig. 4B). These homodimers present the building blocks of the capsid (Zhou and Standring, 1992). In the dimer the α-helical hairpins of two monomers form a four-helix-bundle which appears as a spike on the capsid surface. The structure of the dimer reminds of a pickaxe, the shaft being the four-helix-bundle and the basal region forming the head.

1.1.7.3.1 ***The nucleocapsid***

The icosahedral shell of the HBV nucleocapsid (Fig. 4C) is built by 180 or 240 copies of the core protein resulting in a triangularity of 3 and 4, respectively. Both capsids produced in *E. coli* and capsids isolated from HBV carriers, show mainly the T=4 symmetry while T=3 capsids form the minor species (Roseman et al., 2005). In the infected cell, encapsidation is initiated by binding of the polymerase to the pgRNA (Hirsch et al., 1990; Bartenschlager and Schaller, 1992). The first step in capsid assembly is dimerization of the core monomers to dimers which are the building block of the capsid (Zhou and Standring, 1992). The dimers are further oligomerized to yield the icosahedral capsid. Crystallization of the capsid showed that it is fenestrated containing pores of between 12 and 15 Å (Wynne et al., 1999) which are large enough to enable entry of nucleotides during genome synthesis.

1.1.7.4 E protein

The precore or E protein shares 149 amino acids of sequence with the core protein but has different N- and C-termini. It is translated from the pregenomic RNA containing the additional start codon and gives rise to a protein of initially 25 kDa. It contains an N-terminal signal peptide which the core protein lacks. This signal leads the protein to the secretory pathway of the cell (Ou et al., 1986; Bruss and Gerlich, 1988) and is cleaved off by a signal peptidase. After further processing of the C terminus, the secreted protein has a molecular weight of around 17 kDa. It is an important marker for the diagnosis of HBV infection.

1.1.7.5 Surface proteins

The small, middle and large surface proteins are encoded by a single open reading frame making use of three different start codons and a common stop codon (Heermann et al., 1984). Therefore all three surface proteins share the same C-terminal domain which is referred to as the S domain. The preS1 and preS2 sequences are located upstream of the S domain. Transcription is regulated by a promoter upstream of the ORF (2.4 kb sgRNA) and another one located internally and upstream of the start codon of the middle surface protein (Cattaneo et al., 1983) which initiates generation of the 2.1 kb sgRNA. Translation of the small and middle surface proteins occurs from the 2.1 kb subgenomic RNA by the use of different start codons while the large surface protein is expressed from the 2.4 kb sgRNA. Expression of different amounts of the single surface proteins is due to preferential translational initiation (Sheu and Lo, 1992).

The protein consisting of only the S domain is called the small surface protein (S). It is 226 amino acids long and has a molecular weight of 24 kDa. The middle surface protein (M) contains both the S and preS2 domains and is N-terminally 55 amino acids extended compared to the S protein. The molecular weight of unglycosylated M is 30 kDa. The third surface protein is referred to as the large surface protein and it is the longest of the three surface proteins since it consists of preS1, preS2 and the S domain. With in total 400 amino acids (subtype adw) it is 119 amino acids longer than M and 174 amino acids longer than S. In its unglycosylated form it has a molecular weight of 39 kDa.

All of the surface proteins can be glycosylated at asparagine 146 (Peterson et al., 1982) of the S domain and M additionally at asparagine 4 of the preS2 domain (Stibbe and Gerlich, 1983). Therefore the surface proteins appear in different molecular weights. The S protein is glycosylated at asparagine 146 to an extent of around 50 % and displays molecular weights of 24 kDa (unglycosylated) and 27 kDa (glycosylated). Because of its second glycosylation site

at asparagine 4 of the preS2 domain, the M protein can be found in 3 different forms: An unglycosylated form of 30 kDa, a single-glycosylated form (at asparagine 4) of 33 kDA and a double-glycosylated form of 36 kDa. The most prominent form is the 33 kDa protein. Unglycosylated M proteins are neither found in virions nor in subviral particles. Cotranslational glycosylation of the large surface protein occurs only at asparagine 146 and leads to a molecular weight of 42 kDa.

1.1.7.5.1 ***Transmembrane topology of the surface proteins***

L, M and S are synthesized at the ER and cotranslationally inserted into the ER membrane (Eble et al., 1986; Eble et al., 1990). The transmembrane topology of the small and the middle proteins depends totally on transmembrane domains of the S domain while the topology of the large surface protein is more complex (see Fig. 5).

The S protein contains an N-terminal hydrophobic signal I sequence (transmembrane domain I; TM1) between amino acids 8 and 22 which initiates the insertion into the ER membrane. Between amino acids 80 and 98 there is another signal which is a type II signal (transmembrane domain II; TM2) and anchors the protein in the ER membrane. It leads to translocation of the downstream peptide chain to the lumen of the ER while retaining the upstream part of the protein in the cytosol. Furthermore, the C terminal region of S is very hydrophobic and probably contains another two transmembrane domains (TM3/4). It is therefore assumed to be embedded in the ER membrane with the C terminus looking to the ER lumen. In this way a cytosolic loop between TM1 and TM2 and a luminal loop between TM2 and TM3/4 are formed. The luminal loop carries both the glycosylation site at asparagine 146 as well as the main immunogenic epitope.

The M protein shows identical topology like the small surface protein. The preS2 domain is located on the luminal side of the membrane probably because of the action of the signal I in the S domain (Eble et al., 1990). This topology allows the usage of the second glycosylation site at asparagine 4 which is only modified in context of the middle surface protein.

In contrast to M and S, the N terminus (preS1 and preS2) of the large surface protein is initially located on the cytosolic side of the ER and therefore looking to the inside of the virion (internal preS; i-preS) (Bruss et al., 1994). This is the reason for the partial glycosylation of asparagine 146 in the S domain but not of asparagine 4 located in preS2. Interestingly around 50 % of the L molecules in secreted virions exhibit a contrary topology with the preS1 and preS2 domains located outside of the virions (external preS; e-preS) (Bruss et al., 1994, Ostapchuk et al., 1994, Prange and Streek, 1995). The translocation

process is not fully understood yet. Initially, the heat shock proteins Hsc70 and Hsp40 block the cotranslational translocation of the preS domains by binding to preS1 (Lambert and Prange, 2003). The process of the preS domains crossing the ER membrane depends on the action of the ER chaperone BiP (Awe et al., 2008). By the dual topology of L, the protein is able to fulfil different functions. The i-preS form is assumed to contact the capsid during budding while the e-preS form is important for attachment and entry of the virus. By electron cryo-microscopy, Seitz and colleagues discovered two morphologically distinct phenotypes of virions isolated from chronic HBV carriers: a compact form in which the border between envelope and capsid was hardly recognizable, and gapped particles which show a clear border between these two structures. They hypothesize that the preS domains of L in compact particles might look to the inside of virions while in gapped particles they could be on the outside. Partially gapped particles might be in the process of switching preS to the outside. Interestingly, they furthermore found that highly infectious particles were nearly exclusively gapped (Seitz et al., 2007). The myristoylation at glycine 2 of L (Persing et al., 1987) is assumed to be inserted into the ER membrane. It is dispensable for assembly but absolutely necessary for entry into cells (Macrae et al., 1991).

1.1.7.5.2 ***Subviral particle formation***

Besides virions, infected cells produce an abundant amount of non-infectious subviral particles lacking the nucleocapsid. It has been shown that transfection of cultured cells with plasmids coding for the S protein alone results in secretion of 22-nm spherical SVP which are morphologically and antigenically indistinguishable from those found in serum (Dubois et al., 1980; Moriarty et al., 1981). Therefore, neither M nor L is required for subviral particle formation in cell culture. Subviral particles appear in two different conformations (spherical, filamentous) depending on the L protein quantity and the S / L ratio in the particle. A large proportion of L favours the formation of filamentous SVP (Heermann et al., 1984). Formation of subviral particles takes place in a post-ER pre-Golgi compartment (Huovila et al., 1992). This does not reflect virion formation which depends on the cell machinery responsible for formation of multivesicular bodies (MVB) since the usage of MVB inhibitors led to the selective impairance of virion secretion but did not have an effect on the release of subviral particles (Lambert et al., 2007).

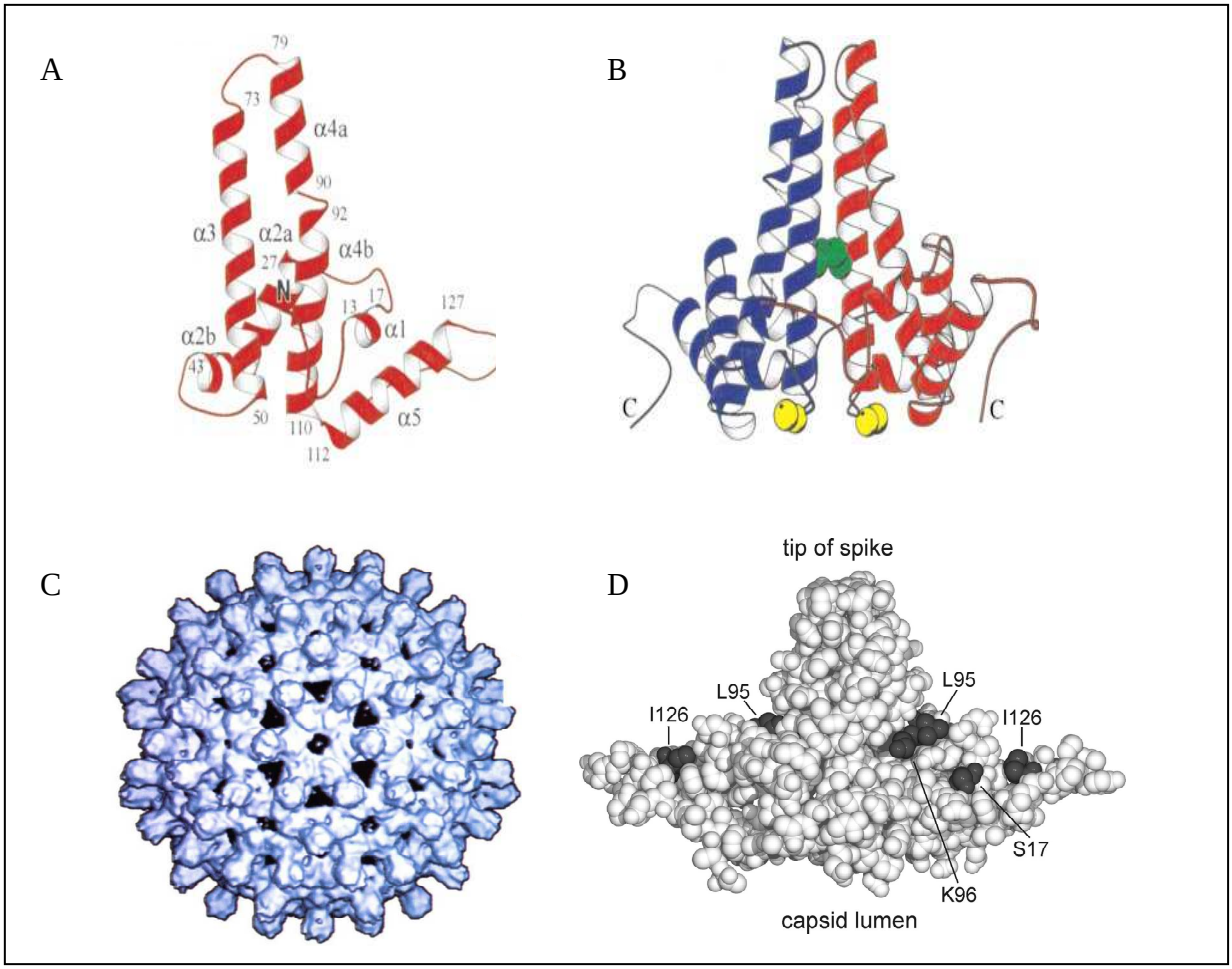

Fig. 4. Structure of the core protein monomer (A), dimer (B) and capsid (C), and positions of residues in the core homodimer that are important for assembly (D). (A) Ribbon diagram of the core monomer with the dimer interface looking to the viewer. The protein is largely α-helical and contains a prominent hairpin structure consisting of the α-helices 3 and 4 (from Wynne et al., 1999). (B) The core protein dimer with the disulfide bridge between the cysteines 61 (green spheres) contains a 4-helix-bundle made up of the two opposite hairpins of the monomers. Yellow spheres present the cysteines at position 48 which do not form disulfide bonds (from Wynne et al., 1999). (C) Cryo-electron micrograph of a T=4 capsid. The spikes are built up of the 4-helix-bundles of core homodimers (from Conway et al., 1997). (D) Amino acid residues important for capsid envelopment (black spheres) are shown in a sphere model of the core homodimer (from Pairan and Bruss, 2009).

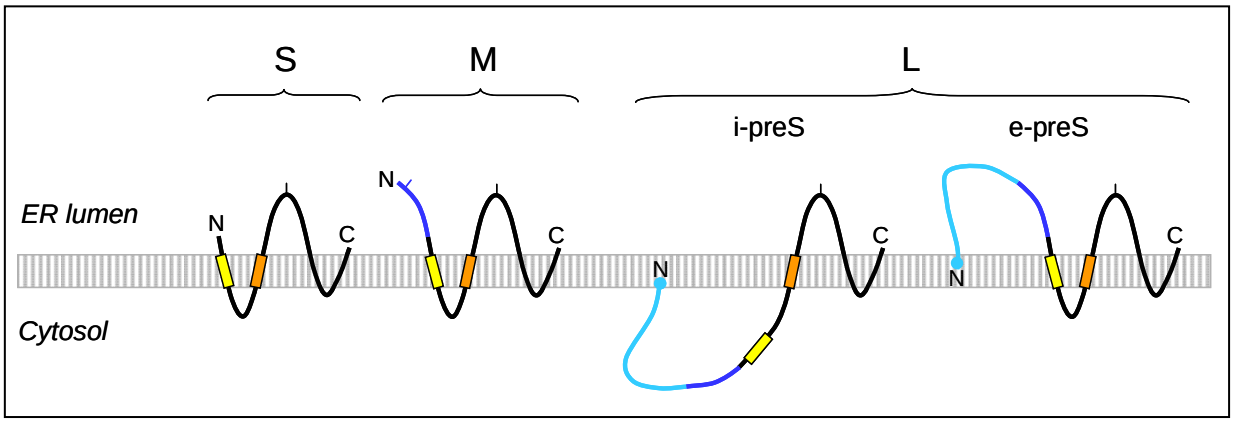

Fig. 5. Transmembrane topology of the HBV surface proteins. The S protein (black line) containing TM1 (yellow barrel) and TM2 (orange barrel), the M protein consisting of S and the preS2 domain (dark blue line) and the L protein with its additional preS1 domain (cyan line) are shown in their assumed transmembrane topologies. Black and dark blue bars indicate glycosylation sites, the cyan dot the myristoylation of L (figure modified from Bruss, 2007).

1.1.8 Life Cycle

HBV is a hepatotropic virus with very narrow host specificity. Besides men, only some primates like chimpanzees are susceptible to HBV infection. Therefore, primary human hepatocytes (PHH) and primary liver cell cultures of tree shrews (Tupaia belangeri) were the only cell culture systems that could be used for infection studies for a long time. In 2002 a new cell line called HepaRG was established which supported HBV infection comparable to PHH (Gripon et al., 2002). Despite this advancement, the receptor necessary for HBV entry into the host cell could not be identified yet. For duck hepatitis B virus, the surface glycoprotein duck carboxypeptidase D was shown to be necessary for entry (Kuroki et al., 1994; Breiner et al., 1998).

HBV entry depends on L. The first 75 amino acids of the protein are important while the matrix domain is dispensable (Blanchet and Sureau, 2007). Furthermore, the myristoylation at glycine 2 of L is essential for infectivity (Macrae et al., 1991; Bruss et al., 1996; Gripon et al., 1995) as well as hydrophobic clusters in transmembrane domain I (Lepère-Douard et al., 2009). Interaction of the preS domains with phospholipid vesicles gave further hints to the involvement of preS in the fusion with the host cell membranes (Nunez et al., 2009).

After entering the cell, the capsid is transported through the cytoplasm to the nucleus. This is accomplished by a nuclear localization signal which is located at the C terminus of the core protein (Eckhardt et al., 1991). Kann and colleagues could show that phosphorylation of the capsid is necessary to induce exposure of the nuclear localization on the capsid surface and thereby allow the capsid to bind to the nuclear pore complexes (NPCs) (Kann et al., 1999). The whole nucleocapsids are transported into the nuclear baskets where they are disassembled and the viral genome enters the nucleus. Immature capsids can also reach the nuclear baskets but cannot release the immature genomes they are containing into the nucleoplasm (Rabe et al., 2003).

The rcDNA in the nucleus is converted to cccDNA by probably both viral and cellular enzymes and persists in episomal form as a nucleosome (Bock et al., 1994). It can also integrate in the host genome which is not necessary for replication but helps in persistence of the virus in the cell. This was found to happen in hepatocellular carcinoma cells (Brechot et al., 1980) and could be an important mechanism in HBV associated carcinogenesis.

The cccDNA is transcribed by RNA polymerase II into pre- and subgenomic RNAs (see 1.1.5) which are transported to the cytosol. Binding of the polymerase to the epsilon structure of the pgRNA initiates capsid assembly and synthesis of the viral genome. Immature capsids are generated that mature during reverse transcription of the pgRNA. The newly formed

capsids can either recirculate to the nucleus to produce more cccDNA (Wu et al., 1990) or be enveloped by the HBV surface proteins. These processes are regulated by the L protein. In early stages of infection there are low levels of L and therefore delivery of the capsid to the nucleus is favoured. In later stages more L is available and the capsids are mainly enveloped (Lenhoff et al., 1990; Summers et al., 1990).

The matrix domain of L is essential for virus assembly probably by directly contacting the capsid and thus serving the function of a matrix protein. It could be shown that a peptide corresponding to the MD could efficiently bind the capsid (Poisson et al., 1997). Furthermore a core mutant which enveloped immature capsids could be rescued by an additional MD point mutation (Le Pogam and Shih, 2002). The minimal distance between MD and TM1 was determined to be 26 amino acids which would fit well to the length of the capsid spike (Kluge et al., 2005). All these results suggest that a direct interaction between capsid and large surface protein takes place which is essential for assembly and budding.

Furthermore it could be shown that both capsid and L bind to γ2-adaptin (Hartmann-Stühler and Prange, 2001; Rost et al., 2006) possibly recruiting the capsid to the budding site and mediating contact with the HBV surface proteins. It is still not clear where budding of virions happens but there are hints that multivesicular bodies are involved since mutants of the ESCRT-complex (endosomal sorting complex required for transport) inhibit virion secretion (Lambert et al., 2007). Subviral particles assemble in a post-ER / pre-Golgi compartment called ERGIC (ER-Golgi intermediate compartment) (Huovila et al., 1992) and are not affected by ESCRT mutants (Lambert et al., 2007).

1.2 Hepatitis B virus morphogenesis

1.2.1 Role of the capsid in viral assembly

While capsids in all maturation stages can be found in infected cells, only mature capsids are found in secreted virions. Immature capsids containing pgRNA cannot be enveloped. A mutant defective in the RNase H function of the viral polymerase allowing production of negative sense DNA by reverse transcription but not formation of dsDNA was still able to secrete virions (Gerelsaikhan et al., 1996). This led to the assumption that minus DNA synthesis changes the nucleocapsid and leads to the appearance of a signal on the capsid that is necessary for envelopment. How this signal looks like is still unclear. Electron-microscopical images of RNA and DNA containing capsids showed some differences

(Roseman et al., 2005) and also the phosphorylation status of the capsid might play a role (Perlman et al., 2005).

Furthermore, several residues located on the capsid surface could be identified which play a specific role in HBV assembly and are referred to as matrix binding domain (MBD). Point mutants of this domain could still form capsids but capsid envelopment was blocked (Ponsel and Bruss, 2003; Pairan and Bruss, 2009). These residues were not located at the tip of the capsid spikes but rather at the base of the spike and in a lateral region of the capsid dimer base forming a ring-like groove (Fig. 4D). Among them are for example the serine at position 17, the lysine 96 and isoleucine 126. Interestingly, K96 was also shown to be essential for recognition of γ2-adaptin (Rost et al., 2006), a protein important in the ESCRT-mediated multivesicular body (MVB) / lysosome sorting pathway (Rost et al., 2008; Döring et al., 2010). Furthermore, the capsid contains the late domain motif PPAY (amino acids 129-132) exposed on the capsid surface which is known from several enveloped viruses like HIV to function in virus budding (Freed, 2002).

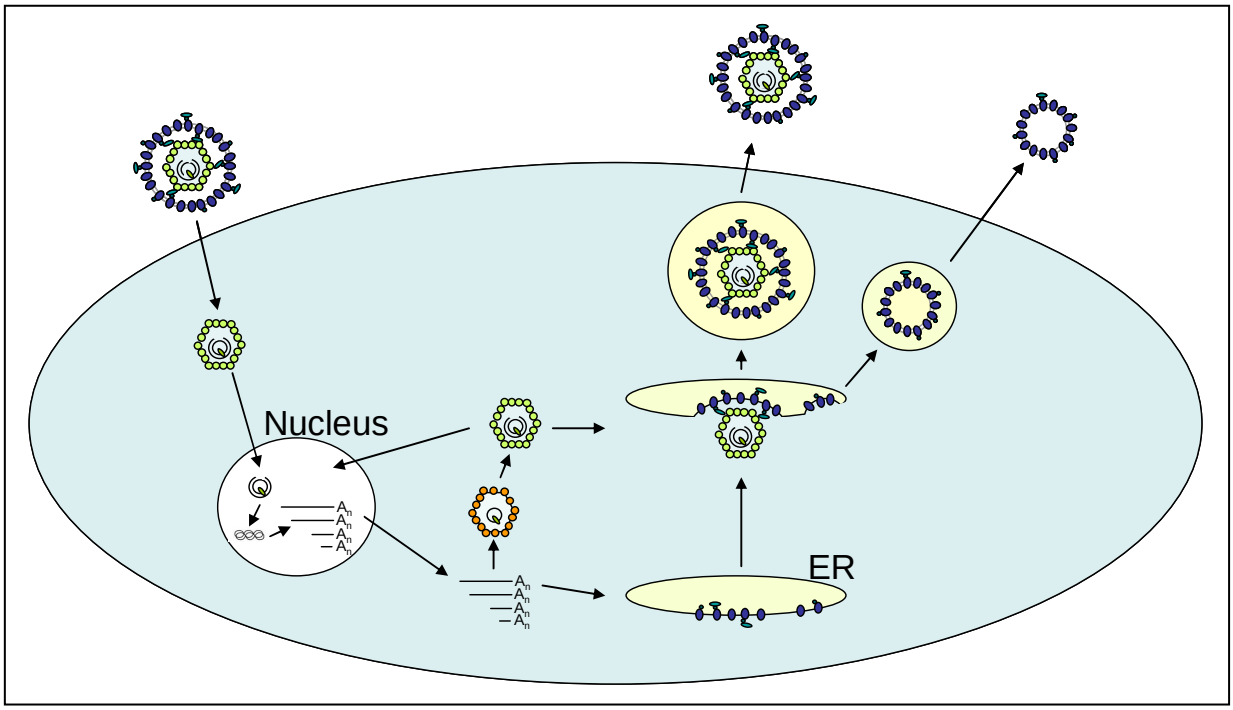

Fig. 6. Life cycle of HBV. The virus enters the cell by receptor-mediated endocytosis and the capsid is transported to the nucleus where uncoating occurs. The rcDNA is converted to cccDNA and transcripts are made which are transported to the cytoplasm. Capsids are formed and either shuttled back to the nucleus to fill up the pool of cccDNA or enveloped by the HBV surface proteins at a yet unknown budding site. Besides virions also subviral particles are secreted from the cells.

1.2.2 Role of the surface proteins in assembly

HBV capsids are not released from infected cells without the expression of surface proteins highlighting their importance for particle morphogenesis. Bruss and Ganem could show that production of infectious virions requires S and L but not M proteins (Bruss and Ganem, 1991). In line with these data, HBV variants defective in M production could be isolated from chronic carriers. Further characterization in hepatoblastoma cells showed that they were replication competent and secreted morphologically normal virions which were able to infect primary hepatocytes (Fernholz et al., 1993). Therefore, M is dispensable for budding and assembly even though it enhances the efficiency of virion secretion if present (Bruss and Ganem, 1991; Fernholz et al., 1993; Garcia et al., 2009). In contrast, the S protein in the absence of any other viral protein is sufficient to drive the formation and secretion of subviral particles. Therefore it seems to play a role in the budding process itself. Nevertheless it does not suffice for Dane particle morphogenesis and Blanchet and Sureau could show that small surface protein residues located on the cytosolic face of the ER membrane do not play a specific role in virion envelopment and secretion (Blanchet and Sureau, 2006). Production of infectious particles is therefore strictly dependent on L, notably the i-preS form. By fusing a secretion signal to the N terminus of L and in this way forcing expression of exclusively the e-preS form of the protein, Bruss and Vieluf could show that preS domains looking into the cytosol are essential for recruitment of mature capsids (Bruss and Vieluf, 1995). Interestingly, overexpression of L inhibits virion release as well as SVP release (Persing et al., 1986; Bruss and Ganem, 1991) due to retention motifs in the preS1 domain (Kuroki et al., 1989; Gallina et al., 1995; Prange et al., 1991). Mutational analyses of the L protein narrowed down the region important for HBV assembly to 22 amino acids containing the 17 C-terminal amino acids of preS1 as well as the 5 N-terminal amino acids of preS2: Characterization of a series of N-terminally truncated L mutants showed that the first 102 amino acids of the preS1 domain were dispensable for virion formation (Bruss and Thomssen, 1991). The investigation of a panel of mutants with linker substitutions demonstrated that constructs between asparagine 98 and threonine 125 did not envelope nucleocapsids (Bruss, 1997). In contrast, mutants downstream of serine 124 did support this process. Further analyses with double-alanine mutants between asparagine 98 and asparagine 123 (Bruss, 1997) and analyses of mutants of the preS2 domain which showed that a mutant lacking the first five amino acids did not support secretion of virions while downstream removal of amino acids was tolerated (Le Seyec et al., 1998) confirmed the location of the so-called matrix domain (MD) which was mapped from arginine 103 to serine 124. It is hypothesized to make contact with the MBD on

capsids thus mediating budding. The MD is conserved in WHV and the preS sequence of this orthohepadnavirus can substitute for the HBV preS in virion assembly, while avihepadnavirus DHBV-preS cannot, indicating relatively precise interactions (Gerhardt and Bruss, 1994). The preS2 domain of L seems to serve as a spacer for assembly (Kluge et al., 2005; Ni et al., 2010). Glycosylation of the envelope proteins also seems to be critical for virion secretion since mutants of the conventional glycosylation site at asparagine 146 were defective in virion secretion (Ito et al., 2010). Restoration of glycosylation although at a different position rescued these mutants.

Notably, the large surface protein can furthermore interact with γ2-adaptin (Hartmann-Stühler and Prange, 2001) which functions in the ESCRT (endosomal sorting complex required for transport) mediated multivesicular body (MVB) sorting parthway (Rost et al., 2008; Döring et al., 2010). Other components of this pathway which is important for the budding of many enveloped viruses have also been shown to be important for HBV assembly (Lambert et al., 2007). Since γ2-adaptin binds to both L and the capsid, it possibly links the two viral subunits for budding.

1.3 Scientific aims of the thesis

The assembly process of hepatitis B virus is far from being elucidated yet. The large surface protein was shown to be essential for virion formation and extensive mutational analyses identified specific residues important for this process. They are located in the so-called matrix domain ranging from amino acid 103 to 124 (HBV adw subtype) and changes of residues in this region specifically blocked capsid envelopment and virion secretion (reviewed by Bruss, 2007).

This work aimed to elucidate the function of the matrix domain in HBV assembly. Which residues are essential and how variable are they? Is it possible to substitute single residues or is the function of the protein strictly depending on the specific WT amico acid? To characterize the matrix domain in more detail, it was planned to produce a panel of point mutants and test them for virion secretion. Eleven of the 22 matrix domain residues should be exchanged against the 19 non-WT amino acids of the respective positions by site-directed mutagenesis and then be tested for virion secretion upon *in trans* complementation of WT capsid, polymerase and X protein in cell culture.

Since the endogenous polymerase reaction which was usually employed to detect virions secreted by cells makes use of radioactive substances and is therefore difficult to handle a new detection method should be established. It should rely on the quantitation of HBV genomic

DNA purified from virions by an HBV genome specific quantitative RT-PCR and allow discrimination of virions from naked capsids.

Furthermore it was planned to test if this new method could be used to solve another yet unsolved question: Does the matrix domain directly interact with the nucleocapsid of the virus and in this way initiate budding? There are many hints in favour of this assembly model. Peptides derived from the preS domains efficiently bound to purified HBV core particles (Poisson et al., 1997). Furthermore a core protein mutation leading to envelopment of immature capsids could be suppressed by a matrix domain point mutation (Le Pogam and Shih, 2002). Last but not least, the so-called matrix binding domain on the core protein could be identified which contains residues which specifically block capsid envelopment upon mutagenesis but not capsid production and could therefore present the binding partner of the matrix domain (Ponsel and Bruss, 2003; Pairan and Bruss, 2009). Identification of a MD mutant which could compensate for an envelopment-negative core mutant would strongly argue for a direct interaction between the matrix domain and the capsid.

