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RECOMBINANT SEMLIKI FOREST VIRUS REPLICON APPROACH IN SEARCH OF AN IMPROVED VACCINE FOR HEPATITIS B VIRUS: INDUCTION OF NEUTRALIZING ANTIBODIES

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ABSTRACT

Most hepatitis B virus (HBV) vaccines consist of viral small surface protein (SHBs), subtype adw2*, expressed in yeast cells, i.e. recombinant HBV surface antigen (rHBsAg). In spite of good efficacy, several concerns exist - non-response and appearance of escape mutants. The vaccine is not immunotherapeutic and cannot induce a curative response. Expression vectors based on replication deficient recombinant Semliki Forest virus vectors (rSFV) were used to transduce mice for HBV surface protein expression, which in turn induced an antibody response. Two subtypes of SHBs protein, subtype adw2 and ayw2, representing HBV genotypes D and A respectively, were cloned in pSFV1 vector. Additionally, in order to combine both determinants of HBV binding, which are also targets of neutralizing antibodies, vectors encoding internally deleted LHBs protein (LHBsΔ49-163, i. e. SHBs (subtype ayw2) linked to 48 aminoterminal amino acids (aa) of preS1) and it's variants were generated. To enhance secretion of internally deleted LHBs protein the myristoylation motif at the amino-terminus of preS1 was inactivated by point mutation. Contrary to the expected results internally deleted LHBs protein with an unchanged myristic acid attachment site formed secretable subviral particles displaying preS1 and S antigenicity with higher efficiency than myristoylation site mutants. Secretion of these particles was dependent on the in-frame expressed SHBs. This was shown by internally deleted LHBs protein protein with a point mutation in the in-frame translational start codon of SHBs protein. Groups of 7 – 9 BALB/c mice were intravenously inoculated with 10^7 replication deficient rSFV encoding both subtypes of SHBs - adw2 and ayw2 and two variants of internally deleted LHBs protein. Three weeks later mice were inoculated with 10^8 rSFV. Antibodies induced to both subtypes of SHBs protein exhibited strong preference to the homologous subdeterminant of the yeast-derived recombinant HBV surface antigen (rHBsAg), however this subtype dependency was not observed with the patient-derived HBsAg. Immunization with rSFV encoding internally deleted LHBs protein resulted in induction of preS1- and S-specific immunoglobulin G (IgG), while immunization with rSFV encoding the mutant without SHBs start codon resulted in preS1 antibodies only. The ratios of subclass IgG1 of immunoglobulin G to subclass IgG2a of immunoglobulin G ranged from 0.28 to 0.88 in the four immunized BALB/c mice groups and were lowest for the group immunized with internally deleted LHBs protein with active translation of SHBs indicating the strongest type 1 T helper cell response .Primary hepatocytes of *Tupaia belangeri* susceptible to infection with HBV were used to determine neutralizing potential of antibodies obtained by rSFV immunization. Antibodies were mixed with highly purified patient plasma-derived HBV (SHBs subtype ayw2), followed by infection of primary *Tupaia belangeri* hepatocytes. Quantitative markers of HBV infection were measured. Complete inhibition of HBV infectio was defined as infection in presence of monoclonal antibodies directed to preS1 and SHBs. The neutralizing power of SHBs antibodies was not dependent on HBsAg subtype and S/adw2 antisera neutralised HBV with SHBs subtype ayw2 with equal efficiency as antisera raised with S/ayw2. Antibodies induced to SHBs alone and internally deleted LHBs neutralized HBV infection completely, while antibodies generated to internally deleted LHBs with inactive in-frame translational start codon of SHBs protein neutralized HBV infection partially.

* In this dissertation the term "subtype" (often referred to as immunological subtype, or serotype of HBV) which is usually in literature referred to HBsAg, will be assigned to SHBs as well as to HBsAg.

ANOTĀCIJA

Lielākā daļa vakcīnu pret B hepatīta vīrusu (HBV) sastāv no vīrusa S proteīna adw2 apakštipa* (genotips A2), kas ar gēnu inženierijas paņēmieniem ekspresēts rauga šūnās. Lai gan šīs vakcīnas ir efektīvas, tomēr ar tām ir saistītas dažādas nepilnības – vāja vai neesoša imūnā atbilde, vīrusa „bēgļu"variantu veidošanās, bez tam vakcīna nav imunoterapeitiska. Uz alfavīrusu pamata izveidotie vektori „svešu" gēnu ekspresijai jau ir lietoti kā rekombinantās vakcīnas un gēnu terapijas līdzekļi vēža terapijai. Promocijas darba izstrādē izmantoti rekombinantie Semliki meža vīrusa (rSMV) vektori. Darba gaitā iegūti rSMV, kas genomā nes gan HBV S proteīna adw2 apakštipu, gan arī ayw2 apakštipu. Turklāt, lai apvienotu abus noteicošos faktorus, kas atbild par HBV saisīšanos pie mērķšūnām, iegūti rSMV, kas kodē saīsinātu L proteīnu, kas sastāv no S proteīna, kam N galā pievienotas preS1 N gala 48 aminoskābes (preS1.1-48/S). Lai palielinātu saīsinātā L proteīna sekrēcijas efektivitāti, izmainīta miristīnskābes pievienošanas vieta preS1 rajona N galā**,** jo miristīnskābe daļēji saistīta ar preS1 rajona aizturēšanu šūnās. Pretēji paredzētajam, saīsinātais L proteīns ar neizmainītu miristilēšanās motīvu, salīdzinot ar šī proteīna mutantiem, visefektīvāk veidoja sekretētas vīrusveidīgās daļiņas ar preS1 un S antigenitāti. Bez tam, proteīna sekrēcija bija pilnībā atkarīga no līdz-ekspresētā S proteīna, kura translācija noris no nākamā iniciācijas kodona.

rSMV replikonus var nogādāt šūnās *in vitro* un *in vivo* ar trim dažādiem paņēmieniem. Promocijas darba izstrādei izvēlēti replicēties nespējīgi rSMV ar vienu infekcijas ciklu. Lai iegūtu antivielas pret S un preS1, BALB/c pelēm vēnā ievadīja 10^7 rSMV, un pēc trim nedēļām 10^8 rSMV.

Antivielas, kas ierosinātas pret abiem HBsAg apakštipiem, efektīvi saistījās ar homologu rekombinantā HBsAg apakšdeterminanti, ar heterologu HBsAg apakšdeterminanti saistīšanās nebija efektīva. Turpretīm, izmantojot no pacientiem iegūto HBsAg, šādu saistīšanos atkarība no HBsAg apakštipa nenovēroja Imunizācija ar rSMV-preS1.1-48/S ierosināja preS1- un S-specifisku G klases imunoglobulīnu (IgG) veidošanos, bet imunizācija ar rSMV, kas kodē preS1.1-48/S_0 proteīnu ar "izslēgtu" S proteīna translāciju - tikai preS1-specifisku IgG veidošanos. IgG apakšklašu IgG1/IgG2a attiecība četrās imunizēto peļu grupas variēja no 0.28 to 0.88, pati zemākā šo apakšklašu attiecība bija ar rSMV- preS1.1-48/S imunizēto peļu grupā, liecinot par visspēcīgāko 1. tipa T līdzētājšūnu (Th1) atbildi.

Lai pārbaudītu anti-S un anti-preS1 spēju neitralizēt HBV infekciju *in vitro*, izmantoja Belangera tupajas (*Tupaia belangeri*) hepatocītus, kurus var inficēt ar HBV. Hepatocītus inficēja ar attīrītu HBV (S proteīna apakštips ayw2), kam iepriekš pievienoja ar rSMV imunizāciju iegūtās antivielas pret HBV virsmas proteīniem. Novērots, ka ierosināto antivielu spēja neitralizēt HBV infekciju nebija atkarīga no S proteīna apakštipa: anti-S/adw2 neitralizēja HBV/ayw2 tikpat efektīvi kā anti-S/ayw2. Anti-preS1.1-48/S un anti-S neitralizēja HBV infekciju pilnībā, turpretīm anti-preS1.1-48/S_0 - daļēji.

Secināts, ka rSMV spēj ierosināt no HBsAg apakštipa -neatkarīgu HBV neitralizējošu antivielu veidošanos, bez tam Th1 tipa atbildes ierosināšana liecina par rSMV pieejas potenciālu imunoterapeitiskas stratēģijas izveidei.

* Promocijas darbā termins "apakštips" (literatūrā lietots arī apzīmējums HBV serotips) attiecināts gan, kā pieņemts, uz HBsAg, gan arī uz HBV S proteīnu.

AIM OF THE STUDY

The aim of the study was generation of antibodies directed to two binding determinants of HBV to hepatocytes: SHBs protein in its natural conformation and combined with aminoterminal 48 aa of preS1 domain (genotype D numeration) and determination of neutralizing antibodies in an *in vitro* HBV infection model. Antibodies to HBs proteins were induced by immunization of an inbred laboratory mice strain with replication deficient recombinant Semliki Forest virus (rSFV) encoding the respective proteins.

OBJECTIVES

1. Clone the coding sequence of internally deleted LHBs (LHBsΔ49-163; consisting of aminoterminal 48 aa of preS1 linked to the amino-terminus of the SHBs protein (further termed preS1.1-48/S)) in pSFV1 vector. Decipher the influence of the myristoylation motif at the amino-terminus of preS1, known to be partially responsible for retention of full length LHBs, on secretion efficiency and cellular distribution of the deleted LHBs. To elucidate the effect of the in-frame expressed SHBs protein in effective formation of preS1.1-48/S subviral particles, abrogate the translation of the SHBs protein from the translational start codon downstream of the preS1 translation initiaton codon.

2. Establish conditions for rSFV production in BHK-21 cells after co-electroporation of rSFV RNA and RNA encoding SFV structural proteins and attain appropriate titer of rSFV (at least 10^9 infectious units/ml) after sedimentation through 20% sucrose (w/v) for immunization of an inbred laboratory mice strain BALB/c.

3. Examine expression *in vitro* of SHBs and preS1.1-48/S and it's variants in BHK-21 and Huh7 cells, as well as in primary hepatocytes of *Tupaia belangeri*. Evaluate ability of preS1.1-48/S protein and it's variants to generate subviral particles displaying preS1 and S antigenicity. From the preS1.1-48/S protein variants, choose proteins with the greatest secretion and efficiency of subviral particle formation for rSFV mediated immunization of BALB/c mice. Obtain rSFV encoding the selected preS1.1-48/S proteins.

4. In preliminary experiments immunize BALB/c mice intravenously (i.v.) and subcutaneously (s.c.) with various infectious units of rSFV encoding SHBs, subtype adw2 (rSFV-S/adw2): 10^6, 10^7 and 10^8 to determine the most appropriate immunization route and scheme for induction of a humoral response to S/adw2. Choose the most efficient route and scheme of immunization of BALB/c mice for generation of antibodies directed to two subtypes of SHBs – adw2 and ayw2, and preS1.1-48/S proteins.

5. Examine the reactivity of the generated antibodies to two subtypes of recombinant HBsAg produced in yeast (rHBsAg) representing homologous and heterologous SHBs subtype determinants. Determine the immunoglobulin class G (IgG) titer of anti-S antibodies and anti-preS1 antibodies in the immunized groups of mice. Detect the ratio

of IgG subclasses IgG1 to IgG2a (IgG1/IgG2a). Assess the binding of antibodies from all immunized groups to patient-derived highly purified HBsAg.

6. Determine the neutralizing potential of antibodies from all immunized mice groups using *in vitro* HBV infection model of primary *Tupaia belangeri* hepatocytes. After incubation of antibodies with highly purified patient-derived HBV and infection of primary *Tupaia belangeri* hepatocytes, detect quantitative markers of HBV infection: viral mRNA, HBsAg and HBeAg.

ABBREVIATIONS

(+) DNA	plus strand (positive strand) DNA
(-) DNA	minus strand (negative strand) DNA
(+) RNA	plus strand (positive strand) RNA
(-) RNA	minus strand (negative strand) RNA
aa	amino acid
cDNA	complementary DNA
CTL	cytotoxic T lymphocytes
EGFP	enhanced green fluorescence protein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
ELISA	enzyme linked immunosorbent assay
ERGIC	endoplasmic reticulum -Golgi intermediate compartment
HBc	core protein of hepatitis B virus
HBeAg	non-particular secreted form of Hepatitis B virus core antigen
HBV	Hepatitis B virus
HBs	surface proteins of Hepatitis B virus
HBsAg	Hepatitis B virus surface antigen (also known as Australia antigen *a* - particulate form of HBV surface proteins)
IFN	interferon
IgG	immunoglobulin G
IgG1	subclass IgG1 of immunoglobulin G
IgG2a	subclass IgG2a of immunoglobulin G
IU	international units of antibodies to HBsAg
i.v.	intravenous
LHBs	large HBs
MAb	monoclonal antibody
MDA	melanoma differentiation-associated factor-5
MEM	minimal essential medium
MHBs	middle HBs
N-glycan	an oligosaccharide added to the amide nitrogen of asparagine side chains
N-glycosylation	attachment of oligosaccharides to the amide nitrogen of asparagine side chains
ORF	open reading frame
OD	optical density
PBS	phosphate buffered saline
P protein	HBV polymerase or reverse transcriptase
PNGase F	peptide - N glycosidase- F
PTH	primary *Tupaia belangeri* hepatocytes
PKR	dsRNA dependent protein kinase
rcDNA	relaxed circular DNA
rHBsAg	HBsAg produced by recombinant DNA technology in yeast
RNase L	ribonuclease L
RIG-1	retinoic acid inducible gene-1
rSFV	recombinant Semliki Forest virus
s.c.	subcutaneous
S/CO	signal/"cut-off" ratio
SD	standard deviation
SFV	Semliki Forest virus
SHBs	small HBs
SIN	Sindbis virus
Th1	type 1 T helper cells
Th2	type 2 T helper cells
PEG	polyethylene glycol
PTH	primary hepatocytes from *Tupaia belangeri*
THM	*Tupaia belangeri* hepatocyte medium
TLR	toll-like receptors
VEE	Venezuelan equine encephalitis virus
wt	wild type

TABLE OF CONTENTS

LIST OF PUBLICATIONS

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3. Hutornojs V., Niedre-Otomere B., Kozlovska T., Zajakina A. 2012. Comparison of ultracentrifugation methods for concentration of recombinant alphaviruses: sucrose and iodixanol cushions. *Environmental and Experimental Biology*, 10: 117-123. *Contribution: established conditions for effective production of rSFV and pelletation through sucrose cushion for rSFV application in vivo.*

4. Niedre-Otomere B., Bogdanova A., Bruvere R., Ose V., Gerlich W.H., Pumpens P., Glebe D, Kozlovska T. 2013. Posttranslational modifications and secretion efficiency of immunogenic hepatitis B virus LHBs deletion variants. *Virology Journal*, 10 : 63.

1. REVIEW OF THE LITERATURE

1.1 Hepatitis B virus (HBV)

HBV is the prototype virus for a family of *Hepadnaviridae*. The hepatotropic DNA viruses of the *Hepadnaviridae* family cause acute and chronic infection of the liver. The mammalian viruses - HBV, woodchuck hepatitis virus and ground squirrel hepatitis virus - form the genus *Orthohepadnavirus*. Genus *Avihepadnavirus* include viruses of birds. The breakthrough leading to the discovery of HBV was the findings of B. Blumberg in 1965 that an antigen in the serum sample of an Australian aboriginee reacted specifically with an antibody in the serum of a hemophilia patient. The antigen was named Australia antigen, now known as hepatitis B virus surface antigen, HBsAg. Krugman and co-workers had by 1967 distinguished two distinct types of hepatitis, the parenterally transmitted „serum hepatitis" is now known as hepatitis B. In 1967 it was postulated that a link exists between the Australia antigen and serum hepatitis. Direct association of Au antigen with serum hepatitis B was put forward by Prince in 1968. The complete hepatitis B virion – double-shelled 42 nm particle (Fig.1A panels 1 and 4) was identified by Dane in the serum of patients with Australia antigen (Hollinger and Lau, 2006;Gerlich, 2013).

HBV virions (Fig. 1A) consist of an outer hepatocyte-derived lipoprotein envelope, which contains three related membrane spanning envelope glycoproteins – large (L), middle (M) and S (small) surface proteins (HBs proteins). The virion contains icosahedral nucleocapsid with T4 or T3 symmetry (Dryden et al., 2006), the majority of capsids in virions bear T=4 symmetry. The capsids which bear T=4 symmetry have a diameter of 36 nm, while capsids with T=3 symmetry are 32 nm (Crowther et al., 1994; Kenney et al., 1995). Details of capsid architecture can be found in excellent studies by Crowther et al. (Crowther et al., 1994), Wynne et al. (Wynne et al., 1999) and Zlotnick et al.(Zlotnick et al., 1996). The capsid encloses partially double stranded circular 3.2 kb DNA genome and an endogenous DNA polymerase – viral reverse transcriptase (Fig. 1B, Fig. 7). According to cryo-electronmicroscopy analysis virions can be divided in two morphologically distinguishable phenotypes: compact particles are characterized by an invisible boarder between nucleocapsid and the envelope (Fig. 1 A panel 4, upper row), whereas gapped particles have a clear "boarder" between the nucleocapsid and envelope (Fig. 1A panel 4, middle row). There are also chimeric particles which are a mixture of both phenotypes (Fig. 1A panel 4, bottom row) (Seitz et al., 2007).

1.1.1. Epidemiology of HBV

With around 240 million persistently infected persons HBV presents an enormous health problem. Each year 650 000 people die from HBV-associated liver failure, cirrhosis and hepatocellular carcinoma (http://www.who.int/mediacentre/factsheets/fs204/en/). In highly endemic areas the prevalent mode of transmission is perinatally from HBV infected mothers, while in low endemic areas the main transmission route is by sexual contact and percutaneously. After contraction of HBV 5%-10 % of adults develop chronic infection, while the remaining clear it. While about 90% of adults clear the infection, 40% - 90% of infected neonates progress to persistent infection (Dienstag, 2008).

1.1.2. HBV surface proteins

HBV envelope proteins are encoded by a single open reading frame. Each of them is produced from alternative translational start sites, but with a common stop codon. SHBs, being most abundant in HBV and subviral particles, is comprised of 226 amino acids (aa) which is also present as S domain in the MHBs and LHBs. M protein contains in addition a preS2 N-terminal extension of 55 aa. The L protein has further N-terminal 108, 118 or 119 aa depending on the HBV-genotype, termed preS1, for schematic representation see Figure 2. All HBs proteins are referred as HBsAg. The proportion of S:M:L in the virion envelope is about 7:2:1 (Heermann et al., 1984). HBV-infected cells and transfected cells secrete nucleocapsid free spherical and filamentous (Fig. ,1A panels 3 and 2) subviral HBsAg particles, 20-22 nm in diameter.Spherical particles consist primarily of SHBs, while filaments have a larger proportion of L protein. The non-infectious subviral particles are produced in up to 10 000-fold excess over virions (Bruss, 2007). The spherical 22 nm subviral particles are composed of dimers and are octahedral (Gilbert et al., 2005).

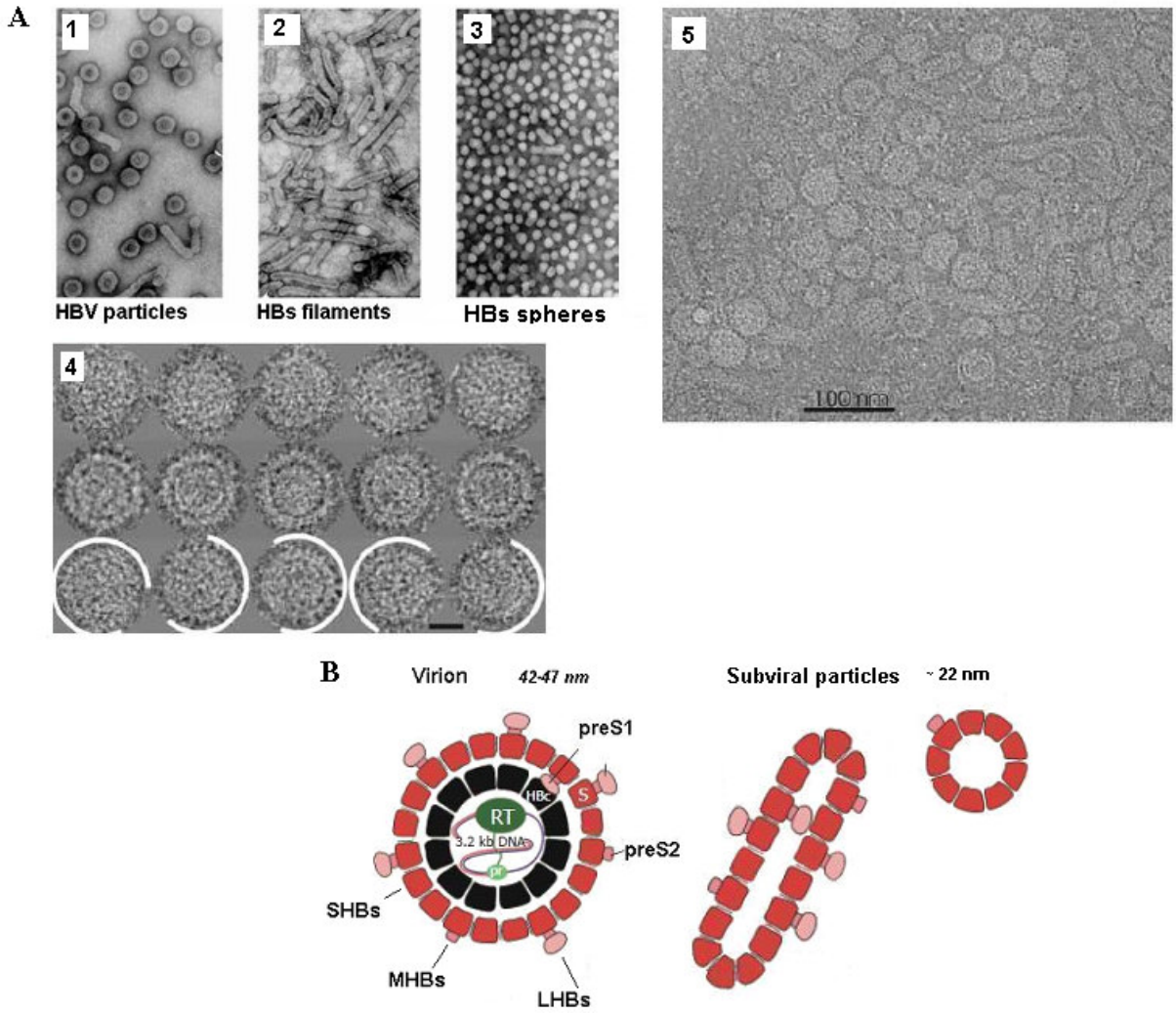

Figure 1. A. Electron micrographs of HBV and subviral particles. (A) 1-3 shows virions and subviral particles purified from HBV carrier serum . The panel 4 and 5 of A shows image processed images of cryo-electron microscopy of infectious HBV virions purified from chronically infected patient. Panel A 4 depicts HBV particles with compact morphology, middle row shows particles with gapped morphology and bottom row - particles with mixed morphology. The white line around particles in the bottom row indicate gapped morphology. Bar: 20 nm. A 5 shows electron micrograph of purified HBV particles, including filamentous and spherical subviral particles. **B. Schematic presentation of HBV.** The LHBs, MHBs and SHBs stand for large, middle and small HBV surface proteins, L consisting of S, preS2 and preS1 domains, while M – of S and preS2. The nucleocapsid (core or HBc) is shown in black. DNA is shown as a single or double line. Subviral HBsAg particles are depicted in filamentous and spherical form. The diameter of the virion and the subviral HBsAg particles in nm is indicated. Fig. 1A panels 1-3 and Fig. 1B adapted from *Gerlich W.H. (2013) Medical Virology of Hepatitis B: how it began and where we are now.Virology Journal 10:239.* Fig.A panels 4 and 5 adapted from *Seitz S. et al. (2007). Cryo-electron microscopy of hepatitis B virions reveals variability in envelope capsid interactions. EMBO J. 19;26(18):4160-7.*

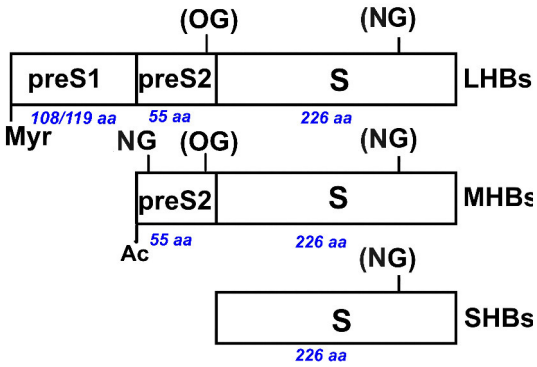

Figure 2. Schematic representation of HBs proteins. The 226 aa S domain is shared by all three proteins. The 55 aa extension, termed preS2 is unique to the M and L proteins, while the preS1 extension is unique to the L protein. The preS1 domain consists of 108 aa, 118 aa or 119 aa depending on the genotype. N-glycosylation is indicated by NG, O-glycosylation by OG. The partial glycosylation is shown by the parenthesis. The L protein is myristoylated at the N-terminus (Myr), and the M protein at the N-terminus is acetylated (Ac). Adapted from *Glebe D., Urban S. (2007). Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol. 13(1): 22-38.*

The three HBs proteins are partially co-translationally N-glycosylated at the Asn146 in the S domain (Figure 2). The preS2 bears a N- glycosylation site at Asn 4, which is employed in M protein, but not in the L protein. The preS2 in the M protein, and in the L protein, can be O-glycosylated at Thr 37 in genotypes B – H (Glebe and Urban, 2007). The preS1 contains an additional potential N-glycosylation site at Asn4, however, the preS1 chains fail to be co-translationally translocated to the ER lumen and due to the orientation of the preS1 domain to the cytosolic side of the ER membrane, these sites remain unmodified (Bruss et al., 1994). An artificially added signal sequence at the N-terminus of L protein forces the co-translational translocation of preS1 to the ER lumen where glycosylation at Asn4 may take place (Bruss and Vieluf, 1995). The N-terminus of the L protein additionally bears a Met-Gly myristoylation motif (Towler et al., 1988), which is recognized by cellular N-myristoyltransferase. Gly 2 of the preS1 is myristoylated and this modification is a common feature of all hepadnaviruses (Persing et al., 1987). Myristoylation of the L protein is indespensible for viral infectivity, since it has been shown that myristoylation-defective virions completely lost infectivity for susceptible cultures (Gripon et al., 1995; Bruss et al., 1996; Glebe et al., 2005).

1.1.3.Topology and function of the HBs proteins

HBV envelope proteins are synthesised at the endoplasmic reticulum (ER), the morphogenesis of HBV S protein was reviewed in 2007 by a study of Patient et al., which, interestingly, resolved that SHBs forms branched filaments of 22 nm in diameter in the lumen of ER, which are transported to ER-Golgi intermediate compartment (ERGIC), packed in folded and bridged crystal-like structures, which are relaxed in ERGIC. However, it remains unresolved how the spherical particles, necessary for the progression through a secretory pathway, are formed (Patiet et al. 2007).

The orientation of the SHBs at the ER membrane results in the region between aa 99 and approximately 169 facing the lumen of the ER, which is most exposed on the surface of virus particles. The “a” determinant of HBsAg is comprised of the antigenic loop in a specific conformation and is located between aa 101 and 164 - the transmembrane domain II and the hydrophobic C-terminus (Salisse and Sureau, 2009). The “a” determinant contains the protective, virus neutralizing B cell epitopes (Ganem and Schneider, 2001). Besides, they are disulphide-dependent (Glebe et al., 2003), with the cysteines having a fundamental role at the viral entry step (Abou-Jaoude and Sureau, 2007). The role of SHBs protein in binding is emphasised by the findings that antibodies against a conformational, but not linear S epitope,

could inhibit HBV infection (Glebe et al., 2003). Amino acids 100 to 170 of the S protein form the hydrophilic loop which is essential for infectivity (Jaoude and Sureau, 2005) and specifically aa 119-128 of the antigenic loop are of crucial importance (Jaoude and Sureau, 2005).

The preS2 domain of the M protein is translocated to the lumen of the ER by signal I in the S domain (Eble et al., 1990; Stibbe and Gerlich, 1983). The preS2 domain does not participate in the binding and is not necessary for virus entry (Ni et al., 2010). M protein is not necessary for virion secretion (Bruss and Ganem 1991, Sureau 1994), however, later work identified that 5 aa (aa109 – 113) at the N-terminus of the pre-S2 region are essential for virion release, while deletions from aa 114 to 163 (numeration from the start of L protein at the preS1) still supported virion secretion (Le et al., 1998). The role of preS2 in virion assembly was revised by a study of Ni et al. which showed that deletions of aa 114 to 143 and 114 – 163 interfered with virion formation. By a frameshift mutation of aa 114 – 143 they demonstrated that this region may serve as a spacer between the capsid binding site in preS2 and the transmembrane domain at the N-terminus of S protein (Ni et al., 2010).

After translation half of the L protein chains position their preS region into the ER. It has been proposed that the ER chaperone BiP regulates the process of post-translational translocation (Awe et al., 2008). The other possible mechanisms of this post-translational translocation are reviewed by Bruss V. (Bruss, 2007). This results in the virions establishing the dual topology of the L protein - the preS1 and preS2 domains face the external and internal side of the viral envelope (Bruss et al., 1994,Lambert, Prange et al. 2001). From the dual topology of the L protein (Fig. 3A) the dual function of the preS domains arise:preS1 mediates binding of the HBV to the susceptible cells (Le Seyec et al., 1999; Engelke et al., 2006; Glebe et al., 2005; Gripon et al., 2005) as well as envelopment of the capsid (Bruss, 2007). The first 75 N-terminal aa are responsible for infectivity (Blanchet and Sureau, 2007), while work by D. Glebe et al. postulates that N-terminal 48 aa are responsible .The essential binding domain of preS1 is comprised of aa 9-18, while aa 28-48 comprise the accessory domain (Glebe et al., 2005). It has been proposed that the attachment site in the preS1 mediates binding of the virus to cell surface heparan sulfate proteoglycans (Leistner et al., 2008). Recently, sodium-dependent taurocholate cotransporting polypeptide has been identified as a receptor for preS1 attachment site (Yan et al., 2012). The signal for encapsidation resides in the C-terminal part of the preS. The exact interaction of the caspid and the HBs still remains unresolved. Sequence between Arg 103 and Ser 124 involving the very N-terminal part of preS1 has been proposed to directly interact with the nucleocapsid (Bruss, 1997). Glu 77 and Asp 78 at the tip of the capsid spike play a role in envelopment (Bottcher et al., 1998). Later work by Bruss (Ponsel and Bruss, 2003) identified 11 aa at the groove around the base of the capsid spike essential for particle envelopment and virion formation (Dryden et al., 2006), although the study by Seitz et al (Seitz et al., 2007) was not able to elucidate that base of the spike is directly interacting with the envelope. Current evidence suggests that binding of HBs to the nucleocapsid is mediated by specific types of electrostatic interactions between the negative charges at the tips of the spikes and cytosolic loop of HBs which contain three arginine residues (Arg 73, 78 and 79) (Seitz et al., 2007). Study by Dryden et al. (Dryden et al., 2006) suggested a random packing of HBs (Fig. 3B panel 1), whereas work by Seitz et al. also recognized some degree of variability in organization of HBs proteins, but proposed a model towards an organized icosahedral packing of HBs in the envelope (Fig. 3B panel 2) (Seitz et al., 2007).

