(I)

Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection

(II)

Generation and characterization of hepatitis B virus surface proteins fused to GFP and RFP

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Abstract I

Dendritic cells (DC), being key players in antigen presentation and initiation of virus-specific T-cell responses, have been reported to exhibit functional impairment in HBV carriers. Possible explanations for this phenomenon are infection of DCs with HBV or alteration of DC function by HBV. Therefore it was analysed whether DCs support the different steps of HBV infection: uptake, delivery of the HBV genome to the nucleus, antigen expression, and progeny virus release. When HBV genomes were artificially introduced into monocyte-derived DCs (moDC) by adenoviral vectors, low-level expression of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) but no HBV replication was detected.

When subjecting moDCs to recombinant HBV expressing *Renilla* luciferase either under a non–liver-specific promoter or under a HBV promoter, no luciferase activity was detected. After incubation with wild-type HBV, intracellular HBV rcDNA was detected in a low percentage of cells, but nuclear cccDNA was not formed. This indicates that either uncoating or nucleocytoplasmic transport were blocked.

To verify the observation in the *in vivo* situation, myeloid (mDC) and plasmacytoid DCs (pDC) were isolated from blood of high viremic HBV carriers, and analysed by quantitative polymerase chain reaction (PCR) and electron microscopy. Although circulating DCs had *in vivo* been exposed to more than 10^4 HBV virions per cell, HBV genomic DNA was hardly detected, and no nuclear cccDNA was detected at all. By using electron microscopy, subviral particles were found in endocytic vesicles, but virions were undetectable, as were viral capsids in the cytoplasm. Quantitative PCR analysis of B cells, monocytes and an enriched T-cell fraction of chronic HBV carriers showed uptake of HBV particles in low amounts, but no establishment of an infection.

In conclusion, circulating DCs may take up HBV antigens, but neither support nucleocytoplasmic transport nor replication of HBV. It can be excluded that HBV infects DCs with a frequency sufficient to explain the functional impairment of the virus-specific T-cell response in chronic HBV carriers. One can hypothesise that the contact of DCs with HBV antigens, which are present in sera of infected persons in high amounts, influences DC and T-cell function.

Abstract II

Fluorescent labelling of viral proteins and viruses, or virus-like particles, has been shown to provide a powerful tool for investigating unexplored aspects in viral life cycles. A commonly used technique is the genetic incorporation of fluorophores into virus proteins. The insertion of GFP into the Gag protein of HIV for instance allowed studying the trafficking of this protein, as well as production of infectious virions (Müller et al., 2004). In case of adeno-associated virus, Lux et al. (2005) were able to directly monitor the cytosolic and nuclear trafficking with GFP-tagged virus particles.

Since many aspects of the HBV life cycle still remain unclear, the visualisation of viral (VP) and subviral (SVP) HBV particles is a promising approach to elucidate different steps of virus entry and egress. GFP or RFP fused HBV S, M and L surface proteins were constructed and analysed for their fluorescence and stability properties. To ensure that the fusion did not alter the characteristic properties of the HBV surface proteins, they were compared to the parental proteins with regard to their localisation in cellular compartments and the ability to form subviral and viral particles. The GFP fused proteins did not co-localise with the parental proteins within the cell, suggesting that they were unstable. The RFP fusion proteins clearly co-localised with the parental proteins. But although they behaved similar to the parental proteins with regard to their distribution in the ER and golgi compartment, neither of them contributed to the formation of SVP or VP.

Thus, GFP and RFP fusion to HBV surface proteins is not suitable to generate fluorescent HBV particles. The main impediment is most probably misfolding of the fusion proteins. The bulky GFP and RFP domains seem to have topological and sterical effects, affecting stability, correct integration into the ER membrane or assembly and secretion of properly formed particles. Labelling with small fluorescent molecules can minimise or overcome these problems and therefore perhaps provides a better strategy.

Zusammenfassung

Die Infektion mit dem Hepatitis B Virus (HBV) stellt weltweit ein großes gesundheitliches Problem dar. Mit mehr als 2 Mrd. Infizierten, darunter ca. 350 Mio. chronische Träger, ist es eine der häufigsten Infektionskrankheiten. Die chronische Hepatitis B führt häufig zu Leberzirrhose und hepatozellulärem Karzinom, an dessen Folgen jährlich schätzungsweise 1 Mio. Menschen sterben. Trotz der Verfügbarkeit eines wirkungsvollen Impfstoffs steigt die Zahl der Infizierten, vor allem in den Entwicklungsländern, stetig an.

HBV gehört zur Familie der hepatotrop-assoziierten DNA-Viren und weist eine enge Organ- und Wirtsspezifität auf. Soweit bekannt ist, kann HBV nur primäre Hepatozyten infizieren und ist im Fall des humanen HBV auf Menschen und humanoide Primaten als Wirt beschränkt. Da HBV über ein RNA-Intermediat mittels reverser Transkription repliziert, wird es zu den Pararetroviren gezählt.

Die 42 nm großen viralen Partikel (VP) enthalten ein 3.2 Kilobasen (kb) großes zirkuläres DNA-Genom, welches nur partiell doppelsträngig und deshalb nicht kovalent geschlossen ist (rcDNA, *relaxed circular DNA*). Es enthält vier sich teilweise überlappende offene Leseraster, von denen durch mehrere Initiationsbereiche während der Translation sieben virale Proteine gebildet werden. Das Genom befindet sich im Nucleokapsid, das aus dem viralen Core-Protein aufgebaut ist. Eine Lipid-Doppelmembran umhüllt das Kapsid. Sie besteht aus einer wirtseigenen Membran, die vom endoplasmatischen Retikulum (ER) stammt. In diese sind die viralen Hüllproteine eingelagert. Die drei Hüllproteine S (*small*), M (*middle*), und L (*large*) sind eng verwandt: M besteht aus der Aminosäuresequenz von S, mit einer N-terminalen Verlängerung um 55 Aminosäuren (aa). Diese zusätzliche Sequenz nennt man PreS2. L besteht aus der Aminosäuresequenz von M, die N-terminal um die PreS1 Sequenz aus 119 aa verlängert ist. Neben den infektiösen viralen Partikeln werden im Rahmen einer Infektion auch nicht-infektiöse Partikel, so genannte subvirale Partikel (SVP), gebildet. Diese bestehen lediglich aus der Lipid-Doppelmembran und enthalten weder DNA noch Kapside. SVP sind im Vergleich zu VP in einem bis zu 10000-fachen Überschuss im Serum Infizierter nachweisbar. Deshalb sind sie stark immunogen. Die Bedeutung der SVP ist bislang ungeklärt, vorstellbar ist aber, dass sie anti-HBV Antikörper abfangen,