2 Materials and methods

2.1 Materials

2.1.1 Eukaryotic cell line

- HuH7

HuH7 is a human liver cancer cell line which was derived from the liver tumor of a 57-year-old Japanese (Nakabayashi et al., 1982). HuH7 are adherent cells and not susceptible to infection with HBV. But upon transfection with the HBV genome they produce viral proteins and secrete virions into the supernatant (SN).

2.1.2 Cell culture media

Product	*Company*	*Stock concentration*	*Working concentration*
DMEM	Lonza		
Fetal bovine serum (FBS)	Sigma	100 %	10 %
MEM Non essential amino acids (NEAA)	PAA	100x	1x
Penicillin-Streptomycin (PS)	PAA	100x	1x
Sodium pyruvate	PAA	100 mM	1 mM
Trypsin/EDTA	BiochromAG	10x	1x

Growth medium used for propagation of cells in cell culture consisted of DMEM, 10 % FBS, 1x NEAA, 1x PS and 1 mM sodium pyruvate.

2.1.3 Bacteria

- *E. coli* BMH 71-18 *MutS* bacterial strain

E. coli strain *MutS* cells (part of the Clontech TransformerTM Site-directed Mutagenesis Kit) were used for the first transformation during the Clontech site-directed mutagenesis protocol. They are deficient in DNA mismatch repair and therefore produce colonies of two morphologies upon transformation with hybrid plasmids.

Genotype: *supE thi* Δ *(lac -proAB) [mutS::*Tn10*] [F' proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15*]*

- *E. coli* DH10B

DH10B is a common *E. coli* laboratory strain and was used for transformation of newly constructed plasmids and for retransformation.

Genotype: *F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG*

2.1.4 Bacterial media and antibiotics

Product	*Company*	*Stock concentration*	*Working concentration*
LB medium	Roth		
TB medium	Roth		
LB Agar	Roth		
Ampicillin	Sigma	100 mg/ml	100 µg/ml
Tetracyclin	Sigma	5 mg/ml	50 µg/ml

2.1.5 Plasmids

2.1.5.1 pSVHBV1.1LE-

Plasmid pSVHBV1.1LE- contains the entire HBV genome of the HBV genotype A, HBsAg subtype adw2 (Valenzuela et al., 1980) with 2 stop codons in the ORF of the surface proteins and a sequence redundancy of 164 bp (see Fig. 7). All capsid mutants were produced in the background of this vector. It was constructed from pSVHBV1.5LE- by removing the last 336 of 500 bp redundant sequence to allow efficient detection of HBV genomes and discrimination from plasmid in the HBV specific qPCR. Expression of the genome is regulated by a SV40 promoter. For selection in *E. coli* bacteria, the β-lactamase gene located on the backbone of the plasmid was used.

2.1.5.2 pSV45-57

Lacking the first 30 codons of the HBV large envelope protein gene this vector codes for all three surface proteins which are under control of a SV40 promoter (Fig. 8). The shortened L sequence allows more efficient secretion of viral particles. All L protein mutants were constructed in this plasmid. The vector backbone also carries an ampicillin resistance for selection in bacteria.

2.1.5.3 pSVBX24H (Gerhardt and Bruss, 1995)

The plasmid contains the ORF for the HBV small surface protein under control of the SV40 promoter. For selection in *E. coli* the β-lactamase gene located on the vector backbone was used (Fig. 8).

2.1.6 Primers

All primers used during this work can be found in the appendix.

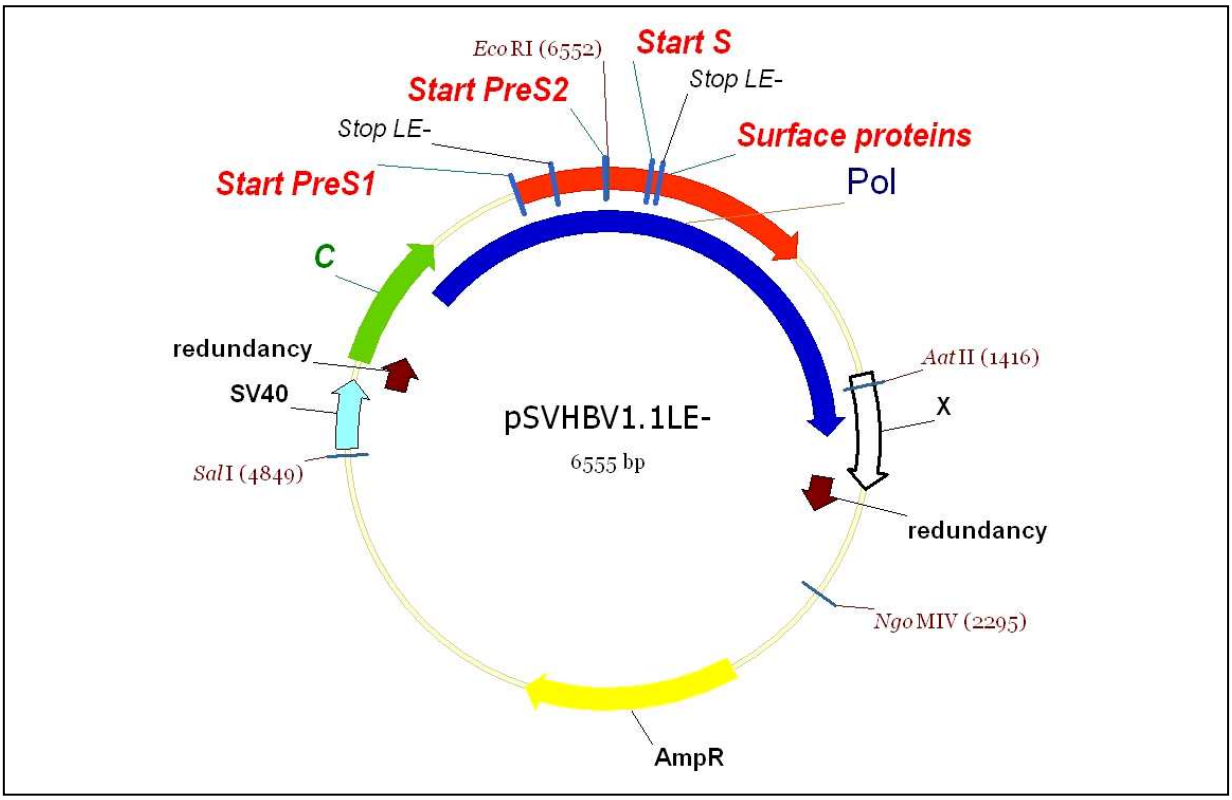

Fig. 7. Scheme of plasmid pSVHBV1.1LE-. This vector was derived from plasmid pSVHBV1.5LE- and carries the entire HBV genome with 2 stop codons in the preS1 and S domains which renders the envelope proteins ORF non-functional (polymerase ORF not affected). Transfection of cells with this plasmid yields only capsids and no virions.

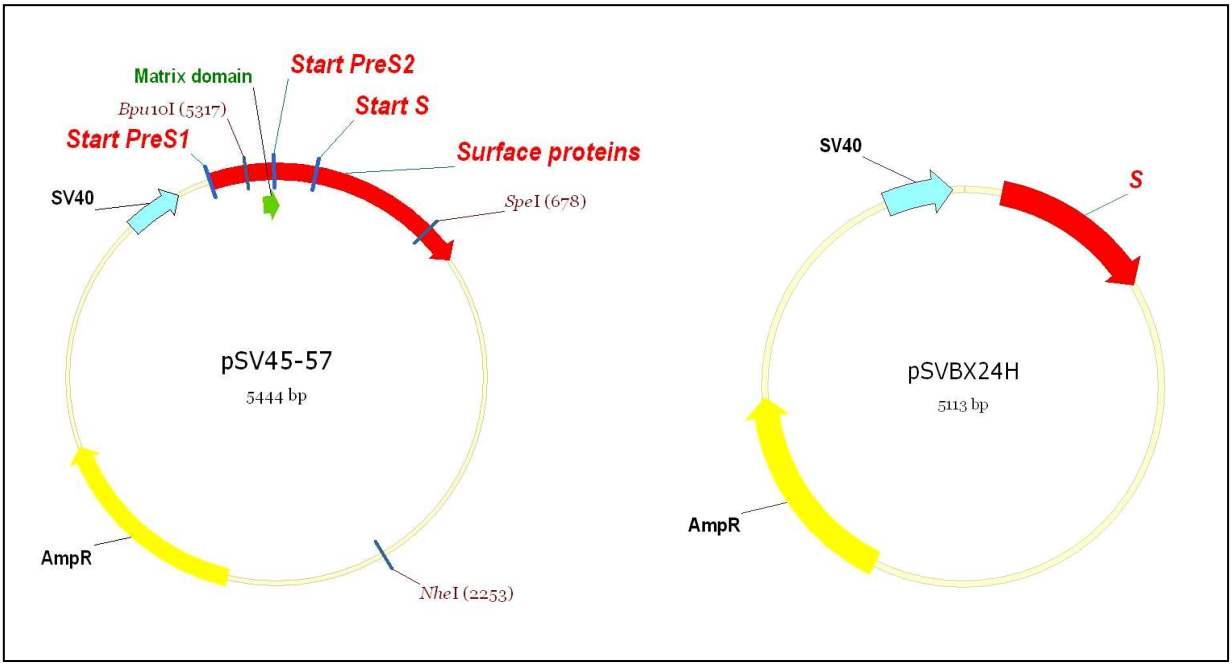

Fig. 8. Schematic drawings of plasmids pSV45-57 and pSVBX24H. These vectors contain the open reading frames for all three HBV surface proteins and the small surface protein alone, respectively. They are used in concert with plasmid pSVHBV1.1LE- for production of virions upon transfection in HUH7 cells.

2.1.7 Enzymes

Enzyme	*Company*	*Concentration*	*Buffer*
T4 polynucleotide kinase	Fermentas	10 U/µl	Buffer A
T4 DNA ligase	Fermentas	5 WeissU/µl	T4 DNA ligase buffer
T4 DNA polymerase	Fermentas	5 U/µl	10x Synthesis buffer
DNaseI	Qiagen	2.7 KunitzU/µl	RDD buffer
Proteinase K	Applichem	10 mg/ml	2x proteinase K buffer
RNaseA	Qiagen	7 U/µl	no buffer used

Restriction enzymes:

Enzyme	*Company*	*Concentration*	*Buffer*
NheI-HF	New England Biolabs	20 U/µl	NEB4
NheI	New England Biolabs	10 U/µl	NEB2 + BSA
SalI-HF	New England Biolabs	20 U/µl	NEB4
SalI	New England Biolabs	100 U/µl	MEB3 + BSA
AatII	New England Biolabs	20 U/µl	NEB4
NgoMIV	New England Biolabs	10 U/µl	NEB4
BsaI	New England Biolabs	10 U/µl	NEB4+BSA
SpeI	New England Biolabs	10 U/µl	NEB4+BSA
Bpu10I	New England Biolabs	5 U/µl	NEB3
EcoRI-HF	New England Biolabs	20 U/µl	NEB4

2.1.8 Antibodies

Antibody	*Application*	*Description*	*Origin*	*Provided by*
HB1	Primary antibody Western blot	Monclonal IgG antibody, anti-HBs (epitope aa 120-125)	Mouse	D. Glebe, Gießen
Anti-mouse-PO	Secondary antibody Western blot	Horseradish peroxidase conjugated anti-mouse antibody	Goat	Dianova, Hamburg
sheep-anti-S	Immunoprecipitation	Polyclonal serum, anti-HBs	Sheep	W. Gerlich, Gießen

2.1.9 DNA and protein markers

DNA markers used in this work were the Lambda DNA/Eco91I (BstEII) Marker, 15 (Fermentas) and the GeneRuler^{TM} 100 bp DNA ladder (Fermentas). The first one was used for expected bands between 1000 and 6000 bp, the latter for bands smaller than 1000 bp. Per lane 0.75 µg marker were applied onto the gel.

For SDS-PAGE, PageRuler^{TM} Plus Prestained Protein Ladder (Fermentas) was employed. Onto a large gel 10 µl of this ready-to-use mixture of 9 recombinant proteins of specific molecular weight were loaded.

Marker	*Company*	*Bands*
Lambda DNA/Eco91I (BstEII) Marker, 15	Fermentas	8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702 bp
GeneRuler^{TM} 100 bp DNA ladder	Fermentas	1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp
PageRuler^{TM} Plus Prestained Protein Ladder	Fermentas	~250, ~130, ~100, ~70, ~55, ~35, ~25, ~15, ~10 kDa

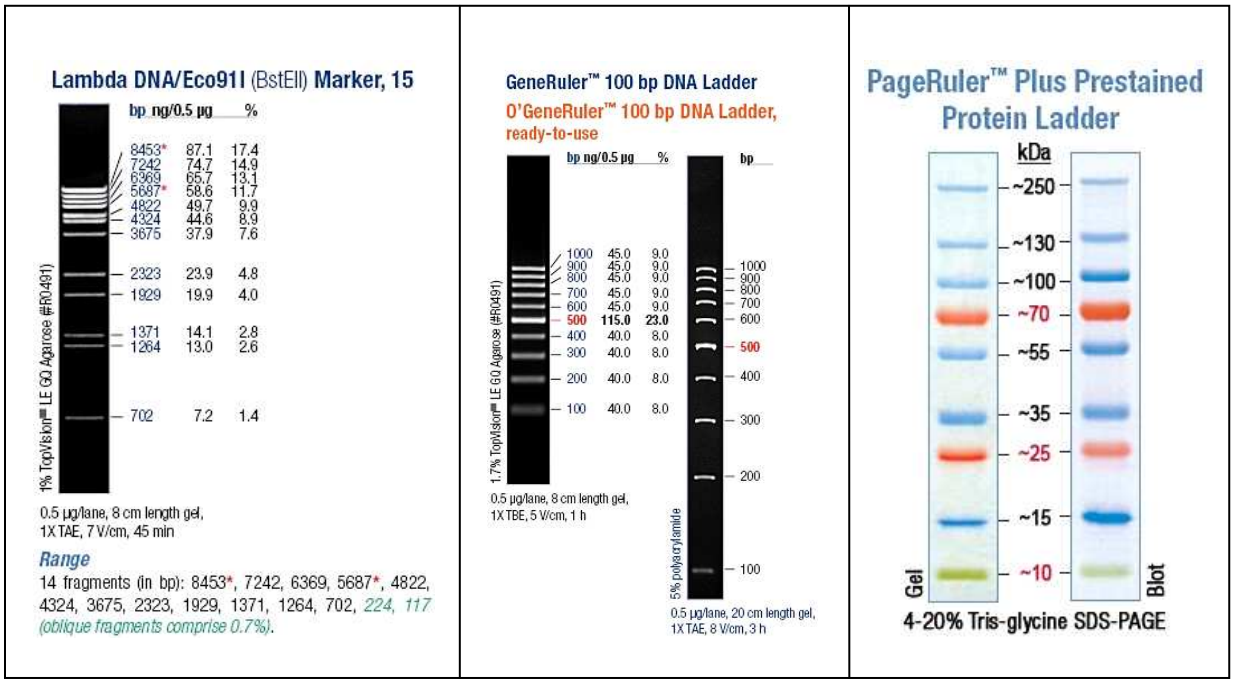

Fig. 9. Markers used for agarose gel elctrophoresis and SDS-PAGE.

2.1.10 Kits

Kit	*Company*
TransformerTM Site-directed Mutagenesis Kit	Clontech
QIAGEN PCR Purification Kit	Qiagen
QIAQuick Gel Extraction Kit	Qiagen
QIAGEN Plasmid Midi kit	Qiagen
QIAGEN Plasmid Maxi kit	Qiagen
Rnase-free Dnase Set	Qiagen
QIAamp DNA Mini Kit	Qiagen
PCR Master	Thermo Scientific
PCR Master	Roche
PCR Master	Promega
SYBRGreen I Master	Roche
Lumi-light Western Blotting Substrate	Roche
Fugene 6 / HD / Extreme	Roche
Protein G PLUS Agarose	Santa Cruz Biotechnologies

2.1.11 Chemicals

Chemical	*Company*
30 % Acrylamide mix	National diagnostics
Adenosine-5'-triphosphate (ATP)	New England Biolabs
Agarose	Biozym
Ammonium persulfate (APS)	Sigma
Bromophenol blue	Sigma
Butanol	Roth
Developer A	Agfa Healthcare
Developer B	Agfa Healthcare
Desoxyribonucleotides (dNTPs)	Promega
Dimethyl Sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Ethanol	Roth
Ethidiumbromide	Roth
Ethylenediaminetetraacetic Acid (EDTA)	Sigma
Glucose	Merck
Glycerol	Applichem
Glycine	Merck
Glycogen	Fermentas
HCl	Sigma
Isopropanol	Roth
KH2PO4	Merck
Lithium chloride	Merck
Methanol	Sigma
Magnesium chloride	Merck
Na2HPO4	Merck
NaCl	Simga
NaOH	Merck
Nonidet P40	Fluka
PEG 6000	Merck
Phenol/Chloroform/Isoamylalcohol	Roth
Potassium acetate	Merck
Rapid Fixer	Agfa Healthcare
Sodium dodecyl sulfate (SDS)	Sigma
Skim milk powder	Fluka
Tetramethylethylendiamin (TEMED)	Sigma
Tris-Base	Sigma
Tris-HCl	Roth
Tween20	Sigma
Xylencyanol	Sigma

2.1.12 Solutions and buffers

Solution or buffer	*Composition*
Annealing buffer	200 mM Tris-Hcl 100 mM $MgCl_2$ 500 mM NaCl pH 7.5

Synthesis buffer	300 mM Tris-HCl 30 mM $MgCl_2$ pH 7.8
50x TAE	2 M Tris-HCl 50 mM EDTA pH 6.8
10x DNA loading buffer	50 % Glycerol 0.5 % (w/v) Bromphenol blue 0.5 % (w/v) Xylencyanol
2x proteinase K buffer	2 % (w/v) SDS 20 mM Tris-HCl 20 mM EDTA pH 7.5
Solution I (plasmid preparation)	50 mM Glucose 25 mM Tris-HCl 10 mM EDTA pH 8.0
Solution II (plasmid preparation)	0.2 N NaOH 1 % (w/v) SDS
Solution III (plasmid preparation)	3 M Potassium 5 M Acetate pH 5.5
Solution IV (plasmid preparation)	13 % (w/v) PEG 6000 1.6 M NaCl
TE buffer	10 mM Tris-HCl 1 mM EDTA pH 8.0
10x PBS	180 mM NaCl 8.5 mM Na2HPO4 2.2 mM KH2PO4 pH 7.4
Lysis buffer	50 mM Tris-HCl 100 mM NaCl 20 mM EDTA 0,5 % (v/v) Nonidet P40 pH 7.5
5x SDS-PAGE loading buffer	250 mM Tris-Base 10 % (w/v) SDS 7.5 % Glycerol Bromophenol blue 25 % (w/v) DTT (added directly before use)
10x SDS running buffer	0.25 M Tris-Base 1.92 M Glycine 1 % (w/v) SDS
4x Running gel buffer	1.5 M Tris-Base pH 8.8
8x Stacking gel buffer	1 M Tris-HCl pH 6.8
10x Salts	0.23 M Tris-Base 1.92 M Glycin
Transfer buffer	1x salts 20 % Methanol
Cathode transfer buffer	Transferbuffer 0.5 % (w/v) SDS

Blocking buffer	1x PBS 10 % (w/v) skim milk powder 0.1 % Tween
PBS-T	1x PBS 0.1 % Tween

2.1.13 Laboratory devices

Device	*Company*
Accu-jet pro	Brand
Agarose gel electrophoresis devices	Angewandte Geltechnologie Systeme
Agarose gel electrophoresis devices	Thermo Scientific
Aspiration system Vacusafe and Vacuboy	Integra Biosciences
Balance 2200-2NM	Kern&Sohn
Biofuge Pico	Heraeus
CO2 incubator HERAcell 150i	Thermo Scientific
Drigalski spatulas	Schütt
E.coli pulser	Biorad
Fastblot Semi-dry Electrophoretic Transfer Apparatus	Biometra
Film cassette 18x24 cm	Agfa Gevaert
Freezer -20℃	Liebherr
Freezer -80℃	Heraeus
Gel documentation system	Biorad
Handy Step Electronic	Brand
Heraeus Fresco 17 centrifuge	Thermo Scientific
Hybridization glass tubes	Biometra
Hybridization oven Compact Line OV4	Biometra
Incubator 37℃ for bacteria	Memmert
Labofuge 400	Heraeus
Labogaz 206	Campingaz
LightCycler 480 II	Roche
Magnetic stirrer Variomag	NeoLab
Microscope Primo Vert	Zeiss
Microwave 9029GD	Privileg
Mini-centrifuge Sprout	Heathrow Scientific
Nanodrop ND 2000c	Peqlab
PCR cycler Mastercycler (Gradient)	Eppendorf
pH meter inoLab	WTW
Pipettes	Abimed, Gilson
Power Pac 300	Biorad
Power pack Standard P25	Biometra
Precision balance AC 100	Mettler
Quartz cuvette Spectrophotometer Cell Micro	Biorad
Refridgerator 4℃	Liebherr
Rotina 420R centrifuge	Hettich
Safety bench Lamin Air HLB 2448	Heraeus
Sample rotator	Fröbel
Shaking incubator	Infors
Sorvall RL-5B Refrigerated Superspeed Centrifuge	Thermo Scientific
Spectrophotometer SmartSpec TM Plus	Biorad
Thermomixer comfort	Eppendorf

Thermomixer compact	Eppendorf
Timer	Roth
Transilluminator (312nm)	Bachofer
Vertical Polyacrylamide Gel Electrophoresis Apparatus	Biometra
Vortexer MS3 basic	IKA
Water bath 37℃	Köttermann
Waving platform shaker Polymax 1040	Heidolph
Western blot developer machine Curix 60	Agfa

2.1.14 Laboratory consumables

Product	*Company*
10 cm cell culture dish	Nunc
6-well cell culture dish	Nunc
Cellstar tubes 50 ml or 15 ml	Greiner bio-one
Cryotubes	Nunc
Culture tube polypropylene round-bottom 14 ml	Falcon
Filter papers	Whatman
Gel Tip 100 ART	Moleculra Bio Products
Gene Pulser / *E.coli* Cuvette	Biorad
Gloves Gentle Skin Aloecare	Meditrade
Gloves Purple-Nitrile powder-free	Kimtech
LightCycler 480 Multiwell Plate 96	Roche
LightCycler 480 Sealing Foil	Roche
Petri dishes	Greiner bio-one
Pipette tips 10 µl, 200 µl, 1000 µl Tip-One	SteriLab
Plastic pipettes 5 ml, 10 ml, 25 ml, 50 ml	Greiner bio-one
Plastic cuvettes	Braun
Quali-PCR tubes	Kisker
Sterile filter (0.22 µm)	Millipore
Syringes	Becton-Dickinson
Trans-Blot Pure Nitrocellulose Membrane (0,45 µm)	Biorad
Tubes 1.5 and 2 ml	Eppendorf

2.2 Molecular biological methods

2.2.1 Mutagenesis strategies

2.2.1.1 Site-directed mutagenesis by unique restriction site elimination

To introduce specific base changes into the L or C gene sequences, the Transformer^{TM} Site-Directed Mutagenesis Kit from Clontech was employed. The system used in this kit is called ‘unique restriction site elimination’ (USE) and relies on the introduction of an additional mutation into the plasmid which makes it possible to distinguish between parental and altered vectors (Fig. 10). In short, there are two phosphorylated primers, one of which carries the desired mutation (mutagenesis primer) and one replacing a unique restriction site on the

vector by another unique restriction site (selection primer). Both primers' directions are the same. They are annealed to the denatured parental plasmid and a polymerase extends the strands until the 5' ends of the other primers are reached. Then the DNA strands are covalently bonded with the phosphorylated primer ends resulting in a hybrid plasmid. Left-over parental plasmid is linearized by cleavage with the unique restriction enzyme which was replaced in the mutagenized vectors and therefore parental plasmids cannot be amplified in the following transformation step. In this transformation a special *E.coli* strain (*MutS*) is employed which cannot repair mismatches in the nucleotide sequence. It amplifies the mutated strand of the hybrid plasmid as well as the parental strand. Therefore, every colony of the bacteria then holds both types of plasmids. These are isolated and once again parental vectors are cut with the enzyme being replaced in the mutant plasmid. After electroporation into a usual laboratory *E.coli* strain only colonies containing the mutant plasmid emerge since parental vectors were linearized and cannot be amplified. This can easily be verified by restriction with the newly introduced restriction site. Plasmids holding the new restriction site are very likely to also hold the desired mutation. Positive clones are sequenced to finally confirm the success of mutagenesis.

2.2.1.1.1 ***Phosphorylation of oligonucleotides***

Oligonuceotides used for mutagenesis according to the Clontech Site-directed mutagenesis protocol were 5' phosphorylated prior to primer extension in order to make them competent for ligation to newly synthesized DNA. For this purpose, 10 U T4 polynucleotide kinase were incubated with 1 µg of the primer, 2 µl of 10x annealing buffer and 2 µl of 10 mM ATP in a total volume of 20 µl at 37°C for 1h. Then the enzyme was inactivated at 70°C for 10'. 0.1 µg (2 µl) of the phosphorylated primes (both mutation and selection primers) were used for the following primer extension.

2.2.1.1.1 ***Primer extension***

To synthesize a strand of DNA with stably incorporated base changes, phosphorylated primers carrying specific mutations were annealed to the denatured template. For annealing, 0.1 µg template, 2 µl of each of the phosphorylated mutation and selection primers and 2 µl of 10x annealing buffer in a total volume of 20 µl were heated up to 99°C for 5', followed by incubation on ice for 5'. To elongate the annealed primers, 5 units T4 DNA polymerase and 3 Weiss units T4 DNA ligase were added as well as 3 µl of 10x synthesis buffer, 1.5 µl of 10 mM ATP, 0.6 µl of 10 mM nucleotide mix and 3 µl of 100 mM DTT. Incubation took place at

37°C for 2 h and the subsequent inactivation of the enzymes was performed by heating the samples to 70°C for 5'. Finally, the reaction batch was precipitated with ethanol and taken up in a suiting volume of water which depended on the buffer demands of the enzyme used for the following first digestion of parental plasmid which was carried out in a total volume of 40 µl. For mutagenesis of the matrix domain in plasmid pSV45-57, the restriction enzyme NheI requiring BSA and buffer NEB2 was used. Precipitated DNA from primer extension was therefore dissolved in 30 µl of water.

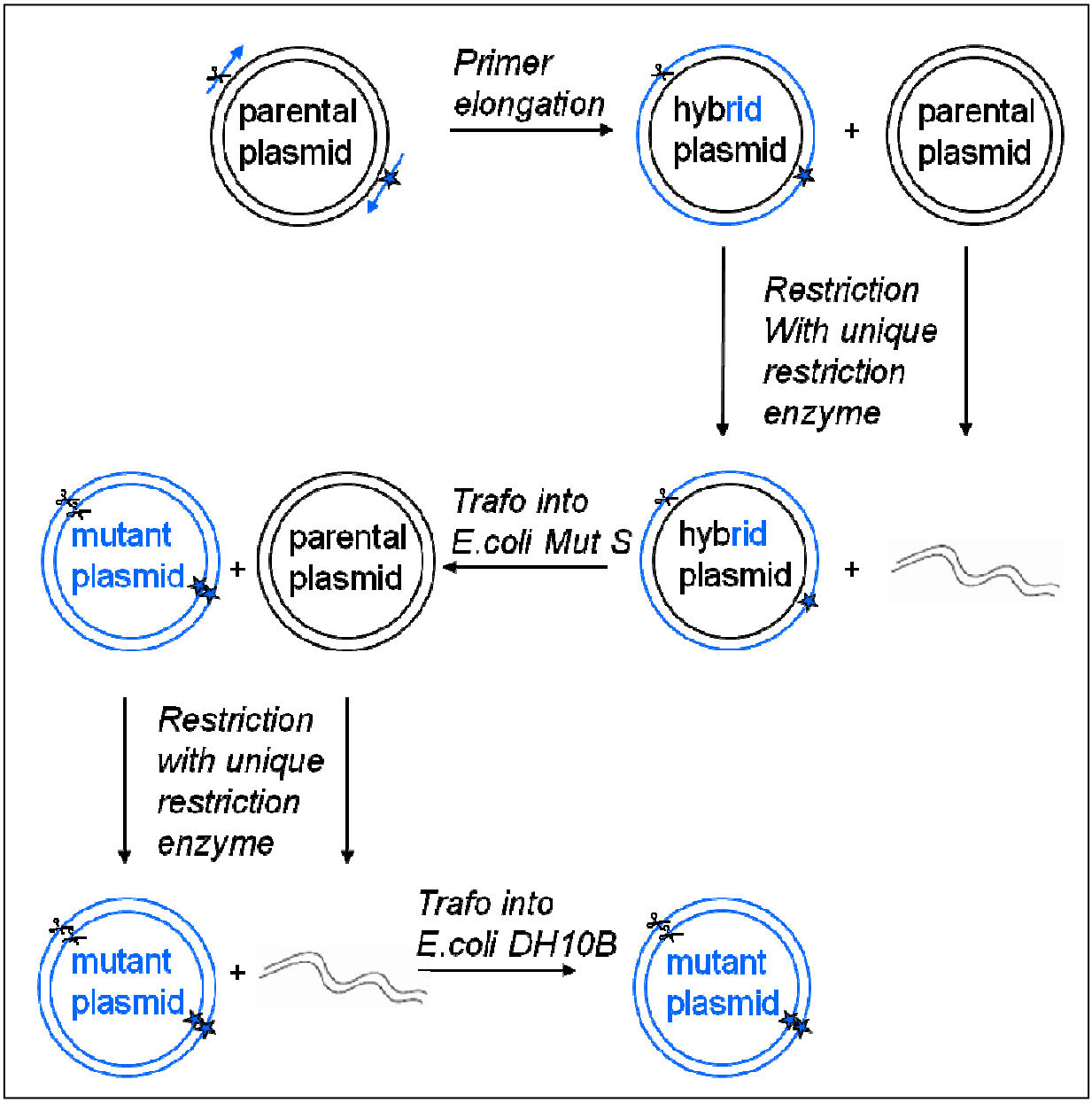

Fig. 10. Mutagenesis strategy with the Clontech TransformerTM site-directed mutagenesis kit. The mutagenesis primer (blue arrow with star) and the selection primer (blue arrow with scissors) were annealed to the parental plasmid and elongated to yield hybrid plasmids containing the mutant sequence (star) and a new restriction site (scissors) on the new strand. After the first restriction and first transformation colonies containing both mutant and parental plasmids were subjected to plasmid isolation. This blend of plasmids was digested another time resulting in colonies carrying only the mutant plasmid after the second transformation.