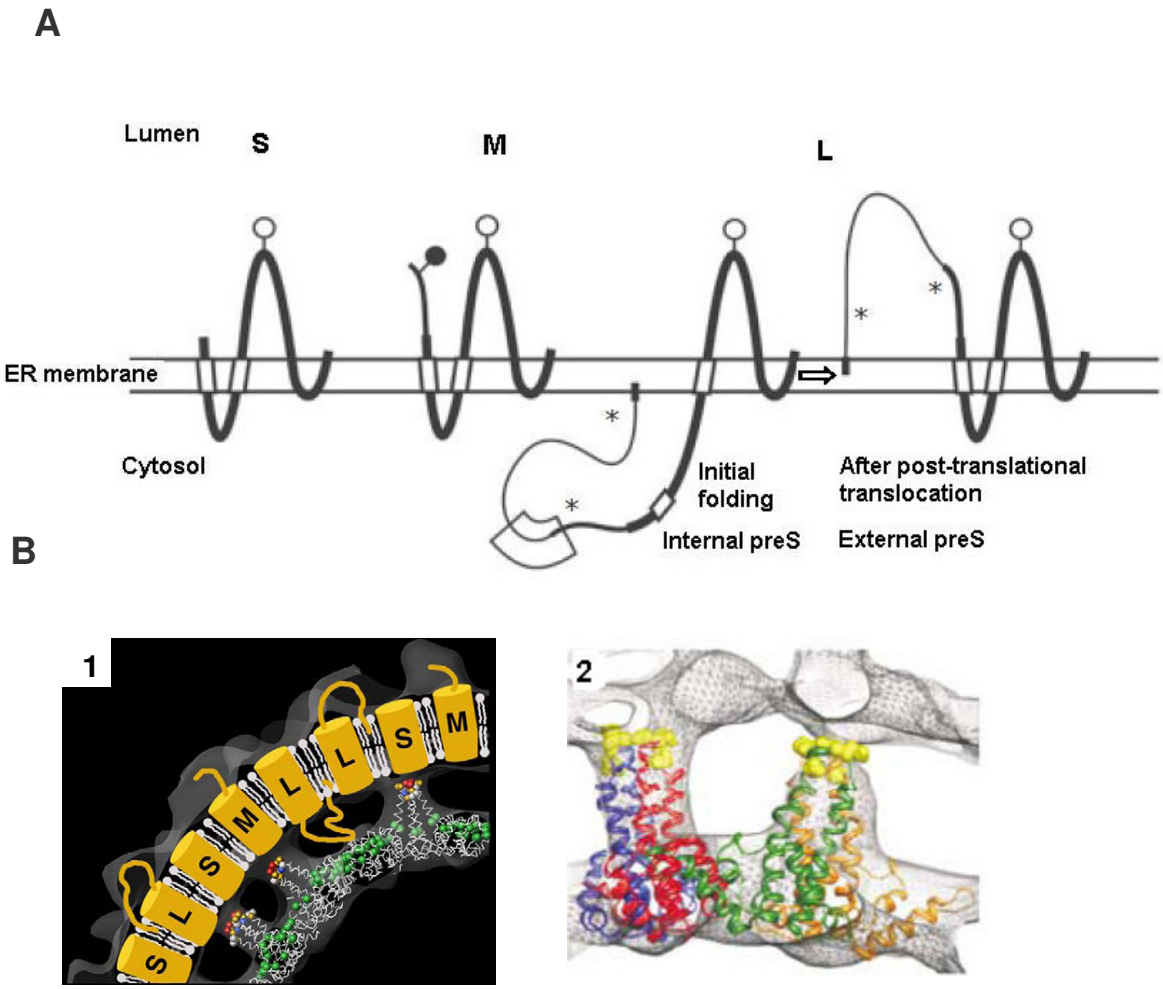

Figure 3. **A. Transmembrane topology of the HBs proteins at the ER.** S, M and L denote the S HBs, MHBs and LHBs proteins. The open boxes stand for N-terminal and internal signals of the S protein determining its transmembrane folding. The thinner line in the M protein represents the preS2 domain.The filled circle denotes N-glycosylation of the preS2 domain, the open circles denote partial N-glycosylation of the S domain. The dual topology of the L protein resulting from ~50% of the molecules post-translationally translocating to the ER lumen during maturation is shown. Internal preS and external preS denotes the orientation of the preS domains in the mature virus particle after budding. The boxed area represents the potential interaction site with the capsid. The proposed position of the N-terminal myristate group (filled box) is shown. Adapted from *Bruss V. (2007). Hepatitis B virus morphogenesis. World J Gastroenterol. 13(1): 65-73.***B. Interaction of preS domains with the capsid. Panel 1.** Cartoon of interpretation of x-ray crystal structure of recombinant capsid docked into the cryo-EM density map of the virion capsid. L, M and S (yellow boxes) denote the HBs proteins. Residues in the capsid tip are colored according to charge and hydrophobicity (negative: red; positive: blue; hydrophobic: gold; hydrophilic:gray). ~50% of the L molecules have an interior loop that according to Dryden et al.(Dryden et al., 2006) is predicted to be disordered, which interacts with specific residues in HBcAg (green spheres) (Ponsel and Bruss, 2003). Adapted from *Dryden et al. (2006). Native Hepatitis B Virions and Capsids Visualized by Electron Cryomicroscopy. Mol. Cell 22: 843–850.* **Panel 2.** The two dimers in the asymmetric unit (Wynne et al., 1999) fitted into the density map of compact virions reflecting the side views of the two types of spikes in contact to the envelope. Negatively charged amino acids at the tips of the spikes are shown in yellow.

1.1.4. Secretion of HBs proteins

Cells infected with HBV as well as cells transfected with HBs encoding constructs secrete spherical and filamentous particles of 20-22 nm in diameter. SHBs and MHBs are efficiently secreted from transfected cells (Ou and Rutter, 1987;Patient et al., 2007)

Although LHBs is abundant on the surface of mature HBV virions, it comprises only 1-2% of the secreted filamentous subviral particles from HBV infected cell (Heermann; 1984). Full length LHBs is not secreted from transfected cells or secreted in minor quantities

(Persing et al., 1986;McLachlan et al., 1987;Ou and Rutter, 1987). Moreover, the secretion of SHBs is impaired if S is expressed simultaneously with full length L or with preS1 domain (Persing et al., 1986; Ou and Rutter, 1987; McLachlan et al., 1987; Chisari et al., 1986;Standring et al., 1986). Specifically the preS1 domain is implicated in retention of the LHBs. Studies on genotype A, HBsAg subtype adw2, mapped the retention signal to aa 6-19 (Kuroki et al., 1989). Gallina et al. (Gallina et al., 1995) postulated that the retention signal resides in the region aa 77-99 of preS1. Another determinant involved in the retention of the L protein is the N-terminal myristic acid (Prange et al., 1991;Gallina et al., 1995;Gazina et al., 1996), while some studies assign only partial role to the N-terminal myristate (Gallina et al., 1995; Gazina et al., 1996). Topology of the L protein at the ER (Bruss and Vieluf, 1995;Ostapchuk et al., 1994;Bruss et al., 1994) is also responsible for the secretion block of the L protein.

1.1.5. Genetic variability of HBV. HBV genotypes and subgenotypes. Subtypes of HBsAg

10 genotypes can be distinguished for human HBV: A, B, C, D, E, F, G, H (Schaefer, 2007), I (Yu et al., 2010) and J (Tatematsu et al., 2009). HBV genotypes differ by more than 8%. HBV genotypes can be divided in subgenotypes, with the exception of genotypes E and G, wich differ by at least 4% (Schaefer, 2007). The geographic distribution of the HBV genotypes and subgenotypes is shown in Figure 4. The HBV genotypes dealt with in this dissertation are genotype D and genotype A.

Figure 4. Geographic distribution of HBV genotypes and subgenotypes. From from *Gerlich W.H. (2013) Medical Virology of Hepatitis B: how it began and where we are now.Virology Journal 10:239.*

HBsAg has nine immunological subtypes, which are represented as a combination of a common immunodominant a with subtype determinants d or y, and w1-w4 or r. HBsAg subtype adr is additionally subdivided into q+ and q- categories The nine HBsAg subtypes are ayw1, ayw2, ayw3, ayw4, adw2, adw4, ayr, adrq+ and adrq- (Norder et al., 2004). Exchange from Lys to Arg at the positions 122 and 160 determines the change from allelic variatons d/y and w/r (Purdy et al., 2007). The d determinant is distinguished by Lys at position 122, while y determinant by Arg at the position 122. Lys at position 160 distinguishes the w determinant, while Arg – r determinant (see Fig. 5).w1-w4 variation is determined by Pro, Thr and Leu at aa 127 and it distinguishes w1/w2, w3 and w4 reactivity respectively. The complete decision tree for HBsAg subtype determination can be found in an article by Purdy and others (Purdy et al., 2007). One subtype of HBsAg may be specified by several HBV genotypes. Subtype adw2 dealt with in this dissertation is associated with genotypes A, B, C, F and G, while HBsAg subtype ayw2, also described here, is specified by genotypes D and E. The complete

association between HBV genotypes and subtypes is described in detail by Norder et al. and Purdy et al. (Norder et al., 2004; Purdy et al., 2007).

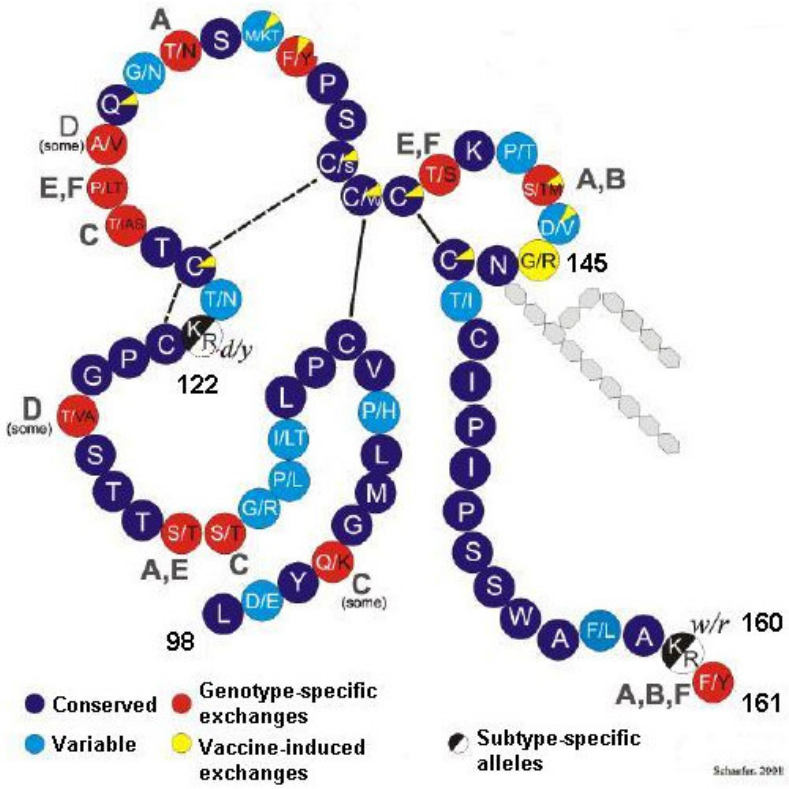

Figure 5. Genotype and vaccine induced specific exchanges in the *a* determinant of SHBs. The a determinant consists of 2 loops: aa 124-137 and aa 138-147 or 149, held together by disulfide bridges, schematically represented by lines. The capital letters in black denote the genotypes of HBV. Adapted from *Schaefer et al. in Doerr & Gerlich: Medizinische Virologie, Thieme 2010, p. 356.*

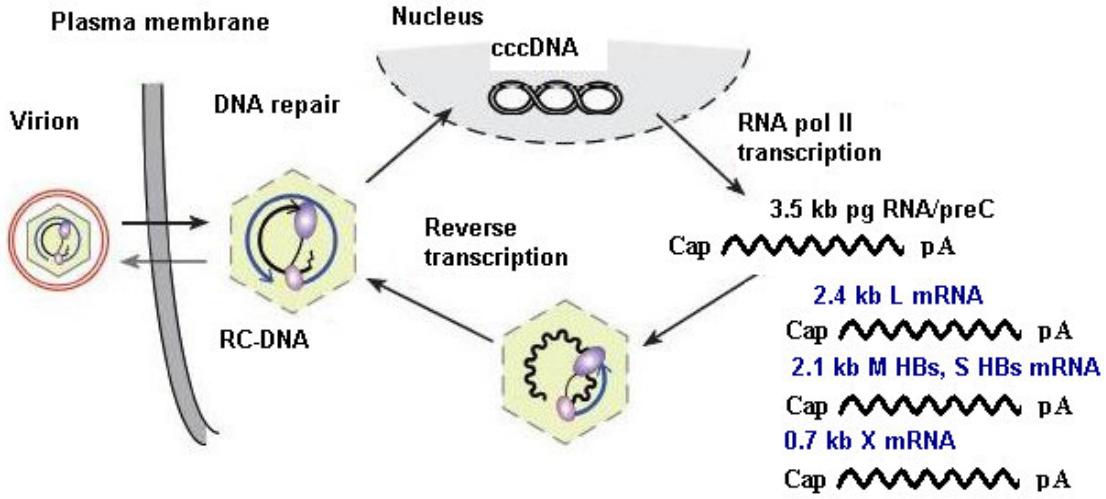

Figure 6. Replication cycle of HBV genome. After binding and uptake of the virions, capsids containing relaxed-circular DNA (RC-DNA) are released in the cytoplasm. RC-DNA is transported to the nucleus and repaired to form cccDNA. Viral mRNAs are transcribed by RNA polymerase II from cccDNA. Pregenome RNA serves as a template for core protein and P protein (reverse transcriptase) synthesis, it is encapsidated with P protein into nucleocapsid where reverse transcription takes place. New RC-DNA is formed by (+) DNA synthesis from the (-) DNA strand. The subgenomic transcripts for translation of HBs proteins and X protein are shown. Adapted from *Beck. J., Nassal M. Hepatits B virus replication. (2007). World J Gastroenterol. 13(1): 48-64* and modified by author.

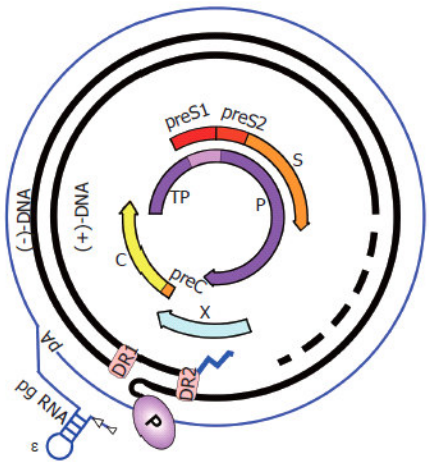

Figure 7. HBV genome and encoded proteins. The thick, black lines denote the partially double-stranded relaxed-circular HBV DNA. The (+)-strand is incomplete. P protein – viral reverse transcriptase is covalently linked to the 5' end of the negative strand DNA, the zig-zag line denotes the RNA primer, which is linked to the 5'end of the positive strand DNA. DR1 and DR2 are the direct repeats. The outer circle shows the terminally redundant pgRNA with hairpin structure (encapsidation signal and takes part in replication initiation) close to the 5' end and poly A at the 3' end. The four open reading frames – C (core), X (X protein), preS/S (HBs proteins) and P (reverse transcriptase) are shown inside. TP – terminal protein domain of P. Adapted from *Beck. J., Nassal M. Hepatits B virus replication. (2007). World J Gastroenterol. 13(1): 48-64.*

1.1.6. HBV entry and replication cycle

Several important findings have predecessed discovery of preS1 receptor on liver surface membranes common to man and *Tupaia belangeri*.– Among them are findings that HBV via preS1 binds to the cell surface heparan sulfate proteoglycans (Leistner et al., 2008). , It has ben shown that cholesterol in the viral envelope is necessary for the HBV entry and proposed that it might play a role in fusion of HBV (Bremer et al., 2009). Insights into early events of hepadnaviral infection have ben gained from experiments with duck HBV (DHBV) using primary duck hepatocytes. DHBV is internalized by endocytosis and acidification is not necessary for fusion with the membrane, which is an unusual mechanism (Kock et al., 1996). Trafficking of incoming DHBV viral particles is dependent on intact and dynamic microtubules (Funk et al., 2004). By using HepRG cells which allow the infection with HBV (Gripon et al., 2002) it was concluded that entry of HBV is mediated by caveolin-1, major constituent of caveolae. Caveolae are small invaginations of the cell surface (Macovei et al., 2010). Experiments with SHBs as a model of an enveloped HBV revelaed that entry of HBsAg occurs via a caveolin-mediated endocytotic pathway and that the movement of internalized particles is actin-dependent (Hao et al., 2011).

The next steps in HBV life cycle are transport of the capsids to the nucleus, disassembly and re-assembly of the capsids and genome replication. Hepadnaviruses employ the step of reverse transcripton for the replication of their genome,but there are a number of fundamental features which differentiate them from retroviruses, which also replicate via reverse transcription. After uncoating and release of the capsid, the capsid is transported to the nucleus. The trafficking of the capsids to the nucleus has been described in fine details by Kann et al. (Kann et al., 2007)., The transport is microtubule-mediated. Phosphorylation of the carboxy-terminus triggers an exposure of the nuclear localization signal (Rabe et al., 2003). The 39 nm diameter of the nuclear pore (Pante and Kann, 2002) allows entry of the capsids, which are complexed with nuclear transport receptors importin α and β. The details of the capsid entry into the nuclear pore and interactions in the nuclear basket can be found in a study by Schmitz et al. (Schmitz et al., 2010). After RanGTP mediated dissociation of importin α and β capsids directly interact with nucleoporin 153 - a protein of the nuclear basket (Schmitz et al., 2010) and disassemble at the karyoplasm side of the nuclear pore to core protein dimers (Rabe et al., 2009). Importantly, only mature capsids (rc genome) disassemble, while immature capsids are trapped (Schmitz et al., 2010) The core protein dimers may reassemble again in the nucleus facilitated by the presence of cellular RNA (Rabe et al., 2009). It is unique among viruses that capsid disassembles and reassembles. The rc genome in complex with polymerase is released from the nuclear basket after which the so called genome repair takes place. The formation of covalently closed circular DNA (ccc) (Fig.

6) requires removal of the P protein attached to the (-)strand and RNA oligonucleotide primer attached to the 5'end of the (+) strand (Fig. 7). The completetion of the (+) strand of the relaxed circular genome requires activity of viral polymerase, host cell specific factors may be involved in the formation of ccc DNA (Beck and Nassal, 2007).From the ccc DNA template the cellular polymerase II transcribes the pregenome RNAs and the subgenomic RNAs. The pregenome RNA relevant for virus replication is translated into the core protein and the P protein (reverse transcriptse) from an overlapping reading frame. The sg RNAs are translated into the three envelope proteins and X protein. P protein binds to the hairpin–like ε structure on pg RNA and triggers the encapsidation of pg RNA. The capsid multimers are recruited to form an intact capsid. The interaction of the extra terminal protein domain of the P protein with the ε initiates reverse transcription of pregenome. The initiation of reverse transcription is also dependent on actvation of P protein by cellular chaperones (Nassal, 2008). The unique feature of hepadnaviruses is that the reverse transcritption can only occur in intact capsids. Pg RNA is transcribed to (-) strand DNA, which serves as a template for (+) strand sythesis generating a rc DNA. Detailed overview of the HBV replication can be found in an article by Beck and Nassal (Beck and Nassal, 2007).

It is important to note that the newly synthesized capsids are structurally identical to the in-coming capsids from the endosome, therefore retrograde transport of the mature genome containing capsids to the nucleus occur (Kann et al., 2007). Mature capsids can be enveloped once the envelope proteins are synthesized in sufficient quantity to generate HBV virions which are released out of the cell. It was shown by Prange et al. that HBV is transported out of the cell by multivesicular bodies assisted by γ2-adaptin (Lambert et al., 2007).

1.1.7. Non-structural HBV proteins

From the precore RNA which is 29 aa codons longer than the pg RNA, the precore precursor protein of 25 kDa is translated, the hydrophobic signal sequence directs the protein to ER and translocates it to the lumen. After cleavage along the secretory pathway 16-18 kDa e protein or HBeAg is secreted (Beck and Nassal, 2007). It serves as a marker of HBV infection of susceptible cells (Gripon et al., 2005;Engelke et al., 2006;Glebe et al., 2003) .

The HBV X protein is a 154 aa protein and its role is still largely unresolved, but it is generally accepted that the protein and its encoding *x* gene of HBV plays a role in the pathogenesis of HBV-induced hepatocellular carcinoma (Kew, 2011). A number of cellular interaction partners have been identified for the HBV X protein, as well as the influence of the protein on HBV replication and pathogenesis has been recognized. A detailed review on the functions of the X protein and its significance in HBV life cycle and pathogenesis has been published (Benhenda et al., 2009).

1.2. Vaccine types

Since the first protocols of Edward Jenner in 1796 vaccination by today has evolved as an important public health instrument for control and prevention of infectious disease. Vaccines include live attenuated, whole killed, subunit vaccines and those produced by recombinant DNA technology. The first vaccines were live attenuated bacteria or viruses. Live attenuated vaccines induce powerful humoral and cellular responses. The presentation of antigen by live attenuated and replicating vaccines mimic the natural viral infection, but the major disadvantage of live attenuated vaccines is the risk of revertion to virulent strains. Although inactivated or killed bacteria or viruses alleviate the biosafety problem, they are less effective. The subunit vaccines include purified antigenic components of a pathogen, proteins produced by recombinant DNA technology and synthetic peptides. Although they

completely eliminate the issue of biosafety and their composition is well charactrerized, they are often poor immunogens, and need to be administered with an adjuvant. Moreover, recombinant proteins induce primarily humoral response (Payette and Davis, 2001). Tremendous advantages have been brought to area of public health by vaccination, for example, vaccination has lead to a complete eradication of smallpox in 1980 (Graham, 2013). Nevertheless, the scientific community is concentrating the efforts on novel improved vaccines, as well as on research and development on vaccines to such important pathogens as HIV and hepatitis C to which a widely available vaccine is not yet available. The activation of humoral and cellular arms of the immune system is a prerequisite for an effective vaccine. Effective vaccine should combine the efficacy of live attenuated vaccines and safety of subunit vaccines. Plasmid DNA vaccines with their ease of manipulation and potent induction of humoral and cellular responses in small animal models appeared as a very promising approach, however, the human clinical trials have been disappointing (Liu, 2011). Besides, plasmid DNA vaccines pose a risk of integration into a host genome, RNA vaccines may overcome this problem, but they are unstable.

1.3. HBV vaccines

1.3.1. Plasma derived vaccines

Due to inability to grow HBV in cell culture and its restricted hoast range to humans and higher primates a different strategy was needed for the HBV vaccine. The first plasma derived HBV vaccines were obtained from the plasma of asymptomatic, human HBV carriers and consisted of purified , formalin or heat inactivated, alum-adsorbed HBsAg (Hollinger and Liang, 2001). In 1981 and 1982 the first plasma-derived hepatitis B vaccines were licensed (Heptavax B®, Merck & Co., Hevac B®, Institut Pasteur) (http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index4.html).

1.3.2.Recombinant yeast derived vaccine

The development and introduction of HBV vaccine based on HBsAg produced by recombinant DNA technology was an important milestone in the vaccine development. The vaccine has been commercially available since 1986. It consists solely of SHBs. The HBsAg is produced from recombinant *Escherichia coli* derived plasmid in transformed yeast *Saccharomyces cerevisiae* cells (McAleer et al., 1984). HBsAg is not secreted from the yeast cells, but released by homogenization or disruption with glass beads. The HBsAg produced in yeast cells is not glycosylated. The vaccine genotype is A2, HBsAg subtype adw2. The two most widely used recombinant HBV vaccines are Engerix-B® (*GlaxoSmithKline*) and Recombivax HB® (*Merck*).The vaccine is moderately immunogenic, 3 doses of 20 µg required for the Engerix-B® vaccine and 3 doses of 5 µg for the Recombivax HB® (http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index4.html). This vaccine is included in worldwide vaccine programs (Komatsu, 2014).

1.4. Significance of SHBs as a HBV vaccine component and rationale for inclusion of preS1 in the HBV vaccine

After vaccination with rHBs, antibodies are induced which recognize exclusively the conformational epitopes of the „a" determinant of SHBs. Although, the predominant role in binding of HBV to hepatocytes is assigned to preS1, SHBs has a secondary role. Aa 119-128 of the antigenic loop are of crucial importance for infectivity (Jaoude and Sureau, 2005), with the cysteines having a fundamental role at the viral entry step and for the structure of the "a"determinant (Abou-Jaoude and Sureau, 2007). The secondary role of SHBs in entry of

HBV could be attributed to attachment, binding, and particle disassembly (Salisse and Sureau, 2009).

It is known that inclusion of preS1 augments anti-S response and can overcome non-responsiveness to SHBs in mice and humans (Neurath et al., 1986a; Neurath et al., 1989; Milich et al., 1986; Shouval et al., 1994; Hellstrom et al., 2009). Significance of preS1 aa residues 21-47 (genotype A numeration, correspond to 10-36 in genotypes D, E and G) in mediating binding to HepG2 cells has long been established (Neurath et al., 1986b). Role of N-terminal preS1 region mediating virus binding has been reinforced by later studies employing cells susceptible to HBV infection (Le et al., 1999; Gripon et al., 2005; Engelke et al., 2006; Glebe et al., 2003). Aa 2-48 of the preS1 domain (genotype D) with the myristoylation at Gly2 have been shown to mediate attachment of HBV to primary hepatocytes of *Tupaia belangeri* (Glebe, 2006). Synthetic myristoylated peptide spanning aa 2-48 of preS1 analogues were able to inhibit the HBV infection in primary *Tupaia belangeri* hepatocytes (Glebe, 2006) and HepaRG cells (Engelke et al., 2006). Moreover, preS1 region is highly immunogenic and contains T and B cell epitopes (Milich et al., 1987; Kuroki et al., 1990; Maeng et al., 2000; Park et al., 2000; Hong et al., 2004). The preS1 region spanning the first N terminal 48 aa residues has been identified as bearing the main immunogenic domains (Milich et al., 1986;Hu et al., 2005), and Hu et al. have identified preS1 23-48 (genotype D numeration) as the major immunogenic region. Recent data of Bremer et al. (Bremer et al., 2009) showed that preS1.1-48 but no other preS sequences carry neutralising B cell epitopes.

1.5. Immune escape mutants

The protection mediated by the current vaccine is based on the recognition and interaction of vaccine induced neutralizing anti-HBs with the tertiary structure of the "a" determinant. Therefore mutations affecting the binding domains of the neutralizing anti-HBs may escape the anti-HBs mediated protection. Immune escape mutants arise as a result of vaccine-induced immune selection, long term use of HBV immunoglobulin and natural infection (Golsaz et al., 2014) . The reverse transcription step of the HBV replication is error-prone and HBV variants associated with immune escape may be appear spontaneously during HBV infection and may be selected in the presence of the host's immune response or as a consequence of appearance of anti-HBs. Reactivation of occult hepatitis B virus infection also may lead to selection of HBsAg escape mutants (Gerlich et al., 2010). Mutations associated with the immune escape frequently occur in the hydrophilic part of SHBs - from aa 99 to 169 (Carman, 1997). The prevailing variant induced by pressure of immune selection isolated from the vaccinated individuals is with a substitution of glycine in position 145 to arginine, G145R (see Fig. 5). It is the first reported escape mutant desribed. The mutant virus is stable (Carman et al., 1990) and horizontal transmission has been reported (Gao et al., 2015). The G145R variant is infectious *in vitro*, with the infection being by 27% more efficient than of *wt* virus (Salisse and Sureau, 2009). Having shown that the G145R variant is pathogenic in chimpanzees (Ogata et al., 1997), Ogata and co-authors then performed a study where they challenged sero-negative chimpanzees with the G145R mutant after vaccination with the licensed HBV vaccine and development of anti-HBs. Animals were not infected and did not develop HBV infection (Ogata et al., 1999), but the challenge with the virus was done shortly after development of anti-HBs. Acute hepatitis B was reported in a vaccinated person, with a mutation Q129H within "a" determinant (Luongo et al., 2015). A repertoire of of monoclonal antibodies to rHBsAg could weakly react with K141E, T143K, D144A and G145R HBsAg variants (Golsaz et al., 2014). Up to date ability of human, HBV vaccine induced atibodies to neutralize virus escape mutants *in vitro* are lacking. Among vaccinated persons with breakthrough infections G145R and T126A/S account for 48% of mutants detected (Chang, 2010). Numerous spontaneously arisen "a" determinant mutations and those selected under

selection pressure are listed with references in Cooreman et al. (Cooreman et al., 1999) and summarized by Pumpens et al. (Pumpens et al., 2002a).

Long-term nucleotide/nucleoside analog therapy of chronic HBV carriers also contributes to the selection of immune escape mutants. Nucleotide/nucleoside analogs affect the catalytic domains of the polymerase, and can introduce changes in the "a" determinant and other regions of HBsAg. Pol and SHBs encoding reading frames overlap (Fig. 7) and therefore alterations in the Pol reading frame not only contributes to the emergence of antiviral resistant HBV variants, but also affect neutralizing antibody binding domains of HBsAg and thus may contribute to the emergence of immune-escape variants. Antiviral therapy induced changes reduce the immunoreactivity of HBsAg to neutralizing antibodies (Torresi et al., 2002; Sloan et al., 2008). It has been shown in a chimpanzee model that despite protective anti-HBs levels achieved by the HBV vaccine, antiviral resistant HBV mutants with the changes in the *pol* gene affecting the HBsAg, were able to successfully infect the experimental animals (Kamili et al., 2009).

1.6. Alternative HBV vaccines

Vaccines have been developed which consist of SHBs with additional preS2 and preS1 components produced in yeast and, as an alternative, in Chinese hamster ovary cells (Shouval et al., 2015). These vaccines have not gained widespread use (Gerlich, 2013), although mammalian cell derived vaccine licensed in Israel and several countries of South East Asia containing preS1, preS2 and S has recently showed promising results in a clinical trial (Shouval et al., 2015).

1.7. Alphaviruses. Semliki Forest virus (SFV) expression system

Alphaviruses are enveloped positive strand RNA viruses of *Togaviridae* family. At least 24 members of Alphaviruses have been described representing seven serocomplexes (Powers et al., 2001). Alphaviruses induce a persistent infection in mosquitoes where they accumulate to high titers in salivary glands (Weaver and Barrett, 2004) and are transmitted to vertebrate hosts - birds, rodents, horses (in case of VEE) or humans. SIN has also been isolated from mites and ticks. In cultured mosquito cells a persisent infection can be established, while in cell lines of vertebrate origin the infection is cytolitic due to the alphavirus induced apoptosis (Strauss and Strauss, 1994). Infection of vertebrate hosts by alphaviruses gives rise to rash, arthritis, or encephalitis.

Two viruses routinely studied in the laboratory are Sindbis virus and SFV. These are considered avirulent for humans, but one fatal case of human encephalitis caused by infection with SFV in a laboratory worker has been described (Willems et al., 1979). SFV has served as a model virus to study the replication of RNA viruses, molecular mechanisms of viral pathogenicity, virus assembly, protein trafficking and transport (Atkins et al., 1999). Alphaviruses are classified in Old World and New World viruses, depending upon the geographic distribution. Interestingly, the symptoms caused by alphaviruses can also be distinguished by this division – New World viruses cause encephalitis, while Old World viruses cause fever, rash and arthritic symptoms (Jose et al., 2009).

Phylogenetically SFV is classified in the Old World Alphavirus branch (Powers et al., 2001). SFV was first isolated from mosquitoes in Uganda in 1944. Most laboratory SFV strains have derived from the two isolates. L10 is the original SFV isolate and is neurovirulent in mice. Aavirulent A7 strain was isolated in 1961. The SFV strains from which the SFV vectors are derived stem from highly attenuated strains (Atkins et al., 1999).

The SFV RNA genome of positive polarity is about 11.5 kB long. Functionally the genome is divided in two parts. Two thirds of the genome code for the replicase complex comprised of non-structural proteins (nsP) 1-4, one third, which is the subgenomic 26S RNA codes for the viral structural proteins C, E3, E2, 6K and E1 (see Fig. 8A) (Schlesinger and Schlesinger, 2001). Structural and non-structural proteins are formed from the polyprotein precursors. E1 is responsible for the membrane fusion during virus entry and is present on the envelope. E3 is not retained in the envelope of most alphaviruses, while E2 is present in the enevelope and responsibe for receptor attachment. 6K protein may contain proton-specific ion channel activity necessary for virus entry/budding. Alphaviral structural and non-structural proteins and their functions are listed in table 1.

The replication cycle of alphaviruses will be overviewed briefly, for an extensive review on alphavirus life cycle and replication see an article by Jose et al. (Jose et al., 2009). After the release of the genome from the capsid in the cell cytoplasm, the replication complex nsP1-4 is translated from the viral genome, which early in infection then synthesizes the (–) RNA strand. Later in infection replicase transcribes the (+) strand subgenomic RNA, from which the precursor polyprotein of the viral structural proteins is translated. The capsid protein (C protein) possesses an autoproteolytic activity and cleaves itself off the nascent polyprotein chain co-translationally. The p62 is a precursor of E2 and E3 forms a heterodimer with E1 in the ER (Barth et al., 1995). 6K in small amounts is incorporated in the virus particle, and soon after synthesis associates with the p62/E1 heterodimer (Jose et al., 2009). At a very late (post-Golgi) stage of transport, the p62 protein is cleaved by host furin-like proteases (Klenk and Garten, 1994) to mature E2 and E3. The packaging signal is located in nsP2 in SFV and nsP1 in SIN (Frolova et al., 1997). It is recognized by the C protein, and assembled nucleocapsids are enveloped by E1/E2 heterodimers and bud from the plasma membrane (Jose et al., 2009). The structure of the mature alphavirus virion has been revealed

by cryo-electron microscopy and further image processing. The mature alphavirus particle is spherical, 70 nm in diameter, and possesses T = 4 icosahedral symmetry (Mancini et al., 2000; Forsell et al., 2000; Mukhopadhyay et al., 2006).

Table 1. Functions of non-structural and structural proteins of Alphaviruses

Non-structural proteins	**Activity**	**Function**
nsP1	Guanine-7-methyltransferase	RNA capping and methylation
	Guanyltransferase enzymatic activity	
nsP2	Helicase activity	RNA duplex unwinding, cleavage of viral non-structural polyprotein
	Protease activity	
	RNA triphosphatase activity	
	Cysteine protease activity	
	Methyltransferase domain	
nsP3	ADP-ribose-1'-phosphate phosphatase	(-) strand and subgenomic RNA synthesis
	RNA binding activity	
nsP4	RNA dependent RNA polymerase activity	RNA replication
Structural proteins	**Function**	
E1	Membrane fusion during entry	
E2	Receptor attachment	
6K	Proton specific ion channel activity	
C	Capsid	

SFV, along with Sindbis virus (SIN) (Xiong et al., 1989) and Venezuelan equine encephalitis virus (VEE) (Pushko et al., 1997), has been manipulated on a cDNA level to develop an efficient expression vector system (Liljeström and Garoff, 1991). Structural proteins of the virus are encoded on a separate ORF in subgenomic RNA (Fig. 8 A), therefore it allows subgenomic RNA to be manipulated without replication capacity of the system being affected. cDNA plasmid contains SP6 or T7 promoter to enable transcription *in vitro* by SP6 or T7 polymerase to produce recombinant RNA (Fig. 9). In the vector system structural genes are replaced by the sequence encoding the gene of interest under the control of subgenomic S26 promoter. The term often used in context of alphavirus derived vectors is "replicon". It denotes the self-amplifying alphavirus genome, which contains all the necessary elements for self-amplification and transgene expression (Piver et al., 2005). The replication deficient SFV expression vectors allows three ways of delivery *in vitro* as well as *in vivo* of the SFV replicon (Fig. 9). SFV replicon DNA which is under control of the RNA polymerase II promoter (CMV promoter) may be transfected *in vitro* or may be delivered to the experimental animals *in vivo* according to conventional plasmid DNA vaccination protocols (Berglund et al., 1998; Nordstrom et al., 2005). In cell nucleus RNA polymerase II transcribes the expression casette to RNA, which is transported to cytoplasm, where it is translated to viral replicase which subsequently takes over the replication. This is the reason why SFV replicon plasmids are referred to as self-replicating DNA plasmids. *In vitro* transcribed, naked SFV replicon RNA can be delivered to cells *in vitro* by electroporation or lipofection, or by injection in experimental animals *in vivo* as RNA vaccine (Fleeton et al., 2001; Andersson et al., 2001).