die vor allem gegen Epitope auf dem S Protein gerichtet sind. Im immunologischen Kontext werden die SVP deshalb auch als HBs-Antigen (HBsAg) bezeichnet. Ein weiteres Antigen, das in hohen Konzentrationen im Serum nachweisbar ist, ist das HBeAg. Dieses virale Protein wird von infizierten Zellen sekretiert, seine Funktion ist unbekannt.

HBV infiziert Hepatozyten über bislang unbekannte Rezeptoren. Nach dem Eintritt in die Zelle wird das Kapsid freigesetzt und das virale Genom, die rcDNA, wird in den Nukleus transloziert. Dort wird das partiell doppelsträngige Genom von der zellulären Polymerase 2 vervollständigt. Dadurch entsteht ein doppelsträngiges, kovalent geschlossenes DNA-Molekül, welches als cccDNA (*covalently closed circular DNA*) bezeichnet wird. Diese Form der HBV-DNA kommt lediglich im Nukleus infizierter Zellen, in Form extrachromosomaler Moleküle, vor. Die cccDNA dient als Matrize für die RNA-Synthese. Virale Proteine werden synthetisiert, und zunächst werden Kapside gebildet. Neben den mRNAs wird auch eine prägenomische RNA synthetisiert, welche zusammen mit der viralen Polymerase in die Kapside verpackt wird. Dort erfolgt die reverse Transkription des RNA-Intermediates in das rcDNA-Genom. Die rcDNA enthaltenden Kapside können entweder ihre DNA wieder in den Kern translozieren und so den Pool an cccDNA auffüllen, oder die Kapside werden am ER umhüllt. Die ER-Membran enthält bereits die viralen Hüllproteine S, M und L, da sie direkt in die ER-Membran synthetisiert werden. Während der Synthese werden die Hüllproteine teilweise glykosyliert. Über Disulfid-Brücken bilden sich zunächst Homo- und Heterodimere, die sich zusammenlagern und entweder in Form von SVP oder nach Kontakt mit dem Kapsid in Form von VP in das ER-Lumen abgeschnürt werden. Die Information für die Bildung subviraler Partikel liegt in der Sequenz des S Proteins, welches alleinig zur SVP Bildung ausreichend ist. Während des vesikulären Transports durch ER und Golgi-Apparat werden die Hüllproteine weiter modifiziert. Über den Exozytoseweg werden die subviralen und viralen Partikel freigesetzt.

Das Virus wird vertikal über Blut und Körperflüssigkeiten übertragen, weiterhin kann eine perinatale Übertragung von der Mutter zum Kind erfolgen. Das Risiko, eine chronische Hepatitis B zu entwickeln ist altersabhängig. Bei Säuglingen liegt die Wahrscheinlichkeit bei 98%, während eine Ansteckung im

Erwachsenenalter nur in 5-10% der Betroffenen zu Ausprägung einer chronischen Infektion führt.
Der Verlauf einer HBV-Infektion hängt von der adaptiven Immunantwort des Wirts ab. Das Virus an sich ruft weder Entzündungsreaktionen noch Leberschäden hervor. Um eine akute Infektion, die meist symptomlos verläuft, zu kontrollieren, ist eine polyklonale zytotoxische T-Zell-Antwort sowie eine starke T-Helferzell-Reaktion notwendig. Eine chronische Infektion ist hingegen gekennzeichnet von einer oligospezifischen T-Helferzell-Antwort, eine virus-spezifische Antwort der zytotoxischen T-Zellen findet meist nicht statt. Neben der eingeschränkten T-Zell-Funktion scheinen auch dendritische Zellen (DC) funktionelle Defizite aufzuweisen. DC präsentieren Antigene und aktivieren T-Zellen, die dieses Antigen erkennen. Können DC diese Funktion nicht oder nur eingeschränkt ausüben, hat dies dementsprechend große Auswirkungen auf die spezifische T-Zellantwort. Eine funktionelle Beeinträchtigung der DC, z.B. ausgelöst durch eine Virusinfektion, könnte deshalb eine Erklärung für die Defizite in der T-Zellantwort chronisch HBV Infizierter sein. Eine Dysfunktion von DC ist bereits von anderen chronischen Virusinfektionen bekannt. Beispielsweise infizieren das Hepatitis C Virus oder das Humane Immundefizienz Virus DC, und entgehen mit dieser Strategie der spezifischen Immunantwort. Eine Beeinträchtigung der DC-Funktionalität muss aber nicht unbedingt durch einen direkten Einfluss des Virus hervorgerufen werden, vorstellbar ist auch eine Interaktion von HBsAg oder HBeAg mit den Oberflächenproteinen der DC.