2.2.1.1.2 ***First restriction***

In order to linearize parental plasmids during mutagenesis to make them incapable of amplification in bacteria upon transformation, the product of a primer extension reaction consisting of hybrid as well as parental plasmids was subjected to restriction with NheI. 20 units of the enzyme, 4 µl of NEB2 and 4 µl of 10x BSA were used in a 40 µl restriction reaction and restriction lasted for 2 hours at 37°C. Then the enzyme was inactivated at 80°C for 20 minutes and the reaction was precipitated prior to transformation. Pelleted DNA was taken up in 10 µl of water.

2.2.1.1.3 ***Transformation in E. coli MutS***

The first transformation in the USE mutagenesis strategy was performed with *E. coli MutS* bacteria because of their deficiency in DNA mismatch repair. 5 µl of precipitated DNA were electroporated (see 2.3.3) into 40 µl of MutS cells, incubated at 37°C for one hour to allow expression of the ampicillin resistance coded for on the plasmids, and plated on LB-Amp plates. After one night at 37°C, colonies of two morphologies (mutant and parental) emerged on the plates.

2.2.1.1.4 ***First plasmid preparation***

The colonies on the plate contained either two different plasmids (mutant and parental) or only parental plasmids which had not been linearized during the first restriction. Therefore all colonies were taken up in LB medium by rinsing the plates with medium until it was cloudy. The plasmid preparation was carried out as described in 2.2.8.1 and the DNA was finally dissolved in 30 µl of water.

2.2.1.1.5 ***Second restriction***

5 µl of the plasmids were subjected to another restriction with NheI to get rid of last traces of parental plasmid. In a total volume of 20 µl, 10 U NheI were used for a 2 hours restriction at 37°C. After that another 10 U were added and restriction continued for another hour.

2.2.1.1.6 ***Transformation in E.coli*** DH10B

The second transformation step during mutagenesis by USE was performed with *E. coli* DH10B. 1 µl of the 1/5 diluted second restriction were used together with 25 µl of cells (see 2.3.3). After incubation of the cells shaking at 37°C for 45 minutes, they were plated on LB-Amp plates and incubated at 37°C o/n.

2.2.1.1.7 ***Second plasmid preparation***

Plasmid preparation of single colonies was performed as described in 2.2.8.1.

2.2.1.1.8 ***Control restriction***

To verify positive colonies, a control restriction with the enzyme SalI cleaving the plasmid at the newly introduced restriction site was performed. In contrast to the parental vectors positive clones were cut and therefore linearized. They were very likely to also hold the desired mutation besides the new restriction site. Restrictions were carried out in a total volume of 20 µl, using 5 U of SalI, 2 µl of NEB3 and 5 µl of the plasmid preparations. After one hour at 37°C the restriction products were monitored on a 1 % agarose gel (2.2.4).

2.2.1.1.9 ***Sequencing***

Success of the mutagenesis procedure was confirmed by sequencing (2.2.9).

2.2.1.2 Site-directed mutagenesis by USE using wobble primers

To reduce the number of mutagenesis reactions, degenerate primers carrying three wobble positions were employed. During oligo synthesis at these positions one of the 4 bases is incorporated randomly and in this way a blend of primers having all different combinations of bases in these degenerate positions is generated. By using degenerate primers as mutagenesis primers it was possible to produce several mutants of one matrix domain residue at the same time. This was verified by final sequencing of all positive clones out of 96 colonies which were subjected to plasmid isolation.

2.2.1.3 Site-directed mutagenesis by USE using primer mixtures

Due to low efficiency of site-directed mutagenesis using wobble primers, the mutagenesis primers were ordered individually and then manually mixed. The mutagenesis procedure was the same.

2.2.1.4 Site-directed mutagenesis via fusion PCR

Fusion PCR is a classical mutagenesis method in which two partly overlapping DNA stretches are synthesized by PCR and are joined by another PCR (see Fig. 11). The mutation is located in the overlap and introduced by mutant primers. These primers have the opposite direction and are the reverse complement of each other. Each of them is used for a first PCR with another primer to produce a piece of DNA which is purified by gel extraction and then used as template for the second PCR. In the second PCR the two pieces formed in the first PCR are

joined and the result is a long stretch of DNA carrying the wanted mutation. After gel extraction, this piece of DNA is restricted with appropriate restriction enzymes and then ligated into the suiting vector backbone (digested by the same enzymes). The newly synthesized plasmid is transformed into *E.coli* DH10B and resulting colonies are inoculated for plasmid isolation. These plasmids are subjected to a control digestion and positive clones are sequenced to confirm the desired mutations.

2.2.1.5 Introduction of a deletion using a type IIS restriction enzyme

Generation of plasmid pSVHBV1.1LE- was performed by deleting a part of the sequence of pSVHBV1.5LE-. This was achieved by making use of primers with special restriction sites. These restriction sites were recognized by a type IIS restriction enzyme which cleaves close to but outside of its recognition sequence. By introducing these sequences into the DNA the cutting points could be precisely determined.

As depicted in Fig. 12, two PCRs amplifying regions flanking the sequence to be deleted were performed (primers VBBS3+4 and VBBS5+6). The primers binding at the edge of the unwanted sequence (VBBS3 and VBBS6) carried the recognition sites for the type IIS restriction enzyme BsaI which were therefore inserted into the amplicons. After PCR, the products were purified and digested with BsaI. After joining the two digested PCR amplicons by ligation, a 3rd PCR using primers VBBS4 and 5 amplified the newly synthesized shortened part of the vector and it was subcloned into the pSVHBV1.5LE- backbone. Successful subcloning yielded the new plasmid pSVHBV1.1LE-.

2.2.2 Polymerase chain reaction

To amplify and / or mutate DNA sequences, the polymerase chain reaction using primers with or without base changes was employed. Different PCR masters were used (ThermoScientific, Roche, Promega) and the standard PCR program was the following: 5 min 95°C/ 30x [30 sec 95°C / 30 sec X°C / 1 min 68 - 72°C]. Primers were used in a concentration of 1 µM. The annealing temperature (X) was calculated by taking the mean value of the melting temperatures of the primers and substracting 5°C. Extension time was approximately 1 minute per 1000 bp and extension temperature depended on the polymerase contained in the PCR masters (72°C or 68°C). The standard number of PCR cycles was 30, and was increased to 35 if only low amounts of PCR product were monitored. Template was used in a range between 10 and 50 ng (usually 1µl of a 1/100 dilution of a large scale plasmid preparation).

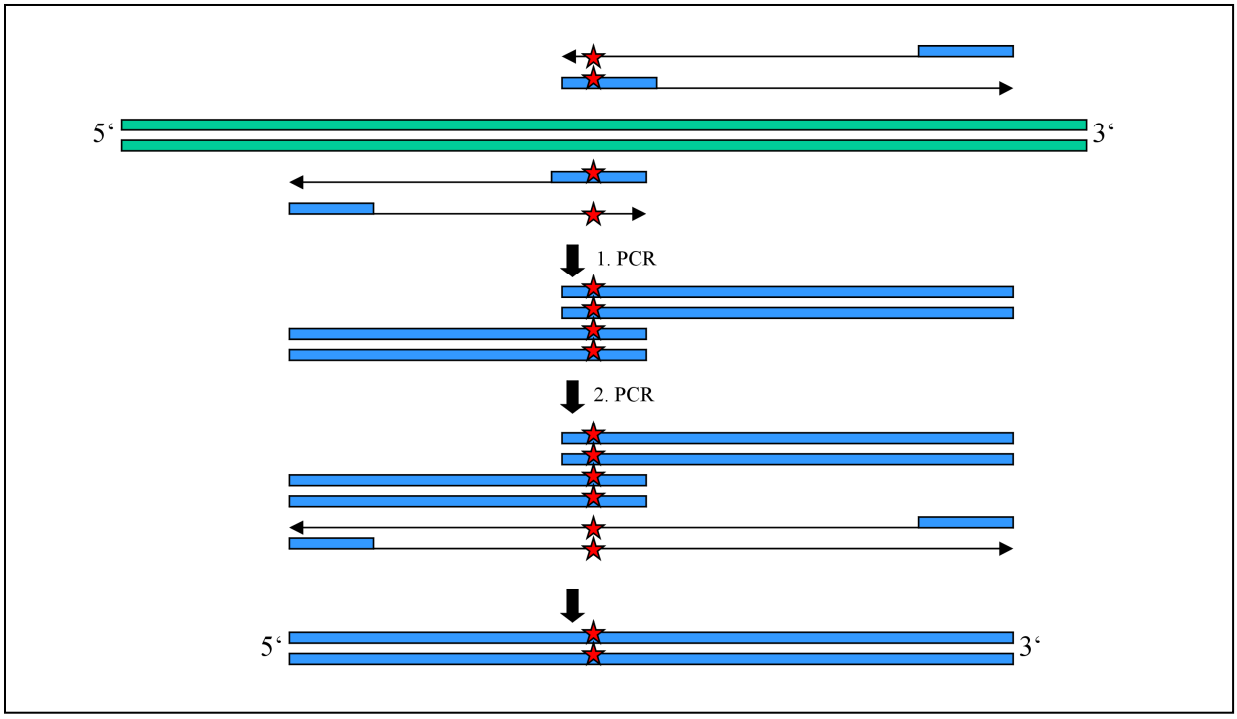

Fig. 11. Principle of mutagenesis by fusion PCR. Primers (blue boxes) carrying the desired base exchanges (red stars) were used for two subsequent PCRs generating amplicons with stably incorporated mutations (blue lines with red stars). Green lines represent the parental DNA sequence.

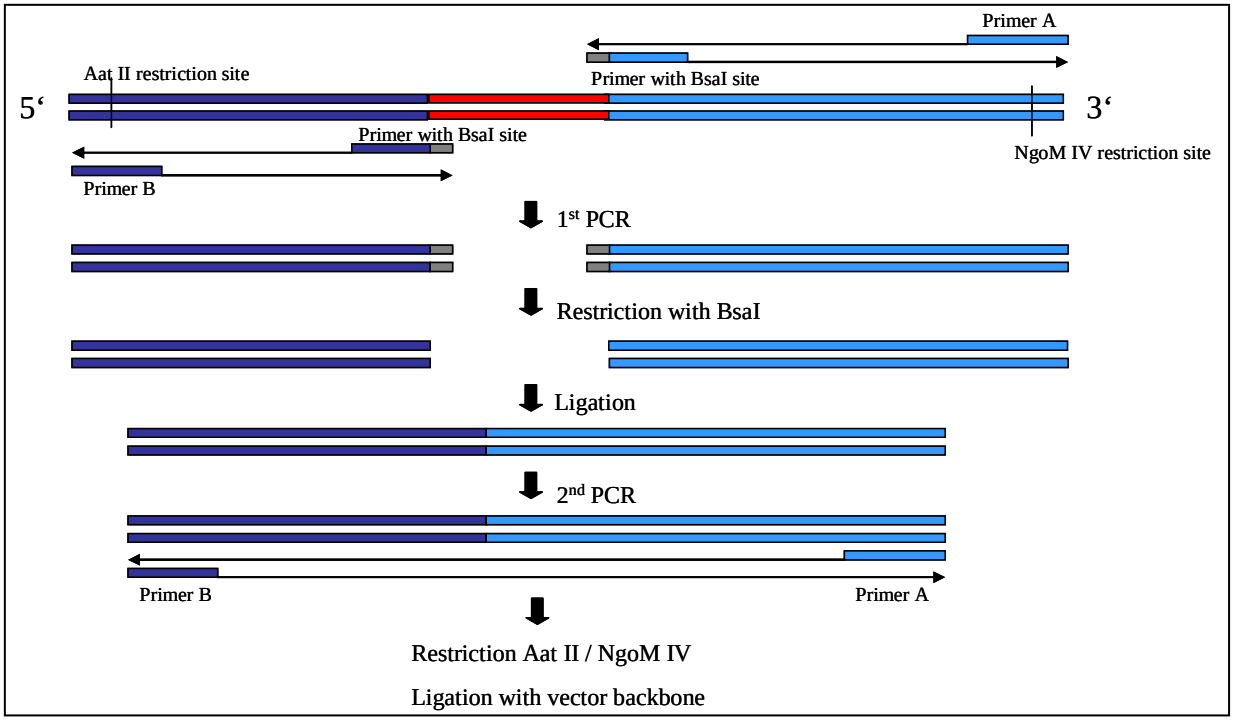

Fig. 12. Introduction of a deletion by a type IIS restriction enzyme. Primers (blue boxes) with or without introduced BsaI restriction sites (grey boxes) were used to amplify the regions flanking the region to be deleted. After restriction of the amplicons with BsaI they were ligated and subjected to another PCR yielding the insert used for further subcloning.

2.2.3 Colony PCR

Colony PCR is a special kind of polymerase chain reaction in which the template DNA is not purified prior to PCR. Single colonies of bacteria transformed with the desired plasmids are taken and suspended directly into the PCR mix. Then, a standard PCR is run. In the initial denaturation step (5' at 95°C) the bacteria are lysed and the DNA becomes accessible for amplification. PCR products are monitored by agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis served two purposes: Estimating the size of the loaded DNA fragments and of the amount of DNA loaded onto the gel. According to the size of DNA fragments that should be monitored, either 1 % (for DNA with more than 1000 bp) or 2 % (DNA smaller than 1000 bp) agarose gels were prepared with 1x TAE buffer and 50 pg/ml ethidiumbromide. Furthermore, different length standards were loaded depending on the expected size of the bands (see 2.1.9). By applying an electric field, the DNA migrated through the maze of agarose and was thereby separated by its length. Running the gel at a voltage of 90 V, the running time varied between 30 minutes and one hour depending on the size of the gel and the purpose of the electrophoresis. Pictures of the gel were taken with the gel documentation system of Biorad.

2.2.5 Restriction

To confirm the success of mutagenesis, create inserts and adequate vector backbones for subcloning, or to linearize parental plasmids during mutagenesis, restrictions using site-specific restriction enzymes were performed. All enzymes were used with buffers and temperatures as described by the manufacturers. The amount of enzyme added to the reactions varied between 5 and 20 Units and depended on the purpose of the digestion: 5 U were used for control digestions and 20 U for all other purposes like preparative restrictions. Preparative restrictions and restrictions of primer extension were carried out in a total volume of 40 µl while control restrictions were made in 20 µl. Digestion duration was 1 h for control and 2 h for preparative restrictions and restrictions of primer extension reactions. The second restriction in mutagenesis by USE lasted 3 hours in total, adding 10 more units of enzyme after 2 hours. Simultaneous double digestions were carried out if the enzymes shared the same buffer. If the enzymes needed different buffers, sequential digestions with an intermediate ethanol precipitation step were performed to obtain the desired restriction product. Success of restriction was monitored on an agarose gel by gel electrophoresis.

2.2.6 Ligation

Covalent linking of two ends of DNA molecules was carried out by the enzyme T4 DNA ligase. The reaction containing insert, vector backbone and 1 µl of T4 DNA ligase was supplied with the corresponding buffer and either performed at 16°C o/n or at 22°C for one hour. The total volume was 20 µl and insert and backbone were used in a molar ratio of around 5:1. Ligated DNA was purified by ethanol precipitation prior to electroporation into electrocompetent bacteria.

2.2.7 Purification and concentration of DNA

2.2.7.1 Phenol-chloroform extraction

To biochemically separate DNA from protein, phenol / chloroform extraction was used. Equal volumes of phenol-chloroform and the DNA containing sample were mixed and formed a biphasic mixture. DNA was located in the upper aqueous phase, denatured protein at the border of the two phases (interphase) and fats in the lower organic phase. Therefore, only the upper phase containing the desired DNA was transferred to a new tube. The lower phase was discarded. This procedure was usually performed three times prior to ethanol precipitation of DNA.

2.2.7.2 DNA precipitation

After phenol-chloroform extraction, restriction or ligation, DNA in aqueous solutions was concentrated or further purified by ethanol or isopropanol precipitation. For ethanol precipitation, the sample was mixed with 1/10 volume of 3 M sodium acetate pH 5.6 and two volumes of 96 % ethanol, put on -20°C for 30 minutes and centrifuged at 13000 rpm for half an hour. For isopropanol precipitation, the same volume of isopropanol was added to the sample and the mixture was centrifuged for 20 minutes at 4 - 15°C after a short incubation at room temperature. In both cases, the DNA pellet was washed with 75 % ethanol, dried and finally taken up in a suiting volume of water (30 µl for qPCR, 45 µl for minipreps, 100 – 300 µl for maxipreps).

2.2.7.3 PCR purification

Purification of PCR products was performed using the Qiagen PCR Purification kit and mainly according to the protocol of Qiagen. Shortly, PCR products were mixed with 5 volumes of buffer PB and 10 µl of 3 M sodium acetate pH 5.0 were added. The mixture was

spinned through a QIAquick column to bind the DNA to the carrier material and then the column was washed with 0.75 ml of buffer PE for 2 times. An additional 1' centrifugation made sure that all buffer PE war removed and the column was dry prior to elution. DNA was eluted from the column with 30 µl of water instead of EB buffer which was contained in the kit. Before centrifugation, the column was incubated for 1' at RT to allow soaking of the carrier material with water.

2.2.7.4 Gel extraction

To isolate DNA (PCR products, restrictions) from an agarose gel, the QIAquick Gel Extraction kit (Qiagen) was employed. Bands of the right size were cut out of an agarose gel and then subjected to gel extraction. The procedure was in large part carried out as recommended by the manufacturer. Firstly, the weight of the excised piece of gel was determined and three volumes of buffer QG were added (for each 100 mg gel 300 µl of QG were added). By incubation at 50°C and intermittent vortexing the gel was dissolved and finally mixed with one gel volume isopropanol. To bind the DNA, the mixture was applied onto a QIAquick column and centrifuged for one minute. This was performed two times. Then, all agarose traces were removed by washing once with 500 µl of buffer QG. Two times washing with 0.75 ml of buffer PE followed and a final centrifugation dried the column. DNA was eluted from the column with 30 µl of water for two times which means that after the first elution the flow-through was applied onto the column for a second time to enhance the DNA yield.

2.2.7.5 DNA concentration determination

The DNA concentration of aqueous solutions was determined either by using a photometer or a nanodrop machine. For photometer measurements, the DNA was diluted 100-fold prior to determination of the concentration and at least 3 measurements were made. In the nanodrop machine, 2 µl undiluted DNA were measured for two times. Calculations were performed by the machines based on the measurement of the absorbance of the sample at 260 nm. The dilution factor and conversion factor for dsDNA ($A_{260\ nm}$ of 1.0 = 50 µg/ml pure dsDNA) were taken into account and therefore the formula underlying the DNA concentration calculations was:

dsDNA concentration (µg/ml) = measured A_{260} x 50 µg/ml x dilution factor

The ratio of the absorbances at 260 nm (DNA) and 280 nm (protein) depicted the purity of the sample, 2.0 being the value for 100% pure DNA.

2.2.8 Plasmid preparation

2.2.8.1 Miniprep

Preparation of DNA on a low scale was performed the following way: 3 ml of overnight cultures of transformed *E. coli* bacteria were pelleted by 3' centrifugation at 9000 rpm and the cells were taken up in either 300 µl of Qiagen buffer 1 (from the Qiagen Plasmid Maxi kit) or 100 µl of solution I. Lysis of the bacteria was achieved by adding lysis buffer (300 µl of Qiagen buffer 2 or 200 µl of solution 2, respectively), inverting the tube a few times to mix and finally incubating them at room temperature for 5'. To neutralize the pH of the solution, either 300 µl of Qiagen buffer 3 or 150 µl of solution 3 were added. The tubes were incubated on ice for 5' prior to sedimenting cell debris and precipitated protein by centrifuging the supernatant for two times. Further concentration of the DNA was achieved by isopropanol precipitation. Finally, the pellet was taken up in 45 µl of water and if not the Qiagen buffers had been used, 0.5 µl of RNase A were added. Prepared plasmid DNA was stored at -20°C.

2.2.8.2 Midiprep

The Qiagen Plasmid Midi kit was used for preparation of plasmids on a medium scale. The day before, 100 ml LB medium with 100 µg/ml ampicillin were inoculated with a single colony containing the desired plasmid. The next morning, the cells were lysed and the DNA was purified as described in the manual. After the final precipitation step, the DNA pellet was dissolved in 100 µl of water.

2.2.8.3 Maxiprep

Large scale preparation of DNA was performed either by using the Qiagen Plasmid Maxi kit (according to the manual) or by the following method: A single colony containing the desired plasmid was inoculated in 30 ml TB-Amp medium the day before the preparation. It was incubated o/n shaking at 37°C. On the next morning, the cells were collected by centrifugation for 15' (6000 g, 4°C) and the cell pellet was resuspended in 5 ml of cold solution I. 10 ml of solution II were freshly prepared and added to the bacteria. The tube was inverted a few times and then incubated at RT for exactly 5' in order to lyse the bacteria. Longer incubation times were avoided to exclude damage of the DNA by the high pH of solution II. Then, 7.5 ml of solution III were put to the lysis mixture to neutralize the pH. Incubation was for 5' on ice. After adding solution III, a white precipitate formed which was removed from the supernatant by two times centrifugation (15', 4000 rpm, 4°C). The

supernatant containing the plasmid DNA was then precipitated using 0.6 volumes (13.5 ml) of isopropanol. After mixing and a short incubation at RT, a final centrifugation was performed (4000 rpm for 15'). The pellet was dried and resuspended in 3 ml of TE buffer. To specifically precipitate the RNA while retaining the DNA in the supernatant, 3 ml of -20°C cold 5 M LiCl were added, mixed and the RNA was sedimented by a 15' centrifugation at 4000 rpm. The DNA in the supernatant was precipitated with an equal volume of isopropanol (15', 4000 rpm). The pellet was dissolved in 500 µl of TE buffer and remaining RNA was digested by adding 1 µl of RNase A and incubating at RT for half an hour. Another precipitation step with 500 µl of solution IV and centrifugation at 13000 rpm for 15' ended with dissolving the pellet in 400 µl of TE buffer and was followed by three times phenol-chloroform extraction (see above). Finally, the upper phase containing the desired plasmid was precipitated by a standard ethanol precipitation (see above). Depending on the size of the pellet, the DNA was taken up in 100 – 300 µl of water and the concentration was determined by either using a photometer or a nanodrop machine.

2.2.9 Sequencing

Sequencing of mutated sequences was performed by the company GATC Biotech. For this purpose, 20 µl of DNA with a concentration between 30 – 100 ng/µl were sent to GATC and were analyzed by them until the next morning. Primers were attached in concentrations of 10 µM or ordered directly at GATC and remained there. Sequencing results were provided online and were aligned with the online available multiple sequence alignment program ClustalW2 of the European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.2.10 Digestion of RNA / DNA

To digest unwanted RNA or DNA in aqueous solutions, RNase A (100 mg/ml; 7 U/µl; Qiagen) and DNase I (Qiagen, RNase-free DNase set), respectively, were used. RNase A was necessary to get rid of RNA in plasmid preparations and was used without buffer. DNase I was needed to remove remaining plasmid DNA from transfections of HuH7 cells. These cells were used to express the HBV proteins and in this way also formed virions which were detected in the supernatant of transfected cells by a qPCR of the viral genome. Plasmid from transfection could disturb in this qPCR and therefore had to be digested. This was achieved the following way: 87.5 µl of an immunoprecipitation sample were mixed with 10 µl of RDD

buffer (contained in Qiagen's RNase-free DNase set) and 2.5 µl of the enzyme were added. This mixture was incubated at RT for half an hour.

2.2.11 Immunoprecipitation

Virions in the supernatant of transfected cells were concentrated and purified by immunoprecipitation using protein G coupled agarose beads and antibodies against the small envelope protein of HBV. The antibody used was a polyclonal serum produced in sheep and directed against the S domain of the three HBV surface proteins. For immunoprecipitation 25 µl of beads (Santa Cruz Biotechnologies) were washed 3 times with PBS and 2 µl of sheep αHBs diluted 1:1 with glycerol were added. The volume in the tube was filled up to ~ 800 µl with PBS to ensure proper mixing. The mixture was preincubated on the sample rotator at 4°C for 6 hours to allow binding of the antibodies to the beads. Then it was washed once again with PBS, added to 750 µl of cell supernatant and finally incubated on the sample rotator at 4°C o/n to precipitate the virions from the supernatant. The beads with the bound virions were washed three times with PBS prior to DNase I digestion to reduce the background plasmid from transfection and to isolation of the HBV genomes out of the virions.

2.2.12 Preparation of HBV genomes

DNA of virions was isolated by disrupting the virus shell and capsid by proteinase K. This enzyme digests protein and therefore can open the capsid and set the viral genome free. Proteinase K was used with the corresponding 2x proteinase K buffer, in a concentration of 500 ng/µl and incubation lasted for 3h at 56°C without shaking. Further purification of the HBV genomes was achieved by three times phenol / chloroform extraction and a final ethanol precipitation.

2.2.13 Isolation of viral DNA from plasma

To isolate viral DNA from the plasma of an HBV positive donor, the Qiagen QIAamp DNA mini kit was employed. 2 µl of the plasma were taken and filled up to 200 µl with PBS. The following isolation was performed according to the manual (protocol 'DNA Purification from Blood and Body Fluids – Spin Protocol' and appendix F 'Protocols for Viral DNA'). If an immunoprecipitation was done prior to use of this kit, the beads were removed after the initial proteinase K digest by sedimenting them and the protocol was continued with the DNA containing supernatant.

2.2.14 HBV genome specific Real-Time PCR

Quantification of viral genomes isolated from virions was performed using quantitative PCR. The dye used was SYBR Green which binds unspecifically to double-stranded but not single-stranded DNA. The problem of background plasmid from transfection being detected by this system was overcome by primers binding very specific locations in the genome and plasmid, respectively (*strategy adopted from Ke Zhang*). Briefly, the primers which were used (VBBS8 and VBBS18) flanked a 222 bp region in the genome, while in the transfected plasmid they were additionally separated by the backbone of the plasmid causing a much longer PCR product. By choosing an elongation time of only 10 seconds the long stretch of DNA produced by the background plasmid could be excluded from amplification while the short DNA made from the genome was multiplied exponentially.

2.2.14.1 PCR standard

Plasmid pSVHBV1.5LE- was used as standard for absolute quantification. A PCR with the qPCR primers VBBS8 and VBBS18 produces the same 222 bp fragment of DNA as the HBV genome and is therefore an ideal standard. After determination of the plasmid concentration by using a spectrophotometer, the number of DNA copies per µl was assessed using the molecular weight of the plasmid, as calculated with the oligonucleotide properties calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). Knowing the number of DNA molecules per µl, $1x10^6$ copies were taken and subsequently 10-fold diluted to gain 5 different dilutions (10^6 – 10^2 copies / µl). These were applied in every qPCR and served as known amounts of target DNA generating a standard curve to which the samples of unknown DNA content were compared.

2.2.14.2 qPCR setup and program

One reaction batch consisted of 10 µl SYRBGreen I Master (Roche), 1 µl of each of the 20 µM primers (VBBS8 and VBBS18) and 8 µl of either template DNA, PCR-grade water (negative control; included in the Roche SYBRGreen kit) or standard (1 µl of plasmid dilutions containing 10^2 - 10^6 copies of pSVHBV1.5LE-, filled up to 8 µl with PCR-grade water). The PCR reactions were carried out in 96well plates which were sealed with adhesive foil (all items from Roche). To run and analyze the quantitative PCR, the Roche LightCycler 480 II and the LightCycler 480 software release 1.5.0 SP4 (version 1.5.0.39) were used. The PCR program chosen was the “SYBRGreen I 96-II” (Roche template) and only minor changes were made. The final program was: 5 min 95°C/ 40x [20 sec 95°C / 10 sec 60°C / 10

sec 72°C]. This was followed by acquisition of melting curves of the PCR products in order to monitor their specificity.