Another method allows to use recombinant SFV. The recombinant SFV are produced after electroporation of BHK-21 cells with the recombinant SFV RNA (Fig. 8 B) and helper vector RNA (Fig. 8 C). The helper vector provides virus structural protein genes in *trans*. The fundamental difference between the formation of fully infectious SFV and recombinant, non-replicative SFV particles, is the packaging step. The packaging of the viral genome in the SFV capsid and formation of fully infectious SFV is dependent upon the interaction between the SFV capsid and a sequence within the nsP2. The helper vector does not contain the "packaging" signal (Fig. 8 C) and thus is not encapsidated. Only the SFV vector RNA bearing the sequence of the desired gene is encapsidated in recombinant SFV particles. When cells are infected with replication deficient viruses progeny virions are not formed, rendering the system suicidal.

Wild type SFV is classified as a Biosafety Level 2 virus in EU, as well as the SFV vectors lacking structural genes (Atkins et al., 1999). Recombinant SFV particles are suicidal and non-replicating, after infection of cells they undergo a single replication cycle. Nevertheless a possibility exists that two RNA strands may recombine during the packaging step resulting in generation of wild-type SFV genomes (Tubulekas et al., 1997). Several approaches have been developed to overcome it. One of them is to use a helper vector encoding structural precursor rotein p62 (of E2 and E3 membrane proteins) with a mutation that prevents the cleavage of it and it has to be cleaved by protease prior to infection (Berglund et al., 1993). Another way is to use two-helper system where spike and capsid proteins are encoded by separate helper vectors (Smerdou and Liljeström, 1999). Alternative approach to exclude the possibility of trans-complementation is to use RNA of a heterologous origin in the packaging step. Vesicular stomatitis viral glycoprotein (VSV-G) has been used to encapsulate full length SFV genome (Dorange et al., 2004). For a detailed review of developments and improvements to the alphavirus expression system, including SFV, see the review by Zajakina et al. 2008. In the work described here first generation SFV-1 vector was used without the modifications to the original vector system (Liljeström and Garoff, 1991).

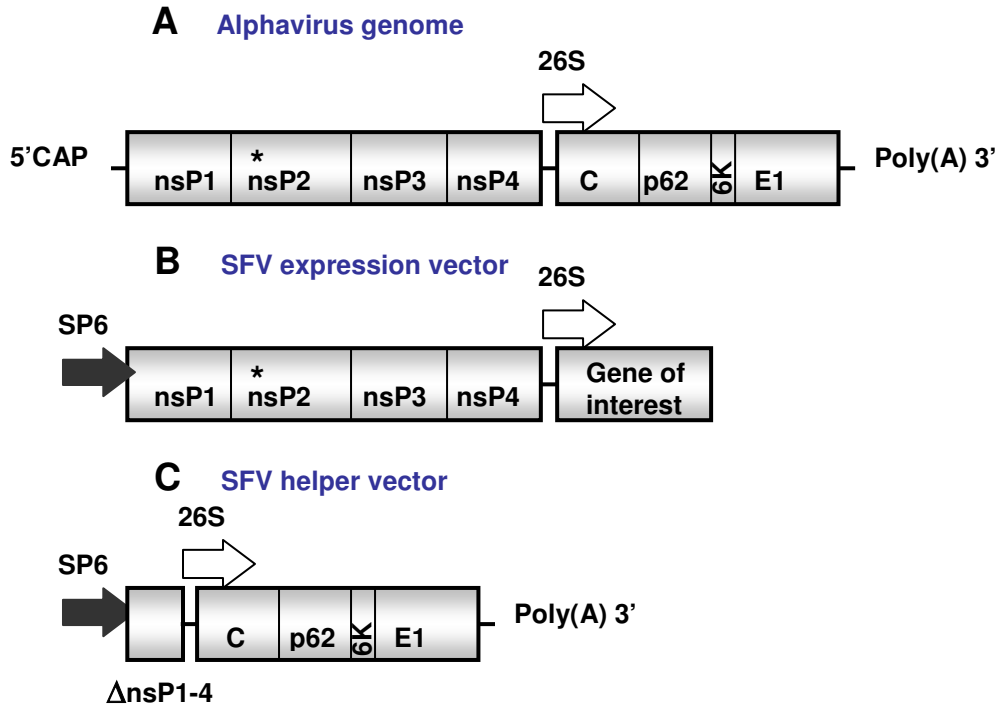

Figure 8. A. Schematic representation of Alphavirus genome organization. B. Replication–deficient SFV expression vector. Asterisk denotes the packaing signal in the nsP2. C. Helper vector, with the the deleted nsP1-4 (ΔnsP1-4) region. SP6 denotes SP6 polymerase promoter (filled arrow), 26S denotes 26S subgenomic promoter (empty arrow).

1.8. Alphavirus replicons for vaccine and cancer therapy purposes

Recombinant Alphaviruses are increasingly used as gene delivery tools for recombinant vaccine or cancer therapy purposes. A wide selection of viral, bacterial and protozoal proteins have been successfully expressed from alphaviral replicons. Their potential to induce specific immune response has been evaluated in numerous studies where potent humoral, cellular and mucosal immune responses have been induced, this has been reviewed by Zajakina and others (Zajakina et al. 2008), and Ljungberg (Ljungberg and Liljeström, 2015).

Alphavirus replicons can be delivered to experimental animals packed in recombinant alphaviruses, in a form of RNA or "layered" DNA plasmid (Fig. 9). Considerable effort has been put into studies employing alphaviral replicons carrying virus antigen genes of viruses against which a vaccine already exists with the objective to improve the existing vaccines. Alphavirus replicons have been used as vaccine tools for pathogens against which there is not yet a vaccine available (Ljungberg and Liljeström, 2015). Although RNA is more unstable compared to recombinant RNA packaged in recombinant viruses or an alphavirus replicon plasmid, vaccination approach employing naked RNA was one of the pioneering studies. rSFV replicon encoding the influenza nucleoprotein gene was inoculated into the quadriceps muscle of mice leading to a strong humoral response (Zhou et al., 1994). Recently intradermal delivery of naked RNA replicons followed by topical electroporation has been described with induction of strong immune responses (Johansson et al., 2012).

rSFV replicons have been employed for delivery of genes encoding numerous important viral proteins – examples include NS3 and E2 of Hepatitis C virus (Vidalin et al., 2000; Brinster et al., 2002), HA and NP of Influenza A (Berglund et al., 1998;Berglund et al., 1999;Zhou et al., 1995; Huckriede et al., 2004) and env and clade C antigens of HIV-1 (Brand et al., 1998; Sundback et al., 2005; Forsell et al., 2007). Among other antigens are E6 and E7 of human papilloma virus (Daemen et al., 2002; Riezebos-Brilman et al., 2005) and F and G

proteins of RSV (Fleeton et al., 2001; Chen et al., 2002). Replicon based on Chikungunya virus cDNA clone, which is an alphavirus, has been examined as a potential vaccine tool for the re-emerging Chikungunya virus, which is a mosquito-borne and mosquito-spread virus (Hallengard et al. 2014).

Another prominent branch of use of alphavirus replicons is for immune therapy. Cytokines and other immunoregulatory proteins that enhance anti-tumor immune responses and/or inhibit tumor cell growth have been delivered by alphavirus vectors (Atkins et al., 2004), for example IL-12 (Rodriguez-Madoz et al., 2009). A single intramuscular injection of SFV-LacZ can prolong the survival time of mice with established tumors and protects from tumor challenge (Ying et al., 1999). SFV based vectors are superior at eliminating tumor to the first-generation adenovirus vector expressing IL-12 (Rodriguez-Madoz et al., 2005). It has been shown that SIN vectors direct transient expression of tumour associated antigens in the lymph nodes draining the injection site. This is followed by long lasting memory T cell response (Granot et al. 2014). Applications of alpavirus vectors in tumour therapy have been extensively reviewed by Lundstrom (Lundstrom, 2015).

Semliki Forest virus replicons and other alphavirus replicons embody a number of advantages which makes them extremely versatile and attractive tools for vaccine purposes. One of the advantages is that immunity to the vector is not widespread, which is a major hurdle in the application of adenovirus-derived vectors (Bangari and Mittal, 2006). The short persistence due to induction of apoptosis is often stated as an advantage, but this depends upon what is wanted to achieve by use of alphavirus replicons. Stimulation of innate immunity mechanisms due to the presence of ds RNA replicative intermediates are among the positive traits of the replicon system. The both latter topics will be reviewed in detail below. The risk of chromosomal integration, which is a concern in case of plasmid DNA administration (Nichols et al., 1995), is alleviated, because the amplification of the replicon occurs exclusively in the cytoplasm. The alphavirus-derived vaccine tools may combine the efficacy of live attenuated vaccines with the safety of RNA vaccination.

1.9 Other Alphavirus-derived vectors

Sindbis virus (SIN) and Venezuelan equine encephalitis (VEE) virus of *Togaviridae* family have also been developed into efficient expression vector systems (Xiong et al., 1989;Pushko et al., 1997). The three vector systems developed from *Togaviridae* family have distinguished characteristics relative to each other, which stem from the “original” viruses they are derived from. Despite of sequence similarity of structural and non-structural proteins, *wt* alphaviruses differ in their pathogenicity (Petrakova et al., 2005). VEE, cause severe encephalitis in humans and animals and has caused serious epidemics with an inclination to reemergence (Anishchenko et al., 2006). VEE system is a basis of a patented Alphavirus replicon vector system developed by the University of North Carolina at Chapel Hill and the U.S. Army Medical Research Institute of Infectious Diseases. The replicon is used by the company *Alphavax*. Several vaccines based on VEE replicon particles have entered Phase I and II clinical trials (http://www.alphavax.com/Pipeline.html). VEE are naturally targeted to dendritic cells. After s.c. injection of recombinant VEE the expression of VEE encoded GFP is observed in Langerhans cells - the resident dendritic cells of the skin and in dendritic cells in the draining lymph node (MacDonald and Johnston, 2000). Delivery of VEE replicons for immunization to achieve immunity mostly to viral targets, has been carried out using recombinantviruses, while SFV replicons have been mostly delivered in form of DNA, recombinant particles, and rarely - RNA. Recombinant SIN virus particles are known to posess affinity to 67 kDa laminin receptors on mammalian cells which are upregulated in several cancers and remain relatively unoccupied by laminin (Wang et al., 1992; Nelson et al.,

2008). SIN can infect human dendritic cells and this ability is conferred by Gly at position 160 of E2 protein (Gardner et al., 2000).

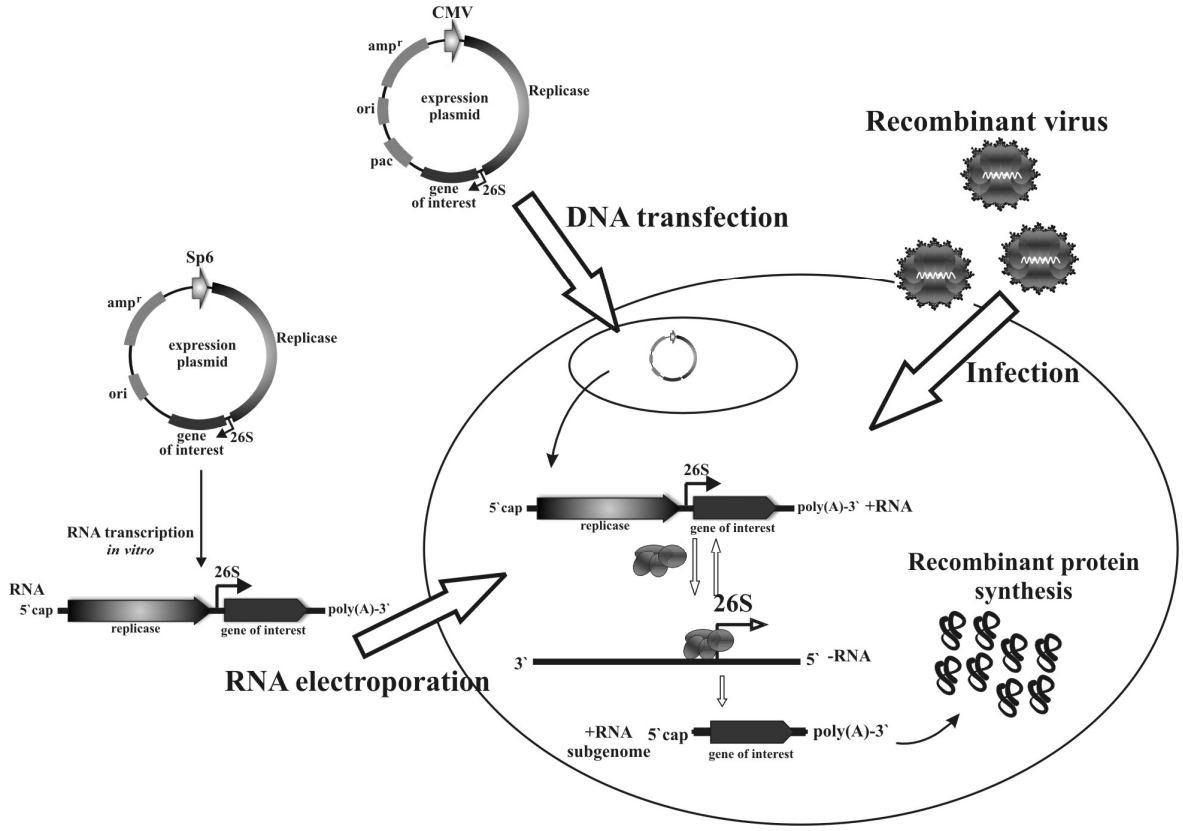

Figure 9. Three modes of delivery of replication deficient Alphavirus-derived vectors. Self-replicating RNA carrying the gene of interest can be delivered into cells by transfection of cells with a self-replicating DNA plasmid under control of CMV promoter, introduction of *in vitro* transcribed RNA by electroporation, and infection of susceptible cells with recombinant alphaviruses. Translation of the in-coming alphavirus recombinant RNA produces non-structural proteins which form the replicase complex. The replicase is responsible for formation of (-) RNA intermediate which serves as a template for (+) RNA synthesis. At the subgenomic promoter (26S) the replicase produces subgenomic RNA from which foreign gene is translated. Adapted from *Zajakina et al. (2008). Alphaviruses: Multiplicity of vectors and their promising application as vaccines and cancer therapy agents.*

1.10. Persistence and distribution of SFV vectors

An important issue when application of SFV vectors *in vivo* is considered, is their persistence and distribution after delivery to an experimental animal. In a study conducted by Morris-Downes (Morris-Downes et al., 2001) SFV based self-replicating plasmid and conventional plasmid were compared after injection in *tibialis anterior* (T.A.) muscle of mice and chickens. Recombinant SFV particles persisted for 7 days at the injection site, while SFV replicon based plasmid 93 days. Conventional plasmid could be detected up to 246 days. Both plasmids could be detected up to 3 months in the tissues distal from the site of injection, indicating dissemination (Morris-Downes et al., 2001). In chickens, however, the transgene could be detected up to 1 day at the injection site in case of recombinant SFV, up to 17 days for the SFV-based plasmid and 25 days for the conventional plasmid. The shortest persistence was shown by rSFV in mice and chickens. Compared to conventional plasmid the SFV-based

replicons showed shorter persistence. Localization and persistence of replicon RNA is also dependent on the route of injection as shown by P. Colmenero et al. (Colmenero et al., 2001). I.v. administration resulted in a systemic distribution, and the reporter gene was detectable in spleen and lymph nodes as well as in non-lymphoid tissues. S.c. injection lead to a local distribution in the draining lymph node and skin surrounding the injection site. Intramuscular injection resulted in distribution in the local lymph nodes and injection site. This study confirmed the transient nature of rSFV *in vivo* – reporter gene was almost undetectable by day 6 after injection by all injection routes. Intratumoral injection of rSFV lead to localization of rSFV-RNA in the tumour cells and draining lymph node only (Colmenero et al., 2002). The short persistence renders the rSFV particles as a safe vaccine tool, but not relevant for applications where prolonged gene expression *in vivo* is desired.

1.11. Alphavirus derived vectors induce apoptosis of the infected or transfected cells

The ability to induce apoptosis or programmed cell death of the infected cells is the inherent propoperty of the alphaviruses and hence the first generation vectors derived from them, including SFV1. The induction of apoptosis of the first generation alphavirus vectors, certainly is a major limitation for the purposes of recombinant protein expression *in vitro*, therefore non-cytopathic vectors have been developed with the aim to improve the survivial of the host cells and prolong the expression of the foreign protein. One of the factors responsible for the cytotoxicity resides in the nsP2 protein of the replicase complex, therefore appropriate mutations bearing mutations in the nsP2 region which overlaps with the nuclear localization signal (Fazakerley et al., 2002) render the vectors con-cytopathic (Dryga et al., 1997; Lundstrom et al., 2003; Fazakerley et al., 2002).

Multiple pathways are held responsible for the induction of apoptotic programme in the infected or transfected cells bearing alphavirus replicons, of which the presence of ds RNA intermediates (Diebold et al., 2009) is the major trigger. Upon copying the (+)-strand RNA alphavirus genome to (–) RNA and back, ds RNA intermediates are produced, this is valid also for Alphavirus-derived vectors. Production of ds RNA after transfection of Vero cells with SIN replicon vector has been shown (Diebold et al., 2009) and in *wt* SFV infected bone-marrow dendritic cells (Schulz et al., 2010). The major role in the execution of the apoptosis is played by the host protein kinase R. This kinase is activated via binding to viral ds RNA. As a result the host cell translation initiation factor, eIF2α, is phosphorylated preventing the translation of the host cell proteins (Stark et al., 1998). Importantly, the eIF2α is not necessary for the translation of viral subgenomic RNA. PKR also directly triggers apoptosis (Tan & Katze 1999), with the involvement of Bcl-2 and caspases (Lee et al., 1997). Additionally, mitochondrial and death receptor pathways (Li and Stollar, 2004 ;Nava et al., 1998) have also been shown to play a role in the execution of the apoptotic programme, namely mitochondrial, Bak-mediated, caspase-8-dependent and Bid-mediated death signalling pathway (Urban et al., 2008).

However, the intrinsic property of the alphavirus vectors to provoke apoptosis of the transduced cells may be advantageous for *in vivo* applications of these vectors for vaccine purposes and cancer therapy. Apoptotic death caused by viral infection has been attributed an immunostimulatory and adjuvant effect (Restifo, 2000). A link between alphavirus-induced apoptotic death of transfected cells and improved efficacy of the alphavirus replicon-based vaccine and enhanced response to an aggressive tumor challenge has been demonstrated (Leitner et al., 2004; Ying et al., 1999). Induction of apoptosis by replicase-based nucleic acid vaccines is essential for activation of antigen presenting cells (Leitner et al., 2004). Processing of apoptotic bodies has been shown to generate T-cell epitopes (Bellone et al., 1997). Acquisition of antigen from the apoptotic bodies by dendritic cells stimulates these cells. They, in turn, stimulate class I-restricted $CD8^+$ CTLs. This is the basis for phenomenon of

cross-priming or cross presentation (Albert et al., 1998). Another hallmark of the presence of ds RNA intermediates in the cell is the induction of 2'-5' oligoadenylate synthetase, which catalyzes formation of short 2'-5' oligoadenylates from ATP. These bind to endoribonuclease L which after dimerization degrades mRNA, thus leading to block of host protein synthesis (Goodbourn et al., 2000). Activation of PKR and 2'-5' oligoadenylate synthetase results in "shut-off" of protein synthesis, leading to apoptosis, which affects level of expressed foreign protein (Terenzi et al., 1999). The apoptosis resulting from virus infection induced cell death and the subsequent activation of dendritic cells by the apoptotic death is classified as "immune apoptosis" (Fig. 10)(Restifo, 2001).

The apoptosis triggered by replicon activity generating biologically active ds RNA definately does not favour the increased or prolonged antigen expression *in vivo* to be dealed by the adaptive immune system (Leitner et al., 2003). However, it is a powerful adjuvant signal and stimulates innate antiviral pathways, where the increased immunogenicity and increased efficacy of alphavirus replicon vaccines may reside (Leitner et al., 2003).

1.12. Stimulation of innate immunity by Alphavirus replicons

Stimulation of innate immune responses by alphavirus derived replicons occurs via ds RNA intermediates, as described above. The major players are the toll-like receptors (TLR), which play a key role in innate immunity and are a bridge between innate and adaptive immunity and recognize molecular patterns of microbial pathogens. Family of TLRs contains 10 members recognized so far in humans and 13 in mice (Thompson et al., 2011). TLR which must be mentioned in context of Alphavirus derived replicons are TLR3, TL3 7 in mouse and TLR 8 in humans. They are membrane associated receptors located on the endosomal membranes. dsRNA is a ligand of TLR3 (Alexopoulou et al., 2001), which is expressed specifically by dendritic cells (Muzio et al., 2000). ss viral RNA is recognized by TLR 7 in mice and TLR 8 in humans (Crozat and Beutler, 2004). While the activation of TLR 3 in response to presence of alphavirus replicon has been investigated (Saikh et al., 2003; Diebold et al., 2009), the reports of TLR7/8 dependent activation in response to *wt* or recombinant Alphavirus are lacking. There is a view that replication of SFV does not trigger TLR depenent pathways (Piver et al., 2005) and it was shown by Naslund et al. that for induction of a $CD8^+$ response by rSFV TLR3 was not necessary (Naslund et al. 2011). For Hepatitis C virus TLR7/8 dependent activation of innate immunity pathways has been shown (Zhang et al., 2009).

Upon activation TLR induce pathways leading to transcription of type I interferon (IFN) response genes, and synthesis of interferon α and β (Bowie and Haga, 2005). Synthesis of type I IFNs is a cell defense reaction of cells in response to virus infection. Interferons contribute significantly to stimulation of adaptive immune response (Tough, 2004). As a consequence of both types of interferon synthesis, cells establish "antiviral sate", either by interfering with virus replication or increasing their susceptibility to apoptosis. Two milestones of the "antiviral state" of the cells induced by IFN is 2'-5'oligoadenylate synthetase and dsRNA dependent protein kinase (PKR). Recombinant SFV is a strong inducer of type I interferon. It has been demonstrated that suicidal rSFV particles can induce rapid and transient IFN α/β response in mice, which can be detected in mice serum at 4 to 6 h after immunization (Hidmark et al., 2005). Immunogenicity of alphavirus replicon based vaccines is dependent on induction of interferon α/β (Leitner et al., 2006). Additionally, cells transfected with alphavirus replicon bearing plasmids show activation of 2'-5'oligoadenylate synthetase system, which is important for immunogenicity and increased efficacy of alphavirus replicon based vaccines (Leitner et al., 2003). The role of PKR in IFN α/β induction has been largely revised, since discovery of cytoplasmic RNA helicases which detect dsRNA - retinoic acid inducible gene (RIG-1) and melanoma differentiation-associated

factor-5 (MDA 5) which lead to IFN α/ β gene transcription (Pichlmair and Reis e Sousa, 2007). The induction of IFN α/ β by SFV is mainly dependent on MDA5, not to a such large extent to RIG-I (Schulz et al., 2010).

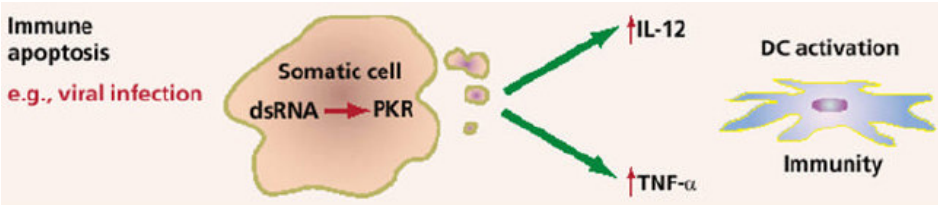

Figure 10. Illustration of immune apoptosis. Immune apoptosis results from viral infection and activation of type I interferon-inducible, double-stranded RNA–dependent protein kinase (PKR), which can induce both death as well as the upregulation of key cytokines like IL-12 and TNF-α. Adapted from *Restifo N. Vaccines to die for: Induction of apoptotic death by modified caspases enhances the function of "naked" DNA vaccination. Nat.Biotechnol. 2001; 19(6): 527–528.*

1.13. Cross priming

The term "cross-priming" is mentioned very frequently in context of alphavirus derived replicon vaccines. The ability of dendritic cells to capture, ingest or acquire and present cellular material to $CD8^+$ cells, which are consequently activated, is referred as cross presentation or coss priming (Albert et al., 1998). Cross presentation is very important in context of Alphavirus replicon bearing cells. Plasmocytoid dendritic cells in mice are the major antigen presenting cells (APC) which drive antiviral CTLs. $CD8\alpha^+$ cells are a subset of cells involved directly in the generation of CTL immunity to viruses (Belz et al., 2004). In mouse the $CD8\alpha^+$ dendritic cells are the mouse antigen presenting cells primarily involved in cross-priming (Villadangos and Schnorrer, 2007). But, SFV is not able to infect *in vitro* $CD8\alpha^+$ subset of mouse spleen dendritic cells when presented as free virus, however, this subset can be activated in TLR3 dependent manner after uptake of material from Sindbis virus replicon based plasmid- transfected cells (Diebold et al., 2009). Dendritic cells can be activated if cultured with SFV infected Vero cells. (Schulz et al., 2005). In case of a virus which does not infect antigen presenting cells cross-presentation is crucial for the development of effective adaptive immunity (Chen et al., 2004; Rovere-Querini and Dumitriu, 2003). Plesa and others (Plesa et al., 2006) have concluded that greater cytopathic capacity lead to a more efficient cross-priming to $CD8^+$ T cells and enhanced short-term humoral and cellular responses, being in line with several other authors (Nowak et al., 2003; Racanelli et al., 2004; Schaible et al., 2003) who have provided evidence that apoptosis induction mediates greater cross-priming.

1.14. Recombinant alphaviruses as adjuvants

Replication deficient rSFV particles which are co-immunized with the protein are able to function as adjuvants and potentiate humoral response to protein antigens by delivering strong adjuvant signals. Additionally, immunization of protein alone results in generation of IgG1 dominance. With the co-delivered rSFV it was possible to shift the response to IgG2a dominance (Hidmark et al., 2006), which indirectly signals about the activation of Th1 cells and hence CTLs. Immunization with the protein vaccines stimulates mainly IgG1, indicating activation of Th2 cell subset, which aids in generation of antibody responses. Several studies

have shown that recombinant Venezuelan equine encephalitis virus (rVEE) can also exert an adjuvant activity by activation of innate immune response (Tonkin et al., 2010).

1.15. *Tupaia belangeri* model of HBV infection

HBV is hepatotropic and extremely species specific virus and early events of infection are strictly host restricted. It was assumed that *in vivo* human HBV infects exclusively humans and higher primates, therefore a small animal model of HBV infection has not been available. Studies on early events of HBV infection have been dependent on the limited availability of primary human hepatocytes.

It has been demonstrated that HBV can specifically infect primary hepatocytes of Asian tree shrews (*Tupaia belangeri*) (Kock et al., 2001; von et al., 2004). The dynamics and course of infection resembles HBV infection of scarcely available human hepatocytes (Gripon et al., 1988; Galle et al., 1994) including the significant early steps of attachment (Glebe et al., 2003). Tree shrews are classified within an order of *Scandentia* and are about 10-15 cm in size, their natural habitat is tropical forests of southeast Asia. After infection of *Tupaia belangeri* with HBV, the virus can replicate *in vivo* with the infection being similar to self-limited, acute HBV infection in humans (Walter et al., 1996). Primary hepatocyte cultures from Asian tree shrews *Tupaia belangeri* livers represents an *in vitro* model for HBV infection. The hepatocytes of *Tupaia belangeri* have been useful in studying the potential of characterized HBV monoclonal antibodies generated against surface proteins of HBV to inhibit binding of the virus (Glebe et al., 2003). Primary Tupaia hepatocytes (PTH) have been employed for investigation of early events of HBV infection (Glebe et al., 2005; Leistner et al., 2008; Bremer et al., 2009). *Tupaia belangeri* can be *in vivo* and *in vitro* infected with hepatitis C virus which is another virus for which a small animal model has not been available. PTH has served as an *in vitro* model for study of early events of HCV infection (Zhao et al., 2002; Tong et al., 2011). PTH model of HBV infection has enabled to elucidate numerous aspects of HBV biology and pathogenesis, for example factors affecting HBV replication (Xu et al., 2011), characterization of clinically significant HBV variants (Baumert et al., 2005) and the inhibitory potential of antiviral drugs for treatment of chronic HBV infection (Kock et al., 2003). Use of PTH has been described for assessment of neutralizing capacity of antibodies induced by immunization of mice with recombinant HBc with inserted C- and N-terminal peptides of preS1 (Bremer et al., 2010).

2. METHODS AND MATERIALS

2.1. Cell lines and primary cells

Cell lines and primary cells employed in this study are summarized in table 2.

Table 2. Cell lines and primary cells employed in the study

Cell line	Origin, organ	Morphology	Propagation
Continuous cell lines			
BHK-21	*Mesocricetus auratus* (Syrian golden hamster), kidney	Fibroblast	Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen), foetal calf serum (FCS), 10% tryptose phosphate broth, 2mM glutamine, 20 mM HEPES, penicillin and streptomycin at 10 U/ml
Huh7	*Homo sapiens*, human hepatocellular carcinoma, liver	Epithelial	RPMI-1640 (Invitrogen), 2mM L-glutamine, 25mM HEPES, 10% FCS, 2% sodium selenite, penicillin and streptomycin at 10 U/ml
HepG2	*Homo sapiens*, human hepatocellular carcinoma, liver	Epithelial	Dulbecco's MEM Nut Mix F-12 (Invitrogen) supplemented with 10% FCS, penicillin and streptomycin at 10 U/ml
Primary cells			
Primary hepatocytes	Asian tree shrews (*Tupaia belangeri)*, liver		PTH medium: DMEM (Invitrogen) supplemented with 0.1% bovine serum albumin (crystallized) 5μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 50 μM hydrocortisone, 0.1μM dexamethasone, 2% dimethyl sulfoxide, 100 μg/ml gentamicin, 0.25 μg/ml amphotericin B.

2.2. Antibodies

Monoclonal and poloyclonal antibodies to HBs proteins used in this study are summarized in table 3.

Table 3. Antibodies to HBs proteins used in the study

Monoclonal antibodies	**Recognize:**
MA 18/7	aa 20-23 (DPXF) of preS1* (Sominskaya et al., 1992; Heermann et al., 1984)
C20/02	correctly folded S domain between aa 118 and 149 subtype-independent (W. Gerlich, unpublished)
1-9C1	Linear epitope in S domain, (Sobotta et al., 2000)
HB1	Linear S domain (A. Zvirbliene, unpublished)
Polyclonal antibody	
H 863**	preS1 and preS2 (H. Schaller)

*Genotype D numeration, correspond to aa 31-34 in genotype A
** Generated in rabbit
All antibodies were a kind gift of Dr. D. Glebe, Justus Liebig University, Giessen, Germany. Polyclonal antibodies H 863 were a gift from Heinz Schaller, Heidelberg, to Dr. D. Glebe and prof. W.H. Gerlich

2.3. Recombinant plasmids

To generate the plasmids the fragments were cloned in pSFV1 vector at the *Bgl* II and *Sma* I sites, except pSFV1-S/adw2. Table 4 summarizes the generated SFV-1 constructs.