Im ersten Teil dieser Arbeit wurde untersucht, ob DC mit HBV infiziert werden können. Dafür wurde zuerst in einem *in vitro*- System analysiert, ob DC die diversen Schritte einer HBV-Infektion unterstützen: Virusaufnahme, Transport des viralen Genoms in der Nukleus, Antigenexpression sowie Produktion und Freisetzung neuer Viren. Dafür wurden Monozyten isoliert und zu unreifen und reifen DC differenziert. Diese aus Monozyten generierten DC (moDC) besitzen viele Eigenschaften der schwer zu isolierenden im Blut zirkulierenden DC und werden deshalb häufig als Modell verwendet. Zunächst wurde das HBV-Genom mittels eines adenoviralen Vektors in unreife und reife moDC eingebracht. Danach konnten im Zellüberstand sehr geringe Mengen HBsAg und HBeAg

mittels ELISA gemessen werden. Replikation und Freisetzung neuer Viren hingegen fand nicht statt, wie eine Untersuchung des Überstandes auf HBV-DNA ergab. Nach Inkubation von moDC mit HBV-basierten Vektoren, die Luziferase entweder unter einem HBV-spezifischen Promoter oder aber unter dem nicht leberspezifischen CMV-Promoter exprimierten, konnte keine Luziferase-Aktivität festgestellt werden. Um auszuschließen, dass Wildtyp-HBV sich anders verhält als rekombinantes HBV, wurden die Zellen auch mit Wildtyp-HBV inkubiert. Mit Hilfe einer quantitativen Echtzeit-Polymerase Kettenreaktion (PCR, *polymerase chain reaction*) wurden die moDC auf die Präsenz der cytoplasmatisch in Viruspartikeln vorkommenden HBV rcDNA und der nukleären cccDNA hin untersucht. Speziell ausgewählte Primer ermöglichten, beide Formen der HBV DNA zu unterscheiden. Während geringe Mengen rcDNA in den Zellen detektiert wurden, konnte cccDNA nicht nachgewiesen werden. Diese Ergebnisse weisen darauf hin, dass zwar eine – vermutlich unspezifische – Aufnahme von HBV in die Zellen erfolgte, eine Infektion jedoch nicht etabliert wurde. Vermutlich waren die Kapsid-Freisetzung oder die Translokation des Genoms in den Nukleus blockiert.

Um diese Daten in der physiologischen Situation zu überprüfen, wurden die Hauptpopulationen zirkulierender DC, die myeloiden und plasmazytoiden DC (mDC, pDC), aus dem Blut chronischer HBV Träger isoliert. Mittels quantitativer Echtzeit-PCR wurden die DC auf das Vorhandensein von rcDNA und cccDNA hin untersucht. Die genomische rcDNA konnte in einigen Fällen in geringer Konzentration in den DC detektiert werden, cccDNA wurde nicht gefunden. In B-Zellen, Monozyten und angereicherten T-Zellfraktionen der chronisch Infizierten befanden sich ebenfalls geringe Mengen HBV Partikel, wie sich in der rcDNA-PCR zeigte, cccDNA war jedoch nicht vorhanden. Elektronenmikroskopisch wurden einige subvirale Partikel in endozytischen Vesikeln in mDC nachgewiesen, das Zytoplasma war jedoch frei von intakten Virionen oder Kapsiden. In pDC wurden keine Hinweise auf virale oder subvirale Partikel gefunden.

Schlussfolgernd wurde in dieser Studie gezeigt, dass zirkulierende DC in geringen Mengen HBV Antigene aufnehmen, einen DNA-Transfer in den Kern sowie die Replikation von HBV jedoch nicht unterstützen. Deshalb ist eine Infektion dieser Zellen mit HBV in einem Maße, welches die Beeinträchtigung

der T-Zell-Antwort in chronischen HBV Trägern erklären könnte, auszuschließen. Diese Arbeit lässt jedoch keine Rückschlüsse darüber zu, ob andere Populationen der sehr heterogenen dendritischen Zellen, wie z.B. leberständige DC, mit HBV infiziert werden können und eine Toleranz gegen das Virus auslösen. Unter Berücksichtigung der Ergebnisse dieser Arbeit sowie Daten anderer Studien erscheint es aber wahrscheinlicher, dass eine Interaktion von HBsAg oder HBeAg mit DC-Oberflächenproteinen einen Funktionsverlust hervorruft.

Der zweite Teil dieser Arbeit beschäftigte sich mit der Generierung fluoreszierender HBV-Hüllproteine durch die Fusion mit GFP (grün fluoreszierendes Protein) oder RFP (rot fluoreszierendes Protein). Dies würde mikroskopische Analysen, z.B. in lebenden Zellen erlauben, die – wie schon für andere Viren gezeigt wurde – Aufschluss über diverse Fragestellungen geben können. Zum einen könnte das Verhalten der Proteine in Zellen verfolgt werden, sowie der Weg viraler und subviraler Partikel aus der Zelle, da beide die Hüllproteine in großer Zahl enthalten. Dies setzt jedoch voraus, dass die Fusionskonstrukte in der Lage sind, Partikelstrukturen auszubilden. Zum anderen könnten mit dieser Strategie auch die frühen Schritte der HBV Infektion visualisiert werden, wenn fluoreszierende VP und SVP aus dem Überstand transfizierter Zellen isoliert und für eine Infektion primärer Hepatozyten eingesetzt werden könnten.
Zunächst wurde GFP sowohl an den N- als auch an den C-Terminus der Hüllproteine fusioniert. Fluoreszenzmikroskopische und Western blot-Analysen zeigten, dass die N-terminale Fusion von GFP an das S Protein in einem instabilen Fusionsprotein resultierte, welches schnell degradiert wurde.
Das kann vermutlich damit erklärt werden, dass das Fusionsprotein entweder gar nicht, oder in einer falschen Topologie in die ER-Membran synthetisiert wurde. Da die Signalsequenz am N-Terminus der Hüllproteine lokalisiert ist, ist sie möglicherweise nach Fusion des globulären GFP nicht mehr funktionell. Eine falsche Lokalisation oder Faltung von Proteinen führt zu einer schnellen Degradierung.
GFP und RFP wurden infolgedessen nur an den C-Terminus der Hüllproteine S, M und L fusioniert. Die Eigenschaften der Fusionsproteine wurden im Hinblick