2.2.15 SDS-PAGE and Western blot

2.2.15.1 Preparation of an SDS polyacrylamide gel

To verify the expression of mutated L protein in cell culture, it was detected by immunostaining against the S domain of the three HBV surface proteins after separating the HBV proteins according to their electrophoretic mobility by a vertical SDS polyacrylamide gel electrophoresis. In order to obtain a high resolution of the HBV envelope proteins, the multigel system (Biometra) was used. Before preparing the gel, the two glass plates (one notched plate with straight edges and one with fixed 1 mm spacers) were cleaned with water, 80 % ethanol and 2 % SDS and mounted with a silicone rubber between them to avoid leakage. Two big and two small clips were used to clamp the two glasses, two small at the bottom and a big one on each side. The comb used (11 wells à 50 µl) was cleaned the same way as the glass plates and it was marked on the glass plates how deep the teeth reached into the gel. Then, 15 ml of 12 % running gel were prepared: 4.9 ml of water, 3.8 ml of 1.5 M Tris pH 8.8, 0.15 ml of 10 % SDS and 6 ml of 30 % acrylamide mix were mixed and 0.15 ml of fresh 10 % APS and 0.006 ml of TEMED were added. This mixture was immediately poured between the glass plates up to 1 cm under the mark and overlaid with 1.5 ml of butanol or 96 % ethanol. The gel was allowed to polymerize for around half an hour and then the overlay was removed with Whatman paper. Next, 5 ml of 5 % stacking gel were mixed (3.4 ml of water, 0.63 ml of 1 M Tris pH 6.8, 0.05 ml of 10 % SDS, 0.83 ml of 30 % acrylamide mix, 0.05 ml of 10 % APS and 0.005 ml of TEMED) and transferred between the glass plates on top of the running gel. To do this, a 10 ml plastic pipette was used to avoid the formation of air bubbles. The comb was instantly inserted before polymerization could take place. After 20 minutes the gel was completely polymerized and ready for use. When used later, the gel was wrapped in moistened papers, put in a plastic bag and stored at 4°C.

2.2.15.2 SDS-PAGE

To prepare the gel for electrophoresis, the comb was removed under running water and the slots were rinsed with 1x SDS buffer. The rubber was removed and the gel fixed onto the electrophoresis apparatus with 4 small clips. Next, the reservoirs were filled with 1x SDS buffer and air bubbles under the gel were removed using a syringe. At the same time, 40 µl of the samples were mixed with 10 µl of 5x SDS loading buffer containing DTT (0.125 g DTT +

500 µl of buffer), heated up to 99°C for 7', put on ice to cool down and shortly centrifuged to remove all drops from the lid. Then they were loaded onto the gel with Gel pipet tips (Molecular Bioproducts). One slot was filled with the marker Pageruler Plus Prestained Protein Ladder (Fermentas). The safety lid was put on the apparatus and the gel was run at 150 V for 3 to 4 h.

2.2.15.3 Electroblot

To make the proteins in the polyacrylamide gel accessible for staining, they were transferred to a nitrocellulose membrane. This was achieved by electroblot using the Fastblot Semi-Dry Electrophoretic Transfer Apparatus from Biometra.

During SDS-PAGE, 8 Whatman blotting papers and the nitrocellulose membrane were cut to gel size. When the 10 kDa marker protein had run out of the gel and the electrophoresis had been ended, the gel was removed from the glass plates and the desired part was cut out. The gel and the membrane were equilibrated for 10' in transfer buffer and 4 of the blotting papers were shortly soaked in the same buffer. The papers were then put onto the anode (+), followed by the membrane and the gel. The remaining 4 blotting papers were soaked in cathode transfer buffer and put on top. Air bubbles between the different layers of the blotting sandwich were removed by rolling them out with a pipette. Then the lid was closed and a weight of around 2 kg was put on top of the lid (usually a large beaker filled with water). Finally, a current of 350 mA was applied for 1 h which forced the negatively charged proteins to migrate out of the gel onto the membrane. The membrane was then removed and stored in blocking buffer o/n at 4°C.

2.2.15.4 Immunostaining

After o/n blocking, the membrane was shaken in blocking buffer for another 10' at RT. Then, it was transferred to a glass tube with the upper side holding the proteins to the inside of the tube permitting access of the antibodies to their antigens. Staining of the proteins on the membrane took place in two steps: First, antibodies directed against an epitope in the S protein bound to the blotted envelope proteins. For this purpose, antibody HB1 was 1/4000 diluted in blocking buffer (15 ml in total) and added to the membrane into the tube. Incubation was for 2 h at RT rolling in a hybridization oven. Then the membrane was washed three times with PBS-T for 10' shaking at RT and put back into the glass tube for the second part of staining. In this step, the secondary antibody conjugated to a reporter enzyme bound to the primary antibody. Since several secondary antibodies can bind to one primary antibody,

the signal is enhanced by this two step detection. The secondary antibody used was a goat anti-mouse horseradish-peroxidase coupled antibody which was diluted 1/3000 in blocking buffer and incubated with the membrane for another 1.5 h. To get rid of unbound antibody, the membrane was washed again for three times with PBS-T and once with PBS prior to development of the blot.

2.2.15.5 Fluorescent detection of blotted proteins

To finally detect the antigen on the membrane, the membrane was placed on transparency foil and 600 µl of substrate (Lumi-light Western Blotting Substrate, Roche) for the horseradish-peroxidase were added dropwise onto the membrane. Then, the membrane was covered with another transparent foil and excess substrate was carefully squeezed out. It was incubated in the dark for 1' and then transferred into a new foil sandwich. Air bubbles were removed and then the membrane was put into a film cassette. X-ray films were exposed for different lengths of time and developed in the Curix 60 developer machine (Agfa).

2.3 Microbiological methods

2.3.1 Inoculation and cultivation of *E. coli*

Single colonies of plasmid carrying *E. coli* bacteria were inoculated with flamed inoculation loops or freshly autoclaved pipette tips in 3 ml of antibiotic containing LB medium (minipreps) or in 30 ml of antibiotic containing TB medium (maxipreps). The culture was shaken at 220 rpm and 37°C for 12 to 16 hours prior to plasmid preparation. For preparation of electrocompetent bacteria, 5 ml o/n cultures were prepared which were used to inoculate larger cultures the next morning (see 2.3.2.1 and 2.3.2.2).

2.3.2 Preparation of electrocompetent bacteria

2.3.2.1 *E. coli* DH10B

E. coli DH10B is a common *E. coli* laboratory strain. It was used to amplify plasmids. To make *E. coli* DH10B cells competent for electroporation, 250 ml of LB medium without any antibiotics were inoculated with 1.5 ml of a DH10B overnight culture (also free of antibiotics) and harvested when the OD at 578 nm reached a value between 0.6 and 1 meaning that the bacterial growth had reached the logarithmic phase. The OD was monitored by frequent measurements using a spectrophotometer. After harvesting, the bacteria were kept on ice for 20' and subsequently sedimented by centrifugation for 15' at 5000 rpm and 4°C. Then, the

cells were washed twice in 250 ml of ice-cold water, and afterwards once in 10 ml of ice-cold, sterile 10 % glycerol. The washing centrifugation steps were performed for 12' with a speed of 5000 rpm at a temperature of 4°C. Finally the cell pellets were resuspended in 1.5 ml of ice-cold and sterile 10 % glycerol and aliquots of 55 µl were stored at -80°C.

2.3.2.2 *E. coli MutS*

To prepare electrocompetent *E. coli MutS* cells, bacteria from a stock supplied by the Clontech kit were streaked on an LB-Tet plate containing 50 µg / ml tetracyclin and kept at 37°C o/n. The next morning the plate was transferred to 4°C and could be kept there for three months. An overnight culture in 5 ml of LB medium with 50 µg / ml of tetracyclin was grown from a single colony of the plate. On the next day, 1 ml of the o/n culture was taken for inoculation of 100 ml LB medium without any antibiotics. It was shaken at 220 rpm and 37°C for 3 – 4 h until the OD at 600 nm reached a value of 0.5 (monitored with the photometer every 5 - 10 minutes starting 3 hours after inoculation). Then the cells were taken out of the incubator, cooled on ice for 20' and collected by centrifugation at 4000 rpm for 5' at 4°C. The pellet was resuspended in 100 ml of ice-cold 10 % glycerol, incubated on ice for another 20' and sedimented at 4000 rpm for 10' at 4°C. This step was repeated once with 10 ml of ice-cold 10 % glycerol and after this washing step the cells were finally taken up in 400 µl of ice-cold 10 % glycerol. Small aliquots of 40 µl were prepared and stored immediately at -80°C.

2.3.3 Transformation of bacteria

DNA samples were diluted 1/10000 (for retransformation of large scale plasmid preparations), 1/5 (for DNA after restriction in context with the Clontech Transformer Site-directed mutagenesis strategy) or used undiluted (precipitated ligations). One µl was finally used for transformation. 25 µl of *E. coli* DH10B or 40 µl of *MutS* cells were thawed on ice and mixed with the DNA before the mixture was transferred into a 0.1 cm pulser cuvette (Biorad). Electroporation was performed with 1.8 kV. Directly after giving a single pulse, 1 ml of LB medium was added to wash the cells out of the cuvette. Then, the bacteria were transferred into a 1 ml Eppendorf tube and shaken at 400 rpm and 37°C for 1 hour. For ligations, all cells were streaked out on plates containing 100 µg/ml ampicillin and for plasmid preparations, 50 µl were plated. The plates were incubated at 37°C o/n or alternatively over the weekend at room temperature.

2.4 Cell culture techniques

2.4.1 Cultivation of HuH7

The doubling time of HuH7 cells under optimal growth conditions (37°C, 5 % CO_2) is approximately 24 hours. To avoid overgrowing, cells which had been allowed to grow to a confluent monolayer were then divided into several plates by splitting in appropriate ratios. Detachment of the cells was accomplished by washing once with PBS (37°C) and once with trypsin/EDTA (RT) and subsequent incubation at 37°C and 5 % CO_2 for 5'. During the incubation the cells detached from the plate and were then taken up in 6 ml of pre-warmed growth medium (DMEM with 10 % FBS, 1x NEAA, 1x sodium pyruvate, 1 % PS), diluted in the desired split ratio and transferred to new cell culture dishes. To maintain the cells in culture, they were split every third to fourth day with a ratio of 1:6 and 1:8, respectively. For transfection, the cells were seeded in 10 cm dishes or 6 well plates. They were either split 1:3 (10 cm dishes) or 1:20 (6 well dishes) the day before transfection. If the cells were not fully confluent or splitting was performed quite late in the afternoon, splitting rates of 1:2 and 1:15 were used, respectively. The total volume of growth medium in 10 cm dishes was 10 ml, in 6 well plates 2 ml.

2.4.2 Freezing of cells

To freeze cells, plates with densely grown cells were trypsinized removing all trypsin before incubation at 37°C and the cells were taken up in 900 µl of pre-warmed FCS and transferred to a cryotube. Then, they were supplemented with 10 % DMSO (100 µl) which is a cryoprotectant and was added slowly and dropwise while swirling the tube to ensure proper mixing. This was important in order to avoid osmotic shock. The cells were firstly frozen in a freezing vessel at -80°C. After a few days, they were transferred to liquid nitrogen where they were stored for unlimited time.

2.4.3 Thawing of cells

Cells frozen in liquid nitrogen were thawed by shaking the vial in a 37°C warm water bath. When only a small frozen remainder was left they were removed from the water bath and transferred to a 15 ml Falcon tube. Warm growth medium was added dropwise and slowly so that the volume doubled in a time of approximately one minute. When the medium was filled up to 10 ml, the cells were sedimented by centrifugation at 1000 rpm for 5' at 4°C. The supernatant was replaced by fresh growth medium and the cells were resuspended and divided

into two or three cell culture dishes. They were incubated at 37°C and 5 % CO_2 until the cell layer was dense. Then they could be split. Splitting was performed at least two times prior to further utilizations.

2.4.4 Transfection of HuH7

In order to introduce plasmid DNA into HuH7 cells, they were transfected using the Roche Fugene system (Fugene 6, Fugene HD or X-tremeGene HP). This reagent forms complexes with DNA and in this way shuttles the vectors into the cells. Two µg (1 µg) of plasmid were used for transfection of cells grown in 10 cm dishes (6 well plates). When cotransfecting several plasmids, they were used in the same molecular ratio making up 2 µg (1 µg) in total. The DNA was diluted in medium w/o any additives to reach 94 µl (47 µl) volume in total, and then 6 µl (3 µl) Fugene reagent were added directly into the liquid without prior touching the tube wall with the pipette tip. The batch was mixed gently and incubated at RT for at least 40' to allow the transfection complex to be formed. For transfection, preconfluent cells (~80 % density) split the day before were used because they show the best transfection rate. The splitting ratio was 1:3 for 10 cm dishes and 1:20 for 6 wells. If the cells were not fully dense and / or seeding was carried out quite late, the splitting ratio was decreased to 1:2 and 1:15, respectively. Furthermore, the growth medium was replaced by 4 ml (0.7 ml) of medium w/o any additives after the cells had been washed once with PBS prior to addition of the transfection complex. The transfection complex was transferred dropwise and evenly onto the cells and the plates were swirled afterwards to ensure even distribution. The cells were then incubated for 6 h at 37°C and 5 % CO_2. After that, the transfection medium was removed and the cell layer was washed once with PBS to get rid of remaining transfection complex. Two ml of fresh growth medium were dispensed and the transfected cells were incubated for 5 days at 37°C and 5 % CO_2 giving them time to express the viral proteins coded for on the plasmids which had been introduced.

2.4.5 Harvest of supernatant

The SN of transfected cells was harvested 5 days after transfection. It was transferred to 2 ml tubes and dead cells were pelleted by centrifugation at 13000 rpm for 10'. 750 µl aliquots of the SN were taken for immunoprecipitations and 40 µl aliquots for analysis by Western Blot. The aliquots could also be stored at -20°C for later use.

2.4.6 Preparation of cell lysates

Five days after transfection cell lysates were made to obtain the viral proteins produced in the cells. To do this, the transfected cells in the wells of 6 well plates were washed once with 1 ml of PBS and then 500 µl of lysis buffer were added. After incubation on ice for 10' the lysis buffer and lysed cells were collected in a 1.5 ml tube and the cell debris was pelleted by centrifugation at 13000 rpm for 10'. The supernatant containing the desired proteins was immediately used for Western blot or stored at -20°C.

3 Results

3.1 Establishment of a new virion detection assay

3.1.1 Overview of the experimental flow

To detect hepatitis B virions in the supernatant of transfected cells, a new assay was created detecting viral genomic DNA by a HBV genome specific qPCR. Since HBV is a non-cytopathic virus not inducing striking morphological alterations of infected cells, classical virological assays like plaque tests could not be used for quantification. The usually employed endogenous polymerase reaction relied on the visualization of radioactively labelled viral DNA and was therefore inconvenient to carry out. In contrast, the newly developed assay was designed to waive the use of radioactive substances and thus facilitated handling. At the same time it was very sensitive and even small amounts of virions could be detected.

The outline of a typical experiment was as follows: HuH7 cells were transfected with pSVHBV1.1LE- (HBV genome carrying the genes for either WT or mutant capsids and two stop codons in the surface proteins ORF so that no envelope proteins can be produced), pSVBX24H (holding the gene for the small surface protein) and pSV45-57 (expressing WT or mutant L besides M and S) in order to analyze the capability of mutant L proteins to form virions with WT or mutant capsids. Transfection of WT capsids with WT surface proteins served as positive control while expression of capsids without surface proteins presented the background of this assay. Secreted virions were purified from the supernatant of the transfected cells by an immunoprecipitation against the HBV surface proteins. Leftover plasmid from transfection was DNase I digested and the virions were opened by proteinase K treatment. Purified viral genomes were subjected to an HBV genome specific qPCR.

This special kind of qPCR was necessary in order to reduce the background resulting from leftover plasmid from transfection. Even though immunoprecipitation and DNase I digest already decrease the plasmid concentration in the samples to a large extent, plasmid would still give a strong signal in a normal qPCR. In the HBV genome specific qPCR, the specific location of the primers in the genome and in plasmid pSVHBV1.1LE- used for transfection (in the latter they are separated by the plasmid backbone) allowed preferential amplification of the genome while amplification of the vector was clearly reduced due to an elongation time of only 10 seconds.

3.1.2 Trans-complementation of the surface proteins

In the HBV genome, the open reading frames are arranged in an overlapping manner which was adopted in the HBV genome containing vectors. In order to be able to ascribe the effects of L mutations only to the alterations in the surface proteins and not to possible changes in the polymerase frame which is overlapping, plasmid pSVHBV1.1LE- carrying stop mutations in the envelope proteins rendering them unfunctional but being silent in the polymerase frame was complemented with vectors coding for L, M and S (pSV45-57 and pSVBX24H) in transfection of HuH7 cells. In this way the phenotypes of the mutants observed during the experiments could for sure be traced back to the specific L protein mutations. Involvement of polymerase changes could be ruled out.

Another main advantage of the trans-complementation approach was the fact that virions emerging from transfection of HuH7 cells could not start a new infectious cycle. The genome contained in the viral particles carried unfunctional surface protein open reading frames which prevented new virions to be formed and secreted without complementation. In this way the risk for the laboratory workers to get infected by HBV during experimental procedures could be minimized.

3.1.3 Separation of virions from capsids

In the supernatant of transfected HuH7 cells naked capsids containing the viral genome can be found besides virions and subviral particles. Since the virion detection assay outlined here relies on the detection of viral genomic DNA by an HBV genome specific qPCR, genomic DNA of naked capsids would give a false positive signal. Therefore, virions were separated from naked capsids in the supernatant by an immunoprecipitation against the HBV surface proteins. While virions carrying an envelope containing the surface proteins L, M and S bound to the bead-coupled αHBs-antibodies and were pulled down, naked capsids remained in the supernatant and could easily be washed away. Thus, only the DNA genome of viral particles was detected in qPCR.

3.1.4 HBV genome specific qPCR

3.1.4.1 Primer design and localization

Design of the qPCR primers to quantitate the viral genomes considered 3 points: Firstly, they should amplify a stretch of DNA with an optimal length of around 100 nucleotides. The location of the primers should furthermore make sure that plasmid used for transfection could

not be detected by qPCR. And last but not least, the primers should not bind to redundant sequences in the plasmids to avoid double binding and unspecific amplification.

In RT-PCR it is favourable to have short PCR products because amplicon length directly correlates to the efficiency of the reaction. Products around 100 bp are optimal but also longer ones are possible, especially if the PCR efficiency of the samples is like that of the standard (see 2.2.14.1), like it is the case in this work. To obtain amplicons as short as possible, plasmid pSVHBV1.5LE- had to be shortened to pSVHBV1.1LE- (as described in 3.2.1).

As explained briefly in 2.2.14, the problem of detection of plasmid left over from transfection could be solved by choosing primers which were separated by the plasmid backbone in the plasmid setting. The primer pair flanks a 222 bp long product on the genome but a more than 3 kb long region on pSVHBV1.1LE-. This fact was utilized to prevent formation of the disturbing amplicon and a very short elongation time during PCR favoured the short genome specific and discriminated against the long plasmid specific product.

Location of the primers had moreover to take into account that there are redundant sequences in pSVHBV1.1LE-. Binding of one of the primers in these regions would have destroyed the strategy of HBV genome specific qPCR by plasmid-backbone separation of the primers. Due to double-binding of one of the primers, the same 222 bp piece of DNA would have been produced like in the genome setting. Therefore, the primers had to be located outside of the redundancies.

Considering all these requirements, the forward primer was finally designed to bind between the two direct repeats DR1 and DR2, and the reverse primer to bind in the core gene region just downstream of DR1 (see Fig. 13). Plasmid pSVHBV1.5LE- was chosen as standard.

3.1.4.2 Efficiency of background reduction in the HBV genome specific qPCR

To test the efficiency of the genome specific qPCR, known amounts of pSVHBV1.5LE- (genome-like amplification) and pSVHBV1.1LE- (plasmid used for transfection) were analyzed. The results demonstrated that a reduction of the signal by more than 1000-fold could be achieved (Fig. 14).

The reason for the remaining background signal was investigated by subjecting the qPCR products to agarose gel electrophoresis (Fig. 15). Surprisingly, a clear band of the same size as the HBV genome amplicon could be detected when amplifying pSVHBV1.1LE-. This was probably due to translocation of the unfinished amplicons containing a part of redundant sequence to the other redundancy on the plasmid. There they could bind and were further

elongated by the polymerase until the second primer binding site and further downstream. In the next cycle of PCR both HBV genome specific primers were able to bind to the newly created DNA and produced PCR products of exactly the same length as PCR of the HBV genome would have generated.

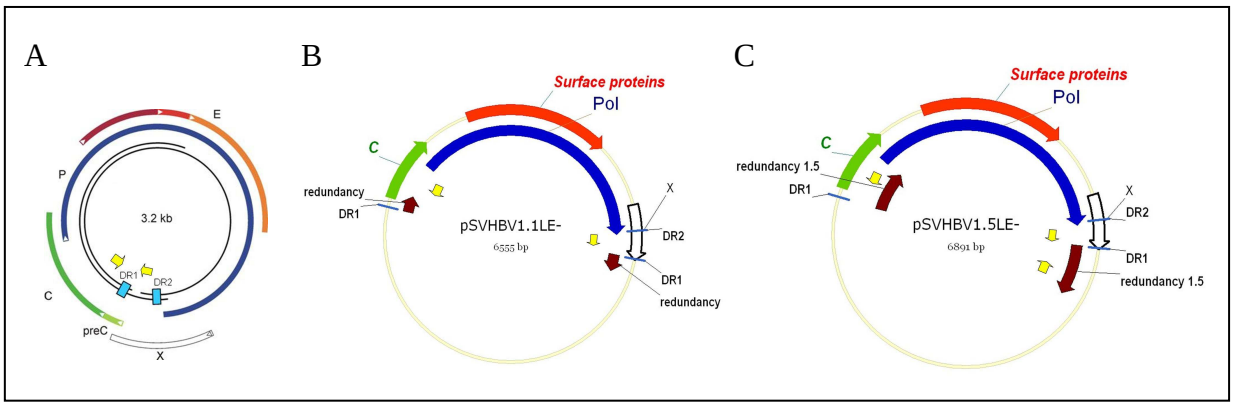

Fig. 13. Location of qPCR primers (yellow) in the genome (A), pSVHBV1.LE- (B) and pSVHBV1.5LE- (C). While just a short amplicon was produced during PCR using the HBV genome as template (A), a long product was synthesized from pSVHBV1.1LE- (B). This could be avoided by using a very short elongation time and in this way the plasmid was almost excluded from amplification. The vector pSVHBV1.5LE- (C) was used as standard because of its long redundant sequences which allowed production of the same amplicon as from the genome.

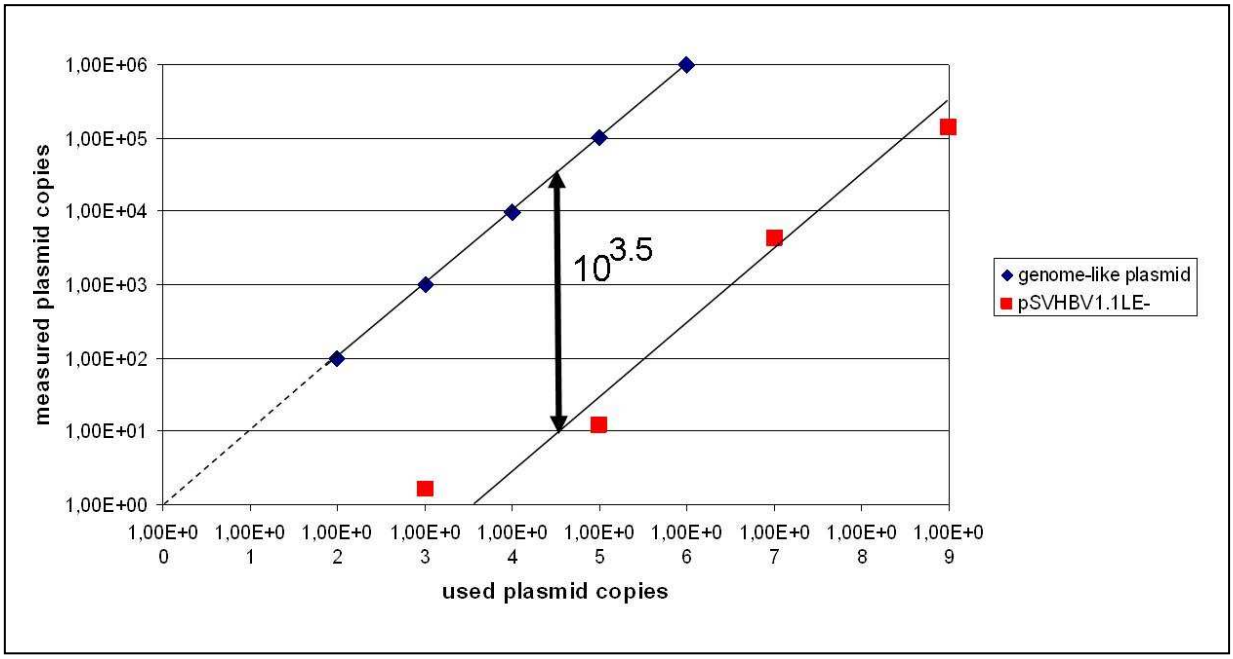

Fig. 14. Efficiency of the HBV genome specific qPCR. Plasmid pSVHBV1.5LE- which produces the same amplicon as PCR of the HBV genome was compared to pSVHBV1.1LE- which was used for transfection of HuH7 cells. Known amounts were subjected to qPCR with the newly designed primers and a reduction of background by 3.5 log was achieved.

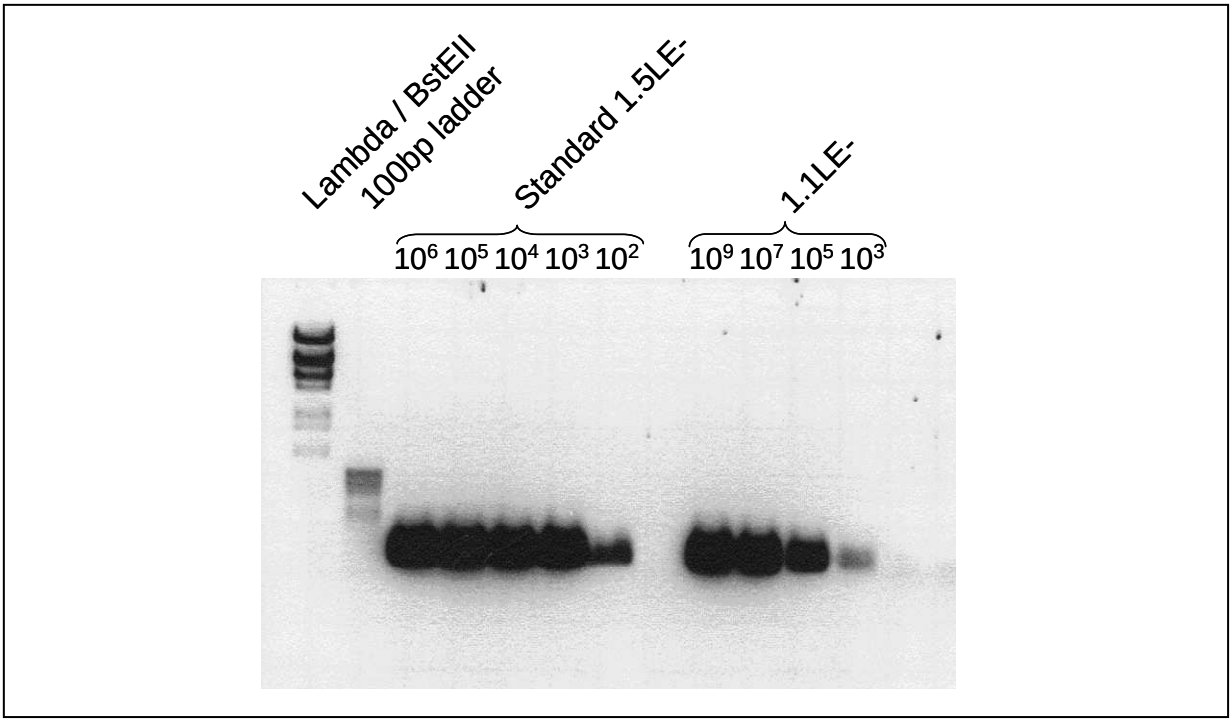

Fig. 15. HBV genome specific qPCR products of pSVHBV1.5LE- and pSVHBV1.1LE- have the same size. The products of qPCR reactions with pSVHBV1.5LE- (standard 1.5LE-) and pSVHBV1.1LE- (1.1LE-) were subjected to gel electrophoresis and bands of the same size could be visualized by ethidium bromide staining.

3.1.5 Choice of immunoprecipitation strategy

Precipitation of proteins can be performed directly (antibodies are immobilized on beads first and added to the proteins later) or indirectly (antibodies are added to the protein solution prior to adding and binding to the beads). Furthermore, it can be advantageous to preclear the protein solution from unspecific material occupying the protein G on the agarose beads and thereby disturbing binding of the specific αHBs antibodies.

Testing was performed with the virion containing supernatant of HuH7 cells transfected with pSVHBV1.1LE-, pSVBX24H and pSV45-57. The preclearance step was carried out by adding 20 µl beads to 750 µl supernatant and following incubation on the sample rotator at 4°C for 2 h. After removal of the beads, immunoprecipitation was performed as described in 2.2.11. The HBV genomes isolated were subjected to HBV genome specific qPCR.

Immunoprecipitation with preceding 6 h of preincubation of beads with antibodies showed the best result and was therefore chosen as standard protocol (see Fig. 16). Without preincubation and with preclearance the IP efficiency decreased by around one third.