Table 4. SFV1- based plasmids employed in the study

pSFV1-EGFP	Described by Zajakina et al., Aleksejeva et al. (Zajakina et al., 2004;Alekseeva et al., 2009)
	SFV1 plasmids encoding SHBs
pSFV1-S/ayw2	Described by Zajakina et al. (Zajakina et al., 2004)
pSFV1-S/adw2	*S/adw2* gene was amplified from plasmid pRVHBV (kindly provided by Volker Bruss, Göttingen) with forward primer 5'TGCCTCTCACATCTCGTCAATCTC3' and reverse primer 5'CCCATGAAGTTTACCCGGGATCCCCATCTTTTTG3'. The resulting segment was cut with *Sma* I and *Avr* II, and inserted into pSFV-1 which had been digested with *Sma* I and *Avr* II.
SFV1 plasmids encoding deleted L protein variants, e.g. fusion proteins of aa 1-48 of preS1 with the S protein (subtype ayw2, genotype D)	
pSFV1- preS1.1–48/S (encodes a fusion protein of aa 1-48 of preS1 with S protein)	Fusion protein of preS1.1 – 48 and S, including a stretch of 6 His at the C terminus of S, from the plasmid pFD GlyPr[13-59]S (kindly provided by Prof. K. Sasnauskas, Vilnius) was amplified by forward primer 5'GACACAGATCTGCCGCCACCATGGGTCAGAATCTTTCCAC3' and reverse primer 5'CTCTGTACCCGGGTTATTAGTGATGGTGATGGTGATGAATG3'. A *Bgl* II site was introduced by the forward primer and a SmaI site by the reverse primer. Vector pSFV-1 was prepared from the plasmid pSFV1-S/adw2 by excision of the *S* gene with *BamH* I and *Sma* I.
pSFV1-preS1.1-48/S_0 (encoding the fusion protein of preS1.1-48 and S_0, internal translation of the S protein abrogated)	Generated by site directed mutagenesis of the template plasmid pSFV1- preS1.1–48/S using the QuikChange II XL site directed mutagenesis kit (Stratagene). Point mutation ATG to GTG in the beginning of the S gene to prevent translation of the S was introduced by the forward primer 5'GTGGATCTGGTGGAGTGGAGAACATCAC3' and reverse primer 5'GTGATGTTCT CCACTCCACCAGATCCAC3', according to PCR conditions indicated by the manufacturer. The introduced mutation was confirmed by sequence analysis.
pSFV1-Ser2.preS1.1-48/S (encoding the fusion protein of preS1.1-48 and S, G at the 2nd position of preS1 substituted by S)	The corresponding gene was amplified from the plasmid pFD Pr[13-59]S (kindly provided by Prof. K. Sasnauskas) by forward primer 5' GACACAGATCTGCCGCCACCATGTCTCAGAATCTTTCCAC 3 and reverse primer 5' CTCTGTACCCGGGTTATTAGTGATGGTGATGGTGATGAATG 3'. A *Bgl* II site was introduced by the forward primer and a *Sma* I site by the reverse primer.
pSFV1-Ala2.preS1.1-48/S (encoding the fusion protein of preS1.1-48 and S, G at the 2nd position of preS1 substituted by A)	Generated by forward primer 5' GACACAGATCTGCCGCCACCATGGCCCAGAATCTTTCCAC 3' from plasmid pFD GlyPr[13-59] (kind gift from Prof. K. Sasnauskas). The reverse primer was as described for the plasmid pSFV1-Ser2.preS1.1-48/S.

2.4. Sequencing

Sequencing of the newly constructed plasmids was performed to verify the sequence of the introduced gene in SFV1 vector and to verify the introduced mutations by site-directed mutagenesis using the Sequencing kit from Applied Biosystems (5x buffer (400mM Tris-HCl, pH 9.0, 10mM $MgCl_2$) and BigDye®). Sequencing was performed in forward and reverse directions, with the forward primer 5'CCATGACCACCTTGGCGAGGGAC3' complementary to the bp 7290 – 7313 in SFV1 vector and reverse primer 5'CCACCGGCGGCCGTAAAACGTTTG3' complementary to nucleotides 7457 – 7434 in SFV1 vector. The conditions for the PCR were as follows: 25 cycles of denaturation at 96 ºC - 10 s, annealing at 50 ºC – 5 s, and extension at 60 ºC - 4 min, using a PCR apparatus GeneAmp PCR System 9700 (Applied Biosystems, UK). Samples were purified by precipitation with 3M sodium acetate and ethanol. Sequence analysis was performed with ABI 3100 Sequencer (Applied Biosystems, UK). Sequencing chromatograms were viewed manually using the Vector NTI Suite v. 6.0 software.

2.5. Transformation of SFV1 plasmids

XL1-Blue cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr)] were thawed on ice, and 4 µl of the PCR reaction (of site directed mutagenesis) mixes were added to 100 µl of XL1-Blue cell suspension and incubated on ice for 10 min. Cells were heat-pulsed for 35 sec at 42 ºC and incubated on ice for 2 min. LB medium with ampicillin (100 µg/ml) was added and incubated for 1 h at 37 ºC with shaking. 200 µl was spread on plates with LB/agar medium with ampicillin (concentration of 200 µg/ml). Plasmid pUC18 was transformed as a control. Transformed clones were selected for ampicillin resistance, picked and grown in 1 ml of 2xTY medium with ampicillin (100 µg/ml) at 37 ºC overnight.
After plasmid DNA extraction according to standard protocol of plasmid DNA isolation, plasmids were retransformed in DH5α cells, clones selected for ampicillin resistance, and grown in 250 ml 2xTY medium with ampicillin (100 µg/ml). Plasmid DNA was extracted according to standard protocol (Sambrook et al., 1985).

2.6. Generation of rSFV particles

2.6.1.Transcription in vitro

SFV1 plasmids were linearized prior to transcription *in vitro* with restriction endonuclease NruI (Bsp681, Fermentas). SFV Helper plasmid was linearized with *SpeI* (Fermentas). Transcription *in vitro* was performed as follows: 5 µl of rNTP mix (1mM ATP, 1mM UTP, 1 mM CTP, 0.5 mM GTP), 2,5 µl 100 mM DTT (final concentration 10 mM), 2,5 µl 10mM Cap (final concentration 1 mM, New England Biolabs), and 5 µl 5× transcription buffer (Fermentas) was mixed and kept at RT for 5 min. 0.75 µg of linearized SFV or helper DNA was added, followed by 2 µl ribonuclease inhibitor (40 u/µl, Fermentas) and 2 µl SP6 polymerase (20 u/µl, Fermentas). Deionized water was added up to 25 µl. Transcription mix was incubated at 37 ºC for 1 h 20 min. Presence of RNA was confirmed by 1% TBE agarose gel electrophoresis.

2.6.2. Electroporation of BHK-21 cells

RNA was transcribed *in vitro* with SP6 polymerase (Fermentas) from SFV1 plasmids, mixed 1:1 with *in vitro* transcribed SFV helper RNA encoding the SFV envelope proteins and

transfected to BHK-21 cells by electroporation as described (Berglund et al., 1993). Briefly, BHK-21 cells were trypsinized, collected and resuspended in 700 µl PBS and transferred to 1, 5 ml Eppendorf tube. 25 µl of transcription mix from SFV RNA and SFV helper RNA was added and the mix transferred to electroporation cuvette (Biorad). Electroporation was performed with two consequent pulses at 850 V and 25 µF (Gene Pulser, Biorad).

2.6.3. Concentration of rSFV particles

After incubation of the cell cultures at 33 ºC for 36-40 h, the medium was transferred to 12 ml tubes and clarified by centrifugation at 40, 000 × g (18, 000 rpm) in a SW40Ti rotor (Beckman) for 30 minutes at 4 ºC. It was followed by sedimentation of rSFV through 5 ml 20% sucrose (w/v) in 35 ml Beckman tubes by centrifugation at 140, 000 ×g (27, 000 rpm) in a SW28Ti rotor for 5 hours at 4ºC. The pellets were resuspended in 500 µl TNE buffer (50 mM Tris-HCl, pH 7.4, 100mM NaCl, 0.5 mM EDTA) and left in the centrifuge tubes on ice at 4 ºC for 12 h. The virus stocks were frozen in liquid nitrogen and stored at -70 ºC. The titer of rSFV stocks was determined as described (Karlsson and Liljeström, 2003).

2.7. Assessment of rSFV-driven SHBs and deleted L protein expression *in vitro*

2.7.1. Cell infection with rSFV particles

For infection of BHK-21 cells, virus stocks were diluted in Eagle minimal essential medium (MEM), (Invitrogen) supplemented with 0.2 % bovine serum albumin (BSA), 2mM L-glutamine and 20 mM HEPES. Prior infection cells were washed with PBS and BHK-21 cells were infected at MOI 20, and incubated for 1 h at 37 ºC. Thereafter virus inoculum was removed, and cells cultured with complete medium containing 1% FCS for 20 h at 37 ºC. At desired time point after infection cells were lysed with buffer containing 1% Nonidet P40 (NP40), 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1 µg/ml phenylmethanesulfonylfluoride (PMSF).

For infection of Huh7 cells at MOI 10, virus stocks were diluted in PBS^{++} (with Ca^{2+} and Mg^{2+}; Invitrogen) supplemented with 1% FCS and 2 mM L-glutamine. After infection, cells were cultured for 20 h at 37 ºC in complete Huh7 medium with 5% FCS. Cells were lysed with a buffer containing 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 2 mM EDTA and 1 µg/ml PMSF.

To infect HepG2 cells, the virus stocks were diluted in PBS^{++} (with Ca^{2+} and Mg^{2+}; Invitrogen) supplemented with 1% FCS and 2 mM L-glutamine and after infection, cells were cultured for 20 h at 37 ºC in HepG2 medium, but with 5% FCS.

For infection of PTH, virus stocks were diluted in PBS^{++} (with Ca^{2+} and Mg^{2+}; Invitrogen) and cells infected at MOI 10 and incubated for 90 min at 37 ºC, after removal of virus inoculum, *Tupaia* hepatocyte medium (PTH) medium was added and cells incubated for 20 h at 37 ºC. After PTH was collected, cells were washed with ice cold PBS++ and lysed with buffer containing 1% Nonidet P40 (NP40), 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, and one Complete Protease Inhibitor Cocktail tablet (Roche) per 10 ml of buffer.

2.7.2. Metabolic labelling of rSFV encoded proteins

Labelling of newly synthesized proteins in infected cells was performed as follows: 20 h after infection, methionine-free medium (MEM, 2mM L-glutamine, 20 mM HEPES) was added and cells incubated at 37 ºC for 30 minutes. Radiolabelling with 100 µCi/ml [^{35}S]-methionine (15 mCi/ml, Amersham Biosciences) was performed for 30 minutes. After removal of medium containing [^{35}S]-methionine, fresh medium (MEM, 2mM glutamine, 10 mM HEPES, 150 µg/ml methionine) was added and cells incubated as required by the

experiment. Thereafter cells were lysed with 1% NP 40 containing lysis buffer supplemented with 1 µg/ml PMSF.

2.7.3. Peptide N-glycosidase F (PNGase F) digestion

500U of PNGase F (New England BioLabs) was added to 10 µl of Huh7 cell lysates diluted in reaction buffer (50mM sodium phosphate buffer, pH 7.5, containing 1% NP40, New England BioLabs), and incubated for 1 h at 37 ºC. To perform reaction under denaturing conditions, glycoprotein denaturing buffer (New England BioLabs) was added to 10 µl of cell lysates and incubated for 10 min at 100 ºC.

2.7.4. Western blotting

Proteins were separated by SDS-PAGE as described (Zajakina et al., 2004) and transferred to Hybond-P membrane (Amersham Biosciences) in the semi-dry electroblotter (Apollo^{TM}, CLP). Membrane was reacted with MAb MA18/7, followed by goat anti-mouse antibodies conjugated with horseradish peroxidase (Pierce). For developing, SuperSignal®WestFemto Stable Peroxide Buffer was mixed with SuperSignal®WestFemto Luminol (Pierce) 1:1, dripped on the membrane and incubated for 5 min at room temperature. After exposure to a photography film (HyperfilmTM, Amersham Biosciences) proteins were visualised by developing solution (Dental Readymatic; Kodak).

2.7.5. ELISA for HBsAg and preS1Ag

96 well flat bottom microtiter plates (MaxiSorp^{TM}; Nunc,) were coated with MA18/7 or C20/02, diluted in 0.1 M sodium phosphate buffer, pH 7.4, at 1 µg/ml, overnight at 4 ºC. The plates were washed twice with 0.1 % Tween 20/PBS buffer, and twice with PBS, and washing performed between each of the steps. After blocking with 10% FCS /TNE, pH 7.4, at 4 ºC overnight 100 µl of sample were added in each well and incubated for 2 h at 37 ºC. Thereafter biotin-labeled anti-HBs (Dade Behring, Marburg, Germany) was added for 1 h at 37 ºC. 100 µl of peroxidase-conjugated streptavidin (Dade Behring) was added, and incubated for 1 h at 37 ºC followed by 100 µl/well of o-phenylenediamine/H_2O_2 substrate (Sigma) in 0.05 M citrate-phosphate buffer, pH 5, and incubated for 15 min at room temperature in dark. The reaction was stopped with 0.5 M sulfuric acid and absorbance was measured at 492 nm. The concentration of HBsAg was calculated according to dilution series of HBV carrier reference plasma ranging from 1 ng to 0.01 ng. ELISA kit Enzygnost® (Dade Behring) was used for quantification of HBsAg expressed in BHK-21 cells using recombinant HBsAg (Rhein Biotech) with a predetermined concentration as a standard as well as for determination of HBsAg in BALB/c mice sera.

2.7.6. Immunofluorescence

BHK-21 cells were infected with rSFV encoding S/adw2, S/ayw2, preS1.1-48/S, preS1.1-48/S_0, Ser2.preS1.1-48/S, Ala2.preS1.1-48/S, Ala2.preS1.1-48/S_0 and L protein (HepG2 cells) at MOI 20 and incubated at 37 ºC. After 20 hours, the cells were fixed with 3.7% formaldehyde (Sigma) for 20 min at 37 ºC and washed with PBS (Invitrogen). The cells were then incubated with PBS for 15 min at room temperature followed by permeabilization with 0.25% Triton X-100 /PBS for 15 min at room temperature. Afterwards, the cells were blocked in a 3% FCS/PBS solution for 20 min at room temperature. Cells were incubated with MAb 1-9C1 or with polyvalent rabbit antibodies H863 (in case of L protein) for 12 h at at 4 °C. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG or tetramethyl rhodamine iso-thiocyanate (TRITC) conjugated anti-rabbit IgG (Sigma) were applied in the dark for 1 h at room temperature. For double staining of HBsAg and preS1 antigen, 1-9C1 antibodies were applied first, followed by FITC-conjugated anti-mouse IgG, then H863 antibodies were applied followed by TRITC-conjugated anti-rabbit IgG. Summary of immunofluorescence analysis is shown in table 5. Cell nuclei were counterstained with DAPI (4 µg/ml; Sigma).

Fluorescent images were generated by microscope Leica DM 6000 B and camera Leica DFC 480 and confocal images with Leica TCS SP2 SE.

Table 5. Summary of immunofluorescence analysis of HBs proteins

Protein	Cell line expressed in	Antibodies
S/adw2	BHK-21	1-9C1
S/ayw2	BHK-21	1-9C1
preS1.1-48/S	BHK-21	1-9C1 followed by H863
preS1.1-48/S_0	BHK-21	1-9C1
Ser2.preS1.1-48/S	BHK-21	1-9C1 followed by H863
Ala2.preS1.1-48/S	BHK-21	1-9C1 followed by H863

2.7.7. Electron microscopy

BHK-21 cells were electroporated by 2 pulses at 850 V/25 μF with recombinant *in vitro* transcribed SFV RNA encoding preS1.1-48/S and Ser2.preS1.1-48/S. After 22 hours incubation at 37 ºC, medium was collected and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore) at 5000 × g for 30 min. The suspension of preS1.1-48/S particles was adsorbed on carbon-formvar coated grids. To detect presence of preS1 antigen, MAb MA18/7 followed by anti-mouse IgG conjugated with 5 nm gold particles (Sigma) was added as described (Louro and Lesemann, 1984). The grids were examined with a JEM-1239 electron microscope (IEOL Ltd., Tokyo, Japan).

2.8. Immunization of mice and evaluation of HBV antibody response

Groups of 6-8 week old female BALB/c mice were obtained from the Animal Breeding Centre of the Institute of Microbiology and Virology, Riga, and kept in the Biomedical Research and Study Centre. For the initial immunization experiments with rSFV-S/adw2 10^6 rSFV were inhjected i.v. and s.c. followed by 10^7 rSFV after two weeks by the same route. Mice were immunized with rSFV-S/adw2, rSFV-S/ayw2, rSFV-preS1.1-48/S, rSFV-preS1.1-48/S_0 and rSFV-EGFP according to the following scheme: 10^7 rSFV particles in 200 μ PBS (Invitrogen) were injected i.v. in the tail vein, followed by booster injection of 10^8 rSFV particles in 200 μl PBS by the same route after 3 weeks. Three weeks after the booster injection, sera were collected.

2.9. ELISA for HBV antibody response

Flat bottom microtiter plates (PolySorb^{TM}; Nunc, Thermo Fisher Scientific, Germany) were coated with recombinant HBsAg of subtypes adw2 and ayw3, expressed in methylotrophic yeast *Hansenula polymorpha* (Janowicz et al., 1991) (Rhein Biotech, Düsseldorf, Germany), at 10 μg/ml in 50 mM sodium carbonate buffer, pH 9.6, for 12 h at 4 ºC. Thereafter plates were washed three times with PBS/0.05% Tween 20 and blocked with PBS/1% BSA for 1 h at 37 ºC. Alternatively, plates were coated with a synthetic peptide corresponding to aa 124 to 148 of S protein, adw2 (CTTPAQGNSMFPSCCCTKPTDGNCT). For detection of anti-preS1, flat bottom microtiter plates (MaxiSorb^{TM}; Nunc) were coated with a synthetic peptide aa 10-36 of preS1 (genotype D numeration - PLGFFPDHQLDPAFRANTANPDWDFNP; both synthetic peptides were from Peptron, South Korea). Sera were serially diluted in PBS/ 0.05% Tween 20/0.5% BSA, incubated for 1 h at 37 ºC and bound antibodies detected as above. Antibody end point titers were calculated

as dilution which gave an absorbance value three times higher than the pre-immune serum. Statistical significance was determined with ANOVA ($p < 0.05$) and Tukey's multiple comparison method ($\alpha = 0.05$).

Additionally, flat bottom microtiter plates (MaxiSorb™; Nunc, Thermo Fisher Scientific, Langenselbold, Germany) were coated with highly purified, human plasma-derived HBsAg particles, subtype ayw2 (genotype D), at 2 µg/ml in 0.1 M sodium phosphate, pH 7.4, for 12 to 18 h at 4 ºC. After washing two times with PBS/0.1% Tween 20 and two times with PBS, wells were blocked for 2 h at 37 ºC with 10% FCS in TNE, pH 7.4, and washed as above. Serially diluted sera in 1%BSA/PBS were applied and incubated for 12 h at 4 ºC and plates subsequently washed. Peroxidase-conjugated anti-mouse IgG in 1% BSA/PBS was added and plates were incubated for 1 h at 37ºC. Thereafter o-phenylenediamine/H_2O_2 (tablets from Dako or Sigma) substrate was added, incubated for 15 min at room temperature, OD was measured at 492 nm.

Additionally, anti-HBs was determined quantitatively in IU/L by ELISA based on microparticles coated with recombinant HBsAg ad and ay (AxSym; Abbot Laboratories, Wiesbaden, Germany) according to manufacturer's instructions.

To determine serum IgG1 and IgG2a titers in the sera of rSFV immunized BALB/c mice, flat bottom microtiter plates (PolySorb™; Nunc) were coated with recombinant HBsAg of subtypes adw2 and ayw3 for the mice sera obtained from rSFV-S/adw2 and rSFV-S/ayw2 immunized mice respectively. For detection of IgG1 and IgG2a in mice sera immunized with rSFV-preS1.1-48/S and rSFV-preS1.1-48/S_0 flat bottom microtiter plates (MaxiSorb™; Nunc) plates were coated with a synthetic peptide aa 10-36 (genotype D numeration). Anti-mouse IgG1 and IgG2a antibodies from goat were used followed by peroxidase-conjugated anti-goat IgG (Sigma). Detection was performed as described above.

Table 6. HBsAg and peptide used in ELISA for detection of anti-HBs and anti-preS1 IgG

Anti-S response	
Recombinant HBsAg/adw2 and HBsAg/ayw3 (Rhein Biotech, Düsseldorf, Germany)	Expressed in methylotrophic yeast *Hansenula polymorpha* (Janowicz et al., 1991)
Anti-S and anti-preS1 response	
subviral HBsAg (subtype ayw2) particles, include preS2 and preS1	Isolated as described (Glebe and Gerlich, 2004) from plasma of an asymptomatic, HBeAg-positive, chronic HBV carrier (ID 326) which contained 8×10^9/ml HBV genome equivalents and 120 µg/ml HBsAg
Anti-preS1 response	
Peptide of aa 10-36 of preS1 (Peptron, South Korea)	genotype D numeration, (correspond to aa 21-47 of genotype A)

2.10. HBV and HBsAg particles from human plasma

Hepatitis B virus (genotype D) and subviral HBsAg (subtype ayw2) particles were isolated as described (Glebe and Gerlich, 2004) from plasma of an asymptomatic, HBeAg-positive, chronic HBV carrier (ID 326) which contained 8×10^9/ml HBV genome equivalents and 120 µg/ml HBsAg. HBV DNA containing fractions at 40-45% sucrose were identified by quantitative real-time PCR (LightCycler system, Roche, Germany) using primers and hybridization probes against the HBV X-region as described (Jursch et al., 2002). The assay was calibrated using the Eurohep reference plasma which has been converted to a WHO international standard sample (Saldanha et al., 2001).

2.11. Isolation and culture of primary *Tupaia belangeri* hepatocytes

Primary hepatocytes of *Tupaia belangeri* were isolated by the modified two-step collagenase method essentially as described (Glebe et al., 2003). Briefly, the livers were perfused via the portal vein with HANKS solution (Invitrogen) containing 5 mM EGTA, followed by perfusion with DMEM (Invitrogen) containing 0.05% collagenase (Sigma). Hepatocytes were separated from other cell types by pelleting 3 times at 40 g for 6 minutes at 4 °C. Hepatocytes were resuspended in PTH and plated on 12-well plates coated with Matrigel^{TM} ,10^5 hepatocytes/well. Plating efficiency and viability were measured prior to infection and at the end of the experiment by a modified MTT assay as described (Glebe et al., 2003). Variability as determined by MTT assay for each preparation was 10% or lower (data not shown). The organ harvest from Tupaias had been approved by the animal protection committee at Justus-Liebig University, Giessen, Germany.

2.12. Detection of HBV neutralizing antibodies

2.12.1. Incubation of HBV with BALB/c mice sera

To remove nonspecific serum factors enhancing the susceptibility of PTH for HBV, IgG was precipitated by addition of Na_2SO_4. In brief, 50 µl of mice sera were mixed with 50µl of 0.2 M sodium phosphate buffer, pH 7.2, and 100 µl saturated Na_2SO_4 solution (36% (w/w) were added and incubated for 15 minutes at room temperature. After centrifugation for 1 min at 20, 000 × g supernatant was removed. The pellet was dissolved in 50 µl 0.1 M sodium phosphate buffer and again precipitated three times with 50 µl sodium sulfate solution. Antibodies were dissolved in 100 µl PBS and immunoglobulin concentration was measured at OD_{280} assuming a factor of 1.35 for 1 mg/ml. For each well of a 12-well plate, 5×10^5 genome equivalents (ge) of HBV were incubated with 50, 100 or 250 µg IgG in 100 µl of PTH for infection (DMEM supplemented with 0.1% bovine serum albumin, 2% dimethyl sulfoxide, 100 µg/ml gentamicin, and 0.25 µg/ml amphotericin B) for 1 h at 16 ºC with mild shaking. Thereafter, 200 µl of PTH medium were added and PTH cultures (10^5 cells/ well) 3 days after plating were infected with HBV/antibody mixture for 18 h at 37 ºC. After removal of the viral inoculum, cells were washed twice with DMEM/ 0.1% BSA/ 20 mM HEPES, and further cultured in PTH medium for 14 days. At days 3, 6, and 10 PTH medium was collected and replaced with a fresh medium.

2.12.2. Assessment of cell viability

Fourteen days after infection, cell viability was measured by a modified MTT assay (assessment of cell viability/metabolic activity)(Glebe et al., 2003) which is based on the metabolic conversion of tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; Roche) producing highly water soluble formazan exclusively in metabolically intact cells, which can be directly measured with an ELISA Reader by 450 nm. Briefly, in every well 300 µl of WST-1 which was diluted in DMEM without the phenolred was added and incubated at 37 ºC for 1 h. 100 µl was transferred to an ELISA plate and absorbance at 450 nm (reference filter 620 nm) measured.

2.12.3. Monitoring the progress of PTH infection/Assessment of HBV infection of PTH

Concentration of newly secreted HBsAg in the supernatant from days 3, 6, 10 and 14 was determined using the ELISA with C20/02 as described above. HBeAg was detected quantitatively in the supernatants from day 14 by a commercially available ELISA (AxSym; Abbott Laboratories, Wiesbaden, Germany). Results were obtained as signal to cut-off (S/CO) ratio. When appropriate, after assessment of cell viability, cells were washed with DMEM without phenol red, followed by two washes with ice cold PBS and lysed with lysis buffer containing 0,5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.6 and 5 mM $MgCl_2$. Cytoplasmic and nucleic fractions were separated by centrifugation at 420 × g for 2 min at 4 ºC. Cytoplasmic and nucleic fractions were frozen in liquid nitrogen and stored at -70 ºC for isolation and quantification of ccc DNA and HBV mRNA.

2.12.4. Isolation of HBV cccDNA

The frozen cell pellets were defrosted on ice and episomal DNA extracted according to Hirt (Hirt, 1967): 400 µl of 0,6% w/v SDS, 10 mM EDTA was added to the cell pellet, mixed carefully by invertion of the tube and left for 20 min at RT. 100 µl of 5 M NaCl was added in order to precipitate chromosomal DNA o/n at 4 ºC. Samples were centrifuged at 4 ºC in a pre-

cooled centrifuge for 4 min at 14.000 rpm (20, 800 × g). From the supernatant fraction ccc DNA was isolated with the High Pure Viral Nucleic Acid Kit (Roche) according to manufacturer's instructions.

2.12.5. Quantification of ccc DNA

For real-time PCR for the quantification of ccc DNA in PTH LightCycler® FastStart DNA Master Hybridization Probes Kit (Roche, Germany) was used. Primers and HybProbes for ccc DNA PCR were from TIB MOLBIOL, Germany. C1 sence primer:
5'-TGCACTTCGCTTCACCT (from nucleotide 1580 to 1596), C1 antisense primer:
5'-AGGGGCATTTGGTGGTC (from nucleotide 2314 to 2298). The primers are specific for the detection of HBV DNA without nick and gap, the ccc DNA. 3FL CB HybProbe: 5' CAATGTCAACGACCGACCTT –FL- 3' (correspond to nucleotide 1678 to 1697, FL – donor dye fluorescein); 5LC CB HybProbe: 5'-LC Red 640-AGGCMTACTTCAAAGACTGTKTGT-PH-3', M=A/C and K=G/T (correspond to nucleotide 1699 to 1722, acceptor dye – Light Cycler Red 640).
PCR conditions: 1 cycle of denaturation at 95 ºC for 10 min; 45 cycles of amplification – 10 s at 95 ºC, 20 s at 57 ºC and 32 s at 72 ºC. The standard for the quantification was the plasmid pBs HB 991 T7 Dimer (10^7 – 10^2 copies). Serum standard - human plasma derived virions ($2x10^5$ ge HBV) was also used, and expected to give a negative signal, since it is not covalently closed.
Afterwards, the amplificate was recovered from capillaries by centrifugation, and 12 µl of the total 20 µl was subjected to 2% agarose gel (Tris-acetate buffer) electrophoresis, and visualized by ethidium bromide staining.

2.12.6. Quantification of HBV mRNA

mRNA was isolated from cytoplasmic fractions of HBV infected PTH with oligo(dT) coated paramagnetic beads (Dynal; Invitrogen) and purified by Dynabeads® mRNA Direct™ Micro Kit (Invitrogen) according to manufacturer's instructions followed by digestion with 1.5 U of RNase-free DNase (Applied Biosystems) for 30 min at 37 ºC. HBV mRNA was quantified using the one-tube real-time reverse transcription kit for LightCycler (Light Cycler® RNA Amplification Kit Hybridization Probes Kit; Roche, Germany) and primers and hybridization probes for the X region. X2 sense primer:
5' GACGTCCTTTGTYTACGTCCCGTC 3' (Y= C/T, correspond to nucleotide 1413 to 1436); X antisense primer: 5' TGCAGAGTTGAAGCGAAGTGCACA 3' (correspond to nucleotide 1579 to 1601). The primers have been described (Jursch et al., 2002;Glebe et al., 2003). 3FL XB HybProbe: 5' ACGGGGCGACCTCTCTTTACGCGG-FL 3' (correspond to nucleotide 1519 to 1543), 5LC XB HybProbe: 5'-LC Red 640-CTCCCCGTCTGTGCCTTCTCATCTGC 3' (correspond to 1545 to 1570). In vitro transcribed X region of HBV from plasmid pCX (Schuster et al., 2000) (transcription using the T7 in vitro transcription kit (Roche) according to manufacturers instructions) was used as a standart. After determining copy number by measuring OD 260 nm it was serially diluted and used as a standard in quantitative RT-PCR. Conditions of RT-PCR: reverse transcription step at 55 ºC for 12 minutes, with the denaturation step at 95 ºC for 10 s and amplification at 95 ºC for 10 s, 64 ºC for 15 s and 72 ºC for 13 s.

3. RESULTS

3.1. Expression of SFV1 vector encoded HBs proteins *in vitro*

The natural and modified HBs proteins expressed from the SFV1 vector will be referred in the text as "HBs proteins". Genes encoding SHBs, subtype adw2 (genotype A2) and ayw2 (genotype D) and five L protein deletion variants generated by fusion of aa 1-48 of preS1 (genotype D numeration, subtype ayw2) to N-terminus of subtype ayw2 S protein (preS1.1-48/S) were cloned in the pSFV1 vector (Fig. 11). The pSFV1 contains SP6 polymerase promoter to enable transcription *in vitro* after linearization of the pSFV1 plasmid, followed by nsP1-4 replicase complex. The gene of interest is preceeded by the SFV 26S subgenomic promoter. The pSFV1 carries gene for ampicillin resistance and site for its linearization with appropriate restriction endonuclease.

The second amino acid of preS1 domain is glycine, which is the myristic acid attachment site, some variants of modified HBs proteins preS1.1-48/S contained unmodified myristic acid attachment site at the 2nd position at the N-terminus. Variants with modified myristic acid attachment site contained serine (Ser2.preS1.1-48/S) and alanine (Ala2.preS1.1-48/S) in the 2nd position. Three of the modified HBs variants of preS1.1-48/S contained the natural codon of translational initiation of SHBs downstream of the codon initiating translation of the deleted L protein. To determine the influence of in-frame expressed SHBs on formation and release of subviral preS1.1-48/S particles, the second initiation codon at the beginning of SHBs was mutated to GTG (variant termed preS1.1-48/S_0). The schematic representation of the SFV1 constructs with the inserted natural and modified HBs protein coding genes is shown in Figure 11. A more detailed scheme of deleted L proteins is depicted in Figure 12. Addionally, the list of the natural and modified HBs proteins cloned in pSFV1 is depicted in table 7.

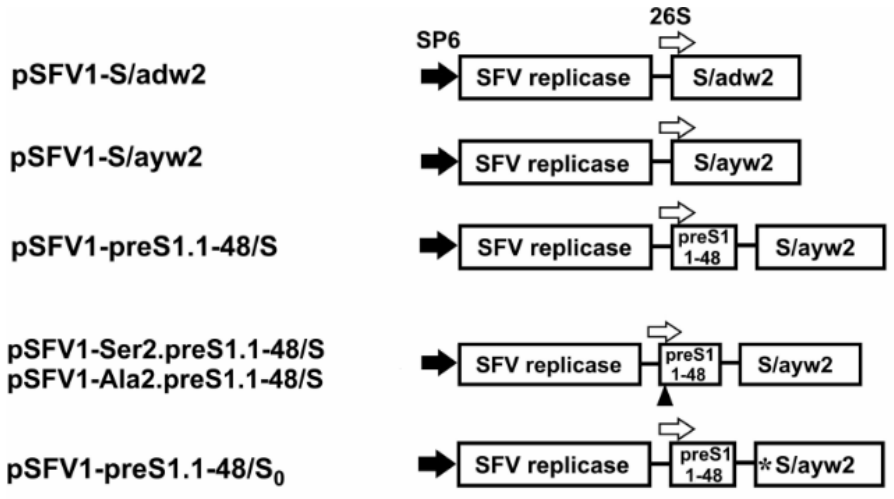

Figure 11. Schematic representation of the SFV1 expression vectors. SP6 RNA polymerase promoter is shown by the filled arrow. HBV surface protein genes are placed under the control of SFV 26S subgenomic promoter (empty arrow). Asterix denotes the missing translation start site of the S/ayw2 encoding sequence. The space between the regions encoding aa 1-48 of preS1 and S/adw2 denotes the "spacer" encoding aa LEGGSGG. The filled triangle denotes the modified myristic acid attachment site, where Gly was replaced with Ala and Ser

Table 7. HBs proteins expressed by SFV1 vector

HBs proteins	Modification
SHBs, adw2 (subgenotype A2)	-
SHBs, ayw2 (genotype D)	-
Deleted L proteins	Fusion of aa 1-48 of preS1 to N terminus of SHBs, ayw2
preS1.1-48/S	As above
preS1.1-48/S_0	Start codon of S (ATG) mutated to GTG
Ser2.preS1.1-48/S	2nd Gly replaced by Ser
Ala2.preS1.1-48/S	2nd Gly replaced by Ala

*HBV L protein coding sequence was cloned in SFVC vector, other HBs protein coding sequences were cloned in SFV1 vector

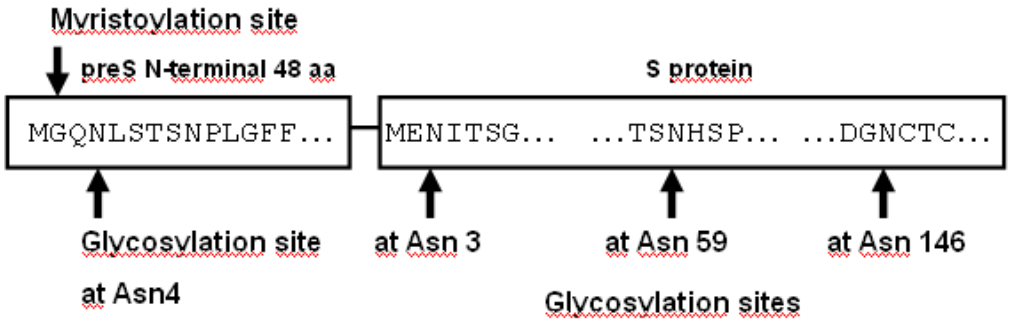

Figure 12. Schematic representation of LHBs deletion variants consisting of aminoterminal 48 aa of preS1 fused to the amino terminus of the S domain showing the myristoylation site and potential glycosylation sites

The sequence of cloned HBs proteins was confirmed by sequence analysis as described in Materials and Methods section. Sequences of the deleted L protein genes beared the desired exchanges. The SFV1 plasmids bearing the HBs protein genes were selected, isolated and amplifed according to standard procedures briefly outlined in Materials and Methods section. After linearization of the SFV1 plasmids with restriction endonuclease NruI, transcription *in vitro* with SP6 polymerase was performed yielding recombinant SFV RNA. Recombinant SFV were produced after electroporation of BHK-21 cells with the *in vitro* transcribed recombinant SFV RNA and helper vector RNA. rSFV were concentrated as described in Materials and Methods section, followed by the determination of the rSFV titer.