auf Veränderungen gegenüber der wildtypischen (wt) Proteine charakterisiert. Dafür wurden die Fusionsproteine einzeln oder in Kombination mit wt-Proteinen in Zellen der humanen Hepatomlinie HuH7 exprimiert. Mittels konfokaler Fluoreszenzmikroskopie und Western blot wurde untersucht, ob die Fusionsproteine fluoreszierten und stabil exprimiert wurden. Alle GFP-fusionierten Proteine waren stark fluoreszierend, wobei die Fluoreszenz sowohl im Zytosol als auch im Nukleus detektiert wurde. Die Färbung von S-GFP mit einem Antikörpern gegen das HBV S Protein ergab eine geringe Kolokalisation von S mit GFP im Zytosol. Dies ließ darauf schließen, dass die Fusionsproteine instabil waren und auch freies GFP oder Fusionsproteinfragmente in Zytosol und Nukleus vorhanden war. Eine Lokalisation im Nukleus ist typisch für GFP, nicht jedoch für die HBV Hüllproteine. Western blot- Analysen bestätigten, dass die GFP-fusionierten Proteine instabil waren, und auch durch die Koexpression der wt-Protein nicht stabilisiert werden konnten.

Im Gegensatz dazu wurde nach Transfektion der RFP-Fusionsproteine keine nukleäre sondern nur cytosolische Fluoreszenz detektiert. Weiterhin kolokalisierte RFP zum größten Teil mit Antikörpern gegen S. Das bedeutete, dass die RFP-Fusionsproteine weitgehend intakt in den Zellen vorlagen.

Im Folgenden wurden nur die RFP-fusionierten Proteine näher charakterisiert. Da bekannt ist, dass die HBV Hüllproteine in die ER Membran synthetisiert und später in Form von SVP ins Golgi transportiert werden, wurde die Lokalisation der RFP-Fusionsproteine in der Zelle genauer untersucht. Eine mittels Konfokalmikroskopie durchgeführte Kolokalisationsstudie der Fusionsproteine mit ER- und Golgi-Markern ergab, dass diese ebenso wie die wt-Proteine zunächst im ER und später auch im Golgi nachweisbar waren. S-Fusionsprotein enthaltende subvirale Partikel wurden jedoch nicht sekretiert. Wurde wt-S koexprimiert, konnten geringe Mengen SVP im Medium durch Immunpräzipitation nachgewiesen werden. Diese enthielten jedoch lediglich S, aber kein S-Fusionsprotein. Im Vergleich zu den Kontrollzellen, die nur mit wt-S transfiziert wurden, schien die Sekretion von wt-SVP in kotransfizierten Zellen eingeschränkt zu sein. Nach Koexpression von Fusionsproteinen und den HBV wt-Proteinen wurden keine Viruspartikel gebildet. Welcher oder welche Schritte in der SVP und VP Bildung beeinträchtigt war, blieb bei diesen Versuchen offen. Eine falsche Faltung der Hüllprotein-Domäne oder falsche Topologie in

der ER-Membran, sowie sterische Hinderungen durch das globuläre Fluorophor könnten einen Einfluss auf verschiedene Schritte der Partikelbildung haben: sowohl eine Behinderung der Glykosylierung als auch der Dimerisierung z.B. durch Verdecken der Bindestellen würde zur Folge haben, dass die Proteine in der Membran verbleiben und später degradiert werden. Alternativ ist denkbar, dass die Proteine zwar in Vesikeln in das ER abgeschnürt werden, diese jedoch nicht sekretiert werden können.

Abschließend kann gesagt werden, dass sich mit den in dieser Arbeit generierten Fusionsproteinen keine fluoreszierenden HBV-Partikel herstellen ließen und diese Konstrukte auch zur Beobachtung der einzelnen Proteine in Zellen nicht geeignet waren. Die Eigenschaften hatten sich im Vergleich zu den wt-Proteinen verändert und spiegelten nicht mehr die natürliche Situation wider. Die Markierung mit kleinen Fluoreszenz-Farbstoffen könnte eine geeignete Alternative zur Fusion mit GFP und RFP darstellen. Ein vielversprechender Ansatz ist die Markierung mit einem so genannten Tetracystein-Tag System. Hierfür muss lediglich eine sechs aa große Erkennungssequenz in die Proteinsequenz eingebracht werden. Dies minimiert die Gefahr, dass wichtige Proteinstrukturen verändert werden. Ein nicht fluoreszierendes Molekül, FlAsH genannt, bindet spezifisch an die Erkennungssequenz und wird durch die kovalente Bindung in ein fluoreszierendes Derivat umgewandelt. Mit diesem Ansatz könnten virale Proteine sowie subvirale und virale Partikel in der Zelle beobachtet werden. Weiterhin könnten mit markierten VP die frühen Schritte einer HBV Infektion visualisiert werden. Eine biochemische Markierung der Virushülle – beispielsweise mit fluoreszierenden Alexa-Farbstoffen über eine Succimidylester-Bindung – stellt eine weitere Alternative zu den Fluorophoren dar.

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1. Introduction

1.1 The Hepatitis B Virus.

The **H**epatitis **B** **V**irus (HBV) is a noncytopathic enveloped **d**eoxyribo**n**ucleic **a**cid (DNA) virus belonging to the family of hepadnaviridae (hepatotrop associated DNA viruses) (Gust et al., 1986). It is highly species and organ specific, and as far as known it reproduces only in hepatocytes. Because DNA synthesis occurs via reverse transcription of a **r**ibo**n**ucleic **a**cid (RNA) intermediate, HBV classes among the pararetroviruses.