3.1.6 Effect of FBS on immunoprecipitation

For many different experiments it is known that fetal bovine serum can disturb further analyses. In order to exclude effects of fetal bovine serum on the virion detection assay this

was tested by adding medium without FBS after transfection of HuH7 instead of normal growth medium. Inspection of the cells under the microscope after 5 days showed increased cell death. Quantification of the virions in the supernatant exhibited a higher amount of virions in the supernatant of cells incubated with FBS containing medium compared to those without (Fig. 17). Therefore, normal growth medium was used for further experiments.

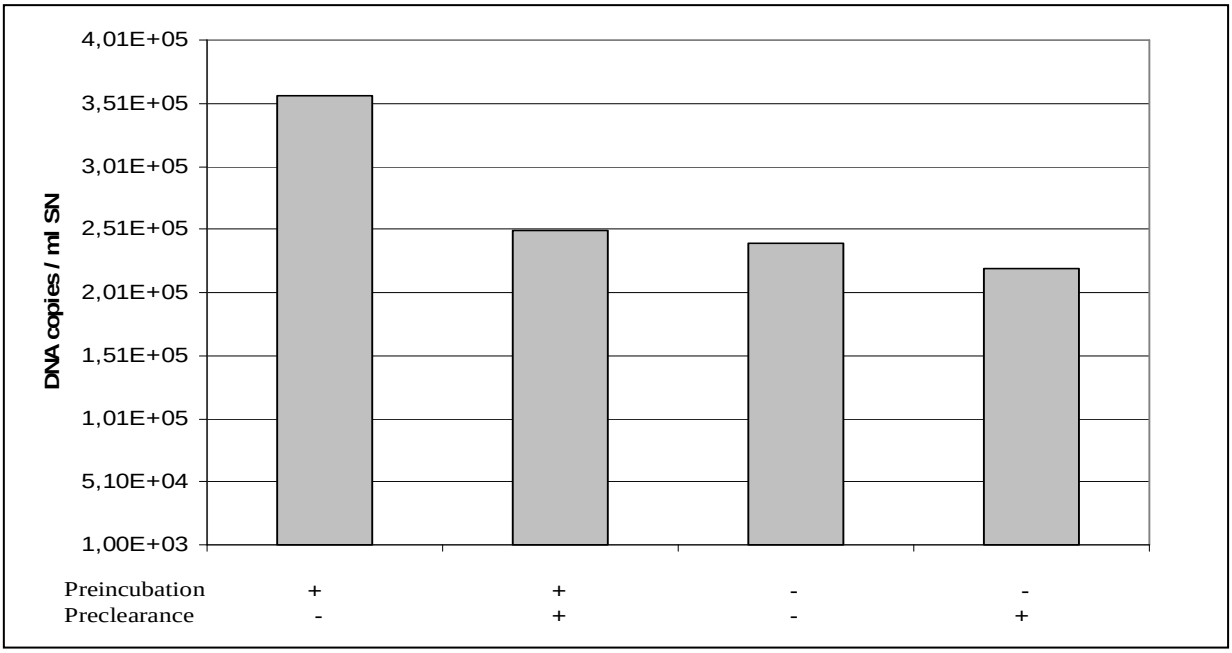

Fig. 16. Comparative analysis of immunoprecipitation methods. The virion containing supernatant of transfected HuH7 cells was subjected to different immunoprecipitation strategies. First, an IP with preincubation of beads and antibodies was performed (first bar). Second, the SN was precleared prior to IP with preincubation (second bar). Next, the IP was performed without preceding preincubation and preclearance (third bar) and finally, precleared SN was subjected to IP without preincubation (fourth bar).

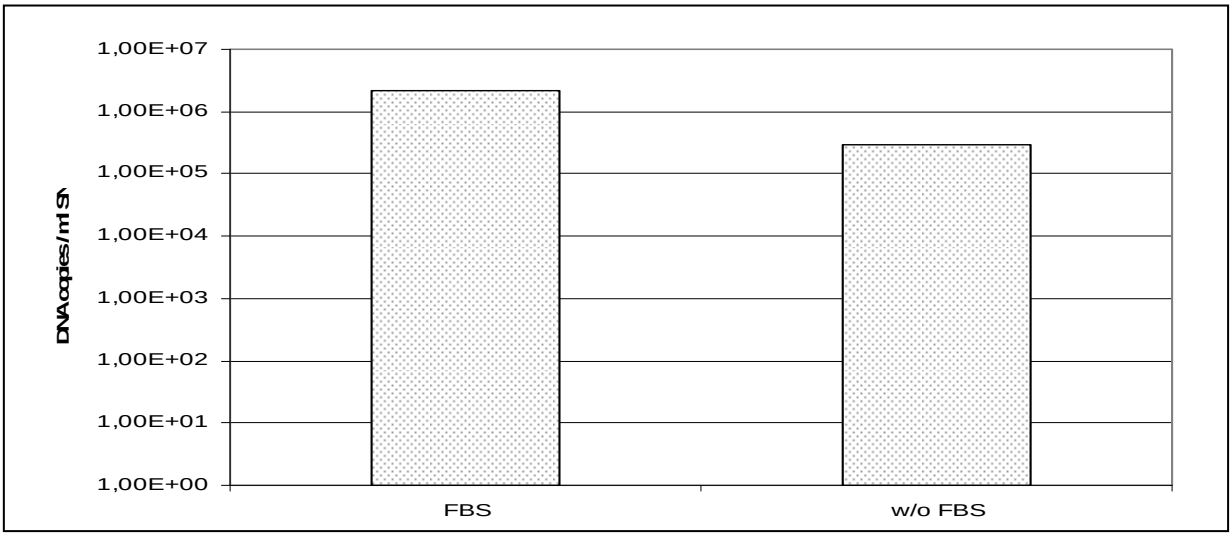

Fig. 17. Comparison of virion amount in supernatants of cells grown in medium with or without FBS. Upon transfection with WT capsid and WT L, HuH7 cells were allowed to grow in presence or without FBS in the growth medium for five days. Then, virions in the supernatant were quantified with the virion detection assay.

3.1.7 Determination of amounts of beads and antibody used for immunoprecipitation

To define appropriate amounts of protein G coupled agarose beads (Santa Cruz) and sheep αHBs antibodies for immunprecipitation of hepatitis B virions, a titration was performed. Identical amounts of virions in 1 µl plasma of an HBV positive donor were precipitated with different amounts of beads and antibodies. Isolation and detection of the viral genome was performed with the new assay. The samples were compared to a sample lacking immunoprecipitation prior to DNA preparation. The results of qPCR showed that 25 µl of beads and 1µl of sheep αHBs (2 µl when the antibody stock was diluted with glycerol) are sufficient for proper immunoprecipitation of virions (see Fig. 18). In sample three (25 µl of beads + 0.5 µl of sheep αHBs) a part of the beads was lost explaining the low value obtained by qPCR.

3.1.8 Comparison of virus production from HuH7 transfections with differently purified plasmids

Because of the fact that large scale preparation of plasmids is quite a time consuming issue and impossible to carry out for over 200 clones produced during this work, it was tested if plasmid minipreps purified by phenol-chloroform extraction can be transfected and expressed in HuH7 as well as maxiprepped vectors. For this purpose, mini- and maxiprepped pSV45-57 were cotransfected with pSVBX24H and pSVHBV1.1LE- into HuH7 cells and the amount of secreted virions in the supernatant was determined (Fig. 19). The results showed that there was no difference in the amount of secreted virions no matter if the plasmids used for transfection were mini- or maxiprepped.

3.1.9 Determination of the level of negative transdominance

MD mutants defective in virion morphogenesis exert a transdominant negative effect on the secretion of WT HBV virions. Cotransfection of envelopment-negative L mutants with WT HBV genomes showed that the release of WT virions was substantially suppressed (Bruss, 1997).

This fact was important for defining the number of L mutants which could be cotransfected at the same time in complementation tests. Since more than 200 MD mutants were produced in this work it was advantageous to transfect them in pools since transfection and virion detection are both money and time consuming. To test the possibility of cotransfecting all 19

mutants of one MD position at once, the envelopment negative large surface protein mutant I1 (Bruss, 1997) was employed. It was introduced into Huh7 in different ratios together with WT L and WT capsid and virion secretion was assayed. qPCR results demonstrated that even 5 % of functional L were sufficient to ensure detection by the newly established assay. Therefore, the L mutants were combined to pools of 19 mutants and used like this for further experiments.

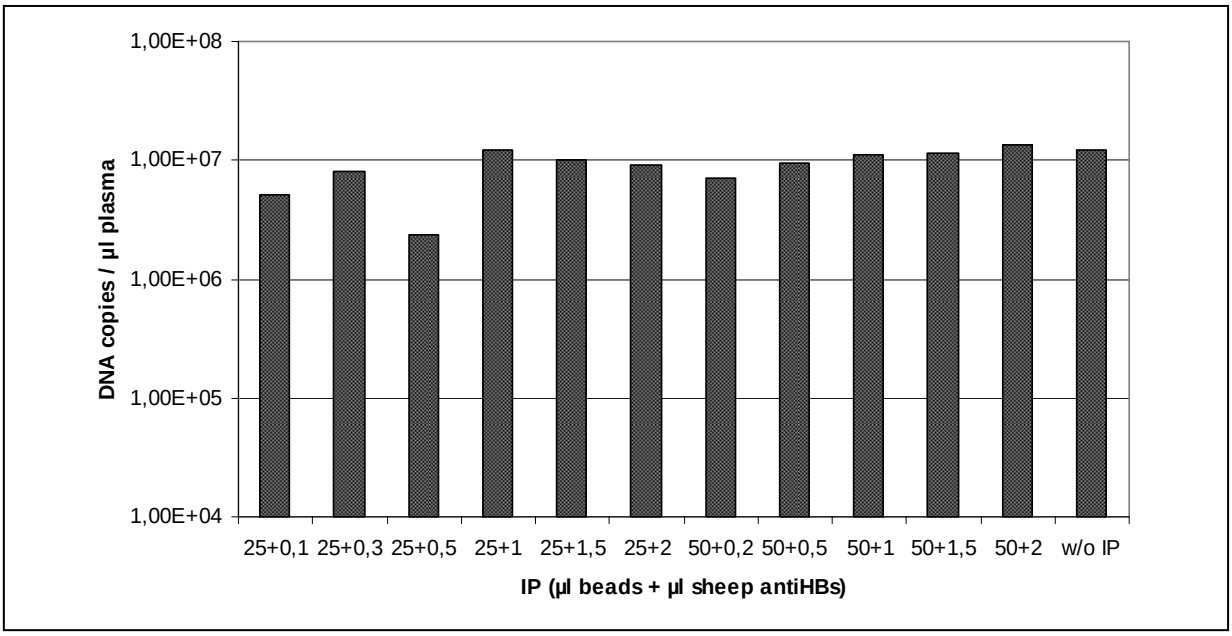

Fig. 18. Titration of agarose beads and αHBs antibodies for immunoprecipitation of virions. Different amounts of beads and antibody were used for pulling down virions from the plasma of an HBV carrier. After purification of the HBV genomic DNA it was subjected to the HBV genome specific qPCR.

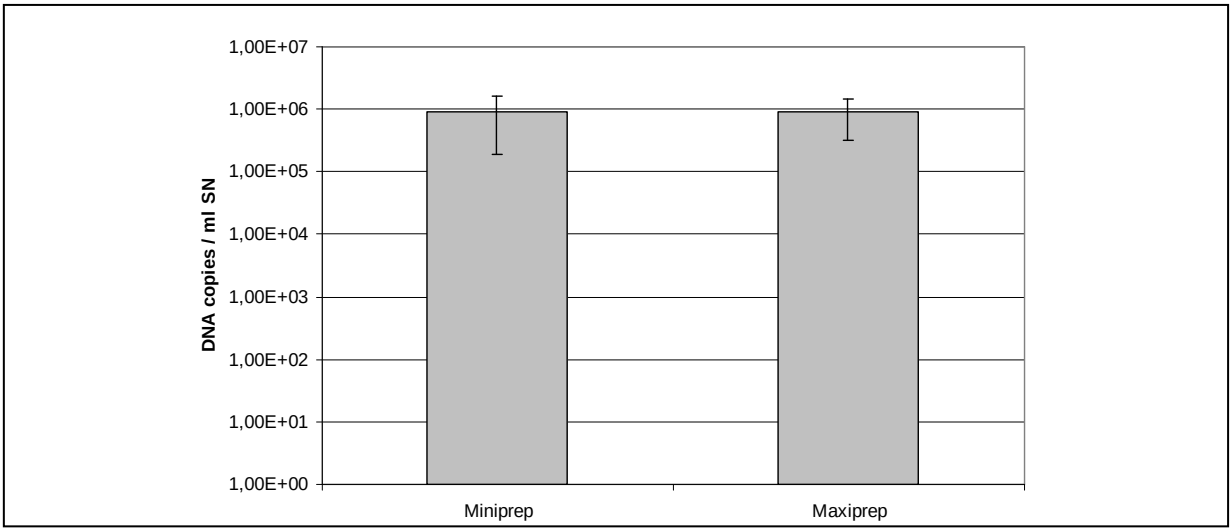

Fig. 19. Comparison of virion secretion upon transfection of HuH7 cells with differently purified plasmids. HuH7 cells were transfected with mini- or maxiprepped pSV45-57 and cotransfected with pSVBX24H and pSVHBV1.1LE to allow virion production. Virions were measured by the new virion detection assay. Mean value and standard deviation were calculated from 18 independent experiments.

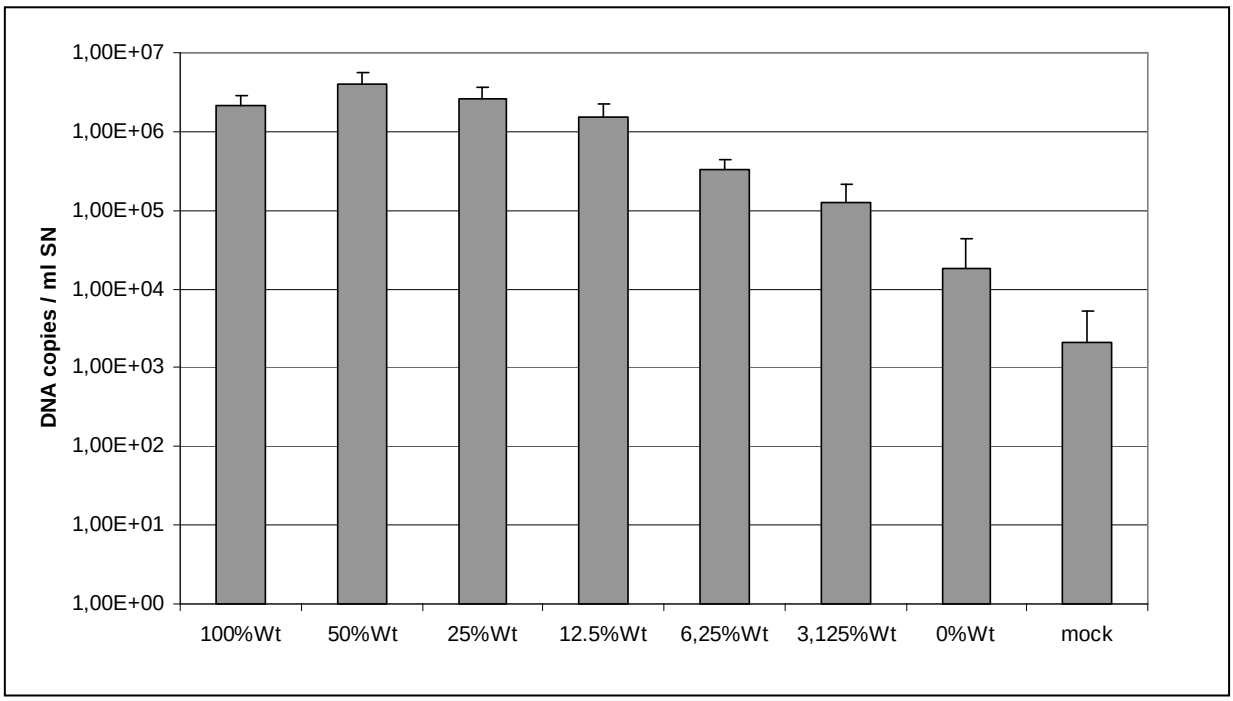

Fig. 20. Transdominant negative effect of L mutants defective in virion morphogenesis. The supernatant of HuH7 cells transfected with WT capsid and different ratios of WT L and the envelopment negative large surface protein mutant I1 (Bruss, 1997) was subjected to virion detection by the HBV genome specific qPCR. The experiment was performed four times.

3.1.10 Demonstration of stable L mutant expression and integration in subviral particles

Expression of WT and mutant L proteins was both driven by SV40 promoters. Therefore, the level of protein production should be equal. Nevertheless, to verify that mutant L proteins were expressed upon transfection like the WT and were also equally stable in HuH7 cells, a random selection of pSV45-57 vectors carrying mutations was introduced into HuH7, the supernatant containing subviral particles was harvested and a Western blot against the S epitope from amino acid 102 to 125 thereby staining all three envelope proteins was performed. Since the L proteins were expressed from plasmid pSV45-57 lacking 30 N-terminal amino acids, the L proteins detected on the Western blot were around 3 kDa smaller than in the natural setting. Therefore, the 36 kDa band showing the double glycosylated M was superimposed by the unglycosylated L band which was found at 36 kDa instead of 39 kDa.

Expression of envelope proteins alone without any other viral components results in the secretion of subviral particles. For this reason, detection of the large surface protein on the Western blot showed that the mutant L proteins were stably expressed and in addition competent for incorporation into subviral particles. Mutants of matrix domain residues that

could support SVP but not virion formation must therefore play a specific role in capsid envelopment and not in association with other envelope proteins.

Western blots of all phenylalanine mutants (Fig. 23) as well as all mutants of matrix domain residue W122 (Fig. 21) were performed and except W122T which was not expressed at all (Fig. 22), all of them formed subviral particles. Repeated sequencing of W122T showed an insertion in L causing a frame shift rendering the protein unfunctional.

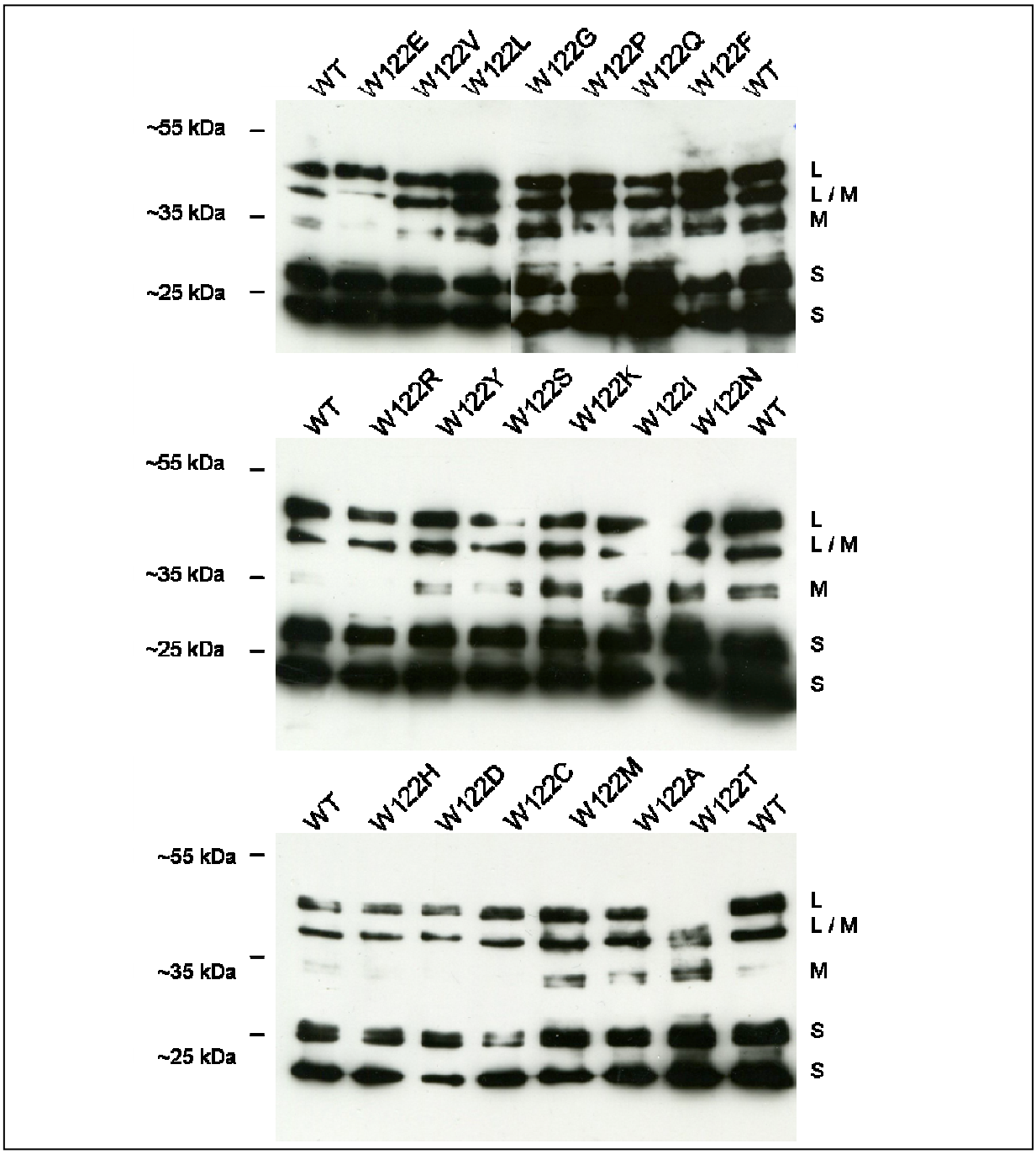

Fig. 21. All mutants of residue W122 except W122T express L protein and form subviral particles. The supernatant of cells transfected with plasmid pSV45-57 coding for the WT or the different W122 mutants was analyzed for the presence of subviral particles by SDS-PAGE. L could be detected for all mutants except W122T. Exposure times were 5' for the upper two blots, 12' for the lower one.

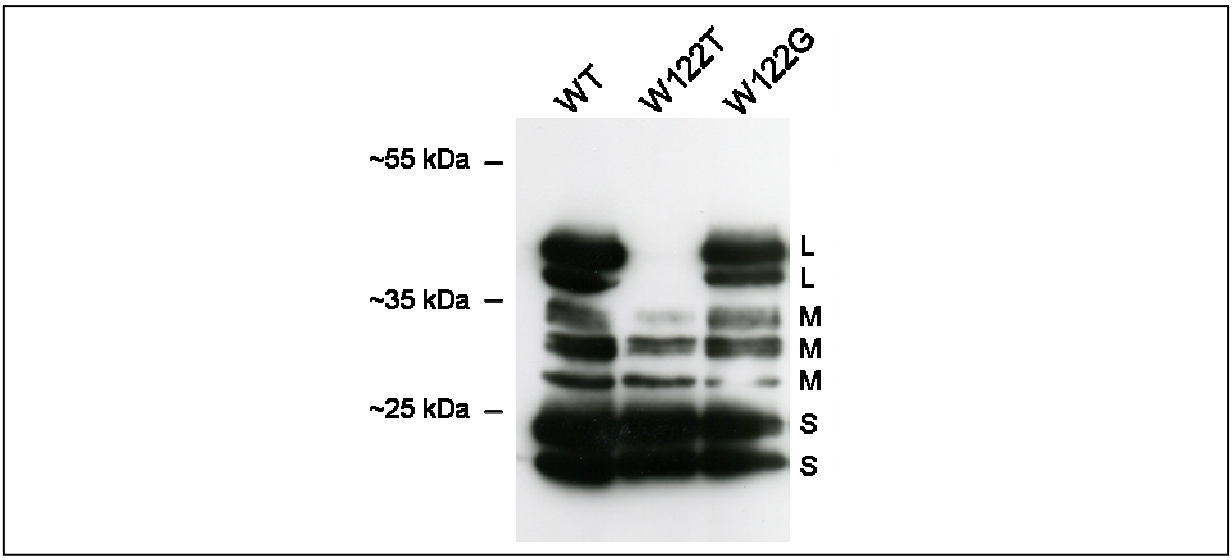

Fig. 22. W122T is not expressed. A Western blot of the lysate of transfected HuH7 cells showed that mutant W122T was not expressed. Exposure time was one minute.

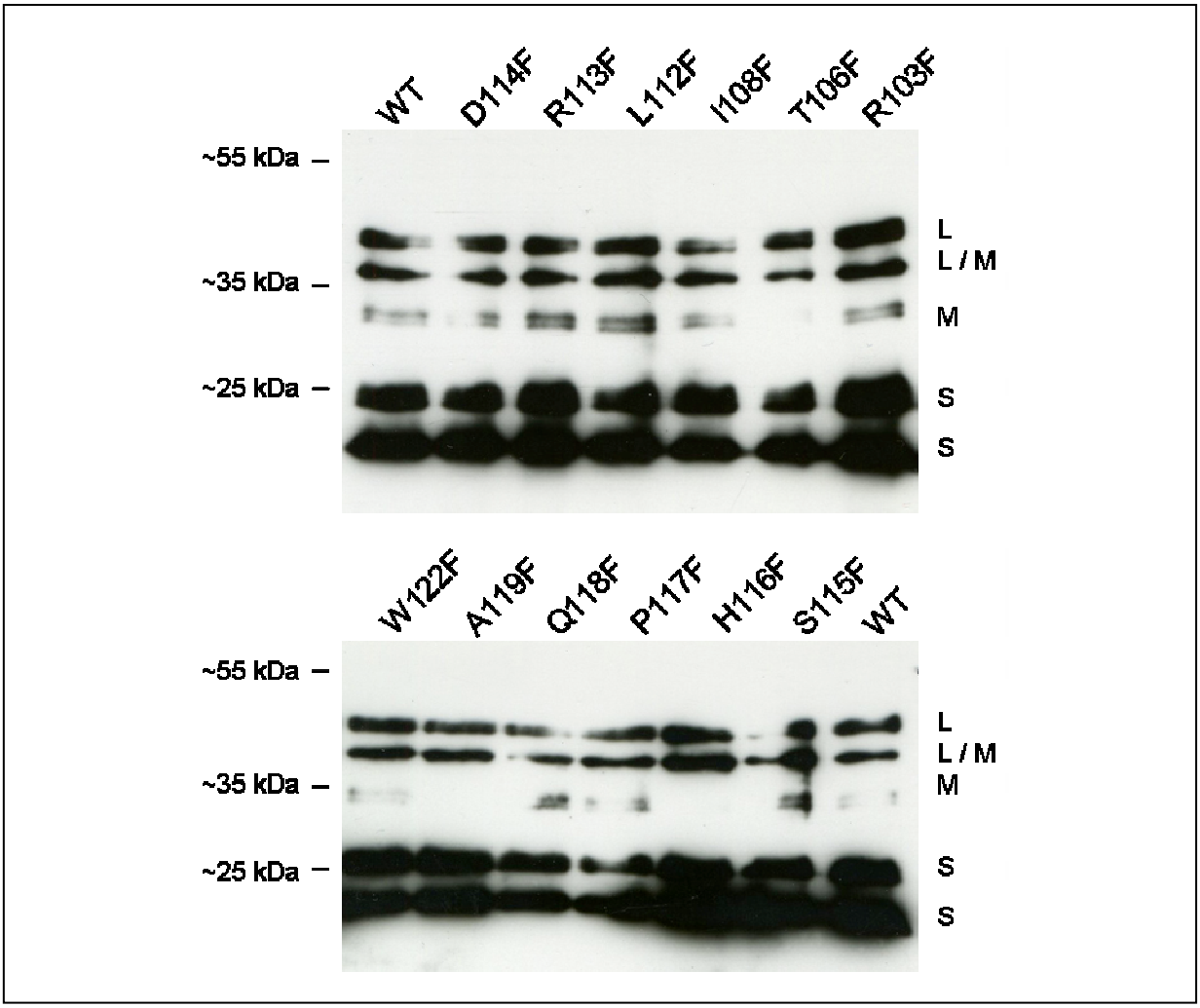

Fig. 23. The phenylalanine mutants of all tested matrix domain residues express L protein and form subviral particles. Western blots of the supernatant of HuH7 cells transfected with the respective phenylalanine mutants proved the expression of mutant L proteins and integration in subviral particles. Exposure times were 1.5' (upper blot) and 2' (lower), respectively.

3.2 Mutagenesis

3.2.1 Generation of pSVHBV1.1LE-

In the HBV life cycle, a pregenomic transcript of the circular genome is produced which is longer than the genome (see 1.1.4). After starting the transcription, the polymerase reads through the polyadenylation signal and continues until it reaches the polyA signal again. In this way it produces an RNA having a short terminal redundancy. The end of the transcript is demarcated by the HBV polyadenylation site. This specific RNA is the substrate for reverse transcription of the pregenome to DNA during nucleocapsid maturation.

Due to the need of this transcript for proper virus replication upon transfection of cells, HBV plasmids always contain at least the 1.1fold version of the genome allowing transcription of the pregenomic RNA. The redundancy carried by the vectors must therefore at least include the sequence until the polyadenylation site.