The expression of HBs proteins was examined mostly in BHK-21 and Huh7 cells. HepG2 cells were used once. Expression of the chimeric preS1.1-48/S proteins was shown also in PTH after infection with rSFV. Cells were infected with rSFV or the recombinant SFV replicon RNA was introduced by electroporation. The transcription of HBs protein encoding sequences is initiated at the 26S subgenomic promoter on the (-) RNA strand, producing (+) RNA subgenomic RNA, from which the HBs proteins are translated (refer to Fig. 9).

It has been established and we have also shown (Zajakina et al., 2004;Braun et al., 2007) that 20-24 hours after infection with rSFV is optimal to assess the expression of the desired protein *in* vitro. 20-24 hours after infection cells were lysed and cell medium was collected. We observed that electroporation with recombinant SFV RNA is more cytotoxic to transfected cells compared to infection with rSFV particles.

3.1.1. Expression in vitro of the SHBs.

BHK-21 and Huh7 cells were infected at MOI 20 with rSFV encoding S/adw2 protein. 20 h after infection cells were pulsed with [^{35}S]-methionine and chased for the indicated time (Fig. 13), up to 24 hours. "Pulse-chase" experiments and examination of cell lysates by SDS-PAGE at indicated times after addition of "chase" medium showed the characteristic pattern of the SHBs - the 27 kDa glycosylated (GP27) and 24 kDa non-glycosylated form (P24) in equal proportions in BHK-21 cell lysates (Fig.12A), while in Huh7 cells the glycosylated form was more prominent (Fig. 13B).

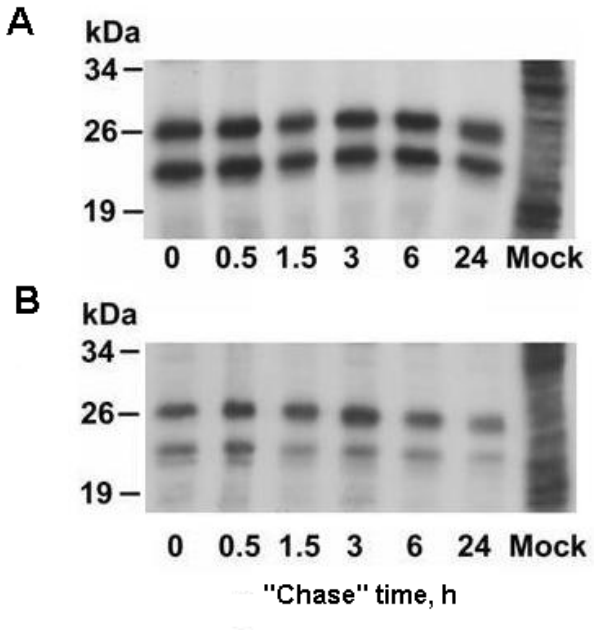

Figure 13. Analysis of rSFV driven SHBs expression in BHK-21 cells (A) and Huh7 cells (B). Cells were infected at MOI 20 with rSFV-S/adw2. 20 hours after infection cells were pulsed with [^{35}S] methionine, and „chased" for the time indicated with the medium containing unlabelled methionine. Cells were lysed and subjected to SDS-PAGE followed by autoradiography.

Secreted [^{35}S] labeled SHBs in BHK-21 or Huh7 medium was detected by SDS-PAGE after precipitation with 20% trichloroacetic acid at 1.5 h, 3 h and 24 h after addition of chase medium (not shown). HBsAg specific ELISA showed a peak concentration in the medium of BHK-21 cells between 36 and 48 h after infection, with the secretion declining at 72 hours after infection, detection was performed by commercially available HBsAg assay (Dade Behring) (Fig. 14).

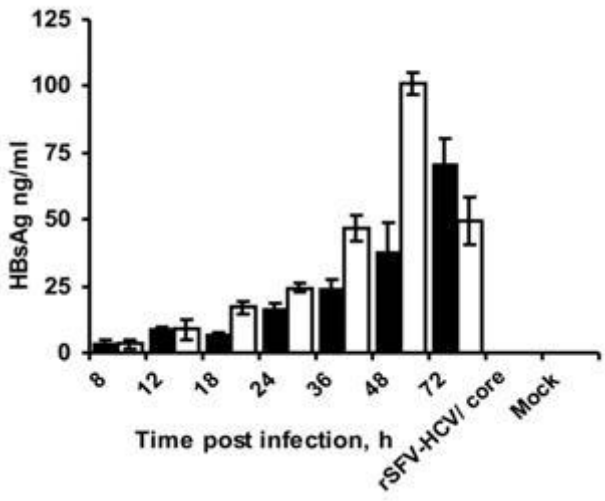

Figure 14. Expression of rSFV driven SHBs expression in BHK-21 cells. At indicated times post infection BHK-21 cell medium and cell lysates were subjected to ELISA using rHBsAg with a predetermined concentration as a standard. rSFV-HCV/core represents cells infected with rSFV particles encoding core protein of hepatitis C virus as a negative control. Mock infected cells during infection were treated with medium. Error bars show standard deviation (SD). Dark bars denote intracellular HBsAg, light bars extracellular HBsAg.

It was not possible to detect S/ayw2 (genotype A), expressed in BHK-21 cells, with the commercial HBsAg assay (Dade Behring), neither in the infected BHK-21 cell lysates, nor in the infected cell medium. However, this commercial assay could detect rHBsAg, subtype ayw2, produced in yeast (not shown). S/ayw2 expressed in mammalian cell lines could be detected, however, with the in-house ELISA using C20/02 antibodies. This was not investigated further.

Immunofluorescence analysis with MAb 1-9C1 of BHK-21 cells infected with rSFV-S/adw2 and rSFV-S/ayw2 demonstrated a cytoplasmic, granular distribution of the SHBs (Fig. 15).

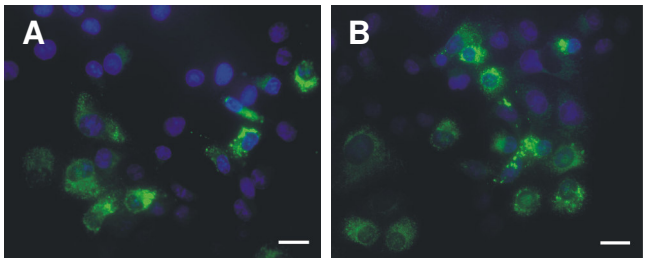

Figure 15. Immunofluorescence analysis of SHBs expressed in BHK-21 cells. Infection of BHK-21 cells and immunofluorescence analysis was performed as described in Materials and Methods section. S/adw2 (A) and S/ayw2 (B) proteins were detected by MAb 1-9C1 followed by FITC conjugated α-mouse IgG. Cell nuclei were stained with DAPI. The size bar corresponds to 20 µm.

3.1.2. Expression of preS1.1-48/S proteins in vitro

BHK-21 and Huh7 cells were infected at MOI 20 and MOI 10 respectively with rSFV encoding the preS1.1-48/S proteins. Analysis of BHK-21 and Huh7 cell lysates for the deleted L proteins and its variants with the changed myristic acid attachment site Ser2.preS1.1-48/S and Ala2.preS1.1-48/S revealed P24 and GP27 bands, indicating an effective internal translation initiation at the second translational start site (Fig. 16A, B). Three bands at approximately 32, 35 and 38 kDa could be detected corresponding to the chimeric proteins preS1.1-48/S, Ser2.preS1.1-48/S and Ala2.preS1.1-48/S (Fig. 16 A, B), and their glycosylated forms as verified by the PNGase F digestion (Fig. 17).

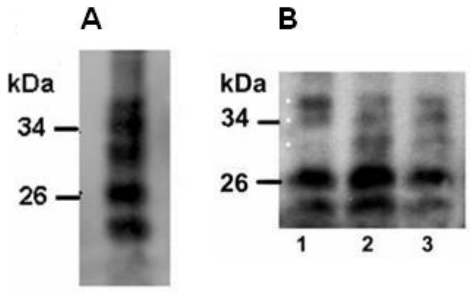

Figure 16. BHK-21(A) and Huh7 (B) cells were infected with rSFV encoding LHBs deletion variants. Cells were lysed 20 hours after infection with 0.5% Triton X-100 containing lysis buffer and subjected to SDS-PAGE and Western blotting with MAbs HB1 recognizing linear S domain. The three bands corresponding to preS1.1-48/S .proteins are marked by the white dots. (A) - preS1.1-48/S; (B) - preS1.1-48/S (lane 1), Ser2.preS1.1-48/S (lane 2) and Ala2.preS1.1-48/S (lane 3).

The modified HBs proteins bear three potential N-glycosylation sites: at Asn4 in the preS1 fragment, and at Asn3 and Asn146 in the S domain (see Fig. 12). In full length L protein the preS1 site is not used due to lack of a signal peptide governing co-translational translocation (Bruss and Vieluf, 1995;Ostapchuk et al., 1994). The Asn 3 site of the S protein is not glycosylated in natural S protein, while Asn-146 is partially used in all 3 HBs proteins. Thus, a doublet of nonglycosylated (ca. 30 kDa) and glycosylated (ca. 33 kDa) fusion protein

was expected. However, Western blot analysis with preS1 specific MAb MA18/7 of Huh7 cell lysates (Fig. 17) unexpectedly revealed three bands at approximately 32, 35and 38 kDa. To confirm the presence of glycosylated forms of preS1.1-48/S proteins were deglycosylated with peptide *N* glycosidase F (PNGase F), which cleaves all N-linked glycans. PNGase F digestion in denaturing conditions shifted the three bands to one 30 kDa position (Fig. 17). This suggested that all three N-glycosylation sites were partially linked with glycans generating a mixture of mono-, di- and tri-glycosylated glycoproteins. PNGaseF digestion of preS1.1-48/S proteins was performed in native conditions in order to distinguish between preS and SHBs linked glycans. SHBs linked glycan at Asn 146 under native conditions is protected from the cleavage with PNGase F in native conformation of SHBs, whereas preS linked glycan is still removed in native conditions (Tolle et al., 1998). Under these conditions, the major product of the digest was the 32 kDa protein, but the nonglycosylated 30 kDa product was also observed. This result suggests that the majority of the preS1.1-48/S proteins is glycosylated at Asn146 and at one or both of the other sites. The preS1.1-48/S proteins with Ser and Ala at position 2, showed identical glycosylation pattern to the protein with the unchanged myristic acid attachment site. Glycosylation pattern of the preS1.1-48/S_0 variant did not differ from the preS1.1-48/S with the unchanged translational start site (Fig. 17). Thus, the presence internally translated SHBs did not change the ER related topology of the preS1.1-48/S protein.

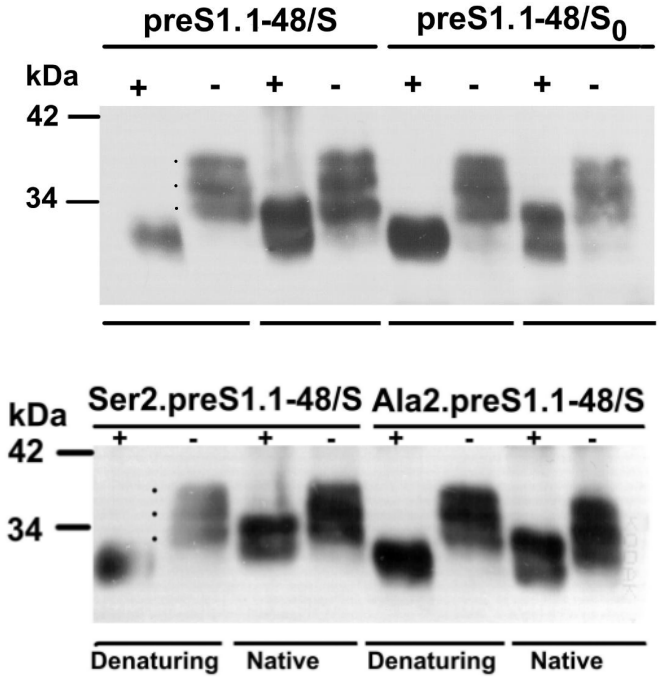

Figure 17. N-Glycosylation pattern of preS1.1-48/S proteins.
Huh7 cells were infected at MOI 10 with rSFV encoding preS1.1-48/S, Ala2.preS1.1-48/S, Ser2.preS1.1-48/S and preS1.1-48/S_0. 20 h after infection cells were lysed with lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 2 mM EDTA and 1 µg/ml phenylmethanesulfonylfluoride. Five-hundred units of PNGase F were added to membrane was reacted with MAb MA18/7, followed by goat anti-mouse antibodies conjugated with horseradish peroxidase. "+", treated with PNGase F; "-", untreated. Black dots indicate the three glycosylated bands of truncated LHBs.10 µl of Huh7 cell lysates diluted in reaction buffer (50mM sodium phosphate buffer, pH 7.5) containing 1% NP40, and incubated for 1 h at 37 ºC. To perform the reaction under denaturing conditions, glycoprotein denaturing buffer was added to 10 µl of cell lysates and incubated for 10 min at 100ºC, whereas for digestion under native conditions denaturing buffer was omitted. After separation by SDS-PAGE, proteins were transferred to a Hybond-P membrane in a semi-dry electro blotter. The membrane was reacted with MAb MA18/7, followed by goat anti-mouse antibodies conjugated with horseradish peroxidase. "+", treated with PNGase F; "-", untreated. Black dots indicate the three glycosylated bands of truncated LHBss.

To detect, if the preS1.1-48/S proteins synthesized in Huh7 cells exhibit preS1 and SHBs antigenicity, Huh7 cells 20 h after infection with rSFV encoding preS1.1-48/S proteins were lysed and cell lysates subjected to ELISA with preS1 specific MAb MA18/7 and MAb detecting correctly folded SHBs - C20/02. The ELISA demonstrated more preS1 antigen than correctly folded HBsAg antigen exposed by the preS1.1-48/S proteins, while the S_0 variant (preS1.1-48/S_0) without start codon for the SHBs showed a very low C20/02 specific signal, which was marginally above the cut-off (Fig. 18A). Aditionally, PTH were infected with

rSFV encoding preS1.1-48/S and preS1.1-48/S_0 at MOI 10 and PTH lysates subjected to .preS1 and S-specific ELISA (Fig. 18B).

3.1.3. Secretion and antigenicity of HBs proteins

To examine the secretory phenotype of the preS1.1-48/S proteins, 20 h after infection of Huh7 cell infection with rSFV encoding the respective proteins the cell medium was replaced with the fresh medium, the medium was collected 24 hours later and examined with ELISA. The proteins exhibited preS1 antigenicity, and to a smaller extent S antigenicity. This was demonstrated by binding to S-conformation dependent MAb C20/02 (Table 8). Binding to C20/02 could originate either from the chimeric preS1.1-48/S particles and/or from the independently secreted SHBs particles. Secretion of the preS1.1-48/S proteins is summarized in table 8. The protein with *wt* myristic acid attachment site was secreted most efficiently, the inactivation of the myristoylation signal had a minor negative effect on the release of the particles. Neither preS1, nor SHBs antigenicity was detectable in the medium of Huh7 cells infected with rSFV encoding preS1.1-48/S_0 Since the preS1.1-48/S_0 was not secreted, it can be assumed that secretion of SHBs particles with preS1 domains on the surface happens only in the presence of independently expressed SHBs.

Huh7 additonally were infected with rSFV encoding LHBs (schematic representation of the construct is shown in Fig. 11). Cell infection with the rSFV particles encoding the L protein served as non-secreted control, since C terminal part of the preS1 domain contains topological (Bruss and Vieluf, 1995; Ostapchuk et al., 1994; Bruss et al., 1994) and retention signals (Gallina et al., 1995) which prevent secretion of the LHBs and SFV-expressed LHBs could not be detected in infected cell medium up to 48 h after infection with rSFV (not shown)

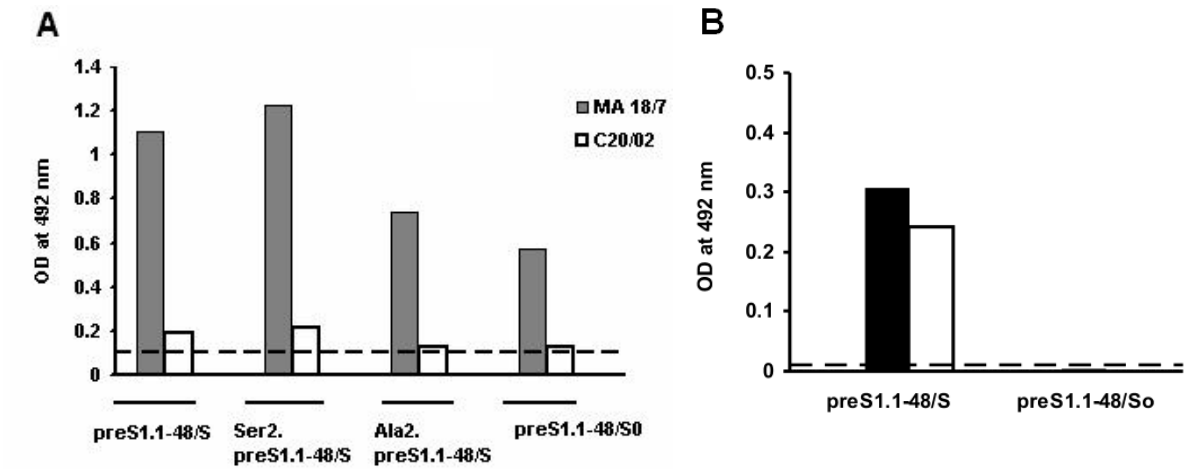

Figure 18. Analysis of preS1.1-48/S and it's variants' expression in Huh7 (A, B) and PTH by preS1 (MA 18/7) and S (C20/02) specific ELISA. Huh7 cells (A) and 10^5 PTH (B) were infected at MOI 10 for 20 h. (A) Huh7 cells lysates with 5 μg/ml cell protein. (B) Lysates of PTH represent material from $5x10^4$ cells. The viability of rSFV infected PTH ranged 20 h after infection from 59 to 68% of mock infected cells. Cut-off is represented by the dashed line and was set as the value shown by rSFV-HCV/core infected Huh7 cell lysate (A) or by mock infected PTH lysate (B), representing $5x10^4$ cells in ELISA employing C20/02. Results of one of three independent experiments showing similar results are shown.

Table 8. Secretion of LHBs deletion variants

L deletion variant	MAbs	
	MA18/7	C20/02
preS1.1-48/S, myr wt	1.10 ± 0.16	1.00 ± 0.12
Ala2preS1.1-48/S, myr$^-$	0.52 ± 0.06*	0.56 ± 0.09*
Ser2preS1.1-48/S, myr$^-$	0.67 ± 0.08*	0.43 ± 0.06*
preS1.1-48/S_0, myr wt	0.17 ± 0.01*	0.14 ± 0.01*

Huh7 cells were infected at MOI 10 with rSFV encoding respective proteins. 18 h after infection cell medium was collected and replaced with a fresh medium, which was collected after 24 h and 100 µl of 2 ml of medium were analyzed by ELISA on microtiter plates coated with MAbs MA18/7 and C20/02. Mean values are shown ± SD. * The difference to preS1.1-48/S, myr *wt* is significant at α=0.05.

To examine if addition of preS1 sequences 1-48 with unmodified and modified myristic acid attachment site to N-terminus of S protein allow formation of intact subviral particles, BHK-21 cells were electroporated with rSFV RNA encoding the respective proteins and after 22 hours the cell medium was collected and concentrated as described in Materials and Methods section. Electron microscopy of the medium revealed spherical, 22 nm diameter HBsAg-like particles (Fig. 19A). Binding of MA18/7 followed by gold conjugated anti-mouse IgG verified that these particles exposed accessible preS1 antigen on the surface. The preS1.1-48 and the Ser2preS1.1-48/S supported the assembly of HBs-like subviral particles, their secretion, and expose preS1 epitopes on their surface recognized by anti-preS1 antibodies (Fig. 19B). Naked SHBs-like particles without the immunogold label were also found (Fig. 19A). Thus, preS1/S particles with preS1 antigen on surface were secreted from the infected or transfected cells, along with unmodified, only SHBs containing particles. Moreover, the secretion of the chimeric preS1.1-48/S proteins occurred only in the presence of in-frame expressed SHBs.

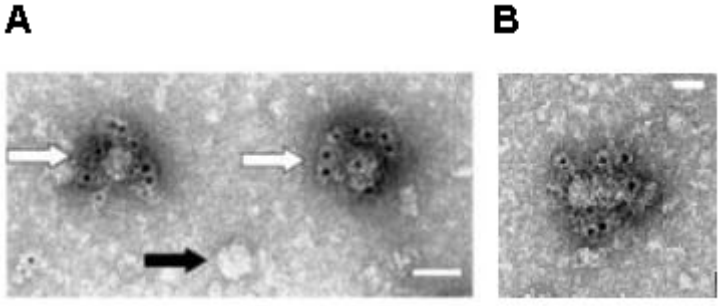

Figure 19. Electron microscopy analysis of immunogold-labelled preS1.1-48/S (A) and Ser2preS1.1-48/S (B) after reaction with MAb MA18/7 recognizing preS1.
The suspension of the particles was adsorbed on carbon-formvar coated grids and incubated with MAb MA 18/7, followed by anti-mouse IgG conjugated with 5 nm gold particles (Sigma), as described (Louro and Lesemann, 1984). The grids were negatively stained with 2% uranyl acetate aqueous solution and examined with a JEM-1230 electron microscope (JEOL Ltd.,Tokyo, Japan) at 100 kV. The scale bar corresponds to 25 nm. There are two immunocomplexes (white arrows) and one naked particle (black arrow).

3.1.4. Immunofluorescence analysis of preS1.1-48/S proteins

To determine the subcellular localization of the preS1.1-48/S proteins and effect of the mutation in the myristoylation site on it, BHK-21 cells were infected with rSFV encoding the respective proteins. Cells were stained with SHBs specific antibodies 1-9C1 directed to a linear epitope in the S domain followed by preS1 specific polyclonal antibodies H863. The anti-S antibodies bind to the in-frame expressed SHBs, as well as to the S domain in the preS1.1-48/S, Ser2.preS1.1-48/S and Ala2.preS1.1-48/S. Immunofluorescence analysis of rSFV-preS1.1-48/S (*wt*) infected cells revealed colocalization of both antigens with a strong perinuclear staining (Fig. 20, upper sections of panels A and B). In contrast to the restricted distribution of preS1.1-48/S protein in the perinuclear area (Fig. 20, section A, upper panel), the Gly 2 mutant proteins showed a granular dispersion throughout the cell cytoplasm (Fig. 20, section A, middle and bottom panels). Anti-preS1 signal displayed brighter intensity at the cell peripheries (Fig. 20, section A, bottom panel), while the perinuclear Golgi-like area showed weaker red staining for rSFV-Ser2.preS1.1-48/S infected cells (Fig.20, section A, bottom panel). To exclude the effect of presence of internally translated SHBs on the localization of the deleted L proteins, we BHK-21 cells were infected with the rSFV-preS1.1-48/ revealed compact staining pattern, which localized strictly around the nucleus in a ring-like pattern (not shown), resembling the pattern of preS1.1-48/S protein with the in-frame expressed SHBs. These results are in line with the matrix protein Z of Lassa virus where mutation of aminoterminal Gly to Ala altered the subcellular localization from a punctuate to a diffuse pattern (Strecker *et al.*, 2006). The stronger colocalization of the preS1 and SHBs epitopes in cells transfected with the *wt* variant than with the variant without myristoylation signal suggests that the myristate supports the interaction between the LHBs and SHBs.

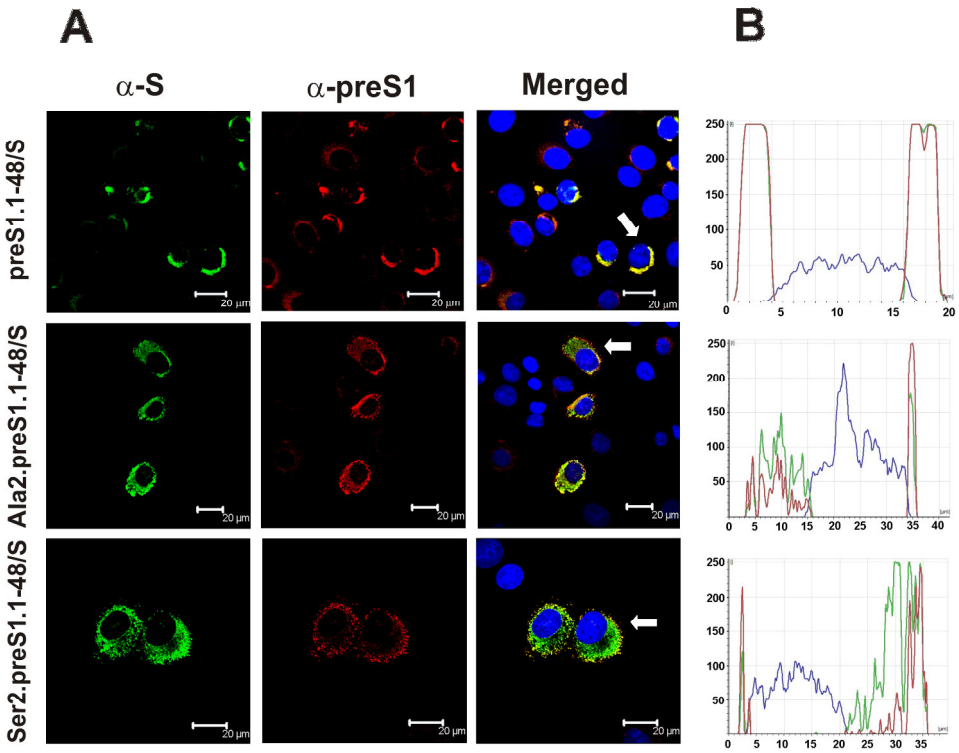

Figure 20. Confocal microscopy analysis of preS1.1-48/S, Ala.2preS1.1-48/S and Ser2.preS1.1-48/S proteins BHK-21 cells were infected with rSFV encoding the respective proteins and stained with MAb 1-9C1 (α-S), followed by polyvalent rabbit antibodies H863 (α-preS1). The cell nuclei were stained with DAPI. Panel A depicts images of cells, panel B shows the fluorescence intensity of each fluorophore - FITC, TRITC and DAPI (y axis, relative numbers) along a line (x axis, □m) drawn across a selected cell which is marked by the white arrow.

3.2. Selection of conditions for rSFV production

rSFV are produced after co-electroporation of BHK-21 cells with the *in vitro* transcribed SFV RNA and helper vector RNA. The intended use of rSFV for immunization of BALB/c mice determined the necessity to obtain rSFV particles in high titres in infectious units/ml after incubation of BHK-21 cells, and concentration of rSFV. One of the essential factors that influence the final outcome of rSFV particles is the temperature of cell incubation after electroporation. 33 ºC was chosen rather that 37 ºC, because at lower temperatures the cytotoxic effects of SFV replicons for the host cells are diminished and incubation at longer times can be performed (A. Merits, personal communication). Incubation of BHK-21 cells at 33 ºC resulted in 10-fold higher titer that incubation at 37 ºC for 24 hours. To increase rSFV particle outcome cells were incubated for 36 – 48 hours at 33 ºC. The experiments for optimization of rSFV production were performed with the rSFV/EGFP particles. Fig. 21 illustrates assessment of rSFV encoding EGFP after optimal conditions for rSFV production were selected.

Figure 21. Infection of BHK-21 cells with rSFV/EGFP. The titer of the rSFV1/EGFP was 2,9 x 10^9 infectious units/ml**.** A. BHK-21 cells were infected with rSFV1/EGFP at MOI 3 and incubated for 20 h. B. BHK-21 cells in the light microscope corresponding to the field of vision depicted in A. The cells are not fixed. C. BHK-21 cells were infected with the rSFV/EGFP at MOI ranging from 0.1 to 50, 6 h after infection the infected BHK-21 cells were labeled with100 µCi/ml [^{35}S]-methionine for 30 min and lysed with SDS lysis buffer, followed by SDS-PAGE. Material corresponding to 1.5 x10^5 cells was loaded in one lane. The band slightly below the molecular weight marker of 34 kDa corresponds to the EGFP protein.

3.3. Immunization of BALB/c mice with rSFV

The initial experiments of immunization of BALB/c mice with rSFV were performed with rSFV encoding S/adw2. The dose of 10^6 rSFV per one animal was chosen according to studies where authors had immunized mice with rSFV encoding other viral antigens or reporter genes (Berglund et al., 1999). The described immunization regimens with rSFV are: 10^6 rSFV followed by 10^6 after two weeks with the detection of immune response 2-3 weeks after the last immunization (Fleeton et al., 2000).

In the current study immunization i.v. and s.c. route with rSFV was chosen. I.v. immunization was performed in the tail vein. I.v. immunization with rSFV enables systemic delivery, while the s.c. route ensures local distribution. In case of systemic delivery the naked rSFV may be subjected to host's acquired humoral immunity to SFV generated after the first administration of rSFV. S.c. route ensures local distribution of the vector (Colmenero et al., 2001). The groups of BALB/c female mice are depicted in table 9, one group was comprised of 6 animals.

Table 9. Groups of BALB/c mice, route of immunization and doses of rSFV

Group	Dose of rSFV, immunized twice at two week intervals	Route of immunization
rSFV-S/adw2		
1	10^6	s.c.
1A	10^6	i.v.
2	10^7	s.c
2A	10^7	i.v.
rSFV-EGFP		
3	10^7	s.c.
3A	10^7	i.v.

2 weeks after immunization mice were repeatedly immunized with the same dose of rSFV. The anti-HBs response was analyzed 2 weeks after the second immunization by ELISA with the serially diluted sera of each individual mouse, using the peptide spanning aa 124-148 of antigenic loop of S/adw2.The serum of BALB/c mice was collected before immunization and 2 weeks after second immunization. Serially diluted serum was applied to microtiterplates coated with the peptide aa 124-148 and the difference between sera collected before immunization and after s.c. and i.v. immunization with rSFV-EGFP was determined as statistically significant with the Student's paired t-test (p=0.05). The result indicates unspecific binding of rSFV-EGFP sera to peptide 124-148 of S/adw2 at a dilution 1:40. The serial dilutions of BALB/c mice sera were prepared: 1:20; 1:40; 1:80; 1:160 and 1: 320. For the statistical analysis dilution 1:40 was chosen. First experiments showed that 2 doses of 10^6 rSFV-S/adw2 at two week intervals by i.v. or by s.c. subcutaneous route were not sufficient to generate a detectable anti-HBs response. A weak response was detected two weeks after two immunziations with 10^7 rSFV injected by the i.v. route, not by the s.c. subcutaneous route. According to the Student's paired t-test (p=0.05) there was significant difference between the groups immunized with with 10^7 rSFV-S/adw2 when compared to mice immunized with 10^7 rSFV/EGFP.

Additionally, rHBsAg, subtype adw2, was used in ELISA to determine group- (a) and subdeterminant (d) specificity. The unspecific binding of rSFV-EGFP immunized mice serum was considerably reduced when rHBsAg was employed for ELISA instead of peptide 124-148 of S/adw2 (Fig. 22).