Infection with HBV can cause acute hepatitis, and in some cases a persistent infection manifests. Pathological consequences of a chronic hepatitis B are often liver cirrhosis and **h**epato**c**ellular **c**arcinoma (HCC).

The human HBV infects only humans and humanoid primates, such as chimpanzees (Vaudin et al., 1988), orang utans (Warren et al., 1999), gibbons (Norder et al., 1996) or gorillas (Grethe et al., 2000). Relatives of the human HBV in the group of *orthohepadnaviridae* are the wooly monkey *(Lagorithrix lagotricha)* hepatitis B virus (Lanford et al., 1998), the woodchuck (*Marmota monax*) hepatitis B virus (Summers et al., 1978), and the ground squirrel (*Xerus inauris*) hepatitis B virus (Marion et al., 1980). The peking duck (*Anas platyrhynchos*) hepatitis B virus (DHBV; Mason et al., 1980), the heron (*Areidea*) hepatitis B virus and the snow goose (*Anser caerulescens*) hepatitis B virus belong to the group of *avihepadnaviridae*.

The establishment of an animal model is hampered by the species specificity of the virus and the fact that the animal species, which can be infected with a human related HBV, are not characterised and difficult to keep. To date, HBV transgenic mice serve as a model for chronic HBV infection. Transduction of mice with adenoviral vectors containing the complete HBV genome or hydrodynamic injection of HBV plasmids mirror an acute HBV infection (Liu et al., 1999; Sprinzl et al., 2001; Yang et al., 2002). The repertory of cells that can serve as a cell-culture model is limited by the hepatotropism of the virus. Human hepatoma cell lines, such as HuH7 or HepG2, are useful to study intracellular facets of the viral replication cycle (Sureau et al., 1986; Sells et al., 1987). However, they cannot be

infected with HBV in culture. The viral genome has to be transferred into the cells via transfection or adenoviral vectors. To gain insight in the early steps of HBV infection, the culture of **p**rimary **h**uman **h**epatocytes (PHH) provides a necessary and valuable tool (Schulze-Bergkamen et al., 2003).

1.1.1 Epidemiology.

HBV was discovered by Blumberg in 1963, in Australian Aborigines and termed Australia antigen (Blumberg et al., 1966). A few years later it was identified as the infectious agent of a viral hepatitis (Blumberg et al., 1969).

Hepatitis B is a major disease of mankind with over 2 billion people infected, predominantly in the developing world. 350 million people are chronic carriers with high risk to develop liver cirrhosis and HCC, diseases that cause approx. 1 million deaths per year (WHO, 2007).

Infection occurs via blood and body fluids and can be transmitted horizontally through sexual contacts or parenteral inoculation from adult to adult, or vertically from the infected mother to the newborn. The course of the disease is variable and depends on age and immune status of the patient.

Primary infection is followed by a six weeks to four months long incubation period. It proceeds either asymptomatically or is accompanied by nausea, fatigue and jaundice. The acute phase is characterised by elevated serum transaminase levels, indicating liver inflammation. Infection is completely cleared in most of the cases due to a multi-specific and polyclonal cytotoxic T-cell response and a strong type 1 T-helper cell response. Thereby patients develop immunity against HBV.

An unusual and severe form of the acute hepatitis is the fulminant hepatitis, leading almost always to death.

Approx. 30% of HBV-infected infants and 90% of perinatal infected newborns develop persistent hepatitis. With increasing age the likelihood of a chronic manifestation of the infection decreases. In 5 to 10% of infected adults the virus is not cleared and the patients become either silent carriers or develop chronic hepatitis. Hallmarks of a chronic HBV infection are active viral replication for more than six months and variable but persistent viral titers for at least twenty weeks. The T helper-cell response is mostly oligoclonal, cytotoxic T-cell activity is weak or undetectable (Ferrari et al., 1990).

HBV prevalence varies and is most endemic in Southeast Asia, China and Africa with about 10% HBV carriers in the population. Intermediate infection rates of 1 to 8% are found in the Middle East, Russia, India and Brazil, whereas developed countries (Europe, Australia, North America) are regions of low prevalence. Despite the availability of a potent vaccine infection levels increase, predominantly in the developing world (WHO, 2007).

1.1.2 Particle structure.

HBV is an enveloped virus with a partially **d**ouble-**s**tranded (ds) DNA genome. The intact virion, also referred to as Dane particle, has a spherical structure with 42 nm in diameter (Dane et al., 1970). The envelope consists of a host derived lipid bilayer with three viral surface proteins integrated: The **s**mall protein (S), the **m**iddle protein (M) and the **l**arge protein (L). The M and L proteins are expressed at levels of about 1 - 2% and 5 - 15% compared to S protein (Robinson, 1995). Homodimers of the viral core protein, arranged with triangulation numbers of three or four, build the 27 nm icosahedral capsid inside the envelope (Crowther et al., 1994). The capsid encloses the 3.2 **k**ilo **b**ase pairs (kb) viral genome, with the viral **p**olymerase (P) covalently linked to the negative strand via its **t**erminal **p**rotein (TP) domain (Bartenschlager and Schaller, 1992).
HBV-infected cells secrete not only infectious virions but also non-infectious **s**ub**v**iral **p**articles (SVP), consisting only of surface proteins without capsid or DNA (Ganem and Prince, 2004). The spherical SVPs, so-called spheres, are small with a diameter of 20 nm and octahedral symmetry. The filamentous SVPs, also named filaments, are present in less quantity and are of variable length (Ganem and Schneider, 2001). They comprise almost only S protein and low levels of M and L. The role of the highly immunogenic SVPs is not yet understood, but since they are present in 100 to 10000-fold excess to virions in serum of infected persons, they may capture and thereby neutralise anti-HBV antibodies (Heermann and Gerlich, 1991). These are predominantly directed against the S protein, which is present in high amounts on the surface of the SVPs. Figure 1 provides an overview of the different HBV particles.