In terms of primer design for the HBV genome specific qPCR it was favourable to keep the HBV insert as short as possible (see 3.1.4.1) and therefore plasmid pSVHBV1.5LE- carrying a 500 bp redundancy was shortened to pSVHBV1.1LE- with the minimum redundancy of 164 bp. This was achieved by deleting part of the redundant sequence using the method described in 2.2.1.5. The final colony PCR employing primers VBBS4 and VBBS5 amplified the region of interest and demonstrated that deletion of part of the redundant sequence had been successful. Sequencing finally verified the correct nucleotide sequence and excluded the rise of additional mutations caused by PCR.

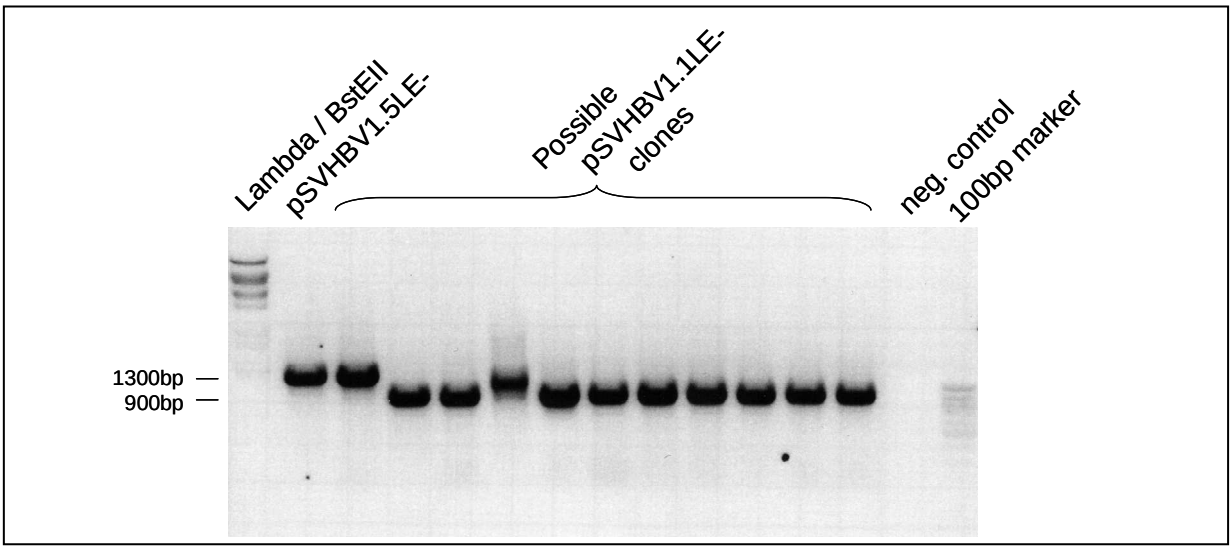

Fig. 24. Colony PCR of the region shortened in pSVHBV1.1LE-. PCR of the region of interest in the parental vector pSVHBV1.5LE- and the newly generated pSVHBV1.1LE- clones showed that 9 of 11 colonies contained the deletion and therefore carried the correct insert.

3.2.2 Choice and generation of capsid mutants

Capsid mutants to be used for complementation assays with MD mutants were chosen according to their phenotype as described by Pairan and Bruss, 2009. Ideally, virion secretion was abolished, but capsids were produced in amounts close to WT level. Position 126 of the capsid had been described extensively and was found to be very restricted to the WT amino acid isoleucin for functional virion assembly. Only methionine could functionally substitute it. When exchanged against alanine, capsids were still abundantly produced but envelopment was disturbed. The same phenotype was detected for the mutants S17T and K96H (Pairan and Bruss, 2009). Therefore these three capsid mutants were chosen for further testings.

Capsid mutant I126A was produced by site-directed mutagenesis by unique restriction site elimination (see 2.2.1.1) using VBBS23 as mutagenesis primer and VBBS24 as selection primer changing the unique restriction site NgoMIV against NheI. S17T and K96H were generated by site-directed mutagenesis via fusion PCR (2.2.1.4), employing the primers VBBS_LErev, FWD_LEfwd, VBBS_S17T, VBBS_K96H, S17T_Rev and K96H_Rev (see Fig. 25). Cotransfection with the three WT surface proteins verified that no virions were secreted (Fig. 26).

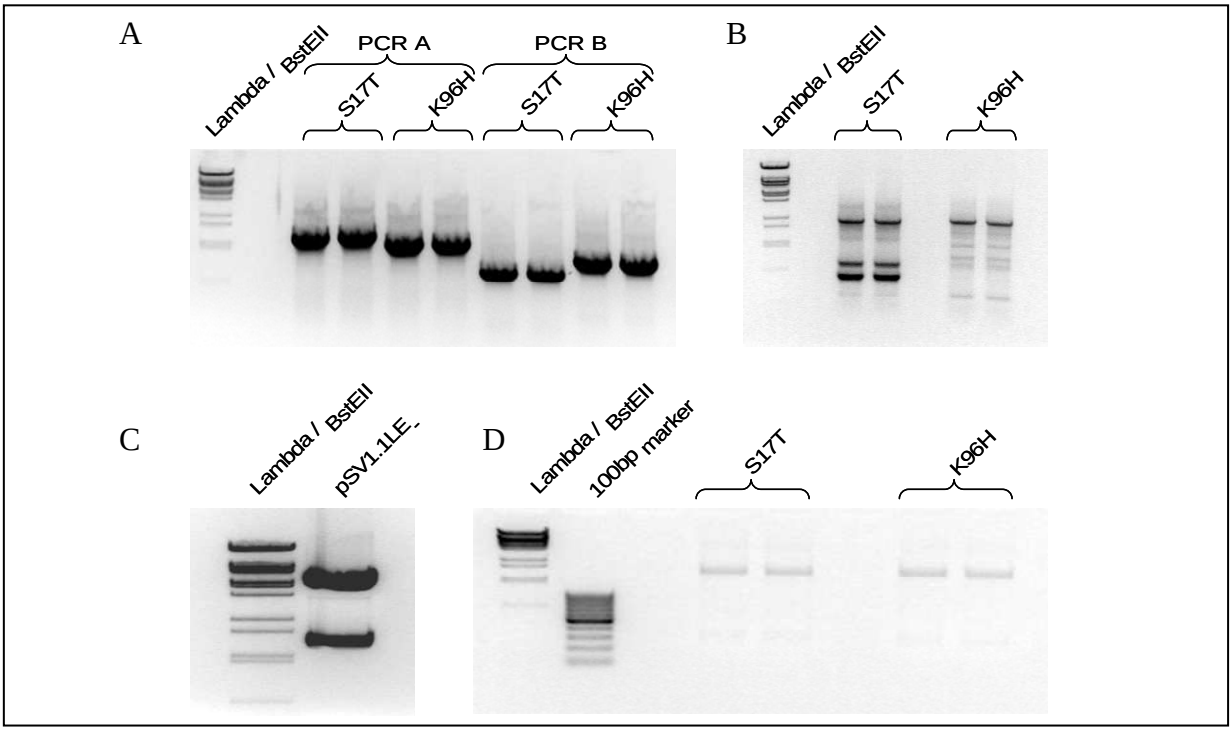

Fig. 25. Generation of pSVHBV1.1LE- with core mutations S17T and K96H. Capsid mutants S17T and K96H were produced by fusion PCR using pSVHBV1.1LE- as template. (A) PCRs A (using primers VBBS_LErev and VBBS_S17T or VBBS_K96H) and B (primers FWD_LEfwd and S17T_Rev or K96H_Rev) generated amplicons of the correct size bearing the S17T or K96H mutation. The two PCR products were ligated by another PCR with the primers FWD_LEfwd and VBBS_LErev (B; upper band). They were subjected to restriction with EcoRI and SalI (D), as well as pSVHBV1.1LE- which was used as backbone for subcloning (C).

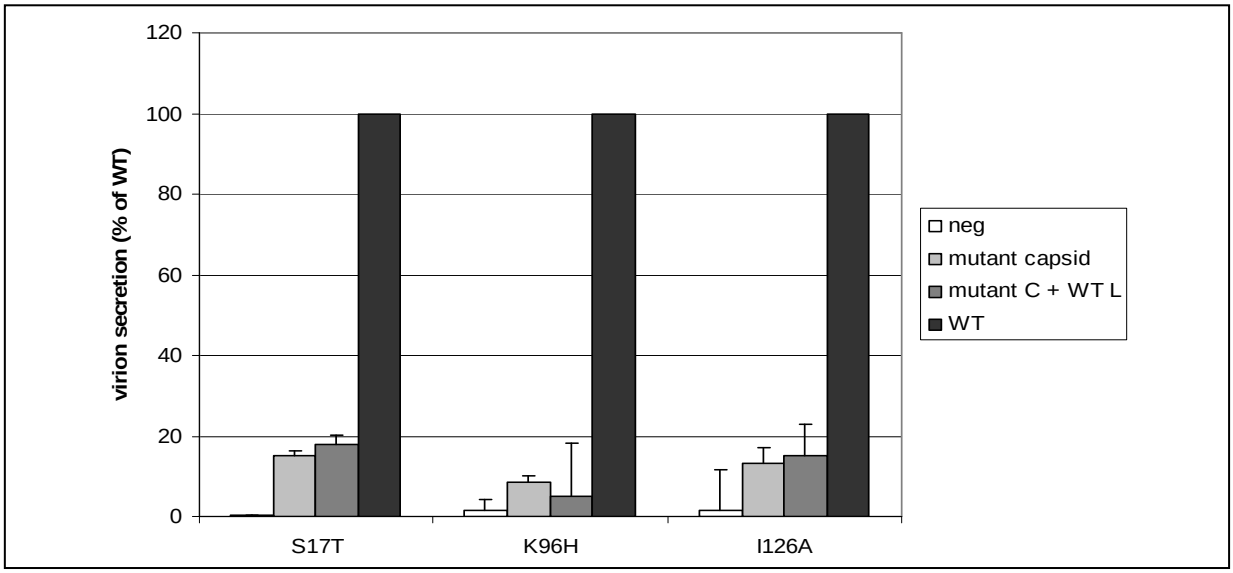

Fig. 26. Capsid mutants S17T, K96H and I126A cannot be enveloped by WT L. Cotransfection of mutant capsids with WT surface proteins (third bar) ruled out the possibility that these mutants can form virions. The background of the assay was transfection with the capsid mutants alone (second bar), positive control was the cotransfection of WT C with WT L (fourth bar). Secretion of virions was monitored by the HBV detection assay.

3.2.3 Production of MD mutants

Matrix domain residues which were chosen to be substituted by all non-WT amino acids were mutated using either the USE mutagenesis method or by mutagenesis involving fusion PCR (see 3.2.3.1 - 3.2.3.4). Fig. 27 highlights the matrix domain residues which were subjected to mutagenesis and further testings.

3.2.3.1 Mutagenesis by USE

Mutagenesis of the L protein matrix domain was mainly carried out with the Clontech Transformer^{TM} Site-directed mutagenesis kit using the method of unique restriction site elimination (2.2.1.1). The largest benefit of this system is the possibility to distinguish between parental and altered vectors by a simple control enzyme digest. For MD mutagenesis, VBBS25 was used as selection primer changing the NheI restriction site to SalI. Before sequencing, clones from mutagenesis were subjected to control digestion with the enzyme for the newly introduced SalI restriction site (see Fig. 28) and only clones being cut were further analysed. Besides the altered restriction site they usually also carried the desired L protein mutation. Because of this pre-selection of positive clones far less clones had to be sequenced than with conventional mutagenesis strategies.

3.2.3.2 Efficiency of mutagenesis by USE using wobble primers

Mutagenesis was initially performed using mutagenesis primers with degenerate nucleotides at defined positions. They were used as described in 2.2.1.1 and 2.2.1.2 to alter the matrix domain sequence in vector pSV45-57. The advantage of this method is the generation of a lot of different mutants of one specific amino acid by a single mutagenesis reaction. It is very timesaving and convenient to handle. Mutants of L112 and A119 were produced in this way. Of 96 colonies prepped, 67 and 62, respectively, were sequenced and 8 / 11 mutants of the desired 19 were detected. Also the WT amino acids were found in high ratios e.g. more than 30 % of the A119 clones sequenced were WT (Fig. 29). This suggests binding of only the selection primer in the initial primer elongation step. Furthermore, insertion or deletion of nucleotides could be observed within the primer binding regions indicating the use of incorrectly synthesized primers. Since sequencing in high extents is quite money consuming and the rate of mutant yield was just around 50 %, the mutagenesis method was finally changed from wobble primers to defined primer mixes.

3.2.3.3 Performance of mutagenesis by USE using primer mixes

The use of defined primer mixes for mutagenesis with the Clontech Transformer^{TM} Site-directed mutagenesis kit was only slightly more effort but proved to be superior in efficiency compared to mutagenesis with wobble primers. The mutant yield was around 75 % ranging from 12 to 17 mutants observed after sequencing of 70 – 80 clones each (see Fig. 30). WT sequences could also be detected, as well as additional mutations. Because of high sequencing costs the lacking mutants (R103, T106, H116 and Q118) were produced individually which finally turned out to be the most moneysaving strategy.

3.2.3.4 Mutagenesis by fusion PCR

Mutants H116D, H116L, H116W and Q118A were produced by classical fusion PCR (see 2.2.1.4 and Fig. 31) since mutagenesis by USE did not result in correct mutants. The primers listed in 7.4 were employed for the 1st PCR (PCR A: VBJN4 + VBBS_H116D / H116W1 / H116L1 / Q118A; PCR B: VBBS_fwd_45H + REV_H116D / H116W1 / H116L1 / Q118A). The 2nd PCR made use of primers VBJN4 and VBBS_fwd_45H (Fig. 31 B) joining the two PCR products resulting from the first PCR. Digestion of the final PCR products and pSV45-57 was performed sequentially with the two restriction enzymes SpeI and Bpu10I to yield the wanted inserts and the backbone for subcloning, respectively (see Fig. 31 C). Mutants of correct sequence were identified by sequencing.

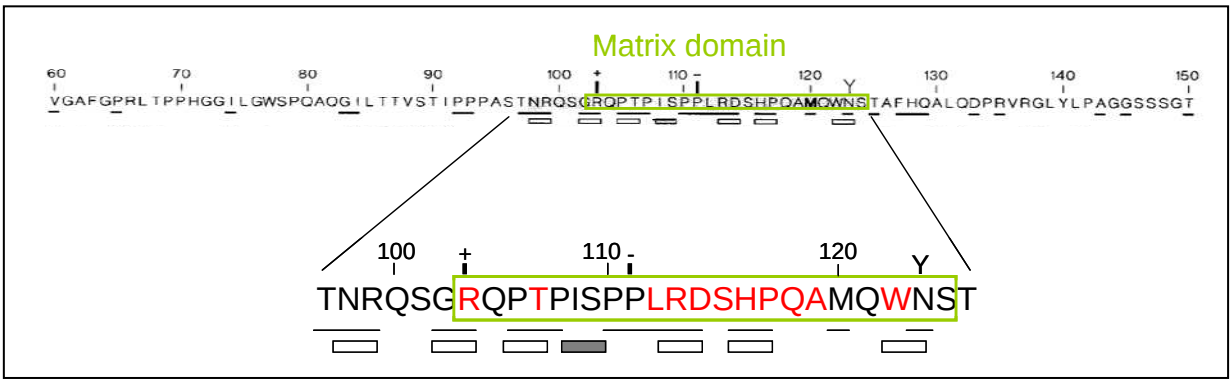

Fig. 27. Scheme of the matrix domain (adapted from Bruss, 1997). The amino acid sequence of L from V60 to T160 is shown with the matrix domain marked by a green box. Matrix domain residues chosen for mutagenesis are depicted in red and include R103, T106, L112, R113, D114, S115, H116, P117, Q118, A119 and W122. The methionine at position 120 is the start codon for the M protein. Underlined residues are conserved in woodchuck hepatitis virus. Double-alanine mutations are depicted by open (envelopment-negative phenotype) and closed (WT phenotype) boxes.

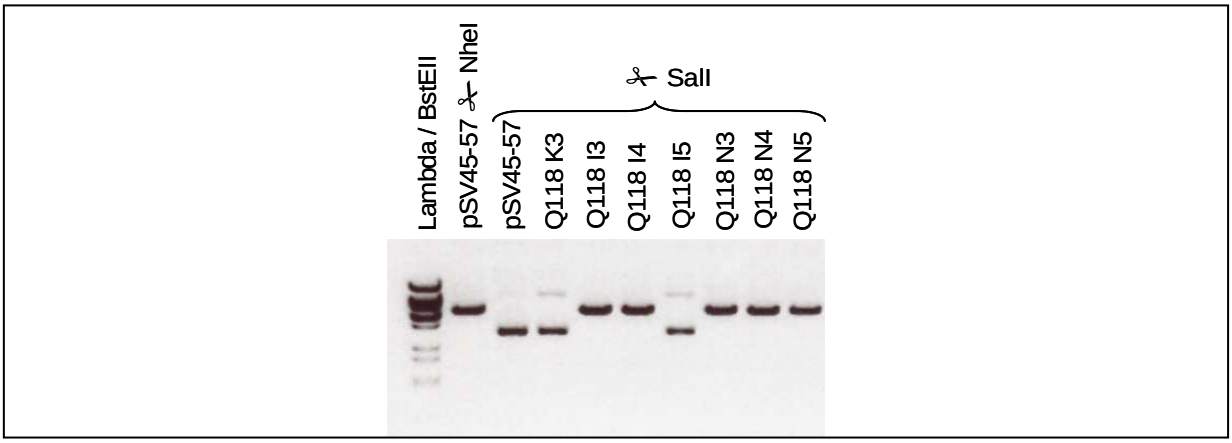

Fig. 28. Control restriction of possible Q118 mutant clones. Possible Q118 mutants were cleaved with the enzyme for the newly introduced SalI cleavage site to prove success of mutagenesis. The parental vector pSV45-57 was cut with NheI (single cutter) and SalI (non-cutter).

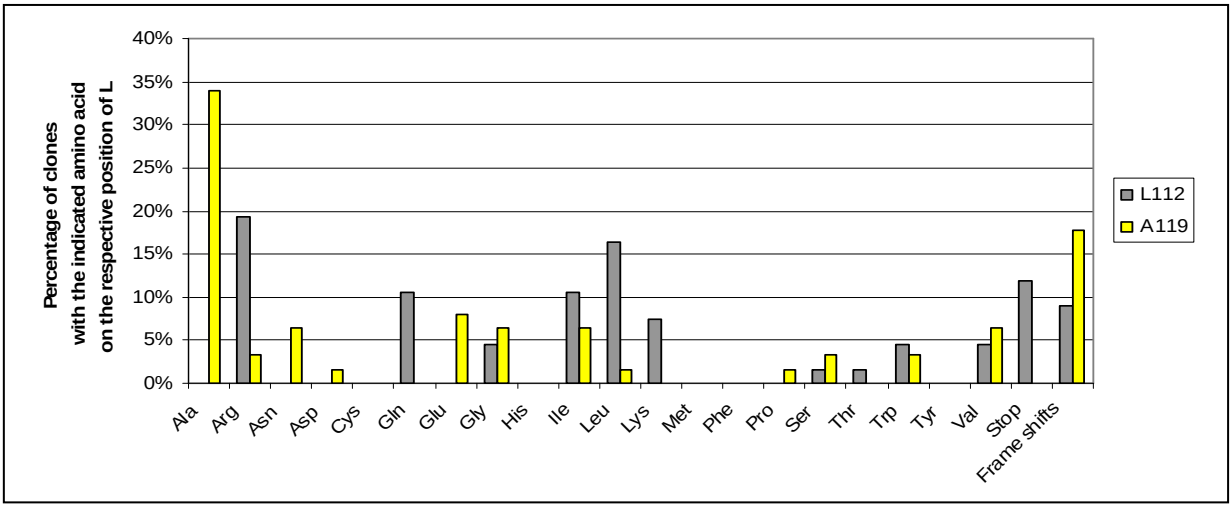

Fig. 29. Distribution and yield of amino acids after mutagenesis by USE using wobble primers. 10 mutants of residue L112 and 12 of A119 could be confirmed by sequencing of 67 and 62 clones, respectively. Besides desired mutations also WT sequences, frame shifts and stop mutations could be detected.

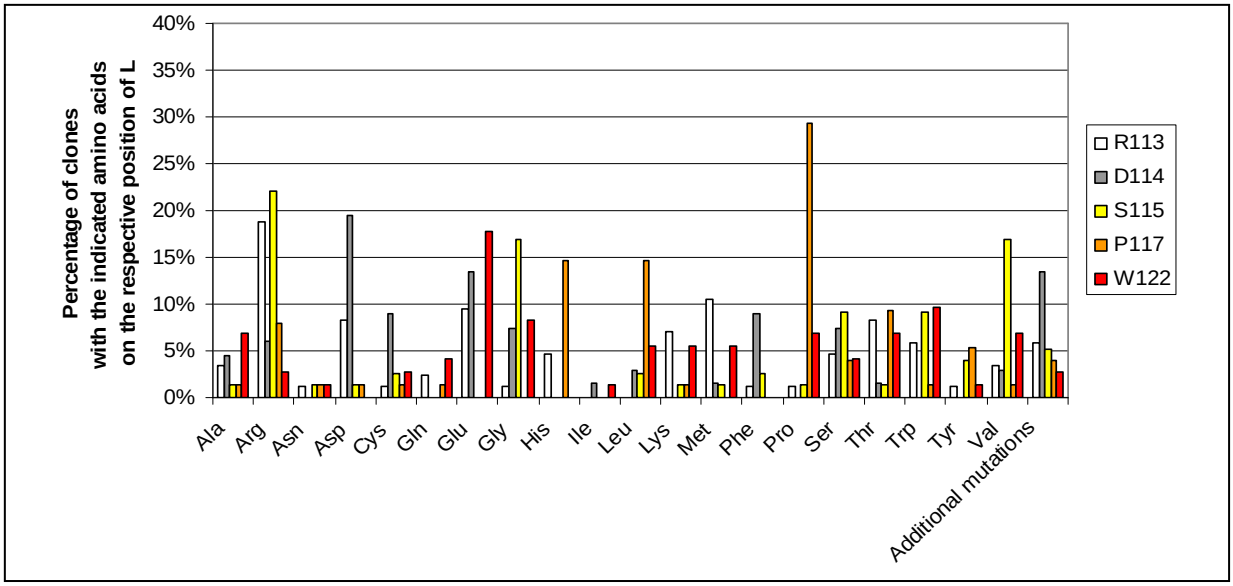

Fig. 30. Distribution and yield of amino acids after mutagenesis by USE using defined primer mixes. Residues R113, D114, S115, P117 and W122 were mutated using mutant primer mixes. Around 75% of the desired mutants could be produced in this way in one single reaction.

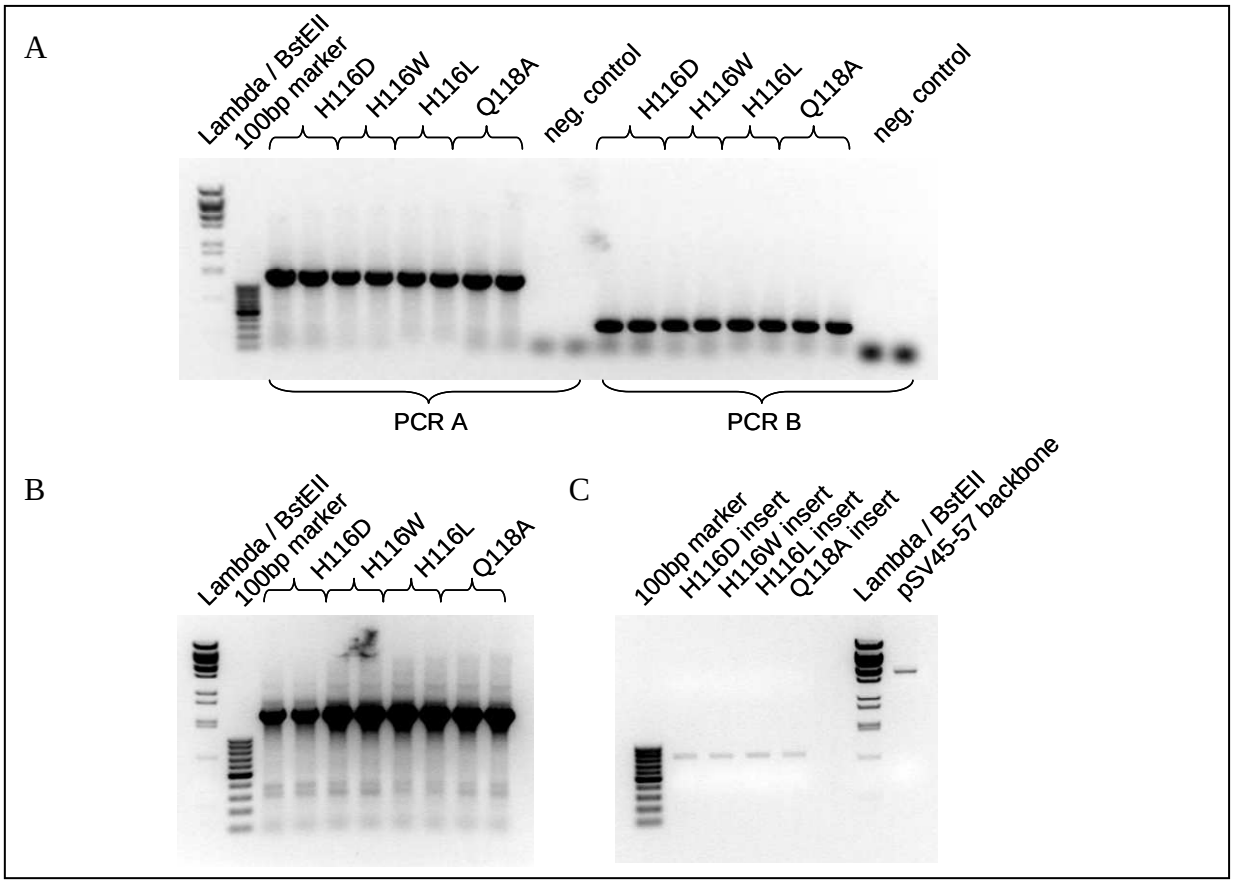

Fig. 31. Generation of MD mutants H116D, H116W, H116L and Q118A by fusion PCR. The matrix domain mutants H116D, H116W, H116L and Q118A were produced by fusion PCR using pSV45-57 as template. (A) PCRs A (using primers VBJN4 and VBBS_H116D / H116W1 / H116L1 / Q118A) and B (primers VBBS_fwd_45H + REV_H116D / H116W1 / H116L1 / Q118A) generated amplicons of the correct size holding the desired mutation. The two PCR products were ligated by another PCR with the primers VBJN4 and VBBS_fwd_45H (B). They were subjected to restriction with SpeI and Bpu10I to obtain the correct insert, as well as pSV45-57 which was restricted to yield the plasmid backbone. Prior to ligation both inserts and backbone were purified by gel extraction (C).

3.3 Complementation tests of MD mutants with mutant capsids

Up to the present it has not been resolved how capsid and envelope make contact with each other during envelopment. An interaction is essential for the budding of capsids but how this interaction could look like is still unclear. It could on the one hand either be a direct interaction between the HBV surface proteins and the capsid or on the other hand cellular proteins could be involved und function as linking molecules.

In order to test the hypothesis of a direct interaction between the capsid and the large surface proteins of HBV, corresponding complementation assays were performed. The envelopment-negative capsid mutants I126A, K96H and S17T were cotransfected with the pools of matrix domain point mutants and virion secretion of the cells was monitored. Identification of a MD mutant able to intergenically complement the capsid mutations would strongly argue for a direct interaction.

The results showed that none of the L mutant pools tested could compensate for the envelopment-negative capsid mutants S17T, K96H (see Fig. 32) or I126A.

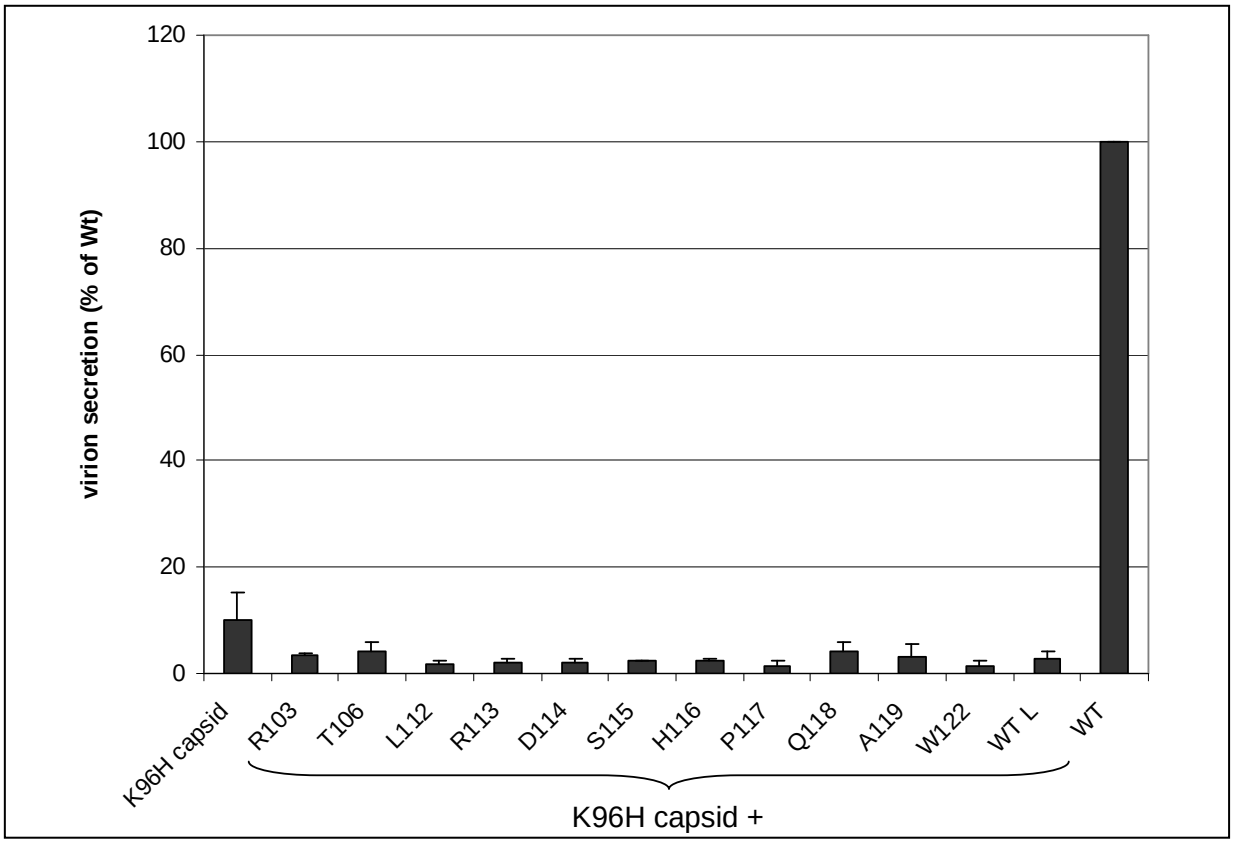

Fig. 32. Capsid mutant K95H cannot be compensated for by any of the tested matrix domain mutant pools. Transfection of HuH7 cells with capsids holding the envelopment negative K96H mutation with matrix domain mutant pools did not show any complementation.