Table 10. Student's paired t-test (p=0.05) of rSFV-S/adw2 immunized mice sera and rSFV-EGFP immunized mice sera

	rSFV-EGFP	
rSFV-S/adw2	10^7 s.c.	10^7 i.v.
10^6 s.c.	Not significant	-
10^7 s.c.	Significant	
10^6 i.v.	-	Not significant
10^7 i.v.	-	Significant

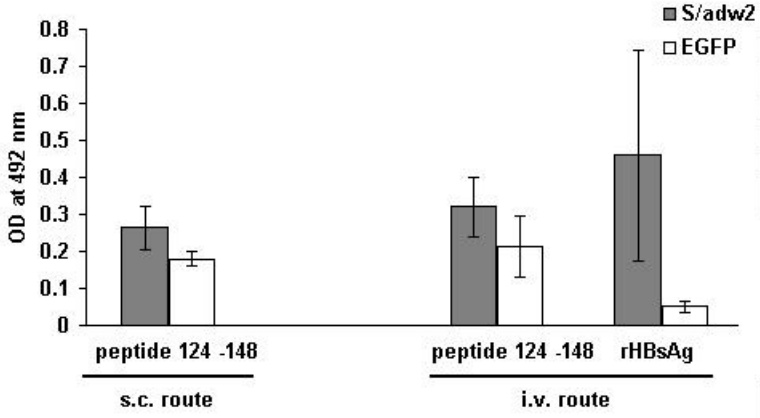

Figure 22. Mice were immunized twice with 10^7 rSFV at two week interval. Sera was collected 2 weeks after the second immunization and tested by ELISA using peptide spanning aa 124-148 of S/adw2 and rHBsAg. The results of the sera diluted 1:40 are depicted. The bars denote SD (standard deviation) of the mean of 6 mice.

The anti-HBs response after two administrations of 10^7 rSFV particles was low. Other authors (Berglund et al., 1999;Fleeton et al., 2000) report markedly higher humoral responses after administration of 10^7 rSFV particles or even 10^6 rSFV particles. Anti-HBs response was not significantly different (p=0.05) from the control group mice (rSFV-EGFP) immunized s.c. and i.v. with 10^6 rSFV. Groups immunized s.c. and i.v. with 10^7 rSFV showed statistically significant difference from the rSFV-EGFP immunized groups (Table 10). The anti-HBs response was variable between individual animals (Fig.23).

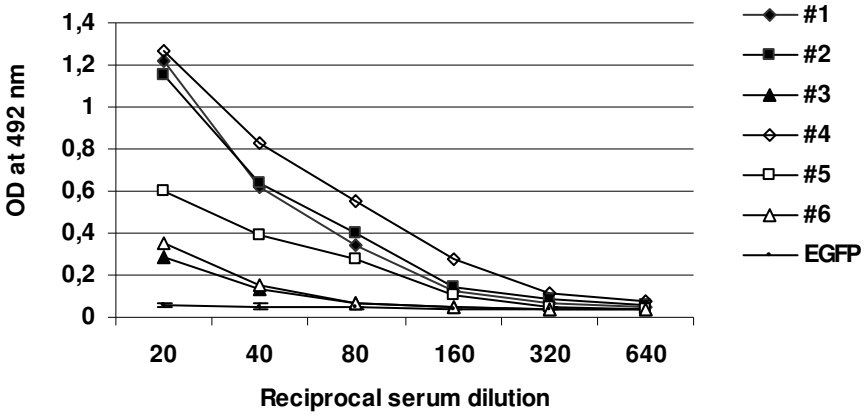

Figure 23. Reciprocal ELISA titers two weeks after the second immunization with 10^7 rSFV via i.v. route. rHBsAg, subtype adw2, was employed for ELISA. For the rSFV-EGFP immunized group mean of six mice is shown, the bars denote SD. The "#" in the legend denotes the numbering of the 6 mice.

Tree weeks after the second immunization two mice of i.v. group (mouse #1 and mouse #3) were administered 10^8 rSFV i.v. and two weeks after the sera was collected and subjected to ELISA using rHBsAg/adw2 (expressed in yeast *Hansenula polymorpha*).

Additionally, sera of one animal who did not receive the boost of 10^8 rSFV was tested with ELISA . The ELISA endpoint titers are depicted in Fig 24. The signal obtained from the serum collected two weeks after immunization with 10^8 rSFV showed pronounced increase in anti-HBs (Fig. 24).

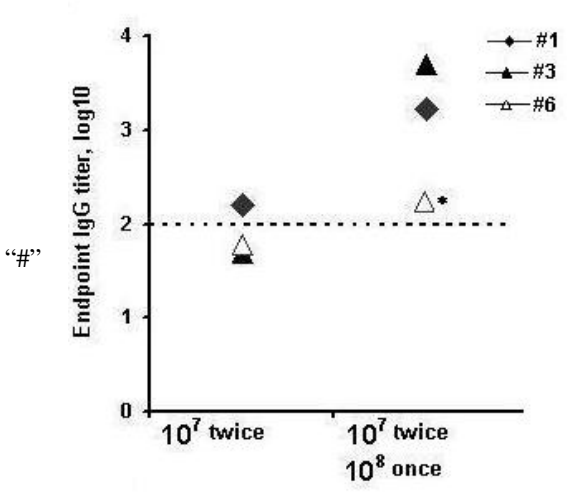

Figure 24. Anti-HBs after immunization of BALB/c mice with 10^7 rSFV at two week interval, followed by immunization with 10^8 rSFV. The sera were tested with ELISA using rHBsAg. The open triangle with an asterisk denotes a mouse which did not receive a "boost" of 10^8 rSFV. The mean of 6 rSFV-EGFP immunized mice twice with 10^7 rSFV was 0.04 OD at 492 nm and the mean of two mice immunized with 10^7 twice, followed by 10^8 rSFV once was 0.048 OD at 492 nm. Anti-HBs titer of 1:100 indicates cut-off, shown by the dashed line. The in the legend denotes the numbering of the animals.

10^7 rSFV immunized by i.v. or by s.c route was necessary to induce a weak, but detectable immune response, while 10^8 were a prerequisite for induction of a pronounced increase in anti-HBs after immunization with 10^7 rSFV. The detection of the anti-HBs response was performed with a homologous subtype of HBsAg - adw2. Subsequently HBsAg subtype ayw3 was used for ELISA. The anti-HBs response was markedly lower and only slightly above the signal obtained with the rSFV-EGFP immunized mice. This showed an exclusive subdeterminant preference of anti-HBs induced by rSFV encoding S/adw2 to rHBsAg of homologous subdeterminant, and a weak reactivity to the HBsAg ysubdeterminant. .

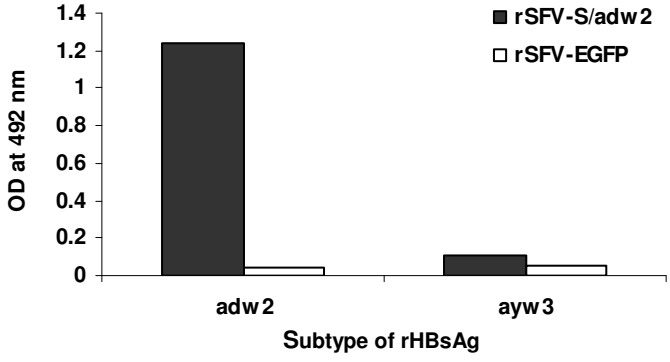

Figure 25. Reactivity of anti-HBs induced in BALB/c mice by rSFV-S/adw2 to rHBsAg of homologous (adw2) and heterologous (ayw3) subdeterminant. BALB/c mice were immunized twice with 10^7 rSFV-S/adw2 at two week intervals followed by 10^8 rSFV-S/adw2 three weeks after the second immunization. Serum was collected two weeks after the last immunization and tested with ELISA using rHBsAg adw2 and ayw3. The result represents serum from one animal (#3), previously being tested with ELISA using rHBsAg, adw2 (see Fig. 24).

It was shown that 10^8 rSFV-S/adw2 were required to elicit a pronounced increase in the anti-HBs generated by immunization with rSFV-S/adw2. The induced antibodies were subdterminant specific. Therefore a question was raised wheather anti-HBs generated by immunization of BALB/c mice with rSFV encoding S/ayw2 would react in ELISA using rHBsAg of homologous and heterologous subdeterminant with the same pattern – i.e. show limited reactivity to HBsAg, subtype adw2. Therefore rSFV encoding S/ayw2 were generated. Subsequently three groups of 9 mice in each group were created. The groups of immunized mice are outlined in table 11. Mice were inoculated with rSFV i.v. First, mice were inoculated with 10^7 rSFV followed by 10^8 three weeks later. Another reason to use an increased dose of rSFV after the first immunization was the eventual anti-SFV response to virus structural proteins which may form after the first administration. Although the general assumption is that since virus structural proteins are not expressed due to one round of infection ensured by the non-replicative nature of the rSFV, justifyiung the use of rSFV in repeated immunizations (Berglund et al., 1999), an increased dose was used three weeks after the first immunization to circumvent the eventual neutralizing effect of potential anti-SFV response. Sera were collected three weeks after the second immunization.

Table 11. Groups of BALB/c mice and their immunization regimen with rSFV encoding S/adw2, S/ayw2 and EGFP

Group	rSFV-	Immunization scheme	Route of immunization
1	rSFV-S/adw2	10^7 followed by 10^8 after three weeks	i.v.
2	rSFV-S/ayw2	As above	As above
3	rSFV-EGFP	As above	As above

3.3.1. HBsAg in BALB/c mice sera after injection of rSFV

HBsAg could be detected in the medium of cells infected with rSFV-S/adw2 *in vitro.* In natural HBV infection hepatocytes secrete HBsAg to the blood stream. Therefore it was assumed that rSFV-transduced cells of BALB/c mice would secrete the HBsAg to the blood stream. We determined whether HBsAg could be detected in the blood of BALB/c mice 18, 48 and 72 h after i.v. injection of 10^8 rSFV-S/adw2. 35 μl of blood from retroorbital sinus of 6 rSFV-S/adw2 imunized mice were collected, pooled, and immediately after collection 100 μl of the pooled sera were tested by a commercially available ELISA (Dade Behring) at a detection limit of 0.2 ng/ml. The HBsAg signal was marginally (13% and 1%) above the cut-off value (100%) at 18 and 48 h after injection respectively and 41% below the cut-off at 72 h post immunization. The HBsAg of the control injection with rSFV-EGFP were 38% to 55% below the cut-off at 18 and 48 h after injection.

3.3.2. Immune responses in BALB/c mice after immunization with rSFV-S/adw2 and rSFV-S/ayw2

Serially diluted sera were tested by ELISA using recombinant HBsAg subtype of adw2 or ayw3 produced in yeast to determine group- (a) and subdeterminant-specific (y or d) end point titers. All nine rSFV-S immunized mice in both groups seroconverted and showed a specific immune response. In line with the previous results, the anti-HBs/adw2 reacted specifically with the homologous subdeterminant d, but showed a very weak binding to a heterelogous subdeterminant y (Fig. 26A). The difference between anti-S/ayw2 sera with

HBsAg adw2 or ayw3 was not so pronounced as with anti-S/ayw2 (Fig. 26A), but it was statistically significant, see the statistical analysis below. The highest reactivity of anti-HBs were displayed when the anti-S/adw2 and anti-S/ayw2 antibodies were tested using the HBsAg with homologous subdterminant.

This is reflected also in Figure 26 B, when anti-HBs titers were determined using rHBsAg of homologous and heterologous subdeterminants.

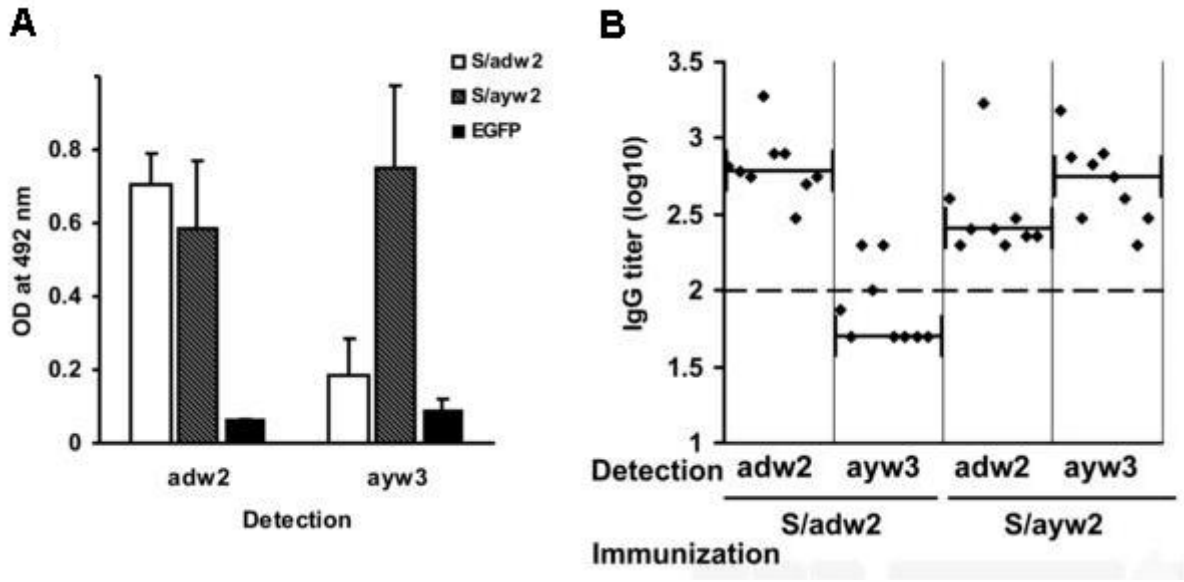

Figure 26. A. . Sera were diluted 1:75. **B.** Anti-HBs end point titers after immunization with rSFV-S/adw2 and rSFV-S/ayw2. Serially diluted mouse sera were tested by ELISA in microtiter plates coated with yeast-derived HBsAg adw2 or ayw3. The horizontal bars represent the median, the dashed line denotes the "cut-off" - anti-HBs titer of 1:100, above which mice were classified as responders.

3.3.2.1. Statistical analysis

To analyse the differences in reactivity of anti-S/adw2 and anti-S/ayw2 sera when HBsAg/adw2 or HBsAg/ayw3 was exploited for ELISA, ANOVA (*Single factor analysis of variance,* One way ANOVA) was used. The test determines if a significant difference exists between the 6 group means (means of the three groups tested using rHBsAg adw2 and ayw2), (see table 12 a). Critical value F was exceeded at 0,05 level of significance, thereforea difference existed between at least two means tested. To determine which groups means are different, Tukey's multiple comparison method (α=0.05) was used to differentiate which group means differ – comparison of sample means with the "control" group (EGFP) and between each other was performed. The calculations were performed manually. The results of the statistical analysis are shown in table 12 b).

A statistically significant difference of OD signals was found with antisera from mice injected with rSFV-S/adw2 or rSFV-S/ayw2 when yeast-derived HBsAg/ayw3 was used as antigen for ELISA. Moreover, the anti-HBs reactivity of anti-S/adw2 sera with HBsAg/ayw2 did not statistically differ with that of negative control mice (see table 12 a)

Table 12. Statistical analysis of anti-HBs response

a) Single factor analysis of variance, One way ANOVA

Immunization, rSFV-	Subtype of rHBsAg used for ELISA	
	adw2	ayw3
	Group means, n=9	
S/adw2	0.71	0.18
S/ayw2	0.58	0.75
EGFP	0.06	0.10
Difference between at least two means exist		

b) Tukey's multiple comparison method

	rHBsAg used for ELISA	
	adw2	ayw3
S/adw2 and EGFP	Different	Not different
S/ayw2 and EGFP	Different	Different
S/adw2 and S/ayw2	Different	Different

To determine binding of anti-HBs to HBsAg purified from a plasma of a highly viremic chronic HBV carrier, microtiter plates were coated with filamentous subviral particles containing all three HBs proteins. The subviral particles represent a source of natural HBsAg particles. The subtype of HBsAg used to determine binding was ayw2, genotype D. Pooled sera were precipitated with 36% Na_2SO_4 and IgG concentration measured at OD_{280} assuming a factor of 1.35 for 1 mg/ml IgG. Sera were tested at 1.5 mg/ml. Binding of IgG to purified filamentous HBsAg particles from the plasma of a chronic HBV carrier was not dependent on SHBs subtype used for immunization (Fig. 27).

Probably, conformation-based differences exist in the natural HBsAg particles compared to yeast-derived HBsAg particles. Natural HBsAg may contain conformational, correctly folded S domain. This can determine the stronger binding capacity of the induced mouse antibodies to natural HBsAg particles which is not dependent on HBsAg subtype. It may be assumed that correctly folded S domain was also present on the rSFV vector-expressed SHBs, but not on the yeast-derived SHBs.

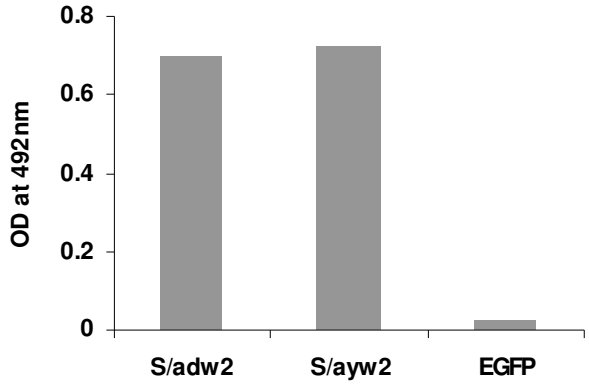

Figure 27. Binding of anti-HBs induced after rSFV mediated immunization to HBsAg ayw2 purified from a plasma of a chronic HBV carrier. Microtiter plates were coated with subviral HBsAg/ayw2 particles purified from plasma of a hronic HBV carrier. IgG obtained after immunization of BALB/c mice with the indicated rSFV was precipitated from the sera with Na_2SO_4 and added at 1.5 mg/ml.

3.3.3. Response to rSFV encoding preS1.1-48/S and preS1.1-48/S_0

BALB/c mice were immunized with with rSFV encoding L protein deletion variants - preS1.1-48/S or its variant preS1.1-48/S_0. Although the increased level of antigen expression *in vitro* by alphavirus replicons does not always correlate with a superior immunogenicity of alphavirus replicon vaccines *in vivo* (Maruggi et al., 2013), the variant with the unmodified myristic acid attachment site was chosen for immunization of BALB/c mice for its superior secretion compared to Gly2 mutants. Animals were immunized according to the regimen described above, see table 13.

Table 13. Groups of BALB/c mice and their immunization regimen with rSFV encoding preS1.1-48/S proteins

Group	rSFV-	Immunization scheme	Route of immunization
1	preS1.1-48/S	10^7 followed by 10^8 after three weeks	i.v.
2	preS1.1-48/S_0	As above	As above

Sera were collected three weeks after the second immunization. First, serial dilutions of the sera were tested by ELISA on microtiter plates coated with a preS1 peptide spanning aa 10-36, genotype D numeration (correspond to aa 21-47 in genotype A, which contains extra 11 aa at N-terminus). Additionally, to determine anti-HBs, microtiter plates were coated with rHBsAg ayw2, because in the deleted L proteins SHBs subtype was ayw2 and it was demonstrated above that anti-S response should be determined using a rHBsAg of a homologous subdeterminant. Along with the sera obtained after immunization with rSFV-preS1.1-48/S and rSFV-preS1.1-48/S_0, sera from rSFV-S/ayw2 mice was tested to compare the anti-HBs titers in order to test the notion that presence of preS1 affects anti-S titer. Anti-preS1 specific responses in rSFV-preS1.1-48/S and rSFV-preS1.1-48/S_0 immunized mice were detected in all of the immunized animals. The anti-preS1 titers were 1:1500 in preS1.1-48/S immunized mice and 1:1000 in preS1.1-48/S_0 immunized animals. Although mice were immunized with an equal number of infectious rSFV, antisera generated to preS1.1-48/S_0 protein contained less anti-preS1 which is in line with its inhibited secretion (Fig. 16B). The preS1 fragment did not significantly increased nor impaired the detectable anti-S titers (Fig. 28). Only 4 of 7 mice immunized with rSFV/preS1.1-48/S showed a detectable anti-S response (titer : >100). Mice immunized with the S_0 protein variant did not develop detectable anti-S titers. Anti-preS1 titer were higher than the anti-S titer in preS1.1-48/S immunized mice (Fig. 28).

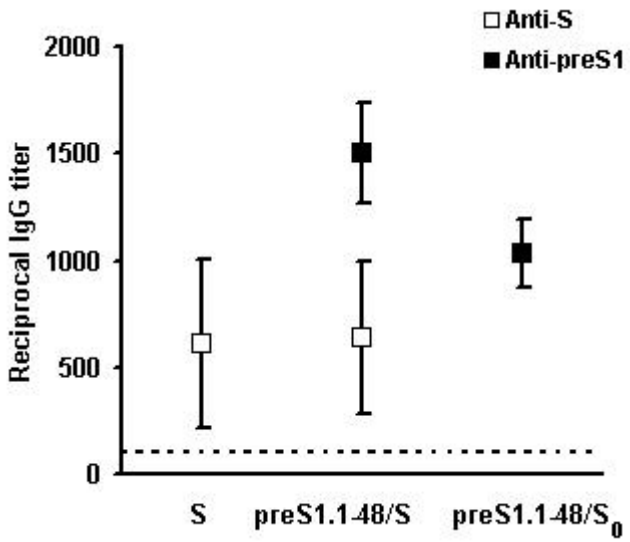

Figure 28. Anti-preS1 (peptide 10-36, genotype D numeration) and anti-S/ayw2 (rHBsAg) end point titers. 9 mice were immunized with rSFV-S/ayw2, 7 mice with rSFV-preS1.1-48/S and 7 with rSFV-preS1.1-48/S_0. In rSFV-S/ayw2 group all mice were responders. In rSFV-preS1.1-48/S or rSFV-preS1.1-48/S_0 group all mice demonstrated specific anti-preS1 titers. Bars represent SD. The horizontal line denotes a titer of 1:100 above which mice were classified as responders.

Antisera to deleted L proteins were subjected to ELISA using HBsAg/ayw2 particles purified from plasma of a hronic HBV carrier. Antibodies to the translational start site mutant protein preS1.1-48/S_0 showed 48.8% less binding to subviral particles than antibodies induced to preS1.1-48/S protein (Fig. 29).

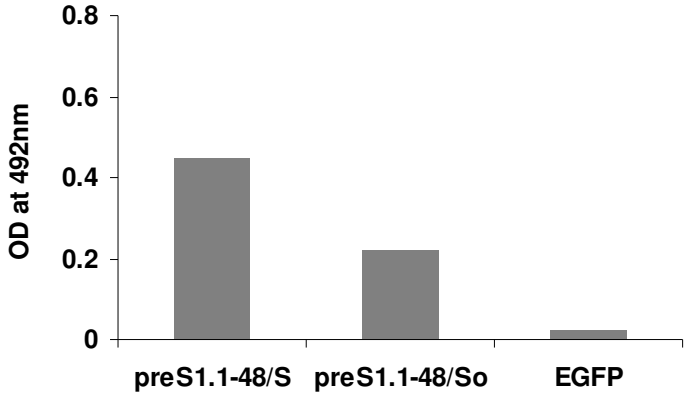

Figure 29. Binding of anti-preS1.1-48/S and anti-preS1.1-48/S_0 to subviral HBs particles purified from purified from a plasma of a chronic HBV carrier. Microtiter plates were coated with subviral HBsAg/ayw2 particles purified from plasma of a hronic HBV carrier. IgG obtained after immunization of BALB/c mice with the indicated rSFV was precipitated from the pooled sera with Na_2SO_4 and added at 1.5 mg/ml.

3.3.4.Isotyping of IgG antibodies

The determination of IgG subclasses IgG1 and IgG2a serves as an indirect tool to evaluate the activation and involvement of Th cell subsets/subpopulations in the generation of the immune response: IgG1 dominance signals that Th_2 cells are activated, whereas IgG2a dominance indicates activation of Th_1 subpopulation. The Th_1 subpopulation assists in generation of antibody mediated immune response, while the Th_2 cells - a CTL response. Therefore dominance of IgG2a serves as an indirect test for the involvement of CTLs. For isotyping of IgG subclasses of rSFV-S/adw2 and rSFV-S/ayw2 immunized mice sera, pooled sera of each group collected 6 weeks after the first immunization were tested using the homologous HBsAg subtype which the group was immunized with. Sera obtained from mice immunized with the preS1.1-48/S fusion proteins were tested with a synthetic preS1 derived peptide 10-36 (genotype D numeration). The ratio of IgG1/IgG2a in all rSFV immunized groups was below one and immunization by rSFV vectors induced more prominent IgG2a than IgG1 responses, but the degree of IgG2a preponderance over IgG1 differed among the immunized groups. IgG2a was most predominant in rSFV-preS1.1-48/S immunized mice (IgG1/IgG2a ratio 0.28) whereas mice immunized with rSFV-preS1.1-48/S_0 showed the lowest proportion of IgG2a (ratio 0.88). The two IgG subclasses were more balanced in the other two immunized groups (ratios 0.54 in both, see Fig. 30).

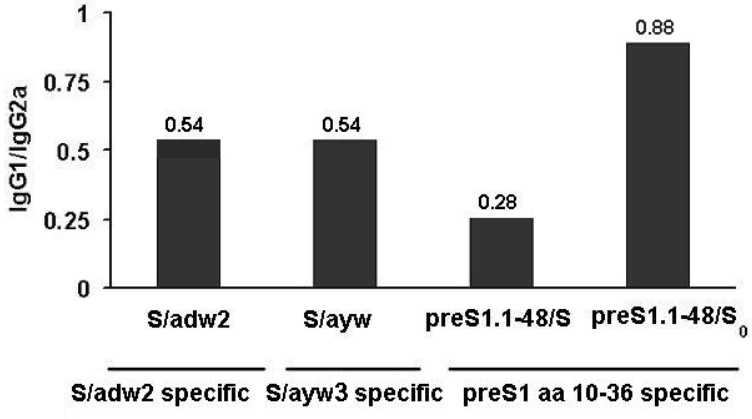

Figure 30. Ratio of IgG1/IgG2a in sera of rSFV-S/adw2, rSFV-S/ayw2, rSFV-preS1.1-48/S and rSFV-preS1.1-48/So immunized BALB/c mice. Microtiter plates were coated with rHBsAg adw2 and ayw3 and with a synthetic peptide aa 10-36 (genotype D numeration).

3.4. HBV neutralizing antibodies

The *Tupaia belangeri* used for the isolation of livers were males or females, and between less than a year and 6 years of age. 3 days after plating PTH could be infected with HBV. To evaluate the HBV infection neutralizing potential of the antibodies induced by rSFV immunization, 5.5 x 10^5 ge HBV were incubated with antisera prior infection of PTH. The capacity of the antisera to inhibit HBV infection of PTH was evaluated for all the sera obtained after rSFV-mediated immunization: S/adw2, S/ayw2, preS1.1-48/S, preS1.1-48/S_0 and EGFP as a control serum. Serum obtained from mice before immunization was also included in the experiments, along with monoclonal antibodies MA18/7 and C20/02. These, antibodies inhibit HBV infection of PTH by 100% (Glebe et al., 2003). After incubation, PTH cultures were infected with virus/antibody mixtures, and after 18 hours the virus inoculum was removed and PTH medium added. The PTH medium was removed at days 3, 6, 10 and 14. Markers of HBV infection – secreted HBsAg and HBeAg were quantitatively determined in the PTH culture medium collected at the above mentioned time points. It must be noted that HBsAg in minor quantities is present in virus inoculum, therefore PTH cultures were extensively washed after removal of the viral inoculum 18 hours after infection. At day 14 after infection the experiments were terminated and WST-1 test was performed (see Materials and Methods section) in order to assess the cell viability, which exclusively distinguishes metabolically intact cells. The HBsAg (ng/ml) and HBeAg (S/CO) accumulated from day 10 to day 14 after infection was normalized to the OD 450 nm values of the WST-1 test. Additionally, after the WST-1 test PTH were extensively washed and lysed, followed by separation of nucleic and cytoplasmic fractions for the detection of cccDNA and HBV mRNA. The duration of each experiment was 17 days. Figure 31 depicts PTH at various time points after isolation.

The quantity of secreted HBV infection markers HBsAg and HBeAg was directly dependent on the quality of the PTH cultures, which was variable between the experiments. For example, the quantity of the secreted HBsAg in PTH between the infection experiments ranged between 35 ng/ml to 5 ng/ml for the pre-immune serum diluted 10 fold in PTH (10 fold diluted pre-immune serum mixed with 5.5 x 10^5 ge HBV), the HBeAg varied from 32 S/CO and 4 S/CO for the afore mentioned serum. The results depicted below (Fig. 32, 33 and 34) showing HBsAg and HBe Ag in the PTH medium are from one of five experiments. The results depicting the quantification of HBV cccDNA (Fig. 34) and mRNA and (Fig. 35) show the results of one of two experiments.

First, 5.5 x 10^5 ge HBV were incubated with BALB/c mice sera diluted in PTH 5 fold, 10 fold, 30 fold and 100 fold. In the first neutralization experiments with the untreated mice sera it could be observed that mouse serum exerts HBV infection enhancing effect. We could observe that 10 fold diluted untreated BALB/c mice serum at day 10 after infection was able to raise HBsAg concentration mored than five fold. 100 fold diluted serum could lead to 3.5 fold increase of HBsAg concentation (Fig. 32A). These findings were confirmed by determining HBeAg in the hepatocyte medium at day 14 after infection (Fig. 32B). Therefore IgG were semi-purified from mice sera by precipitation with 36 % Na_2SO_4 and 0.5 mg/ml, 1.0 mg/ml and 2.5 mg/ml total IgG from the precipitated sera were incubated with 5.5 x 10^5 ge HBV. The enhancing effect on HBV infection was also observed with Na_2SO_4 precipitated mouse sera (Fig. 32C) in a dose dependent manner. Dynamics of HBV infection during 14 day period reflected that already at day 6 post infection concentration of HBsAg was markedly higher in primary hepatocyte medium infected with HBV which was incubated with 1.0 and 0.2 mg/ml mice sera than in the medium from hepatocytes infected with HBV in the presence of PTH medium only (Fig. 32C). Therefore, in the light of these observations, it has to be taken into account that in the given experimental model, the component in the BALB/c mice sera was present which was able to enhance HBV infection of the primary *Tupaia*

hepatocytes. Thus, to determine HBV infection neutralization capacity of the tested antisera, infection in the presence of pre-immune serum or in the presence of EGFP antiserum (in case when it exceeded the pre-immune serum) was referred as 100% infection.

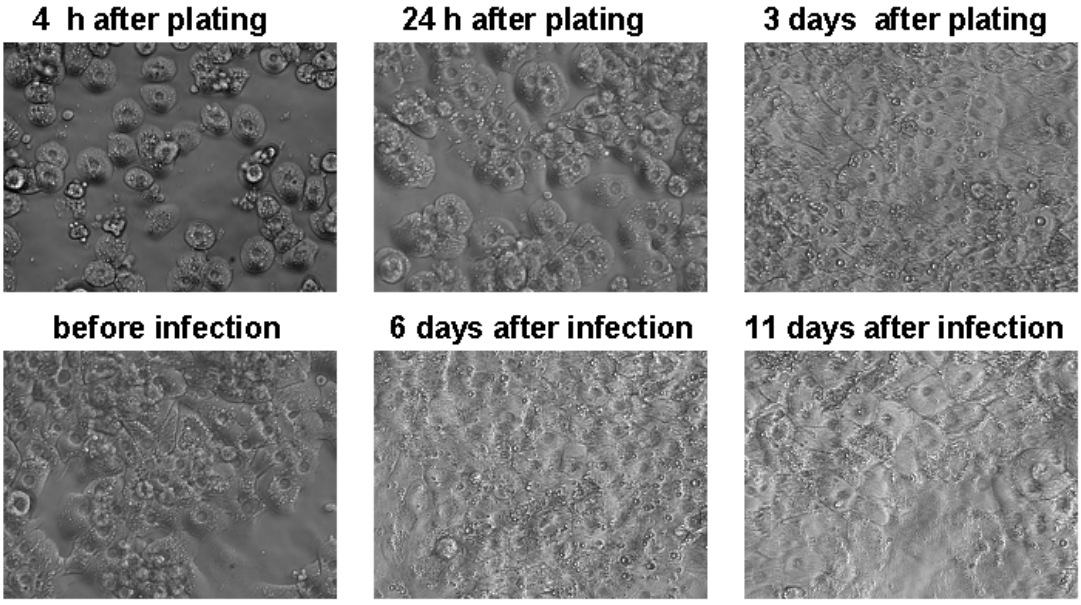

Figure 31. Primary hepatocytes of *Tupaia belangeri* at various time points after plating on Matrigel^{TM} -coated 12-well plates. (Magnification, x 320).