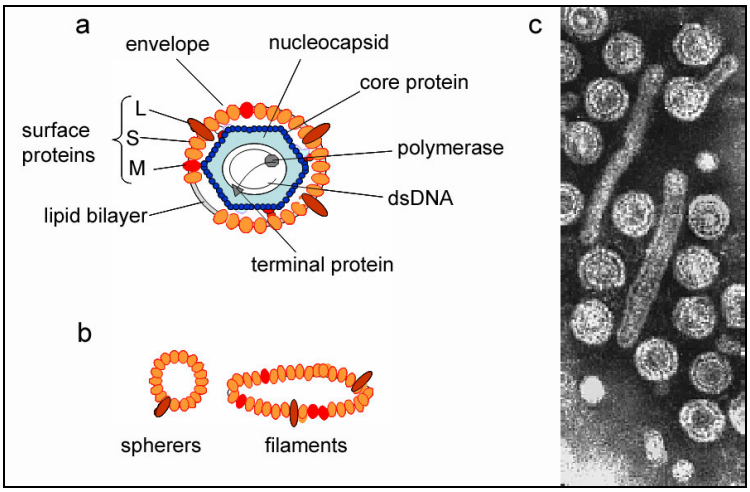

Fig.1: Structure of hepatitis B virus particles.

Fig.1a) shows the structure of an infectious 40 nm Dane particle, in b) the two forms of subviral particles (spheres and filaments) are illustrated. c) Electron microscopic image of HB virions, filaments and spheres (Copyright Linda M. Stannard, Department of Medical Microbiology, University of Cape town, South Africa).

1.1.3 Genome and proteins.

The 3.2 kb DNA genome present in the virion is a partially ds **r**elaxed **c**ircular (rc) molecule. The minus strand is of unit length and linked to the viral polymerase via the terminal protein at the 5` end. The 5` end of the plus strand is of variable length so that a part of the minus strand is **s**ingle **s**tranded (ss). Fig. 2 provides an overview over the viral DNA and RNAs. The gap in the duplex genome is repaired in the host-cell nucleus by a host-specific polymerase, generating a fully duplex genome. This form of the hepatitis B viral genome exists only inside the host-cell nucleus and is termed **c**ovalently **c**losed **c**ircular DNA (cccDNA). It serves as a template for the viral **m**essenger **RNA**s (mRNA). The small genome is organised compactly with four partially overlapping **o**pen **r**eading **f**rames (ORF), without non coding regions (Ganem and Schneider, 2001):

1) Product of ORF P is the viral **p**olymerase (P).
2) ORF C encodes the **c**ore protein (C) and the precore protein.
3) ORF S/pre-S products are the surface proteins S, M and L.
4) ORF X codes for the **X** protein (X).

Four promoters (preC/C, preS1, preS2/S and X promoter) regulate transcription, whereby the resulting transcripts have to be distinguished into pre- and

sub-genomic RNAs. The former is bi-functional, being a template for reverse transcription as well as a message for viral proteins. The latter have exclusively messenger RNA functionalities. mRNA transcription starts at the different promoters and stops at one common polyadenylation signal (Cattaneo et al., 1984).

Two **enh**ancer elements (enh1 and enh2) influence the promoters. Enh1 increases transcription from all four promoters, in contrast to enh2, which only up-regulates transcription from the preS2/S promoter. Enh2 activity requires liver-specific transcription factors, and is therefore restricted to hepatic cells (Hu and Siddiqui, 1991; Yuh and Ting, 1993).

The longest transcript is the 3.5 kb **p**re-**g**enomic RNA (pgRNA). It includes the complete genomic information of the virus and serves as a template for reverse transcription of the HBV genome. Furthermore, it encodes the polymerase, precore and core protein. Essential for transcription of the pgRNA is the transcription factor **h**epatic **n**uclear **f**actor (HNF) 4 (Raney et al., 1997). Therefore, the transcription of pgRNA is liver-specific.

The protein product of the polymerase gene encompasses three functional domains, the RNAseH domain, the **r**everse **t**ranscriptase (RT) domain, and the TP domain. The polymerase drives packaging of the pgRNA into the capsids by binding to the encapsidation signal (ε) of the RNA. As soon as the RNA-polymerase complex is encapsidated, the polymerase reverse transcribes the pgRNA into DNA and removes the RNA with its RNAseH activity.

Expression of preC and C proteins is highly liver specific, because liver-enriched transcription factors are required. PreC protein, in the immunological context also referred to as HBeAg, contains the identical **a**mino **a**cids (aa) as the core protein plus additional 29 aa at the N terminus. It is a non-structural protein, which is secreted in high amounts from infected cells (Seeger and Mason, 2000). Because serum levels of HBeAg correlate with viral replication levels, it is a serological marker for infection. The function of HBeAg is yet unknown.

The core protein is the structural component of the viral capsid. It possesses self-assembly properties, building first homo-dimers, which then aggregate to form capsids (Zhou and Standring, 1992).

Subgenomic RNAs of 2.4 kb and 2.1 kb encode the L protein, and the S and M protein, respectively. Whilst the preS2/S promoter is active in a wide range of

cells, the preS1 promoter is liver specific, requiring, amongst others, the transcription factor HNF 1 (Courtois et al., 1988). All surface proteins share the S-protein sequence. The N terminus of the M protein is extended by the hydrophilic 55 aa long preS2 domain. L contains the S and preS2 domain and additionally the preS1 domain.