3.4 Flexibility of MD residues

The matrix domain of the HBV large envelope protein has been analyzed by double alanine substitutions and linker substitutions (Bruss, 1997). In this work, the flexibility of the matrix domain was characterized by investigating the ability of single MD mutants to envelop WT capsids. To accomplish this task, cotransfections of WT capsid and L mutant pools were performed and virion secretion was monitored. While transfection of mutant pools of restricted residues should not result in the release of virions into the cell culture supernatant, flexible residues should give a strong signal in the HBV genome specific qPCR because also a non-WT amino acid at the corresponding position could take over the function in envelopment of the capsid. By this experimental setting it could be demonstrated that only two residues tested were flexible while envelopment strictly depended on the WT amino acid at the other 9 positions (see Fig. 33).

3.4.1 Restricted residues

To validate the data obtained by cotransfections of WT capsids and L mutant pools, one pool was chosen and its mutants transfected individually with WT capsid. In this way it should be verified that none of the mutants contained in the pool could envelop WT capsid. The residue chosen was the tryptophan at position 122 and the results of the virion detection assay impressively showed that none of the mutants of this residue allowed virion formation.

3.4.2 Flexible residues

Cotransfection of MD mutant pools of positions 114 and 118 of L showed that virions are secreted. Therefore at least one mutant in these pools was able to take over the tasks of the WT amino acids of these positions. To find out which amino acid can substitute for the WT amino acid the mutants were transfected singly with WT capsid and virion secretion was analyzed. Interestingly, a range of non-WT amino acids proved to be functional in capsid envelopment. For residue D114, glutamic acid, isoleucine, histidine and glutamine turned out to work best, while for Q118, substitution by tryptophan, glutamic acid and tyrosine was superior. Besides these amino acids also others could form virions while some were shown to clearly inhibit envelopment of the capsid.

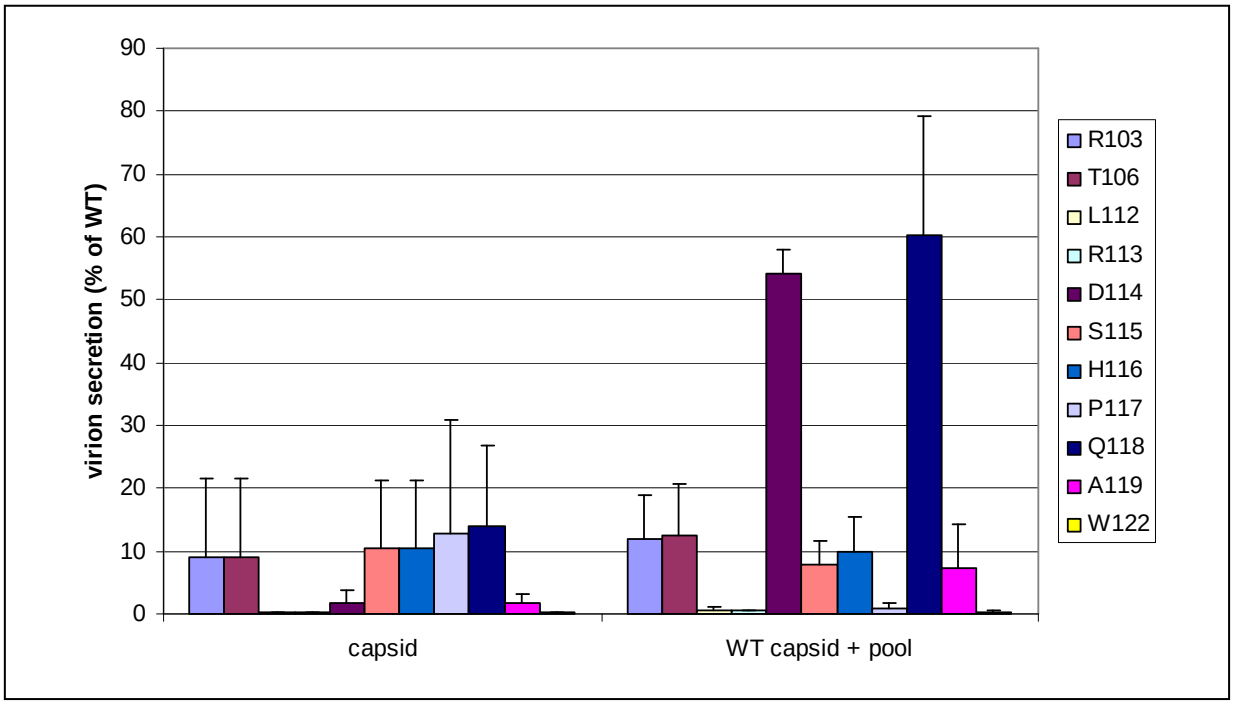

Fig. 33. Characterization of the ability of different matrix domain mutant pools to envelop WT capsids. To test the flexibility of the matrix domain, MD mutants were cotransfected with WT capsid and virion secretion was measured. Two pools (D114 and Q118) were able to support virion formation. Mean values and standard deviations were calculated from at least two independent experiments.

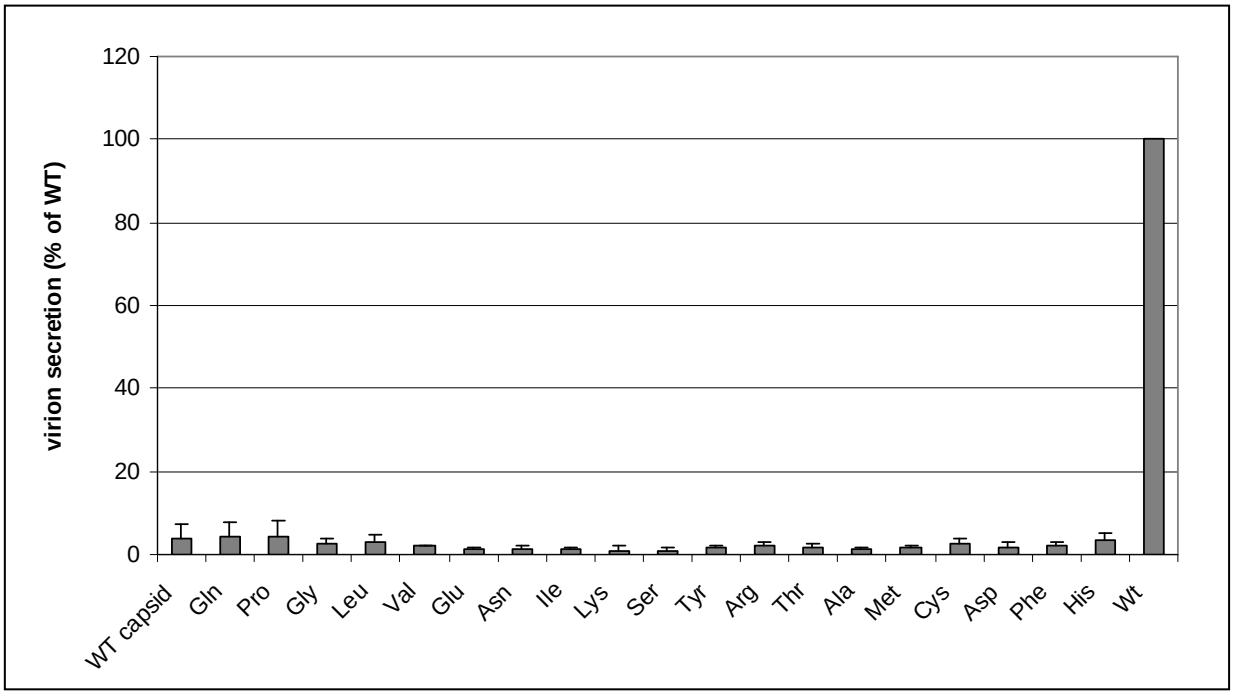

Fig. 34. Tryptophan 122 is essential for capsid envelopment and cannot be substituted by any other amino acid. Single mutants of MD position 122 were cotransfected with WT capsids and virion secretion was measured. Capsid alone gives the background of the assay. Mean values and standard deviations were calculated from at least two independent experiments.

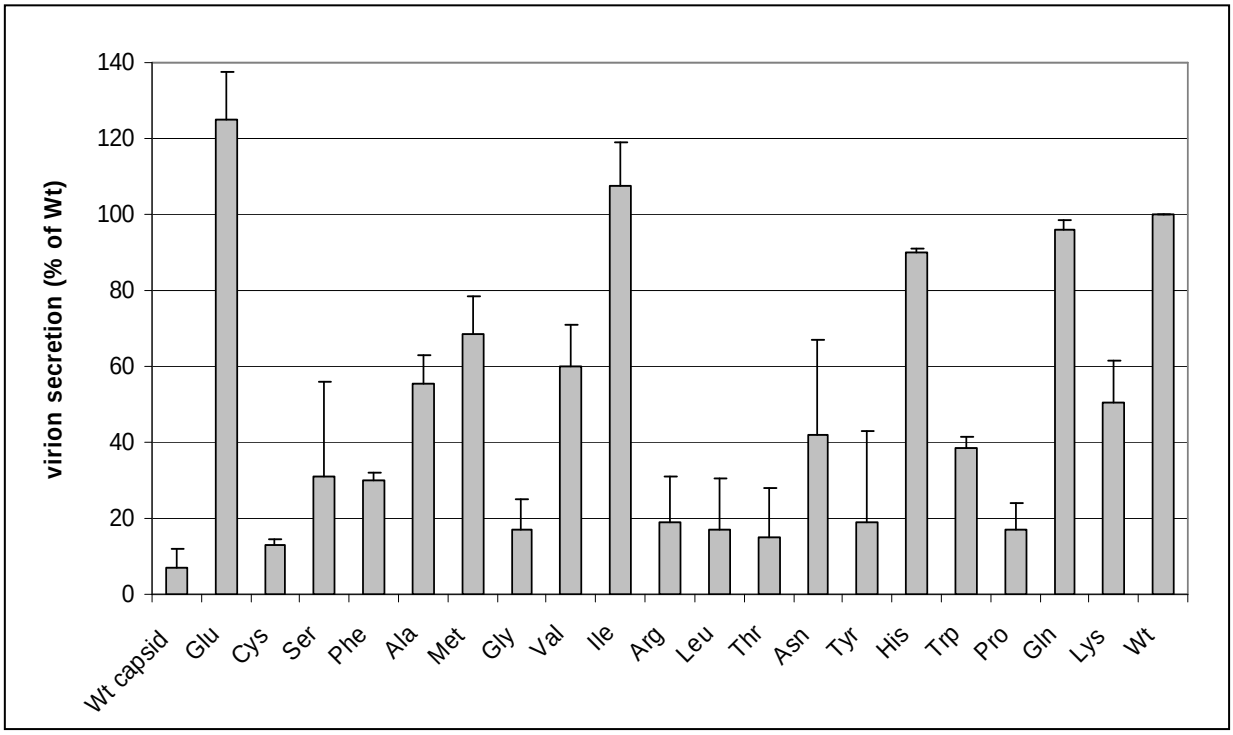

Fig. 35. Position 114 of the L protein is highly flexible. Single mutants of MD position D114 were cotransfected with WT capsids and virion secretion was monitored. Mean values and standard deviations were calculated from at least two independent experiments.

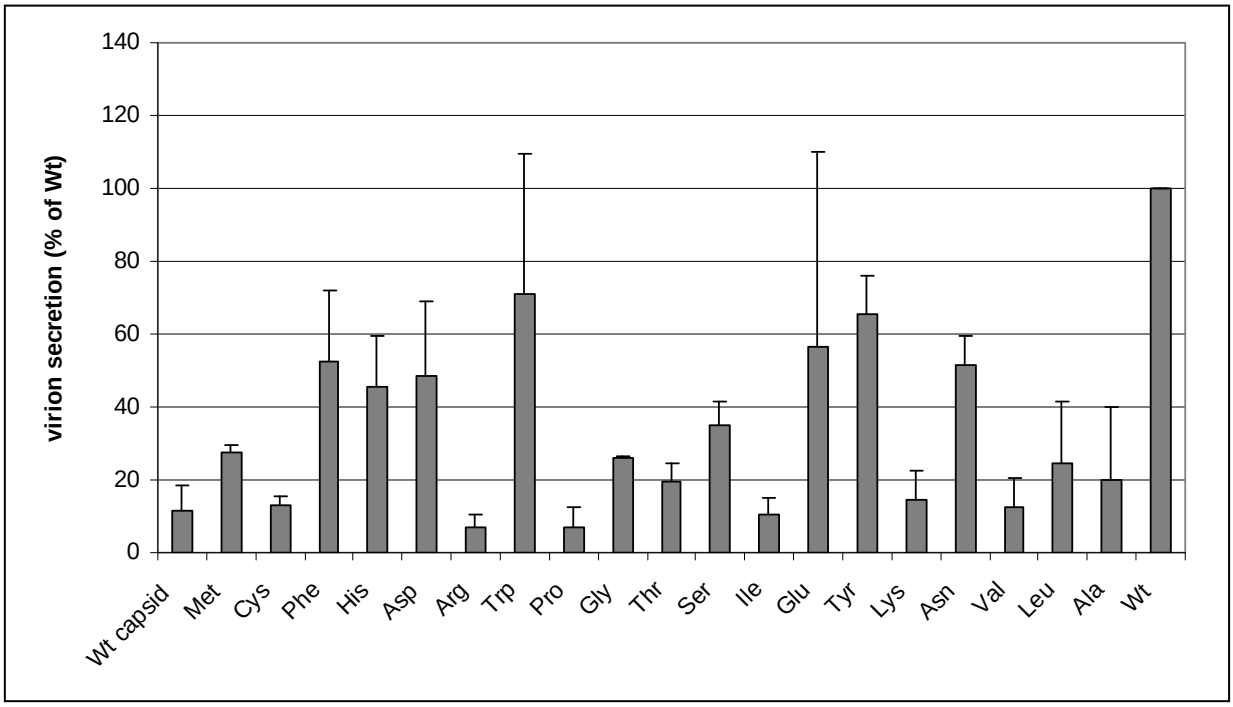

Fig. 36. Glutamine 118 can be substituted by many residues for functional capsid envelopment and virion secretion. Single mutants of MD position Q118 were cotransfected with WT capsids and virion secretion was monitored by the virion detection assay. Mean values and standard deviations were calculated from at least two independent experiments.

3.4.3 Test of single MD mutants occurring in natural mutants

HBV isolated from patients sometimes contains natural mutations, also in the matrix domain. Very prominent ones are L112G and D114N. These were tested separately for their function of the matrix domain by cotransfection with WT capsid.

Interestingly, L112G seems to be envelopment-negative in our assay. Residue D114N which has been shown before to allow virion secretion (Fig. 35) clearly showed its ability to envelop nucleocapsids.

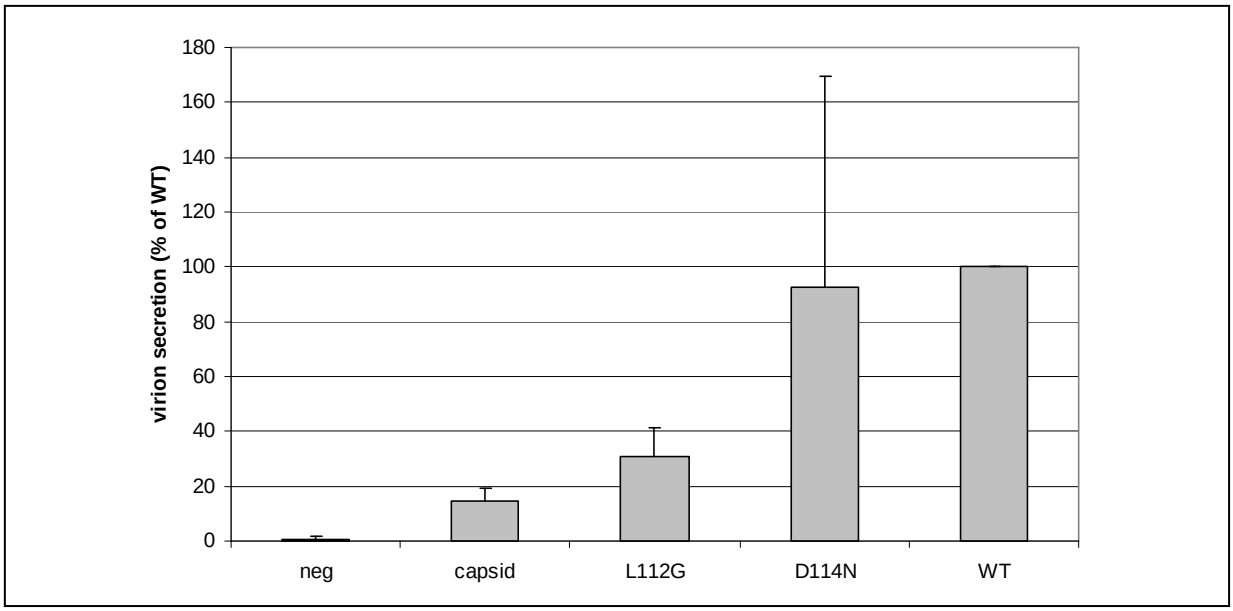

Fig. 37. Virion secretion of the naturally occuring MD mutants L112G and D114N. Cotransfection with WT capsid showed that D114N but not L112G allowed virion formation.

4 Discussion

4.1 Establishment of a new virion detection assay

To test hepatitis B virus mutants for their budding and assembly ability, it was necessary to monitor the secretion of virions in the supernatant of transfected cells. The hitherto existing method of choice for detection and quantification of mature virions was the so-called endogenous polymerase reaction (Landers et al., 1977) and based on the visualization of radioactively labelled genomes purified from the capsids of secreted virions. It exploits the facts that the viral genome contained in mature capsids consists of an incompletely double-stranded DNA and that the viral polymerase is packed into the capsid. For these reasons it is possible to carry out an *in vitro* endogenous polymerase reaction in which the gap in the genome is filled up with radioactively labelled nucleotides. The labelled genome can then be detected after gel electrophoresis by autoradiography. Unfortunately, this method is very inconvenient due to the need of handling of radioactive substances.

A far more convenient way to demonstrate the secretion of virions from cells would be the use of a reporter cell line. The supernatant of transfected cells could be used to infect a cell line which thereupon expresses a reporter protein e.g. the firefly luciferase. In this way the secretion of virions could be detected in an indirect way. The problem in this system is that besides primary hepatic cell cultures there is only one cell line which is susceptible to HBV infection and in this cell line, the infection efficiency is only around 10 % (Gripon et al., 2002). Furthermore, mutations of the large surface protein could have a negative effect on the infection capability of the virions. In this work, a shortened version of the L protein was expressed lacking 30 N-terminal amino acids in order to release the intracellular retention of the surface proteins and in this way obtain a higher virion yield. Since the first 75 amino acids of L are essential for the viral infectivity (Blanchet and Sureau, 2007) the virions produced were probably not infectious and could therefore not be used in a reporter system.

For all these reasons, a new method was created in this work which directly measured the virions in the supernatant and abandoned the use of radioactivity. It relied on the separation of virions from naked capsids by an immunoprecipitation against the HBV surface proteins and was very sensitive because of a final quantification of the viral genomes purified from the virions by a RT-PCR discriminating between genomic and plasmid DNA (see 3.1.4). Only 5% of normal virion production was sufficient to get a clear positive signal enabling the detection of small amounts of virions. Furthermore, the little volume of cell supernatant

needed allowed convenient handling and the performance of duplicates. The deviations in one experiment were very low and comparisons between two experiments were made by expressing the values as percentage of the WT. This assay was used to assess the ability of matrix domain mutants to form virions with WT capsids or capsids bearing envelopment-negative mutations upon cotransfection in HuH7 cells.

4.2 Analysis of the negative transdominant effect of an envelopment-negative L mutant

Shortly after synthesis of the surface proteins and their cotranslational insertion into the ER membrane, dimers between S, M and L can be detected. Homodimers are formed as well as heterodimers without preference for pairing of glycosylated and unglycosylated proteins (Wunderlich and Bruss, 1997). Besides homodimerization, L proteins form mixed dimers with S as well as with M. The dimers are stabilized by intra- and intermolecular disulfide bridges between cysteines in the S domain which is important for assembly and secretion of the particles (Mangold et al., 1997). Later in assembly, the dimers are linked to oligomers which are assumed to be necessary for virion assembly. For subviral particles, it could be shown that this must happen in a post-ER pre-Golgi compartment since treatment with brefeldin A inhibiting the transport from the ER to the Golgi caused accumulation of surface dimers and prevented oligomer formation (Huovila et al., 1992). Furthermore, while the ER resident protein disulphide isomerase catalyzes the formation of dimers in the ER it would resolve higher aggregates and therefore oligomers could not be built in the ER but must first be transported to another compartment (Huovila et al., 1992). Further steps in particle assembly are not clear yet. Nevertheless, the assumed necessity of L oligomers for capsid envelopment implies that envelopment-negative L mutants could exert a transdominant negative effect on WT L by disturbing the dimer- and oligomerization. This was substantiated in a publication in which the WT virion release upon cotransfection of WT and envelopment-negative L mutants dropped to around 10 % (Bruss, 1997).

In this work, one of the mutants of the publication mentioned above (Bruss, 1997) exerting a strong negative transdominant effect on WT L was characterized in more detail. Different ratios of WT L and mutant I1 (linker substitution in L from amino acid 98 to 104 plus insertion of 2 amino acids) which did not support virion release were cotransfected in HuH7 cells and the suppression effect was monitored (see Fig. 20). Interestingly and in contrast to the results obtained by V. Bruss, cotransfection of WT and mutant L with a ratio of 1:1 led to an increase in virion production instead of a clear decrease. These diverging results can be

explained by (1) the different transfection strategies used in the two experimental settings and (2) the use of different L constructs. In this work, the HBV genome with an unfunctional surface protein open reading frame was complemented by the vector pSV45-57 coding for all three envelope proteins (WT or I1 mutant) and plasmid pSVBX24H holding only the S gene. In the original experiment, a WT HBV genome had been cotransfected with a vector coding for the three surface proteins and the L gene being mutated, but no additional S vector had been supplied (Bruss, 1997). Since the L / S protein ratio is quite critical for virion secretion and relative overexpression of S is favourable (Garcia et al., 2009), the system used in this work stronger supported efficient virion release and therefore the transdominant effect was not that pronounced and delayed to lower mutant / WT L ratios. The rise in virion secretion during the drop of WT L from 100 % to 50 % can be traced back to the same reason. In the transfection system used here, 50 % of WT L seem to be optimal for virion formation, implying that 100 % functional L is too much L relative to S. The second point explaining the diverging results from this and previous works is the use of an L construct in this work which lacks 30 amino acids on its N-terminus and with it the myristoylation signal, a retention signal reducing the efficiency of virion secretion. In this way the release of virions was enhanced and the transdominant negative effect exerted by the envelopment-negative L mutant I1 was shifted to lower mutant / WT L ratios.

4.3 Choice of matrix domain mutants

The matrix domain of the HBV large surface protein has been mapped to reach from amino acid 103 to 124 (Bruss and Thomssen, 1994; Bruss, 1997) since N-terminal truncations up to amino acid 102 were tolerated in virion formation (Bruss and Thomssen, 1994) as well as substitution mutations downstream of amino acid 124 (Bruss, 1997). Double-alanine mutants of this region were mostly not tolerated (Bruss, 1997). All these results imply an important role of the matrix domain in virion formation and so it was chosen for an extensive mutational analysis. Half of the residues were changed against all non-WT amino acids and then tested for their ability to envelop capsids and secrete virions.

In contrast to the capsid, no crystal structure of the large surface protein is available. Furthermore, preS1 seems to be intrinsically unfolded (Seitz et al., 2007). For this reason, no predictions of possible binding sites to the capsid could be made on which the choice of residues to be mutated could be based on. Selection of the matrix domain residues further analyzed in this work was therefore performed because of other characteristics like conservation of the corresponsive residues in woodchuck hepatitis virus or the phenotype of L

mutants characterized before. R103, T106, R113, D114, H116 and P117 were selected because they were conserved in woodchuck hepatitis virus (Bruss 1997; see underlined residues in Fig. 27) and furthermore showed a negative phenotype when examined in a series of double-alanine mutants (Bruss 1997; open boxes in Fig. 27). Furthermore, the arginine at position 103 seems to be particularly important for assembly as determined by binding assays of L point mutants (Tan et al., 1999). L112 exhibited conservation with the corresponding woodchuck hepatitis virus residue but had not been tested in double-alanine mutations. Le Pogam and Shih attested this residue to be important for particle formation (Le pogam and Shih, 2002). Residue W122 showed a negative phenotype in the double-alanine mutants but was not conserved in WHV (Bruss, 1997; Fig. 27). S115, Q118 and A119 were neither conserved nor had been tested as double-alanine mutants. A119 seems to be important for envelopment and mutant A119F could restore the WT phenotype of capsid mutant F97L (subtype *adr*) causing envelopment of immature capsids (Le Pogam and Shih, 2002). Other residues like the prolines on positions 110 and 111 were excluded because changing a proline in a protein probably results in an altered secondary structure and could possibly render the whole protein unfunctional. The methionine located at position 120 and marking the beginning of the M protein was not replaced in order to sustain proper expression of the middle envelope protein. All phenotypes seen during testing should be able to be led back to matrix domain mutations only.

4.4 Characterization of single positions of the matrix domain

The matrix domain residues chosen for detailed characterization were mutated to all non-WT amino acids of the corresponding position. In this way a large panel of mutants was generated whose analyses should give a deep insight into the function of MD. Overall inspection of the matrix domain showed 4 prolines in this 22 amino acids long region which is nearly 20 %. Proline-rich regions are known to hint to protein-protein interacting regions (Ball et al., 2005; Kini et al., 1996) which further strengthens the importance of the matrix domain.

Concerning the mutational approach, it has to be mentioned that mutations can also affect e.g. the positioning of neighbouring residues as well as distant ones. This has been shown for the S protein where the defective virion secretion of several S point mutants could be rescued by the additional mutation M133T which created a novel glycosylation site (Ito et al., 2010). Another example is the capsid mutation I97L which could functionally be complemented by the distant P130T mutation (YUAN & SHIH, 2000). Therefore it is questionable to consider one

mutation as the exclusive change of the respective residue and it is important to keep that in mind when interpreting the phenotype of a point mutant.

4.4.1 Restricted residues

Nine of 11 residues tested turned out to be very restricted regarding their amino acid sequence. Pools of all 19 non-WT amino acids of the respective residue could not envelope WT capsids implying that only the WT amino acid on that position was allowed for proper virion assembly. One of the restricted residues, W122, was more closely investigated. The single mutants of this position were characterized regarding their ability to envelop nucleocapsids and to be incorporated in subviral particles. As expected from the envelopment-negative phenotype displayed from the W122 mutant pool, none of the mutants supported virion formation. Checking the surface proteins of subviral particles produced by the single mutants on a Western blot showed that nearly all of them were integrated in subviral particles and therefore did not have a block in budding. Therefore it can be concluded that the restricted residues are specifically important for the envelopment of the nucleocapsid and not for budding.

The nine residues shown to be highly restricted did not show a recognizable pattern. Some residues were positively charged (e.g. R103), some were uncharged (e.g. T106 and P117), some were hydrophobic (e.g. L112), some hydrophilic (e.g. H116), some small (A119) and some bulky (W122). Therefore no conclusions about the kind of interaction (e.g. electrostatic) could be drawn.