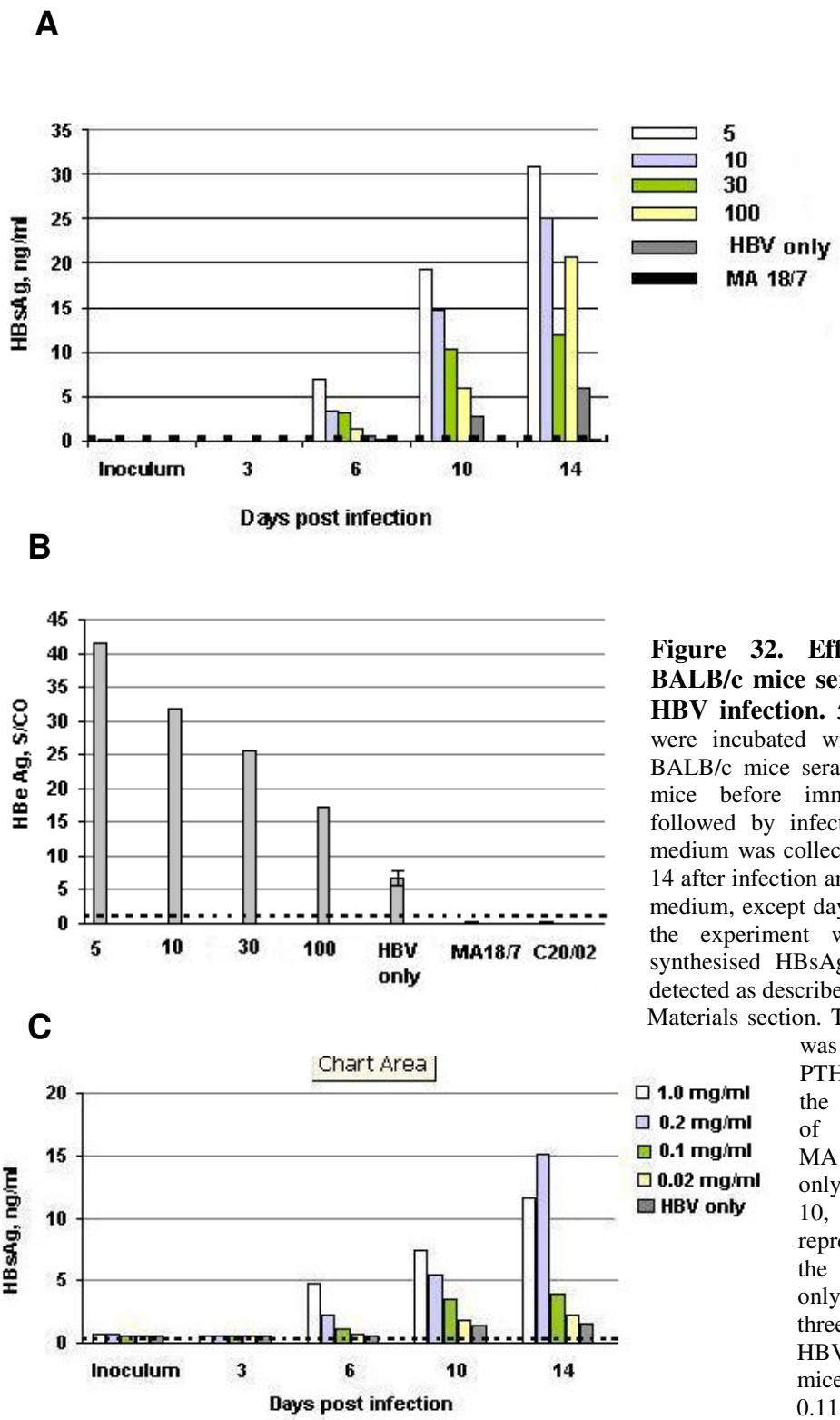

Figure 32. Effect of pre-immune BALB/c mice serum on the course of HBV infection. 5 ge of HBV/hepatocyte were incubated with various dilutions of BALB/c mice sera in PTH, obtained from mice before immunization with rSFV, followed by infection of PTH. (A) PTH medium was collected on days 3, 6, 10 and 14 after infection and replaced by fresh PTH medium, except day 14 after infection when the experiment was terminated. Newly synthesised HBsAg in PTH medium was detected as described in Methods and Materials section. The HBsAg and HBe Ag was below the cut-off for the PTH infected with HBV in the presence of 0.02 mg/ml of monoclonal antibodies MA18/7 and C20/02 (shown only in B). The numbers (5, 10, 30, 100) in the legend represent "fold" dilutions of the mice sera, "HBV only"denotes the mean of three wells, infected with HBV without the presence of mice serum, the SD was ± 0.11 for day 6, ±1.27 for day 10 and ±3.14 for day 14. (B) HBe Ag, accumulated from day 10 to day 14 in PTH medium was measured as described in Materials and Methods section. The bar denotes the SD of the three wells. The numbers in the legend of (A) and on the x axis of (B) denote the fold dilutions of mice sera in PTH.(C) Pre-immune BALB/c mice sera was precipitated with 36% Na_2SO_4 as described in Materials and Methods section and added at 1.0, 0.2, 0.1 and 0.02 mg/ml total IgG to 5 ge of HBV/hepatocyte prior infection of PTH. PTH were obtained from a male animal, 4 years old (A and B) and from a 6 years old female animal (C).

Antisera obtained with all four rSFV vectors contained well detectable amounts of HBV neutralizing antibodies, but the HBV infection inhibition potential differed among the four antisera. Antisera obtained with vectors for HBsAg/ayw2 and HBsAg/adw2 neutralized completely at 2.5 and 0.5 mg/ml as shown by complete suppression of HBsAg or HBeAg secretion (Fig. 34) and HBV mRNA synthesis (Fig. 36). Antiserum to preS1.1-48/S inhibited

completely the infection of HBV at 2.5 mg/ml, and the HBsAg and HBe Ag was only marginally above the cut-off at 0.5 mg/ml (Fig. 33A, B, Fig. 34). According to quantitative determination of HBV mRNA, the afore mentioned antiserum neutralized completely at 1.0 and 2.5 mg/ml (Fig. 36).

The absence of the conformational anti-S antibodies in the serum generated after immunization with preS1.1-48/S_0 lead to the weakest HBV infection inhibition capacity at 0.5 mg/ml (Fig. 33A, Fig. 34). The difference in neutralization capacity between antibodies induced to two variants of the preS1.1-48/S fusion protein could be diminished if antibodies to preS1.1-48/S_0 were incubated in 5 fold higher concentration (2.5 mg/ml) with HBV, the inhibitory capacity being completely neutralizing and comparable to antibodies generated to preS1.1-48/S protein (Fig. 32B, Fig. 34B). This result suggests that the anti-preS1 antibodies alone mediated the neutralization if present in sufficient concentration since the antisera did not contain anti-S antibodies (Fig. 26).

Surprisingly, antibodies generated to S/adw2 neutralized infectivity of HBV of genotype D, subtype ayw2, with an equal efficiency as the antibodies to S/ayw2. This is in line with the ELISA data of Figure 25 obtained with natural HBsAg particles but in contradiction with the ELISA data obtained with yeast-derived HBsAg (Fig. 26A). Inclusion of the preS1 region directly responsible for the binding of HBV to hepatocytes did not favour more potent infection neutralization capacity of the generated antibodies.

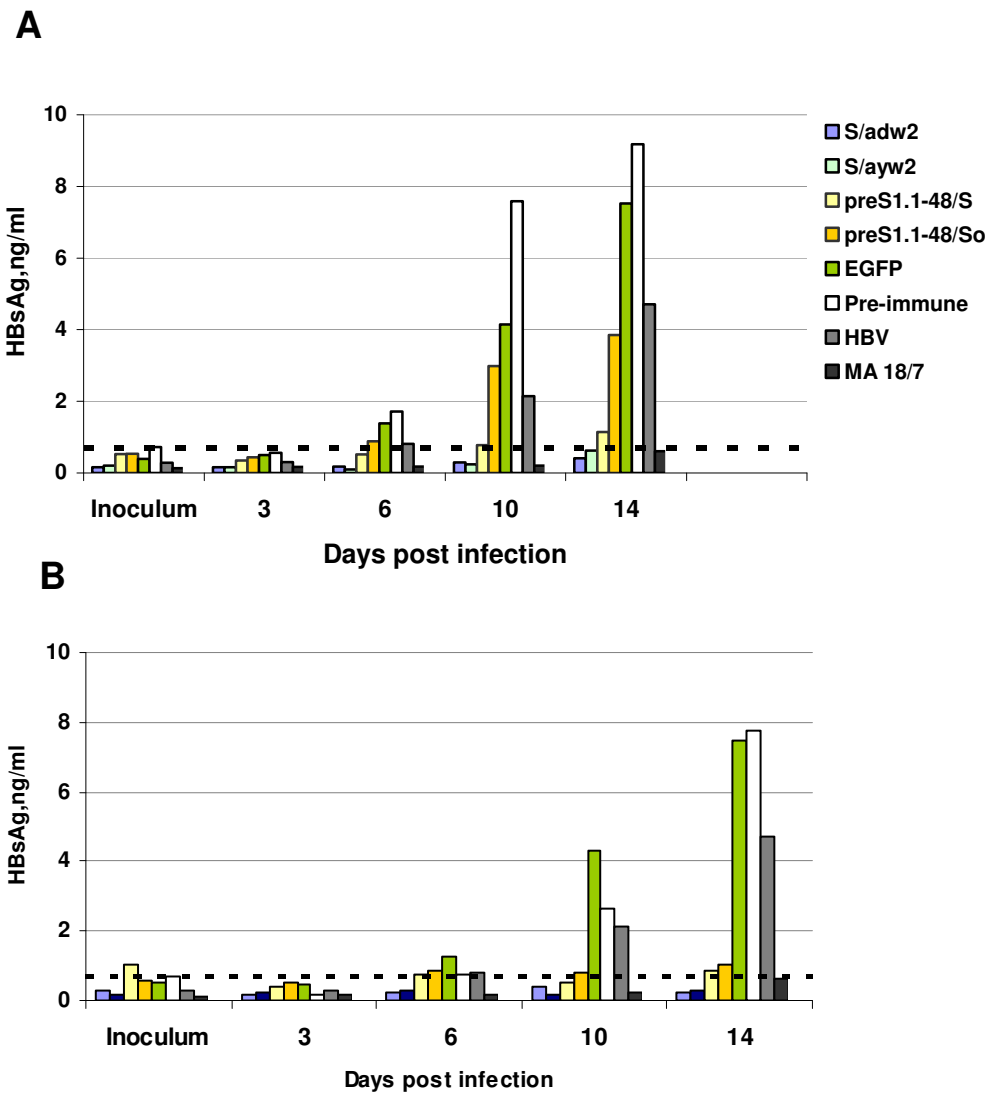

Figure 33. HBsAg detected in PTH medium. 5 ge of HBV/hepatocyte were incubated with 0.5 mg/ml (A) and 2.5 mg/ml (B) total IgG from the Na_2SO_4 precipitated sera, with PTH only (HBV), or 0.02 mg/ml of monoclonal antibodies MA18/7 or C20/02 followed by infection of PTH cultures. PTH medium was collected at indicated days post infection and HBsAg was measured by ELISA as described in Methods and Materials section. HBsAg was measured also in the inoculum, which was removed from PTH 18 hours after infection.

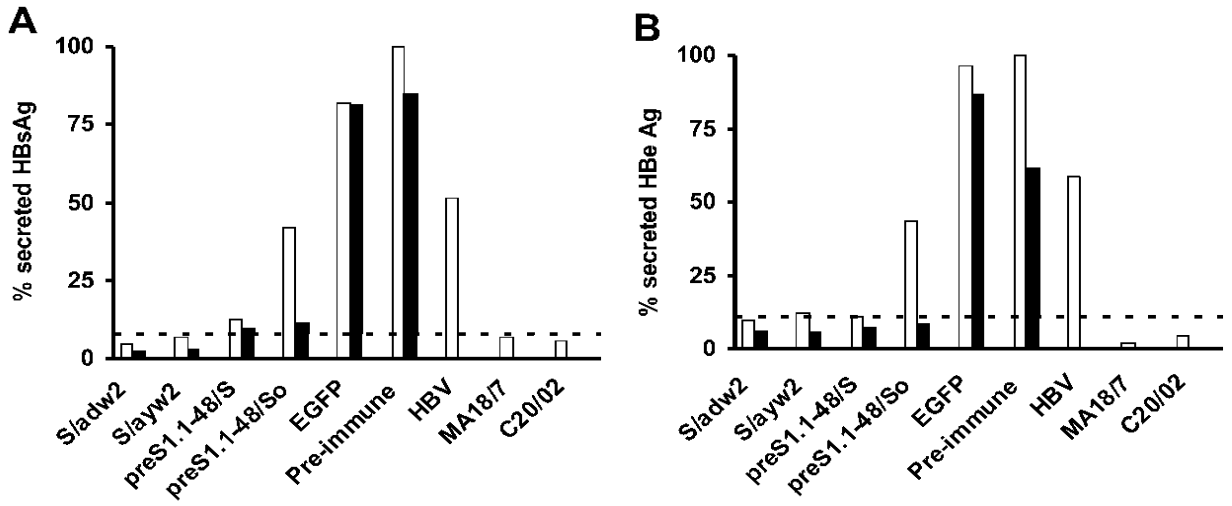

Figure 34. Markers of HBV infection in PTH cultures after inoculation with virus/antibody mixtures. Results are represented as percentage of the control infection with HBV in the presence of 0.5 mg/ml pre-immune serum. HBsAg (A) and HBe Ag (B) accumulated from day 10 to day 14 after infection in the medium of PTH cultures were measured by ELISA. HBV (5 ge/hepatocyte) was incubated with 0.5 mg/ml (light bars) and 2.5 mg/ml (dark bars) total IgG from the Na_2SO_4 precipitated sera before infection, or with PTH only (HBV), or 0.02 mg/ml of monoclonal antibodies MA18/7 or C20/02. The horizontal dashed line denotes cut-off, which was 0.7 ng/ml for HBsAg and 1 S/CO (signal/cut-off ratio) for HBeAg. The PTH were isolated from male *Tupaia belangeri*, less than a year old.

Upon infection of hepatocytes, including primary *Tupaia belangeri* hepatocytes, ccc DNA is formed in infected hepatocytes from the relaxed circular DNA which is a sign of infection and start of the HBV replication cycle. It is the earliest marker of HBV infection. The discrimination of relaxed circular DNA and ccc DNA depends on the use of a special temperature protocol with very rapid temperature changes in the quartz capillaries (Glebe et al., 2003). Only the cloned dimeric HBV DNA samples within the plasmid (log 2 – log 7) could be quantified in the experimental conditions described. Purified HBV from human plasma (2×10^5 ge) served as a serum standard and, as expected, was negative (Fig. 35A), since the virion DNA is relaxed circular with a nick-and-gap. In the experimental setting described where viral DNA had been extracted from PTH initially infected with 5 HBV ge per one hepatocyte the detection limit for the quantification of cccDNA could not be reached. Glebe et al. (Glebe et al., 2003) have described detection of cccDNA from PTH infected with 10 ge/hepatocyte, but not with 1 ge/hepatocyte. It was demonstrated by the negative signals obtained from viral DNA extracted from PTH infected with HBV in the presence of PTH medium only. The infection of PTH hepatocytes with HBV in the presence of pre-immune serum, which showed and HBV infection enhancing effect also failed to reach the detection limit necessary for cccDNA quantification.

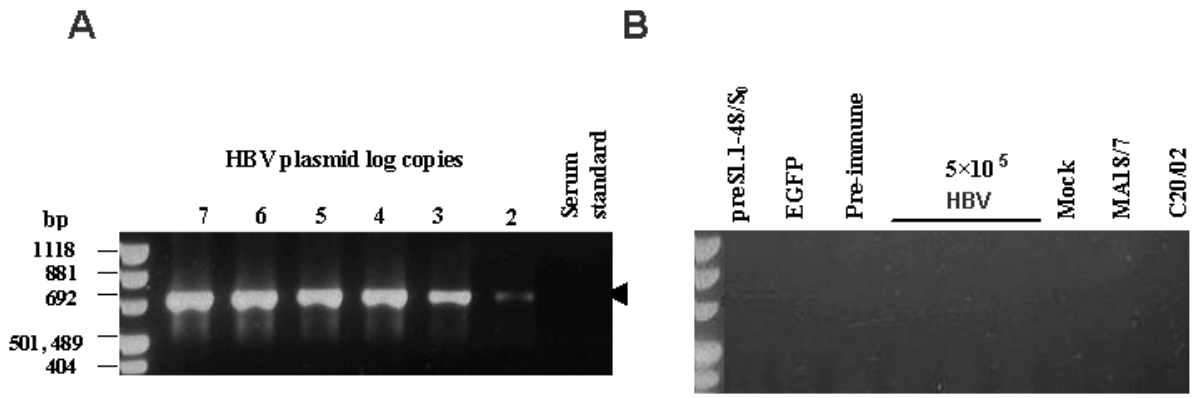

Figure 35. **2% agarose gel electrophoresis of real-time PCR reaction mixes after amplification and quantification of ccc DNA in *Tupaia belangeri* primary hepatocytes infected with HBV, which was preincubated with the antisera**. 734 bp amlplificate could be detected only in HBV plasmid samples, indicated by a triangle in panel **A**. The viral DNA isolated from 10^5 PTH initially infected with 5 x 10^5 ge HBV and amplified as described in Materials and Methods section was too low to be detectable by real-time PCR in the above described experimental conditions. Agarose gel electrophoresis confirms only presence of HBV plasmid standart pBs HB 991 T7 Dimer (10^7 – 10^2 copies), (see panel **A**). Panel B – only samples obtained from 10^5 PTH infected with 5 x 10^5 HBV ge in the presence of preS1.1-48/S_0, EGFP and pre-immune serum are shown. Next three lanes (5 x10^5 HBV) represent PTH infected with HBV in the presence of PTH medium, MA 18/7, C20/02 - PTH infected with with 5 x 10^5 ge HBV, but in the presence of HBV infection inhibiting MAbs MA18/7 (anti-preS1) and C20/02 (anti-S). 735 bp amplificate could also not be detected from PTH infected with 250 µg IgG from precipitated sera and 1:10 diluted sera during the incubation step of HBV with the antisera (not shown).

With 5 ge/cell HBV mRNA was detectable in HBV-infected PTH 14 days after infection. 1 ge/hepatocyte is enough to detect mRNA in cytoplasmic extracts of PTH 12 days p.i. (Glebe et al., 2003). The detection limit of HBV mRNA real-time PCR is 10^4 copies per 10^5 cells. For quantification of HBV mRNA 5 ge/hepatocyte were incubated with antisera diluted 1:10 with PTH, as well as with 1.0 mg/ml and 2.5 mg/ml of IgG from the Na_2SO_4 precipitated sera. HBV was incubated with PTH only, and additionally with 0.02 mg/ml MAbs MA/187 and C20/02. The results of the HBV mRNA quantification are shown in Figure 26 A and B. The mRNA analysis shows that the four anti-rSFV sera encoding HBV surface proteins were completely HBV infection neutralizing, when applied in dilution 1:10. The mRNA isolated from PTH infected with HBV incubated in the presence of anti-rSFV/EGFP sera showed the highest number of HBV mRNA copies in the cell cytoplasm.

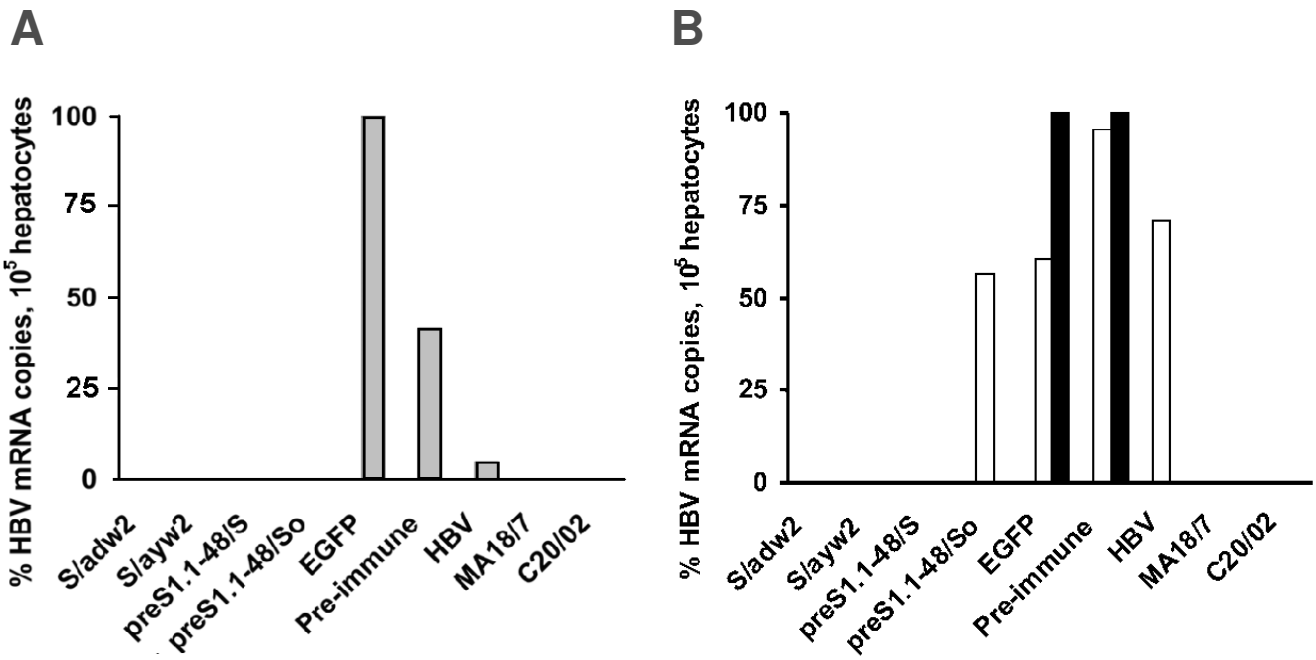

Figure 36. Quantification of HBV mRNA by real-time RT-PCR from cytoplasmic fractions of PTH 14 days after infection. 5 ge of HBV per hepatocyte before infection were incubated with sera diluted 10 fold with PTH (A), and with 1.0 mg/ml (light bars) and 2.5 mg/ml (dark bars) of IgG from the Na_2SO_4 precipitated sera (B), with PTH only (HBV) and with 0.02 mg/ml monoclonal antibodies MA/187 and C20/02. No bar means that the mRNA was below the detection level of 10^4 copies per 10^5 cells or 1% of the anti-rSFV-EGFP value for A and 1.5% for B. The PTH were isolated from male *Tupaia belangeri*, less than a year old.

4. DISCUSSION

The aim of the study was the generation of constructs based on the vector derived from SFV for immunization against HBV which would present broadly neutralising epitopes of SHBs and LHBs with the background of strong innate immune reactions provoked by the vector.

The SFV1 vectors encoding two subtypes of SHBs were selected for immunization along with vectors coding for two variants of deleted L proteins. Antibodies to HBsAg subtypes adw2 or ayw2, and to deleted L proteins with wt myristic acid attachment site have been generated via two i.v. injections of rSFV to inbred mouse strain BALB/c. The neutralizing potential in an *in vitro* HBV infection model using primary *Tupaia belangeri* hepatocytes was investigated.

Antibodies formed after vaccination with the currently available HBV vaccine (MacAleer 1984) consisting of recombinant SHBs produced in yeast are completely infection neutralizing and protective. Moreover, monoclonal, conformation dependent anti-HBs antibodies can neutralize HBV infection with an equal efficiency as monoclonal antibodies recognizing preS1 aa 20-23 in vitro (Glebe et al., 2003). But, interestingly, subviral particles consisting of only SHBs do not bind to primary Tupaia belangeri hepatocytes, while particles with added 48 N-terminal aa of preS1 exhibited efficient binding (Glebe et al., 2005).

There are numerous reasons for improvement of the current HBV vaccine. One of the problems is that HBsAg is highly variable under immune selection and the inclusion of the HBV infection neutralizing preS1 epitope is of major significance because the current vaccine cannot provide protection to escape mutants with the exchanges in the antigenic loop (Grethe et al., 2000). Indeed, widespread HBV vaccination is one of the major driving forces for selection and propagation of immune-escape mutants. Appearance of HBV mutants with exchanges in the SHBs antigenic loop has been frequently reported from areas of high endemicity with high coverage of immunization programs (Lazarevic, 2014).

One of the crucial drawbacks is the non-respnse and/or poor anti-HBs response to the vaccine - anti-HBs levels of 5-10% of the vaccinated individuals (Alper, 1995) do not exceed the borderline of sufficient immunoprotective level of anti-HBs which is generally considered as 10 mIU/ml. The occurrence of non-response or poor response is even more pronounced in elderly people (Wolters et al., 2003), where it can reach up to 60% (W.H.Gerlich, personal communication) and immunosuppressed, for example HIV infected individuals (Wolters et al., 2009; Landrum et al., 2010). Other concerns associated with the vaccine are vaccine breakthrough infections which are continually observed despite successful implementation of HBV vaccination programmes (Stramer et al., 2011). Appearance of *S* gene mutants has been detected in breakthrough infections in vaccinated individuals (Chang, 2010). Breakthrough HBV infections of non-vaccine genotype have been also documented (Abushady et al. 2011; Seed et al. 2012). Escape mutants with aa exchanges in the antigenic loop of SHBs which may evade anti-HBs mediated protection establish ground for addition of preS1 epitopes. Studies in highly endemic regions of South-East Asia showed that breakthrough infections may occur in the second decade after vaccination which had been performed in infancy, with incidence of occult infections (Poovorawan et al., 2011;Su et al., 2012). This shows waning HBV immunity over time. In the light of newly discovered zoonotic reservoir of HBV-like virus in tent-making bats which is able to infect human hepatocytes via HBs protein-mediated infection and cannot be neutralized by sera from vaccinated humans (Drexler et al., 2013), search for improvements of the current HBV vaccine appear even more significant and relevant.

PreS containing vaccines, expressed in mammalian cells, have already been successfully used as vaccines in humans (Shapira et al., 2001; Bertino, Jr. et al., 1997),

(Shouval et al., 1994; Zuckerman et al., 2001;Hellstrom et al., 2009). It is not known whether their superior anti-HBs induction is due to the inclusion of preS or the better folding of the conformational S epitopes by mammalian cells.

Numerous studies have been carried out towards an improved HBV vaccine so far. Various immunization platforms or carriers for delivery of HBs proteins have been employed in small animal models. The vaccination platforms may be distinguished as those enabling "direct" delivery to experimental animals of purified HBs proteins. The other group is the vaccine vehicles allowing for intracellular or endogenous expression mimicking a "natural" infection and processing of HBs proteins in the experimental animal. HBs proteins have been delivered solely as SHBs particles, and with preS2 and preS1 domains. PreS2 and preS1 containing HBsAg has been expressed in Chinese hamster ovary cells as an alternative HBV vaccine and delivered to human recipients (Shapira et al., 2001). Immunization of experimental animals with HBsAg has often been carried out together with oligonucleotides containing immunostimulatory CpG motifs as adjuvants (Weeratna et al., 2003; Malanchere-Bres et al., 2001; Siegrist et al., 2004). Vesicular carriers, like liposomes and ethosomes, have attracted attention as vehicles for delivery of antigens, including also HBsAg (Mishra et al., 2008). HBsAg has been efficiently encapsulated into nanoparticles based on polymers of polylactic acid and PEG (Jain et al., 2009) and delivered to experimental animals by s.c. and oral routes. HBs Ag can also be encapsulated by lectin-linked polylactic-co-glycolic acid nanoparticles specifically targeted for oral administration (Mishra et al., 2011). Importantly, HBsAg loaded liposomes and ethosomes can be efficiently taken up by human dendritic cells (Mishra et al., 2010).

Virus-like particle carriers play a prominent role as non-infectious vaccine carriers and gene therapy tools of particulate nature. They are virus-protein derived multimeric structures that lack the virus genome. Among the VLPs, which have been developed from more than 30 viruses (Roldao et al., 2010). Among them HBc protein has evolved as a promising carrier of immunological epitopes (Pumpens and Grens, 2001). For example, preS1 aa 13-59 linked to 90-119 (genotype A numeration) have been inserted into the major immunodominant region of HBc protein (Skrastina et al., 2008). Due to ability to incorporate foreign epitopes without hindering the self-assembly ability SHBs itself has served as a vaccine carrier of foreign epitopes (Vietheer et al., 2007; Netter et al., 2001; Pumpens et al., 2002b).

Genetic immunization has evolved rapidly since the first studies in 1990's and involves delivery of antigen coding gene as plasmid DNA or mRNA for expression and processing of the antigen in the experimental animal. Several studies describe immunization with plasmid DNA encoding SHBs (Davis et al., 1994; Michel et al., 1995). DNA may be delivered naked or encapsulated into liposomes. Intranasal delivery of plasmid DNA encoding HBsAg encapsulated into glycol chitosan coated liposomes (Khatri et al., 2008) resulted in systemic and mucosal humoral specific immune response. While studies in small animals have been promising, the experiments with chimpanzees have not been satisfactory (Payette et al., 2006). Although, there are clear benefits of the approach, like ease of production, ability to prime both $CD8^+$ and $CD4^+$ cells, a potential risk of integration into host cell genome exists (Nichols et al., 1995). Alternatively, use of whole bacteriophage lambda particles containing expression cassette of a DNA vaccine has been reported (March et al., 2004). HBsAg has been successfully expressed in transgenic plants and delivered to human recipients as an edible HBV vaccine (Thanavala et al., 2005; Guan et al., 2010), but has delivered disappointing results with 40% of the volunteers being nonresponders (Thanavala et al., 2005).

Viruses are excellent stimulators of immune system due to a number of features. Vaccines based on attenuated viruses have proven to be extremely successful (Röhn and Bachmann, 2008). Therefore viral vectors have evolved as potential delivery tools of antigens or immunodominant epitopes. The immunogenicity of live recombinant human adenoviruses Ad4 and Ad7 encoding HBsAg in a chimpanzee model has been tested (Lubeck et al., 1989),

unfortunately the use of recombinant adenoviruses is accompanied by significant humoral response to the adenovirus (Lubeck et al., 1989) and very often a strong acute inflammatory response (Bangari and Mittal, 2006). Immunogenicity of adenovirus vector encoded antigens is significantly impaired by adenovirus protein specific T-cell immunity (Schirmbeck et al., 2008). Application of Ad5 in human recipients is hindered by the widespread immunity in population (Seregin and Amalfitano, 2009). Modified vaccinia virus Ankara (MVA) vectors, based on a highly attenuated strain of vaccinia virus encoding MHBs (Hutchings et al., 2005; Wu et al., 2007) induced a vigorous T cell response, but a negligible anti-HBs titer when used as a "booster" immunization after priming BALB/c mice with a DNA vaccine. By simultaneous intradermal administration of rHBsAg (5 µg/mouse) and MVA (5 x 10^6 pfu/animal), and boosting animals with the same combination, anti-HBs titer was increased about 6-fold compared to rHBsAg alone. Cell mediated response as measured by IFN-γ secreting cells from spleen and distal lymph nodes was considerable , while rHBs Ag alone administered as a vaccine formulation resulted in a negligible number of IFN-γ secreting cells after specific stimulation with a peptide. Importantly, as shown by co-administration of rHBsAg and non-recombinant, "empty" MVA, the enhanced $CD8^+$ responses were due to intracellular production of the antigen. Thus, the co-administered recombinant MVA encoding the M protein of HBV can exert a potent adjuvant effect on the co-administered HBsAg (Hutchings et al., 2005). A single intramuscular injection of 10^7 integrating and non-integrating lentivirus particles vectors encoding HBsAg resulted in an elevated humoral response compared to a DNA injection (Karwacz et al., 2009). The antibody response remained stable up to 8 weeks after injection, but decreased in mice immunized with the plasmid DNA.

It has long been recognized that for efficient induction of robust immune response of both arms of the immune system other factors than a presence of antigen encoding sequences is required. Although, plasmid DNA contains immunostimulatory motifs like unmethylated CpG dinucleotides (Klinman et al., 1997), additional adjuvant signals are necessary to mimick a "natural" infection. In concert with the search for novel vaccine strategies employing the adjuvant signals, a study with the aim to generate a live attenuated vaccine described an attenuated strain of *Mycobacterium smegmatis* which is transformed with the genes for fusion protein consisting of a truncated core protein and preS1 peptide aa 1-55. The study found that after vaccination with live recombinant *M. smegmatis* a stronger cellular immune response was induced accompanied by a longer duration of humoral immune response compared to the DNA vaccination (Yue et al., 2007). Attempts have been made to enhance the immunogenicity of DNA vaccines by co-transfection of interferon regulatory factors (Sasaki et al., 2002) to resemble a virus infection.

rSFV vectors deliver potent adjuvant signals to the immune system – induction of type I interferons (Hidmark et al., 2005) and induction of apoptosis. rSFV replicons generate robust and protective immune responses to important human viral pathogens in various animal models including mice, rabbits and rhesus macaques (Ljungberg and Liljeström, 2015). In addition, alphavirus replicons have been used as vaccine platforms to extremely pathogenic viruses, like Ebola, Lassa and Marburg virus (Hevey et al., 1998; Pushko et al., 2001; Hevey et al., 2001). Immunization with recombinant alphavirus replicons has been addressed primarily to viral targets, although there are several studies describing use of alphavirus vectors for bacterial targets. SIN replicon has been used in a study contributing to research in the improvement of vaccines to *Mycobacterium tuberculosis*. C57BL/6 mice were injected twice with an interval of two weeks subcutaneously with SIN replicon plasmid encoding antigen 85A. 28 days after the last injection mice were challenged with *M. tuberculosis*. An enhanced long-term protection against M. tuberculosis was achieved compared to the conventional DNA vector. Sindbis replicon-based plasmid was highly immunogenic at much lower doses than the conventional DNA plasmid: doses from 2 to 5 µg the IFN-γ production equaled to that of the conventional DNA plasmid at 100 µg. Attempts

have been undertaken to develop alphavirus vector-based malaria vaccine. However, a study by Andersson C. (Andersson et al., 2001) showed that the conventional DNA plasmid elicited a higher antibody response than SFV RNA or rSFV encoding part of Plasmodium falciparum antigen Pf332. On the other hand, use of replication-competent recombinant SIN expressing a $CD8^+$ T-cell epitope of the circumsporozoite protein of *Plasmodium yoelii* showed that the $CD8^+$ response achieved in this study exceeded that of studies using recombinant vaccinia, influenza, and adenovirus vectors (Murata et al., 1996; Tsuji et al., 1998). The expressed SHBs and preS1.1-48/S proteins by SFV1 vectors did not interfere with the packaging and release of the recombinant SFV particles, and rSFV could be recovered in sufficient titers. It has been described that Rift Valley fever virus glycoprotein strongly intervened with the packaging and release of the recombinant VEE (Gorchakov et al., 2007). The titers of recombinant SFV encoding HBV surface proteins were up to 10^9 infectious units/ml, while titers of replication proficient VEE virions, reach 10^7- 10^8 and in some cases 10^{10} plaque forming units/ml (Gorchakov et al., 2007).