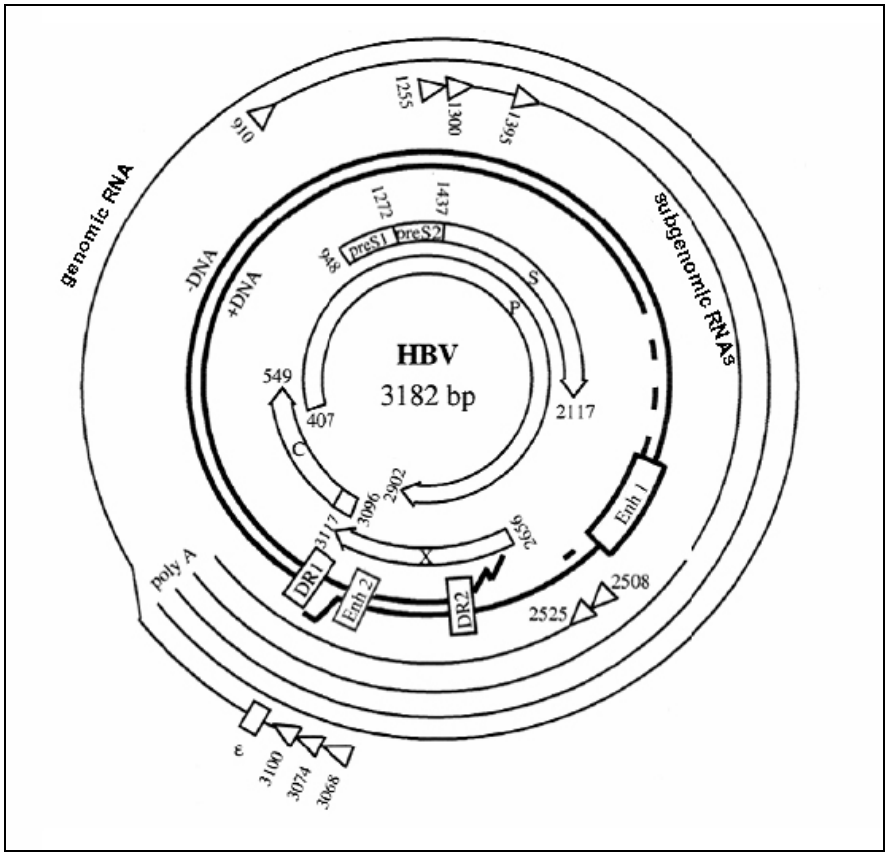

Fig.2: Genome organisation of HBV.

Outer circles depict the transcripts (genomic and subgenomic RNAs). The triangles indicate the transcription starts, the square symbols the encapsidation signal (ε). The bold black lines represent the partially ds genome. Enhancers (Enh1, Enh2) and direct repeats (DR1, DR2) are outlined in squares. The arrows illustrate the four open reading frames (ORF) with their protein products X, C, P and the surface proteins S, M and L. (Modified from Protzer et al., 1999).

The S protein is expressed in vast amounts in comparison to M and L, and the distribution of the surface proteins differs between virions and subviral particles. While the virion envelope is enriched for L, the SVP are composed of S protein with variable amounts of M and L. It is known that S and L protein are necessary for virus assembly and binding to cellular receptors, respectively. M protein is not required for infection and its function remains to be elucidated.

The 0.7 kb subgenomic RNA encodes the X protein. Its function is controversially discussed. On the one hand, it is a prerequisite for HBV and WHV infection *in vivo,* on the other hand, it is not conserved throughout the family of hepadnaviridae, implying a minor role in the HBV life cycle. Involvement in liver cancerogenesis is likely (Su et al., 1998).

1.1.4 Replication cycle.

The early steps of HBV infection are still obscure, but current data suggest that L protein is responsible for attachment and binding to still unknown cellular receptors. Results obtained with the DHBV model demonstrated that the entry occurs via endocytosis of the virion and fusion of the viral envelope with the endosomal membrane in the acidic milieu (Kock et al., 1996; Breiner and Schaller, 2000; Funk et al., 2004; Stöckl et al., 2006). This process seemed to be pH independent (Rigg and Schaller, 1992; Kock et al., 1996). The replication cycle of HBV is schematically outlined in fig. 3.

After uncoating the capsid translocates microtubule dependent to the nucleus, likely via a nuclear localisation signal harboured at the C terminus of the core protein. Whether the capsid releases the rcDNA genome at the nuclear pore, or whether it translocates in intact form into the nucleus, remains to be investigated. Considering the sizes of the nuclear pore (25 nm) and the intact capsid (30 to 34 nm), it is likely that capsid disorganisation is induced, so that only the DNA-polymerase-complex enters the nucleus.

Within the nucleus, the gap in the rcDNA is repaired by cellular enzymes, resulting in the covalently closed circular form (cccDNA). In rare cases, HBV DNA integrates into the host genome. This event does not have an impact on the virus life cycle, but is proposed to promote the development of HCC (Ganem, 1990; Wang et al., 1991; Tu et al., 2006).

The cellular RNA polymerase transcribes the four viral mRNAs, which are transported into the cytoplasm without splicing. Translation of the viral surface proteins S, M and L takes place at ribosomes at the ER, where they integrate into the ER membrane. The products of the pgRNA (core, precore and polymerase) are synthesised at free ribosomes (Ganem and Schneider, 2001).

The TP domain of the polymerase recognises a stem-loop-formation at the 5` end of the pgRNA. This structure serves as an encapsidation signal (ε) and is covalently bound by the polymerase-protein-complex (Ganem and Schneider, 2001).