4.4.2 Variable amino acids

Two of the residues tested for their ability to envelop WT capsids showed certain variability and were not restricted. At the one hand, this was the aspartic acid at position 114, and on the other hand the glutamine at position 118. The fact that these amino acids could be exchanged against some other amino acids implies that their role in envelopment is not as pronounced as the role of the restricted residues. D114 could be replaced by e.g. glutamic acid, isoleucine, histidine and glutamine, being amino acids with very different properties. Q118 could functionally be substituted by e.g. tryptophan, glutamic acid and tyrosine. No pattern in these substitutions could be recognized preventing further insight into the interactions needed for capsid envelopment. Nevertheless, since all of the variable residues had mutants that could not take over the function of the WT residue and abolished virion formation, the MD positions 114 and 118 are not fully variable and dispensable for virion formation but must

fulfil some function in the assembly process. Interestingly, one of the two residues (Q118) was not conserved in woodchuck hepatitis virus while the other one was. This could be due to the genome organization of HBV in which the open reading frames are overlapping. The conservation of residue D114 could result from an important sequence of the overlapping polymerase frame which cannot be changed without loss of function. Closer inspection of the region overlapping with the matrix domain showed that it is the spacer region of the polymerase. This region is generally very flexible but also lethal point mutations have been identified (Faruqi et al., 1991).

As mentioned before, the matrix domain of HBV is highly conserved in WHV and the preS sequence of this Orthohepadnavirus can substitute for the HBV preS domains in virion assembly, while the unconserved DHBV-preS cannot, indicating relatively precise interactions necessary for virion formation (Gerhardt and Bruss, 1994). This assumption is substantiated by our findings that only some amino acids can take over the function of the WT amino acid while others can not.

4.4.3 Comparison to NCBI database

In the NCBI database more than 3000 different HBV sequences can be found that occur naturally. Some of them contain natural subsitutions. One of these natural mutants was tested in our system for its functionality. Surprisingly the mutant from the database was not viable in our system and not functional in virion secretion. Residue L112G showed very impaired assembly ability even though nearly one fifth of the sequences contained in the database carried the substitution at this position by glycine. The reason for this phenomenon could also be further changes in the genome which compensate for the mutation (see 4.4). The database furthermore does not state if the sequences contained are viable and replication competent or not.

4.5 Complementation tests of matrix domain mutants with capsid mutants

Mapping of domains of critical importance for nucleocpsid envelopment on both the L protein (matrix domain) and the capsid side (matrix binding domain) led to the hypothesis of a direct contact between these regions mediating budding. This theory is substantiated by the absolutely necessary i-preS form of L (Bruss and Vieluf, 1995) where the preS domains face the cytosol which is the compartment containing the capsid. The necessity of i-preS for virion assembly has been shown by fusing a secretion signal to the N terminus of the large surface

protein and in this way forcing expression of exclusively the e-preS form which resulted in no secretion of virions (Bruss and Vieluf, 1995). In vitro binding assays of capsid particles with peptides derived from L further hinted to the MD binding directly to the capsid (Poisson et al., 1997). Kluge and colleagues determined the minimal distance which is needed for proper virion assembly between the matrix domain and transmembrane signal II and came to the conclusion that it is around 26 amino acids. Since the matrix binding domain on the capsid is located at the base of the spike and in a ring-like structure around the pores (Ponsel and Bruss, 2003) and the spike of the capsid has a length of 24 amino acids in alpha-helical conformation (Wynne et al., 1999), the mimimal distance determined by Kluge et al. would fit perfectly for the matrix domain to still reach the putative binding sites on the capsid. Even though peptides binding to the tip of spike inhibited virion secretion (Böttcher et al., 1998), the tip does not seem to be involved in virion assembly since triple-point mutations did not abolish virion secretion (Ponsel and Bruss, 2003). The phenomenon observed by Böttcher et al. is therefore probably due to sterical hindrance. Last but not least, a very interesting compensation experiment has been performed, in which the capsid mutant F97L was compensated by the A119F mutation in the matrix domain of L (Le Pogam and Shih, 2002). The substitution of phenylalanine with leucine at position 97 in the capsid (subtype *adr*) led to the envelopment and secretion of immature capsids while the A119F mutant in context of the WT capsid exhibited a low-secretion phenotype. Combination of these two mutants led to WT-like virion release, but the possibility exists that this compensation is just the sum of two mutant phenotypes and not necessarily a real compensation via the direct interaction between C and L. To really strengthen the direct interaction theory, compensation of a mutant with loss-of-function phenotype would be a better proof than of one with loss-of-specificity phenotype.

Therefore, in order to confirm the hypothesis of direct interaction between the large surface protein and the capsid, an intergenical complementation approach was employed using loss-of-function capsid mutations. Peptide binding assays performed previously in the working group had given very unspecific results and the possibility to carry out yeast two-hybrid screens was discarded due to the high rate of false positives that transmembrane proteins usually give in these experiments.

The capsid mutants chosen were S17T, K96H and I126A, being mutants from MBD residues very well described in the literature (Ponsel and Bruss, 2003; Pairan and Bruss, 2009). They formed comparable amounts of mature capsids to the WT but could not be enveloped. Interestingly, K96 was also shown to be essential for recognition of γ2-adaptin (Rost et al., 2006). However, testing of the single MD mutant pools with these capsid mutants did not

reveal any functional complementation. This can be due to many reasons. First, it could be possible that there are cellular proteins mediating the contact between large surface protein and capsid instead of a direct interaction. Several publications hint to that direction (Hartmann-Stühler and Prange, 2001; Rost et al., 2006). Second, the capsid protein residues chosen for this assay could fulfil other functions than directly contacting the envelope and can therefore not be complemented at all. They could e.g. play a role in displaying the maturation signal on the capsid surface or be part of an assumed membrane targeting signal which has been shown to exist in the DHBV capsid (Mabit and Schaller, 2000). Lingappa and colleagues supposed such a signal also for human HBV (Lingappa et al., 2005). Furthermore, the transport of the capsid to the budding site could also be affected by substitution of capsid residues in this way disabling assembly. Since the role of the residues of MBD is not fully understood, these could also be plausible reasons for the failing of the intergenical complementation approach.

Another important point is that the assembly process is very complex and presumably more than one amino acid is involved in the putative interaction with the capsid. This assumption is substantiated by the fact that both the MD and the MBD consist of several amino acids and not just one. Maybe more than one substitution in the matrix domain would be necessary to compensate for envelopment-negative capsid mutants.

4.6 Budding model for HBV

HBV is an enveloped virus which means that it covers its nucleocapsid with an envelope which is derived from a host cell membrane where the viral surface proteins are embedded. The process of acquiring the envelope is called budding and in the case of HBV not fully understood yet. Neither the location nor the way of initiating the whole process is clear. Since only 1 to 10 virions are released per day from a single hepatocyte (Nowak et al., 1996) microscopical analyses are quite difficult to carry out and most findings were made by other techniques like mutational analyses.

Generally, a variety of budding mechanisms are used by the different virus families. Alphaviruses belonging to the family of *Togaviridae* are a prominent example for viruses with the need for a direct interaction between core and envelope proteins for virion formation. The cytoplasmic portion of the alphaviral E2 surface protein makes contact with a hydrophobic pocket of the capsid and thus drives budding. Structural evidence of this molecular link formed has been gained recently by cryo-electron microscopy (Tang et al., 2011). In contrast, retroviral Gag is able to form viral particles without the presence of surface proteins (Gheysen

et al., 1989; von Schwedler et al., 2003). And coronaviruses can release membrane particles which are morphologically indistinguishable from virions without the need of a nucleocapsid (Vennema et al., 1996). Hepadnaviruses are assumed to bud similar to alphaviruses.

The envelopment of the HBV capsid is strictly depending on the envelope proteins. While the S protein is sufficient for driving budding of subviral particles lacking the nucleocapsid, the L protein is essential for nucleocapsid envelopment. The M protein is dispensable (Bruss and Ganem, 1991). L is found in two different transmembrane topologies but only one of them is necessary for capsid envelopement which is the i-preS form with the preS-domains facing the cytosol and with it the capsid (Bruss and Vieluf, 1995). The matrix domain on the preS domains of L was identified by different mutagenesis analyses (Bruss and Thomssen, 1991; Bruss, 1997; Le Seyec et al., 1998). Many residues necessary for proper capsid envelopment are clustered in this domain. To emphasize the importance of single residues of the matrix domain, it is noteworthy that the well conserved WHV L can substitute for HBV L, but not DHBV L which is not conserved (Gerhardt and Bruss, 1995). The restriction was also confirmed in this work by the detection of several residues which were absolutely restricted to the WT amino acid for functional virion assembly. Detailed analysis of W122 which is one of these restricted residues showed that most of the single mutants are still incorporated in subviral particles but no virions are formed. Therefore, this residue must play a specific role in envelopment of the capsid and not in budding itself.

The question about initiation of the assembly process was also addressed in this work. Whether it is a direct interaction between envelope and capsid or an interposed host cell protein which initiates budding could not be resolved (see chapter 4.5). Since it was shown that both the capsid and the L protein can bind to γ2-adaptin (Hartmann-Stühler and Prange, 2001; Rost et al., 2006) it is possible that this protein recruites the nucleocapsid to the budding site where the envelope proteins are. Then a direct interaction could take place which initiates budding (see chapter 4.5). The variable MD residues determined in this work (see 3.4.2) each showed a specific substitution pattern with defined amino acids enabling virion formation implying that the interaction – no matter if it is with the capsid or a host cell factor – must be very specific.

Budding occurs at an internal membrane which is not clearly defined yet. Both L and C have been found in the late endosomal compartment (Rost et al., 2006).). Furthermore, the so-called ESCRT (endosomal sorting complex required for transport) complex driving the MVB machinery and required to generate multivesicular bodies plays an important role in virion secretion (Lambert et al., 2007; Rost et al., 2006; Watanabe et al., 2007). The use of MVB

inhibitors led to the selective impairance of virion secretion but no effect on the release of subviral particles was observed (Lambert et al., 2007). Therefore a possible budding place is the MVB while budding of subviral particles takes place in a post-ER pre-Golgi compartment (Huovila et al., 1992) and does not reflect virion formation. Further support for budding at MVB comes from the capsid containing the sequence PPAY (amino acids 129-132) which resembles a late domain motif and is exposed on the capsid surface and accessible from outside (Wynne et al., 1999). It is known that in some enveloped viruses (Freed, 2002) these late domains are important for interaction of the capsid with the host cell machinery which is necessary for budding of vesicles into the lumen of multivesicular bodies. Taken together all the observations mentioned above, it is likely that HBV also uses this pathway (Rost et al., 2006).

4.7 Outlook

Concerning the transdominant effect of envelopment-negative L mutants on WT virion release, some other interesting aspects would be worth to look at e.g. the morphology of the virions which are secreted. Envelopment-negative L mutants are still able to bud and are found in subviral particles but how about the virions? Do they contain both kinds of L protein (mutant and WT) or only the WT form? Is there a certain threshold or percentage of WT L that is needed to build and secrete a virion? These questions could be examined by tagging the mutant L proteins e.g. with an HA tag and analyze the surface proteins of secreted virions on a Western blot. Because of the tag the L protein bands of WT and mutant L would differ in size and could be easily distinguished from each other. However, a good separation of virions and subviral particles would be necessary for this analysis which is very difficult to achieve. If both L forms would be found on the Western blot density measurements of the bands would give further hints to the relative ratio.

Regarding the proof of the hypothetical direct interaction between capsid and envelope proteins driving budding further complementation attempts on a large scale could be made. It would also be interesting to check secreted virions for the presence of γ2-adaptin. This could be tested by a Western blot staining against γ2-adaptin upon immunoprecipitation of virions.

5 Summary

The hepatitis B virus (HBV) causes a major health problem with more than 350 million chronic carriers worldwide who are at high risk to developer liver cirrhosis or cancer. HBV is a small, liver specific, enveloped DNA virus consisting of a nucleocapsid and a surrounding envelope with the inserted small (S), middle (M) and large (L) surface poteins. The N-terminal preS domains of L can adopt two different topologies, either facing the inside (i-preS) or outside (e-preS) of the virion. While the i-preS form is assumed to contact the capsid during budding, the e-preS form is essential for entry into new hepatocytes through binding to a receptor. One region of L has been implicated to play a role in virion formation serving the function of a matrix protein. Mutations in this so-called matrix domain (MD) reaching from position 103 to 124 (subtype adw) nearly always abolished secretion of virions.

In this work the function of the matrix domain during envelopment of the capsid in virion formation was characterized by testing a panel of MD mutants for functional virion secretion. Eleven residues of MD were chosen and substituted by all 19 non-WT amino acids. The mutants were cotransfected with an envelope-negative HBV genome into the human hepatoma cell line HuH7 and analyzed for virion formation. Furthermore, complementation attempts with capsid mutants were made to test for a direct interaction between capsid and L in virus assembly. For quantification of secreted virions, a new virion detection assay was established. It detected viral genomes isolated from virions by an HBV genome specific quantitative PCR measuring HBV genomes but excluding plasmid used for transfection from amplification by specific location of the primers.

The intergenical complementation approach using MD mutants with capsid mutants could not reveal a direct interaction between capsid and surface proteins. Upon cotranfection of MD mutants with WT capsids, we could identify variable residues (D114 and Q118) which were not absolutely necessary for functional envelopment of the capsid because besides the WT residue also some non-WT amino acids supported virion secretion. On the other hand highly conserved, invariant residues were detected (R103, T106, L112, R113, S115, H116, P117, A119 and W122). The WT amino acid was essential at these positions and since these mutant L proteins still were secreted as subviral particles these residues must play a specific role in nucleocapsid envelopment. At least nine residues tested belonged to this group which makes the matrix domain a highly conserved region and turns it into a very interesting potential target for an antiviral therapy.

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7 Appendix

7.1 Primers for generation of pSVHBV1.1LE-

Bold letters indicate introduced BsaI restriction enzyme recognition sequences.

Name	*Sequence 5' -> 3'*	*Purpose*	*Primers used with*
VBBS3	CAGATC**GGTCTC**AATTCGCCCTATAGTGAGTCGTAT	Introduction of BsaI restriction site	VBBS4
VBBS4	GGGAAAGCCGGCGAACGT	Generation of pSVHBV1.1LE-	VBBS3, VBBS5
VBBS5	TCGCGGGACGTCCTTTGT	Generation of pSVHBV1.1LE-	VBBS4, VBBS6
VBBS6	CAGATC**GGTCTC**CGAATGACGGAAGGAAAGAAGTCAGAA	Introduction of BsaI restriction site	VBBS5

7.2 Primers for generation of capsid mutants

Bold letters indicate nucleotide changes compared to the WT sequence.

Name	*Sequence 5' -> 3'*	*Purpose*	*Primers used with*
VBBS23	CGGAGTGTGG**GCT**CGCACTCCTCC	Mutagenesis primer for pSVHBV1.1LE- I126A	VBBS24
VBBS24	CCACGTTC**GCTAGC**TTTCCCCGT	Selection primer in plasmid pSVHBV1.1LE-	VBBS23
VBBS_LErev	CGGTATTGTGAGGATTCT	Mutagenesis of pSVHBV1.1LE- by fusion PCR	VBBS_S17T, VBBS_K96H, FWD_LEfwd
FWD_LEfwd	AGGAAGCGGAAGAGCGCC	Mutagenesis of pSVHBV1.1LE- by fusion PCR	S17T_rev, K96H_rev, VBBS_LErev
VBBS_S17T	GGAGTTACTC**A**CGTTTTTGCCTTCT	Generation of capsid mutant	VBBS_LErev
VBBS_K96H	CATGGGTTTA**C**A**C**ATCAGGCAACT	Generation of capsid mutant	VBBS_LErev
S17T_Rev	AGAAGGCAAAAACG**T**GAGTAACTCC	Generation of capsid mutant	FWD_LEfwd
K96H_Rev	AGTTGCCTGAT**G**T**G**TAAACCCATG	Generation of capsid mutant	FWD_LEfwd

7.3 Primers for MD mutagenesis by USE

Bold letters indicate nucleotide changes compared to WT, N stands for any nucleotide.

Name	*Sequence 5' -> 3'*	*Purpose*	*Used with*
VBBS25	AGGGTCATC**GTCGAC**CAGATCCTC	Selection primer in pSV45-57	all below
VBBS_R103S	GGCAGTCAGGA**AGT**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103K	GGCAGTCAGGA**AAG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103N	GGCAGTCAGGA**AAC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103T	GGCAGTCAGGA**ACC**CAGCCTACTCC	Generation of MD mutant	VBBS25

VBBS_R103M	GGCAGTCAGGA**ATG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103I	GGCAGTCAGGA**ATC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103Q	GGCAGTCAGGA**CAG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103H	GGCAGTCAGGA**CAC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103P	GGCAGTCAGGA**CCG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103L	GGCAGTCAGGA**CTG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103W	GGCAGTCAGGA**TGG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103C	GGCAGTCAGGA**TGC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103Y	GGCAGTCAGGA**TAC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103F	GGCAGTCAGGA**TTC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103G	GGCAGTCAGGA**GGC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103E	GGCAGTCAGGA**GAG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103D	GGCAGTCAGGA**GAC**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103A	GGCAGTCAGGA**GCG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_R103V	GGCAGTCAGGA**GTG**CAGCCTACTCC	Generation of MD mutant	VBBS25
VBBS_T106M	GAAGGCAGCCT**ATG**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106I	GAAGGCAGCCT**ATC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106R	GAAGGCAGCCT**CGC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106Q	GAAGGCAGCCT**CAG**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106H	GAAGGCAGCCT**CAC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106P	GAAGGCAGCCT**CCT**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106L	GAAGGCAGCCT**CTC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106W	GAAGGCAGCCT**TGG**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106C	GAAGGCAGCCT**TGC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106Y	GAAGGCAGCCT**TAC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106S	GAAGGCAGCCT**TCT**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106F	GAAGGCAGCCT**TTC**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106G	GAAGGCAGCCT**GGT**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106E	GAAGGCAGCCT**GAG**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106D	GAAGGCAGCCT**GAT**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106A	GAAGGCAGCCT**GCG**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106V	GAAGGCAGCCT**GTT**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106K	GAAGGCAGCCT**AAG**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS_T106N	GAAGGCAGCCT**AAT**CCCATCTCTC	Generation of MD mutant	VBBS25
VBBS22	CTCTCCACCT**NNN**AGAGACAGTC	Generation of MD mutants at L112	VBBS25
VBBS_L112F	CTCTCCACCT**TTC**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112Y	CTCTCCACCT**TAC**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112C	CTCTCCACCT**TGT**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112P	CTCTCCACCT**CCG**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112H	CTCTCCACCT**CAT**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112M	CTCTCCACCT**ATG**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112N	CTCTCCACCT**AAC**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112A	CTCTCCACCT**GCC**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112D	CTCTCCACCT**GAC**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112E	CTCTCCACCT**GAA**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_L112S	CTCTCCACCT**AGT**AGAGACAGTC	Generation of MD mutant	VBBS25
VBBS_R113L	CTCCACCTCTA**CTA**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113P	CTCCACCTCTA**CCA**GACAGTCATCC	Generation of MD mutant	VBBS25

VBBS_R113H	CTCCACCTCTA**CAC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113Q	CTCCACCTCTA**CAA**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113W	CTCCACCTCTA**TGG**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113I	CTCCACCTCTA**ATC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113M	CTCCACCTCTA**ATG**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113T	CTCCACCTCTA**ACG**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113N	CTCCACCTCTA**AAC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113K	CTCCACCTCTA**AAG**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113S	CTCCACCTCTA**TCA**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113V	CTCCACCTCTA**GTA**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113A	CTCCACCTCTA**GCA**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113D	CTCCACCTCTA**GAT**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113E	CTCCACCTCTA**GAA**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113G	CTCCACCTCTA**GGC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113F	CTCCACCTCTA**TTC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113Y	CTCCACCTCTA**TAC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_R113C	CTCCACCTCTA**TGC**GACAGTCATCC	Generation of MD mutant	VBBS25
VBBS_D114L	CCACCTCTAAGA**CTC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114P	CCACCTCTAAGA**CCC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114H	CCACCTCTAAGA**CAC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114Q	CCACCTCTAAGA**CAG**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114W	CCACCTCTAAGA**TGG**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114I	CCACCTCTAAGA**ATC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114M	CCACCTCTAAGA**ATG**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114T	CCACCTCTAAGA**ACC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114R	CCACCTCTAAGA**CGC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114K	CCACCTCTAAGA**AAG**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114S	CCACCTCTAAGA**AGC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114V	CCACCTCTAAGA**GTG**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114A	CCACCTCTAAGA**GCA**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114N	CCACCTCTAAGA**AAT**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114E	CCACCTCTAAGA**GAG**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114G	CCACCTCTAAGA**GGT**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114F	CCACCTCTAAGA**TTC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114Y	CCACCTCTAAGA**TAT**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_D114C	CCACCTCTAAGA**TGC**AGTCATCCTCAGGC	Generation of MD mutant	VBBS25
VBBS_S115L	CCTCTAAGAGAC**CTT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115P	CCTCTAAGAGAC**CCT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115H	CCTCTAAGAGAC**CAT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115Q	CCTCTAAGAGAC**CAA**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115W	CCTCTAAGAGAC**TGG**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115I	CCTCTAAGAGAC**ATC**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115M	CCTCTAAGAGAC**ATG**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115T	CCTCTAAGAGAC**ACC**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115R	CCTCTAAGAGAC**AGG**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115K	CCTCTAAGAGAC**AAG**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115N	CCTCTAAGAGAC**AAC**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115V	CCTCTAAGAGAC**GTT**CATCCTCAGGCC	Generation of MD mutant	VBBS25

VBBS_S115A	CCTCTAAGAGAC**GCT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115D	CCTCTAAGAGAC**GAT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115E	CCTCTAAGAGAC**GAG**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115G	CCTCTAAGAGAC**GGA**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115F	CCTCTAAGAGAC**TTT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115Y	CCTCTAAGAGAC**TAT**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_S115C	CCTCTAAGAGAC**TGC**CATCCTCAGGCC	Generation of MD mutant	VBBS25
VBBS_H116P	CTAAGAGACAGT**CCA**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
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VBBS_H116Q	CTAAGAGACAGT**CAG**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116I	CTAAGAGACAGT**ATT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116M	CTAAGAGACAGT**ATG**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116T	CTAAGAGACAGT**ACT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116R	CTAAGAGACAGT**AGG**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116K	CTAAGAGACAGT**AAG**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116S	CTAAGAGACAGT**AGT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116V	CTAAGAGACAGT**GTT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116A	CTAAGAGACAGT**GCT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116E	CTAAGAGACAGT**GAG**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116G	CTAAGAGACAGT**GGT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116F	CTAAGAGACAGT**TTT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116Y	CTAAGAGACAGT**TAC**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_H116C	CTAAGAGACAGT**TGT**CCTCAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117L	GAGACAGTCAT**CTC**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117H	GAGACAGTCAT**CAC**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117N	GAGACAGTCAT**AAT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117Q	GAGACAGTCAT**CAG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117W	GAGACAGTCAT**TGG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117I	GAGACAGTCAT**ATT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117M	GAGACAGTCAT**ATG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117T	GAGACAGTCAT**ACG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117R	GAGACAGTCAT**CGC**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117K	GAGACAGTCAT**AAG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117S	GAGACAGTCAT**AGT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117V	GAGACAGTCAT**GTC**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117A	GAGACAGTCAT**GCG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117D	GAGACAGTCAT**GAT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117E	GAGACAGTCAT**GAG**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117G	GAGACAGTCAT**GGT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117F	GAGACAGTCAT**TTT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117Y	GAGACAGTCAT**TAT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_P117C	GAGACAGTCAT**TGT**CAGGCCATGC	Generation of MD mutant	VBBS25
VBBS_Q118H	CAGTCATCCT**CAC**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118P	CAGTCATCCT**CCA**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118L	CAGTCATCCT**CTG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118W	CAGTCATCCT**TGG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118C	CAGTCATCCT**TGC**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118Y	CAGTCATCCT**TAC**GCCATGCAGTG	Generation of MD mutant	VBBS25

VBBS_Q118S	CAGTCATCCT**TCG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118F	CAGTCATCCT**TTC**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118G	CAGTCATCCT**GGT**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118E	CAGTCATCCT**GAG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118D	CAGTCATCCT**GAC**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118V	CAGTCATCCT**GTG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118R	CAGTCATCCT**AGG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118K	CAGTCATCCT**AAG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118N	CAGTCATCCT**AAC**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118T	CAGTCATCCT**ACG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118M	CAGTCATCCT**ATG**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBBS_Q118I	CAGTCATCCT**ATC**GCCATGCAGTG	Generation of MD mutant	VBBS25
VBSK1	GTCATCCTCAG**NNN**ATGCAGTGGA	Generation of MD mutants at A119	VBBS25
VBBS_A119F	GTCATCCTCAG**TTC**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119Y	GTCATCCTCAG**TAC**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119C	GTCATCCTCAG**TGT**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119H	GTCATCCTCAG**CAC**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119Q	GTCATCCTCAG**CAG**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119M	GTCATCCTCAG**ATG**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119T	GTCATCCTCAG**ACG**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_A119K	GTCATCCTCAG**AAG**ATGCAGTGGA	Generation of MD mutant	VBBS25
VBBS_W122L	GGCCATGCAG**CTG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122P	GGCCATGCAG**CCG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122H	GGCCATGCAG**CAC**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122Q	GGCCATGCAG**CAG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122R	GGCCATGCAG**CGT**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122I	GGCCATGCAG**ATC**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122M	GGCCATGCAG**ATG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122T	GGCCATGCAG**ACG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122N	GGCCATGCAG**AAC**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122K	GGCCATGCAG**AAG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122S	GGCCATGCAG**AGT**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122V	GGCCATGCAG**GTG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122A	GGCCATGCAG**GCG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122D	GGCCATGCAG**GAC**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122E	GGCCATGCAG**GAG**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122G	GGCCATGCAG**GGA**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122F	GGCCATGCAG**TTC**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122Y	GGCCATGCAG**TAC**AATTCCACTGCC	Generation of MD mutant	VBBS25
VBBS_W122C	GGCCATGCAG**TGC**AATTCCACTGCC	Generation of MD mutant	VBBS25

7.4 Primers for MD mutagenesis by fusion PCR

Bold letters indicate nucleotide changes compared to the WT sequence.

Name	*Sequence 5′ -> 3′*	*Purpose*	*Primers used with*
VBJN4	CGAGCAACGGGGTAAAGG	Mutagenesis of pSV45-57 by fusion PCR	VBBS_H116W1, VBBS_H116L1, VBBS_H116D, VBBS_Q118A, VBBS_fwd_45H
VBBS_fwd_45H	CTCGGCCTCTGAGCTATTCCAGA	Mutagenesis of pSV45-57 by fusion PCR	REV_H116W1, REV_H116L1, REV_H116D, REV_Q118A, VBJN4
REV_H116W1	ACTGCATGGCCTGAGG**CCA**ACTGTCTCTTAGA	Generation of MD mutant	VBBS_fwd_45H
REV_H116L1	ACTGCATGGCCTGAGG**GAG**ACTGTCTCTTAGA	Generation of MD mutant	VBBS_fwd_45H
REV_H116D	ACTGCATGGCCTGAGG**GTC**ACTGTCTCTTAGA	Generation of MD mutant	VBBS_fwd_45H
REV_Q118A	CACTGCATGGC**CGC**AGGATGACTG	Generation of MD mutant	VBBS_fwd_45H
VBBS_H116W1	TCTAAGAGACAGT**TGG**CCTCAGGCCATGCAGT	Generation of MD mutant	VBJN4
VBBS_H116L1	TCTAAGAGACAGT**CTC**CCTCAGGCCATGCAGT	Generation of MD mutant	VBJN4
VBBS_H116D	CTAAGAGACAGT**GAC**CCTCAGGCCATGC	Generation of MD mutant	VBJN4
VBBS_Q118A	CAGTCATCCT**GCG**GCCATGCAGTG	Generation of MD mutant	VBJN4

7.5 Primers for qPCR

Name	*Sequence 5′ -> 3′*	*Purpose*	*Primers used with*
VBBS8	GCTGAGGCGGTGTCTAGGAGA	HBV genome specific qPCR and qPCR for pSVHBV1.1LE-	VBBS18, VBBS19
VBBS18	GGCATAAATTGGTCTGCGCACC	HBV genome specific qPCR	VBBS8
VBBS19	TGTAAAACGACGGCCAGTGAATTG	qPCR specific for pSVHBV1.1LE-	VBBS8
VBBS20	CTGCACTCAGGCAAGCCATTCT	qPCR specific for core gene	VBBS21
VBBS21	GCGAATCCACACTCCGAAAGAGA	qPCR specific for core gene	VBBS20

7.6 Primers for sequencing

Name	*Sequence 5′ -> 3′*	*Purpose*
VBBS_sequ_neu	TCGGAGCCAACTCAAACAATCCAGA	Sequencing MD mutants
VBBS1	CTTAGAGTCTCCTGAGC	Sequencing capsid mutants
VBBS2	TGTTGATAAGATAGGGGC	Sequencing capsid mutants
VBBS5	TCGCGGGACGTCCTTTGT	Sequencing pSVHBV1.1LE-
VBBS10	TAGGGCTTTCCCCCACTG	Sequencing pSVHBV1.1LE-
VBBS9	ACTGCCTTCCACCAAGCT	Sequencing capsid mutants and pSVHBV1.1LE-
VBBS15	GGGTGTGGAAAGTCCCCA	Sequencing capsid mutants and pSVHBV1.1LE-
VBBS16	CGGAGTGTGGATTCGCACT	Sequencing capsid mutants and pSVHBV1.1LE-
VBBS17	CTCTTTGGAAGGCTGGTATTCT	Sequencing capsid mutants and pSVHBV1.1LE-

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