The intracellular distribution of the SHBs expressed by rSFV was diffuse (Fig 15). Chua et al. showed a granular staining pattern of SHBs in transfected Huh-7 cells (Chua et al., 2005). In contrast, Patient et al. (Patient et al., 2007) demonstrated a compact, perinuclear localization of SHBs, colocalization with an ER marker, and ERGIC53, a specific ERGIC marker. We observed an efficient secretion of SHBs by SFV1-S/adw2 infected BHK-21 (Fig. 14) and Huh-7 cells (not shown), while Patient et al. report limited secretion of SHBs after BHK-21 cell electroporation with SFV1 encoding SHBs, compared to HBsAg signal provided by the MHBs protein transfected cells (Patient et al., 2007). In this study MHBs showed a diffuse distribution within the cell. The authors attribute the limited secretion of SHBs to the cytotoxicity of SFV1 expression system that prevents culture longer than 24 hours after transfection, fast protein synthesis and accumulation of SHBs in the early compartments of the secretory pathway. Patient et al. (Patient et al., 2007) used electroporation with recombinant RNA, whereas in the study described here infection with rSFV was employed. We noted that electroporation with recombinant SFV RNA is more cytotoxic to transfected cells compared to infection with rSFV particles.

We observed internal translation initiation at the next translational start codon at the beginning of SHBs coding sequence, as shown by expression of the characteristic doublet of glycosylated and non-glycosylated SHBs in Huh7 cells infected with rSFV-preS1.1-48/S proteins and detection by specific antibodies (Fig. 16). Patient et al. could show internal initiation of SHBs and its secretion by MHBs expressing SFV1 vector .

The expression of preS1.1-48/S protein *in vitro* unexpectedly revealed presence of three N-glycans. While the partial glycosylation at Asn146 of S was expected, the glycosylation at Asn4 of preS1 was unexpected. In natural LHBs the entire preS is not translocated to the ER and not N- glycosylated (Bruss et al., 1994). In contrast, an artificially added signal sequence at the N-terminus of L protein enables the translocation of preS1 to the ER lumen where glycosylation at Asn4 takes place (Bruss and Vieluf, 1995). The preS1.1-48/S proteins resemble M protein, where the 55 aa preS2 sequence of M can be cotranslationally translocated to the ER lumen under the control of signal I in S (Eble et al., 1987; Eble et al., 1990), and is glycosylated at Asn4. Apparently, this signal can also translocate the shortened preS1 sequence of the preS1.1-48/S proteins. The preS1 sequence of wildtype LHBs bears a Met-Gly-myristoylation motif and is modified by N-myristoyl transferase by addition of myristic acid (Persing et al., 1987). Likewise, Prange et al. reported secretion and glycosylation at the Asn 4 of a protein consisting of the first 41 aa of preS1 (subtype ayw) joined N-terminally to the S protein (Prange et al., 1995), but with already substituted N-terminal Gly with Ala to prevent myristoylation, which is implicated in retention of the L protein (Prange et al., 1991). Chai et al., however, did not detect glycosylation at the Asn4 (Asn 15 in subtype adw2, genotype A, used in the study) in the chimeric preS1 proteins consisting of aa 1-59 and 1-30 of preS1 fused to Fc domain of a rabbit immunoglobulin G

(Chai et al., 2007). Moreover, the chimeric preS1 proteins are efficiently secreted by unconventional pathway, which by-passed ER and glycosylation. Our data obtained with preS1.1-48/S_0 mutant suggest that the linkage of preS1.1-48 to S suppresses this unconventional pathway and leads, in contrast, to inhibition of the S domain-mediated secretion. Importantly, Chai et al. report myristoylation at preS1. Thus, it appears that myristoylation and glycosylation of preS1 at Asn4 in these constructs is mutually exclusive (Chai et al., 2007). This is noteworthy in light of the results demonstrated here, which showed that all preS1.1-48/S proteins are glycosylated at Asn4. Although, myristoylation directly was not shown, the intracellular distribution of the preS1.1-48/S bearing N-terminal Gly showed clear difference to Gly2Ala and Gly2Ser mutants. The glycosylation pattern shown in Fig. 4 suggests that the glycosylation site at Asn3 of S in the preS1.1-48 protein with or without SHBs start codon is partially occupied. Possibly, the 7 aa-spacer between preS1 and SHBs has increased accessibility of this site for the N-glycan transferase.

It appears that there are two determinants affecting the intracellular distribution of the preS1.1-48./S proteins – the myristoylation motif at the N-terminus and the presence of the in-frame expressed SHBs. These two determinants also affect the release of the subviral particles. The change of Gly in the myristoylation motif to Ala ensures myristoylation block (Towler et al., 1988). The presence of the myristoylation motif at the N-terminus of the preS1.1-48/S protein seems to facilitate release of subviral particles, because the secreted Ser2.preS1.1-48/S protein was not released so efficiently from the cells as the *wt* protein (Fig. 18B) with the myristoylation motif, although was efficiency of expression was equal of both wt and Gly2Ser mutant BHK-21 (not shown) and Huh-7 cells (Fig. 18A). These results are in line with the published studies that myristoylation is necessary for VLP formation by several viruses. Myristoylation is formation by Moloney murine leukemia virus (Rein et al., 1986) formation and release of VLPs of Arenavirus Z protein (Strecker et al., 2006), and significant for virus particle assembly of poliovirus (Marc et al., 1991). N-terminal myristic acid serves as a membrane anchor for a number of proteins and confines affinity to membrane bilayers (Resh, 1999). With the present evidence it is not known whether absence of the myristoylation motif interferes to a more extent with subviral particle formation or with release. For the Gly2Ser mutant the preS1 signal was in a similar level as for the wt protein, while for the Gly2Ala mutant there preS1 signal was considerably less (Fig. 18A). But, both mutants displayed reduced release of subviral particles compared to *wt* protein. Whether the N-terminal myristic acid facilitates the passage of subviral particles from the site of their formation to ERGIC, Golgi, constitutive secretory pathway or at the final stage of budding of subviral particles at the plasma membrane, must be investigated.

Of the two determinants affecting secretion of subviral preS1.1-48./S particles, the presence of in-frame expressed SHBs is essential and was shown to rescue the secretion of prS1.1-48/S, possibly the presence of internally translated SHBs facilitates the trafficking of preS1.1-48/S subviral particles. Presence of the myristoylation motif plays a secondary role, and the myristic acid possibly facilatites the interaction with the cellular membranes at the sites of subviral particle fomation. Importantly, myristoylation is not necessary for formation of complete HBV virions (Gripon et al., 1995), but myristil anchor is necessary for HBV infectivity. Transfer of myristic acid attachment site to MHBs lead to loss of infectivity of hepatitis D virus (Abou-Jaoude et al., 2007).

Another factor which can possibly affect the secretion of preS1.1-48/S proteins are the presence of N-glycans. N-glycosylation is necessary for the release of virions (Mehta A. 1997). Glycosylation did not affect secretion of preS1.1-48/S, because preS1.1-48/S_0, which was completely retained, was glycosylated identically to the wt protein (Fig. 17). MHBs, which largely resembles the preS1.1-48/S, and is glycosylated at the Asn4 in the preS2, requires the glycan at Asn 4 for efficient secretion, while glycan at the S domain is not necessary (Werr and Prange, 1998). However, the release of preS1.1-48/S was completely blocked by abolishing internal translation initiation of S, while it does not block the secretion

of M protein (Werr and Prange, 1998). Since it is known that the glycan at the S domain is dispensible for release of subviral particles, the effect of the two glycans of the preS1.1-48./S protein on secretion in the presence of internally translated SHBs is a subject of future studies. It appears that the preS1 stretch is sufficient for translocation, but does not govern release, which requires internally translated SHBs. N-glycan at Asn-4 in the preS2 domain mediates binding of MHBs with ER chaperone calnexin, it is known that calnexin assists formation of HBV viral envelope. Therefore, role of the N-glycan at Asn-4 in the preS1.1-48/S protein must be investigated. Interestingly, Lambert et al. could identify two additional forms of L protein of about 48 kDa and 51 kDa by analyzing microsomal fractions obtained from transfected Cos-7, Hek 293T and Huh-7 cells. Digestion with PNGase F and Endo H confirms that the isoforms have resulted from N-glycosylation of Asn 4 and Asn112. The authors propose unconventional posttranslational N-glycosylation, which is segregated from protein synthesis (Lambert and Prange, 2007).

Although alphavirus vector system enables three ways of replicon delivery both *in vitro* and *in vivo*, we chose rSFV for delivery of HBs encoding genes, since studies have indicated the superiority of injection of rSFV in inducing immune responses and protecting mice from lethal challenge over injection of naked SFV RNA or rSFV replicon plasmids (Fleeton et al., 2000; Brand et al., 1998). Delivery via rSFV to mice ensures relatively short time of antigen persistence and exposure in the lymph nodes and spleen - 1 day and 16 h respectively (Morris-Downes et al., 2001). The disappearance of HBsAg in mice sera 72 h after systemic delivery is in conjunction with the published data. Assuming that presence of circulating HBsAg indicate SFV replicon driven synthesis of HBsAg *in vivo*, which can be detected in the bloodstream, our findings can be put in line with Colmenero et al. (Colmenero et al., 2001) who detected the presence of abundant vector RNA at day 1 post immunization which decreased by day 3 in spleen, local lymph nodes and brain, being almost undetectable by day 6. Significant levels of HBsAg up to 3 ng/ml are detected in murine sera after immunization with 100 µg of plasmid DNA immunization encoding S protein, but the amount of injected nucleic acid (Davis et al., 1993; Geissler et al., 1997) exceeded the amount of nonreplicating vector RNA in the study described here by a factor of ca. 100,000. The relatively short time of persistence of HBV surface proteins in BALB/c mice was sufficient to induce neutralizing HBV neutralizing antibodies. The desirable length of antigen exposure is dependent on the aim of the study. For an effective $CD8^+$ response longer persistence of antigen would be favourable (Rohn and Bachmann, 2008).

Virus particles injected into the bloodstream are susceptible to inactivation by complement proteins, uptake by the reticuloendothelial system, and neutralization by circulating antibodies (Power et al., 2007). The second dose of rSFV was higher in order to circumvent the eventual humoral immunity to SFV which might have evolved after the first injection of rSFV. Although rSFV are not replicating, response to structural proteins and replicase is acknowledged, but development of immune responses directed against the vector itself do not inhibit generation of immune response after subsequent immunization (Berglund et al., 1999). The anti-SFV response of BALB/c mice after administration of rSFV was not determined in this study. Generally, rSFV is successfully used in repeated administrations, while repeated administration is not possible for adenovirus derived viral vectors, because of the evolved potent immunity after the first administration prevents repeated administrations. Another hurdle for adenovirus is the pre-existing immunity (Worgall et al., 1997; Tsai et al., 2004), while pre-existing immunity to vaccinia virus can lower the magnitude of immune response in case of recombinant modified vaccinia Ankara (Gudmundsdotter et al., 2009). Several techniques have been described so far to conceal the virus vector from the circulating neutralizing antibodies (Power et al., 2007). Use of carrier cells as delivery platforms to deliver oncolytic VSV to the dessiminated tumor sites is reported. Other reported strategies to encapsulate viruses are coating with polymers (Fisher et al., 2001), complexing with polyethylene glycol and cationic lipids (Chillon et al., 1998). Encapsulation of SFV replicon

particles coding for IL-12 in cationic liposomes has been taken to phase II clinical trials (Ren et al., 2003) in adult patients with glioblastoma multiform, an incurable brain tumor.

BALB/c mice are considered good responders to SHBs, but Geissler et al. (Geissler et al., 1997) report that anti-HBs titer was 1.5 – 2 fold higher in C57BL/6 (H-2^b) mice than in BALB/c (H-2^b) mice. As expected, anti-HBs titers after two immunizations with rSFV could not reach the magnitude of anti-HBs titers which are generated by immunization of mice with plasmid DNA encoding SHBs. Reported titers after plasmid DNA based immunization are variable and not well standardised. Michel et al. (Michel et al., 1995) report anti-HBs titer more than 1:10,000 6 weeks after immunization of C57BL/6 mice with SHBs encoding CMV based plasmid. Wu et al reported that 6 weeks after initial immunization the the anti-HBsAg titer achieved by two injections of plasmid DNA (50 µg in each T.A. muscle) was 1:100, while the titer generated by initial immunization of recombinant vaccinia virus vaccine followed by two booster immunizations at weeks 2 and 5, was 1:1000 and could be comparable with the titer when mice were boosted with DNA after priming with the recombinant vaccinia vaccine (Wu et al., 2007). Weeratna R. et al. show that (Weeratna et al., 2000a) 6 weeks after immunization with 10 µg DNA plasmid encoding HBsAg/ay, a titer of 1:500 between 1:1000 could be achieved, addition of immunostimulatory motifs did not significantly increase the anti-HBs titer. The anti-HBs titer could be significantly increased with DNA prime-protein boost (1 µg HBsAg) strategy (Weeratna et al., 2000a). Immunization three times at two week intervals with 50 µg recombinant HBs resulted in anti-HBs response of up to to 1:10,000 (Skrastina et al., 2008). Single intramuscular immunization of CB6F1 mice with recombinant VSV encoding preS2 resulted in anti-HBs of up to 1000 IU/L (Cobleigh et al., 2010). In our study the end point titers of anti-HBs against S protein with the same subtype were between 1:200 and 1:1800 (Fig. 26B), the quantitative determination of anti-HBs in rSFV-adw2 immunized mice resulted in 1150 IU/L anti-HBs. The lower magnitude of the preS1 specific response generated by rSFV encoding preS1.1-48/S_0 may be linked to non-secretability of the protein. Geissler et al. reported also that nonsecreted LHBs was less immunogenic than a secreted version of HBsAg (Geissler et al., 1999). The comparatively low dose of the HBsAg deliverd by rSFV and the short life span of the transduced cells must be taken into account, therefore immunogenicity appears to be good in our system. However, induction of the anti-HBs and anti-preS1 titers seems to require higher doses of rSFV particles than reported for other viral antigens (Berglund et al., 1999; Fleeton et al., 2000).

rSFV system could be exploited to increase the magnitude of the humoral anti-HBs response after rSFV mediated delivery of HBs coding sequences. SFVC vector contains the translational enancer element which consists of the first 103 nt of SFV subgenomic RNA capsid–coding region (Sjoberg et al., 1994). It is placed downstream of the subgenomic promoter, upstream of foreign antigens. The vector provides higher expression level of foreign antigens. However, there is a view that the translational enhancer element derived from the SFV C coding sequence is not desirable for rSFV vectors intended for application *in vivo* due to possibly undesirable immune response to virus structural proteins. Nevertheless, a study by Forsell et al. describes insertion of internal signal sequence, derived from the SFV E1 spike protein, for increased expression and secretion of solube antigens between the enhancer element and the gp120 of HIV-1 and administration of 1 x10^7 rSFV s.c. at 3 week interval to mice. The authors observed considerable increase in ELISA end-point titres, compared to the parental vector without the enhancer element and internal signal sequence. Markedly higher levels of gp120 were synthesized in case of the enhanced rSFV vectors, which subsequently produced 1-3 logs higher humoral response, additionally 90-100% of immunized animals had responded, compared to 14-17% respondees immunized with the parental vector (Forsell et al., 2007).

Humoral response to HBs proteins was determined 3 weeks after the second injection with rSFV, therefore it is not possible to evaluate kinetics of antibody production and

sustainability of the induced anti-HBs response over longer period of time. Detection of specific IgGs indicate a class switch from IgM. The anti-S antibodies generated via rSFV mediated immunization exhibited a strong subdeterminant preference in ELISAs employing yeast-expressed HBsAg (Fig. 26A, B). Davis and others (Davis et al., 1995) also showed higher anti-HBs titers after DNA immunisation when using the homologous HBsAg subtype for assessment of anti-HBs response. Such differences between the HBsAg subtypes were not observed in this study when human plasma-derived HBsAg was used for ELISA of the rSFV induced anti-HBs. Although the generated anti-HBs/adw2 antibodies in this study showed preference to recombinant adw2, they neutralized the binding of HBV/ayw2 to primary *Tupaia belangeri* hepatocytes equally well with anti-HBs/ayw2 (Fig. 33A, B). These results must be considered in context of the present vaccination policy and the protective power of the current HBV vaccine. The most widely used vaccine bears HBsAg subtype adw2 (genotype A2) (De et al., 1985;McAleer et al., 1984), but the prevailing HBs subtype is ayw, genotype D. It is generally considered that 10 IU/L anti-HBs are believed to protect against infection of all HBV subtypes (Fitzsimons et al., 2005), however, recent findings suggest that the protective titer of 10 IU/L anti-HBs is only able to completely protect against genotype A2 (vaccine genotype), whereas against other genotypes >100 IU/L anti-HBs were required. Importantly, only 47% of the vaccinated American blood donors had >100 IU/L anti-HBs (Stramer et al., 2011). The results presented in this study suggest that the yeast-derived HBV vaccine bears a strong subtype dependency, which, possibly, could be overcome with mammalian cell-derived or vector-type vaccines. The difference between yeast and mammalian-derived HBsAg rests presumably in the folding of the HBsAg loop. Anti-HBs from yeast–dervied vaccine recipients is highly reactive with denatured SHB or with SHBs peptides in Western blots, while anti-HBs induced by mammalian-derived HBs is non-reactive (W.H. Gerlich, personal communication, C. M. Bremer, unpublished data).

It is known that the deficient response to current HBV vaccine of non-responders, who develop less than 10 IU/L anti-HBs has been attributed to the absence of HBsAg-specific Th1-like cells or their anergy (Chedid et al., 1997). Disposition to Th1 activity shown by IgG1/IgG2a sublass ratios was shown in this study. The response was IgG2a biased, but to a different scale for each of the immunized groups. The IgG2a dominance is in line with other studies employing rSFVvectors (Berglund et al., 1999;Andersson et al., 2001). IgG2a is dominant after a single intramuscular injection of integrating and non-integrating lentivirus vector (Karwacz et al., 2009). rSFV-preS1.1-48/S_0 does not allow the secretion of HBsAg particles, and mice immunized with this rSFV induced the most prominent IgG1 while the isogenic vector with expression of SHBs had most IgG2a. In contrast, VLPs, including recombinant HBs induce predominantly Th2-type response (Skrastina et al., 2008). Th1 dominance after immunization with SHBs could be achieved only after co-administration with synthetic oligodeoxynucleotides containing CpG motifs (Weeratna et al., 2000b), while delivery of SHBs by polylactic acid and PEG copolymer nanapoarticles resulted in a mixed Th1/Th2 response (Jain et al., 2009).

Primary Tupaia hepatocyte cultures of *Tupaia belangeri* were successfully used in this study as *in vitro* model for evaluation of the neutralization capacity of antibodies generated to HBs via rSFV mediated immunization. Elevated efficiency of HBV infection of primary *Tupaia belangeri* hepatocytes using pre-immune BALB/c mice sera and sera from rSFV-EGFP immunized mice compared to infection with HBV in the presence of PTH medium only was observed, while, interestingly, in presence of human serum the HBV infection of PTH is inhibited (Kock et al., 2001). Numerous serum associated proteins have been found to interact with HBV, including polymerized serum albumin (Imai et al., 1979; Dash et al., 1991) and apolipoprotein H, which have been shown to be involved in HBV surface antigen (Mehdi et al., 1996) and HBV particle binding (Stefas et al., 2001). It has been suggested that lipoprotein lipase, an enzyme involved in lipoprotein metabolism might bind with preS and HBV particles (Deng et al., 2007).

The preS1.1-48 component reduced the secretion of the HBsAg and resulted in slightly lower anti-HBs titers in immunized mice. Such a negative effect of reduced HBsAg secretion on immunogenicity of HBsAg expression vectors was reported previously (Geissler et al., 1999). The HBV neutralization capacity of preS1.1-48/S antisera was equal with that of antisera generated to anti-S only. This is in line with the observation that monoclonal, conformation dependent anti-HBs antibodies can neutralize the HBV infection with an equal efficiency as monoclonal antibodies recognizing preS1 20-23 *in vitro* (Glebe et al., 2003). The results obtained here with the rSFV-preS1.1-48/S_0 indicate that for efficient neutralization of HBV infection presence of anti-S is necessary. Although, the primary determinant of HBV infectivity is preS1, the efficient neutralization potential of anti-S observed here underlines the significance of antigenic loop as the second infectivity determinant of HBV. Antigenic loop contains conformation dependent HBV entry determinant. The aa residues responsible for infectivity are conserved in the sequences of all human HBV genotypes and are clustered in close proximity of cysteines forming the disulfide bridges. The infectivity determinant can be separeted from antigenicity of „a" determinant, as shown by the most frequently occuring HBV „escape mutant" G145R. Aa exchange has detrimental effects on antigenicity, without affecting the infectivity: the G145R mutant was even more infectious than *wt* (Salisse and Sureau, 2009). Therefore inclusion of the HBV infection neutralizing preS1 epitope is of major significance, since anti-preS1 can interfere with another entry determinant - N terminal part of preS1.

For a long time primary human hepatocytes have served as *in vitro* model for HBV-related research. The availability of primary human hepatocytes was scarce, the low infection efficiency of *in vitro* cultures with HBV had to be increased by use of dimethylsulfoxide (DMSO) and polyethyleneglycol (PEG) (Gripon et al., 1988; Gripon et al., 1993). The quality of hepatocytes was extremely heterogenous as well as susceptibility to infection between preparations (Glebe, 2006). In 2002 a permissive cell line HepaRG was developed, however, before infection cells have to be cultivated in presence of DMSO and hydrocortisone to promote and sustain their differentiation and HBV infection is effective in presence of PEG only (Gripon et al., 2002). For efficient infection of PTH with HBV the fusogenic agent PEG and DMSO is not used (Glebe et al., 2003), nor it was used in this study. Efficiency of PTH infection with HBV also depends on the quality of isolated hepatocytes, which is variable between experiments, although the reproduceability between experiments is greater, compared to primary human hepatocytes (D. Glebe, personal communication). Cell lines stably transfected for HBV receptor sodium taurocholate co-transporting polypeptide can now also serve as an *in vitro* model of HBV infection (Ko et al., 2015).

In this study hepatocytes of Asian tree shrew have been used for studies *in vitro*, for considering studies *in vivo* using *Tupaia belangeri* model of HBV infection it has to be taken into account that infection with HBV does not result in a chronic carrier state (Walter et al., 1996). Primary hepatocytes of *Tupaia belangeri* have contributed in establishment of a model of chronic HBV infection – for repopulation of immunodeficient urokinase-type plasminogen activator mice in order to obtain a model for assessment of antivirals (Dandri et al., 2005) and HBV entry inhibitors (Petersen et al., 2008). Woodchuck (*Marmota monax*) model presents another small animal model of hepadnavirus infection, infection of woodchucks with Woodchuck Hepatitis virus results in a chronic infection (Menne and Cote, 2007). Woodchuck model has served to unravel many aspects of disease pathogenesis, immune response of the host, as well as for evaluation of therapeutic vaccines (Roggendorf et al., 2007), antivirals and immunomodulators in a setting of chronic hepatitis and hepatocellular carcinoma (Tennant et al., 2004).

5. CONCLUDING REMARKS AND OUTLOOK

Prophylactic vaccination of HBV with the current vaccine is aimed at generation of neutralizing antibodies able to prevent the spread of the virus. This study has outlined approaches for improvement. The rSFV replicon may contribute to design and development of a therapeutic approach for chronic hepatitis B. Chronic hepatitis B is one of the major public health threats, since the chronic carriers represent a reservoir of HBV and a permanent source of infection. Significant proportion of chronic carriers develop liver cirrhosis and hepatocellular carcinoma, which is one of the most common cancers worldwide (Tan, 2011). Current therapy using IFN-α is able to reduce viral load only in 30 – 40% chronic HBV patients and is dependent on HBV genotype as one of the factors affecting the outcome (Buster et al., 2009), while use of HBV polymerase inhibitors result in emergence of resistant HBV variants (Tillmann, 2007), and recurrence after completion of a long-term therapy (Reijnders et al., 2010). An effective therapeutic strategy for chronic hepatitis still remains an important goal. Chronic carrier state is linked to weak and narrowly directed virus specific $CD8^+$ and $CD4^+$ cell response (Chisari and Ferrari, 1995; Rouse and Sehrawat, 2010). T cell-mediated response to HBV antigens, including HBs, is crucial in clearance of HBV from the liver. The weak T cell responses has been attributed to T-cell tolerance or exhaustion due to high viral load (Bertoletti and Gehring, 2013). Broadly specific $CD8^+$ cells are essential for elimination of HBV infected hepatocytes, assisted by $CD4^+$ cells (Yang et al., 2010). Induction of HBV-specific T cells is a goal of therapeutic vaccination (Bertoletti and Gehring, 2009). The current and other conventional HBsAg vaccines, even with preS antigens, which contain additional T cell epitopes, are not able to induce curative Th1 or CTL immune response in HBV carriers (Pol et al., 2001; Jung et al., 2002; Yalcin et al., 2003). rSFV might contribute to development of immunotherapeutic strategies with the well documented ability of SFV replicons to provide enhanced antigen presentation by cross-priming (Huckriede et al., 2004) and to break immunological tolerance and anergy (Leitner et al., 2003). rSFV replicons were shown to induce HBs specific IgG2a in this study - an indirect indication of a Th1 dominant immune response. This is crucial for viral clearance in a persistent infection (Rossol et. al. 1997). Moreover, evidence about the suppression of innate immune response signalling (induced via pattern recognition receptors), among which are blockage of TLR receptor, RIG, -1 and IFN-β signalling pathways, during persistent HBV infection is mounting (Wu et al., 2009; Kondo et al., 2011; Busca and Kumar, 2014). rSFV may represent an excellent tool for activation of innate antiviral pathways (Leitner et al., 2003) due to activation of pattern recognition receptors (Schulz et al., 2005). Generation of neutralizing antibodies was shown in this study, importantly, neutralizing antibodies play an important role in a persistent HBV setting by neutralizing the circulating virus particles and thus preventing the infection of yet non-infected hepatocytes (Chisari and Ferrari, 1995). High viral load of chronic HBV patients was found to be linked with non-reactive virus-specific $CD8^+$ cells (Webster et al., 2004), therefore elimination of virus particles and circulating HBsAg by neutralizing antibodies is crucial. Kutscher et al. conclude that vaccines aimed at T cell induction were not effective in recipients with high viral and antigenic load and propose a two-step strategy: first reducing the high viraemia and antigenaemia by generation of neutralizing antibodies with the protein-based vaccine, which would be followed by viral vector-based vaccines known to induce HBV-specific CTLs (Kutscher et al., 2011). Generation of neutralizing, HBs subtype independent antibodies was shown in this study, along with the indication of a Th1 dominant response. rSFV replicons may offer an immunization strategy of a single modality without the need of protein-based priming. However, the potential of rSFV replicons to provoke HBV-specific CTLs in a chronic HBV infection animal model has to be yet investigated. Some promising results have been delivered by Rodriguez-Madoz et al. (Rodriguez-Madoz et al., 2009), who showed transient reduction of intrahepatic tumours and viraemia in woodchucks treated intratumorally with rSFV encoding immunostimulatory cytokine IL-12. To achieve

broad CTL responses, additional HBV antigens must be included, for example HBc, either by insertion in the existing constructs after introduction of the second subgenomic promoter, or by additional administration of rSFV encoding HBc. The potential of rSFV replicon approach for the development of a therapeutic approach for chronic HBV infection must be further carefully evaluated.

6. CONCLUSIONS

1. Recombinant SFV encoding two SHBs subtypes adw2 and ayw2 representing HBV genotypes A2 and D were produced effectively in BHK-21 cells, along with rSFV encoding internally deleted LHBs protein (LHBsΔ49-163, i. e. preS1.1-48/S and it's variants), and resulted in titres up to 10^9 infectious units/ml of rSFV after sedimentation through 20% (w/v) sucrose. SHBs proteins and deleted LHBs proteins were expressed *in vitro* by various continuous cell lines and primary *Tupaia belangeri* hepatocytes after infection with rSFV at MOI 10-20. The SHBs protein had appropriate post-translational modifications and was efficiently secreted.

2. The LHBsΔ49-163 protein (preS1.1-48/S) and it's variants demonstrated presence of two N-linked glycans present at ectopic sites (N4 in preS1 and N3 in S) and one N-linked glycan at the normal site Asn146 in the S domain. Glycosylation occurred in presence and absence of the aminoterminal myristoylation motif of LHBs. The S domain retained the native conformation in preS1.1-48/S and preS1.1-48/S_0 proteins.

3. Co-expressed LHBsΔ49-163 and SHBs proteins were capable of efficient formation of subviral particles, displaying preS1 antigenicity on the surface and to a lesser extent SHBs antigenicity. The efficiency of subviral particle formation and secretion was dependent on the presence of SHBs protein expressed from the second internal initiation codon downstream of the preS1 start codon and was enhanced by the presence of the myristoylation motif at the amino-terminus of LHBs. Contrary to expectations proteins with an inactivated myristoylation motif displayed reduced efficiency of subviral particle formation and secretion.

4. Immunization of BALB/c mice with 10^7 and 3 weeks later with 10^8 rSFV encoding SHBs/adw2 and SHBs/ayw2, preS1.1-48/S and preS1.1-48/S_0 generated after three weeks end-point antibody titres in enzyme-linked immune assays up to 1:1,800 to SHBs proteins.

5. The anti-preS1 titers were 1:1,500 in preS1.1-48/S immunized mice and higher than the anti-SHBs titers. Mice immunized with an equal number of infectious units of rSFV encoding preS1.1-48/S_0 protein produced less, but still detectable anti-preS1 which is in line with inhibited secretion of this protein.

6. The antibodies induced to SHBs/adw2 and SHBs/ayw2 reacted preferentially with homologous subtype determinants of yeast-derived rSHBs, demonstrating a strong rSHBs subtype dependency. However, anti-SHBs/adw2 and anti-SHBs/ayw2 reacted equally well with patient-derived HBsAg, subtype ayw2. Here, the reactivity was not HBsAg subtype-dependent. This suggested a lack of group-specific a-determinants in the yeast-derived rHBsAg.

7. Inclusion of the preS1 aminoterminal 48 aa did not elevate the anti-SHBs titre in mice immunized with rSFV vectors. Immunization with the preS1.1-48/S_0 without the SHBs start codon did not induce detectable amounts of SHBs specific antibodies.

8. The *in vitro* model of primary *Tupaia belangeri* hepatocytes infected with highly purified patient-derived HBV provided a valuable tool for assessment of the neutralizing potential of the induced L- and S-HBs specific antibodies. The infection of these cells was measured by detection of the typical markers of an established HBV infection: secreted HBsAg, HBeAg and viral mRNA. Antibodies induced to SHBs/adw2 or SHBs/ayw2 neutralized HBV with the SHBs subtype ayw2 with equal efficiency. The potential of antibodies against preS1.1-48/S to neutralize HBV infection was comparable to anti-SHBs antibodies. Antisera obtained with vectors for SHBs/adw2, SHBs/ayw2, and preS1.1-48/S neutralized 5 HBV particles/hepatocyte completely at 1.0 and 0.5 mg/ml IgG. The antibodies generated to preS1.1-48/S_0 lacking the presence of conformation dependent anti-SHBs neutralized only partially at 1.0 mg/ml, but could neutralize completely when present at 2.5 mg/ml IgG.

9. rSFV replicon-mediated generation of antibodies to SHBs led predominantly to SHBs subtype-independent neutralizing antibodies. Addition of the aminoterminal 48 aa of preS1 - the most essential hepatocellular HBV attachment site - to the neutralizing epitopes of SHBs protein in the rSFV vector may provide escape-mutant resistant antibodies.

10. The IgG1/IgG2a ratios of the induced antibodies indicated IgG2a dominance and thus a Th1 type response. The indirect evidence presented for a Th1 type dominant response after rSFV-mediated immunization may suggest use of this vector for studies on resolution of non-response or hypo-response to HBV.

7. THESES FOR DEFENCE

1. The formation of secretable subviral particles by LHBs protein deletion variants (preS1.1-48/S) is more efficient in presence of the myristoylation motif at the amino-terminus of preS1, and dependent on the in-frame co-expressed SHBs protein. Inactivation of the myristoylation motif did not enhance secretion in LHBs protein deletion variants consisting of aminoterminal 48 aa of preS1 and SHBs protein as opposed to full length *wt* LHBs protein.

2. Reactivity of the anti-SHBs/adw2 and anti-SHBs/ayw2 generated by rSFV-mediated immunization of BALB/c mice with rHBsAg from yeast is strongly rSHBs subtype dependent, while binding of anti-SHBs/adw2 and anti-SHBs/ayw2 to patient-derived HBsAg is not dependent on SHBs subtype.

3. The neutralizing potential of SHBs antibodies induced with rSFV mediated immunization is not SHBs subtype dependent. Sera containing antibodies to SHBs/adw2 and SHBs/ayw2 neutralize with equal efficiency as serum containing anti-SHBs and anti-preS1 neutralizing epitopes. PreS1-specific antibodies alone generated after rSFV mediated immunization also can confer protection.

4. The well-documented evidence of alphavirus replicons in activating innate and cellular immunity in conjunction with the presented data on humoral immunity and Th1 type dominant response may provide a platform for immunotherapy of chronic hepatitis B.

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