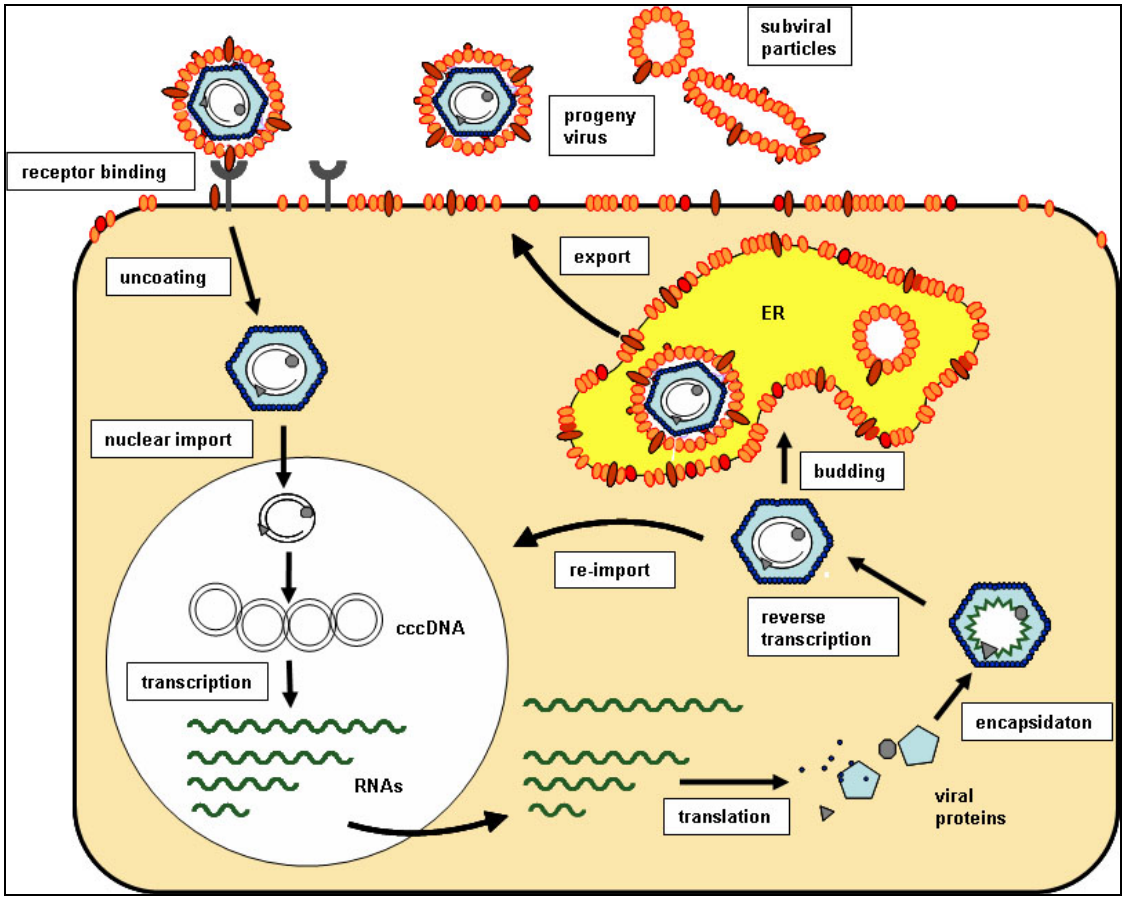

Fig. 3: Replication cycle of HBV.

Virions bind to a receptor on the cell surface and enter. The nucleocapsids uncoat and the partially ds DNA genome is imported into the nucleus. DNA reparation leads to cccDNA formation and transcription of viral RNAs. After translation and capsid formation, the pgRNA is encapsidated. Reverse transcription takes place within the capsids. Nucleocapsids are either re-imported into the nucleus or bud into the ER, obtaining their envelope. Virions, as well as budded SVP, are transported to the cell surface and are secreted.

As soon as a sufficient amount of core protein has been produced, it self-assembles into capsids. The pgRNA-protein-complex is packaged into the capsids, and the polymerase starts to reverse transcribe the DNA minus strand. A tyrosin residue at the terminal protein serves as a primer. Simultaneously, the RNA plus strand is eliminated by the RNAse H activity of the viral polymerase. Then synthesis of the DNA plus strand, initiated at **d**irect **r**epeat 2 (DR2), occurs,

resulting in the rcDNA genome with an overlapping minus-strand segment. A short capped oligoribonucleotide at the 5` end of the pgRNA is required here for priming. Complementary sequences at the 5` end of the plus strand and the 3` end of the minus strand lead to circularisation of the DNA molecule (Ganem and Schneider, 2001).

The originated mature nucleocapsids can now follow two different pathways. Some translocate to the nucleus and re-import the DNA to refill the cccDNA pool. Others bud into the ER, thereby receiving their envelope, consisting of the viral surface proteins S, M and L integrated into the ER lipid bilayer. SVP self-assembly is thought to occur at a post-ER/pre-golgi compartment, the so-called **ER**-**g**olgi **i**ntermediate **c**ompartment (ERGIC; Huovila et al., 1992). Both VPs and SVPs traverse the golgi before being exocytosed. Little is known about the transport and sorting mechanisms, which HBV uses to be efficiently exported in order to infect neighbouring cells and new hosts. However, it is likely that cell polarity plays a role in this process. Hepatocytes are polarised cells with distinct apical and basolateral sides, facing bile canaliculi and hepatic sinusoid, respectively (Wisher et al., 1975). For DHBV, Funk et al. (2004) assumed that the virus is transported predominantly to the basolateral sites of primary duck hepatocytes.

1.1.5 The HBV surface proteins.

The HBV envelope consists of three closely related viral surface proteins, integrated into a host derived lipid bilayer. The S, M and L surface proteins are expressed from one ORF. A promoter upstream the ORF and another internal promoter initiate transcription and give rise to L and M protein. An additional translation initiation site within the smaller transcript leads to the formation of the small S protein. S, M and L share the common C terminus containing the S polypeptide, which harbours self-assembly properties. M is extended by the 55 aa preS2 sequence, L contains the preS2 sequence and an additional 108 or 119 aa sequence called preS1. The surface proteins are synthesised at the ER as integral membrane proteins. Their topology is not completely elucidated, but experiments with cell-free systems revealed that both N and C termini of S locate in the lumen of the ER, implying at least two membrane-spanning domains (Eble

et al., 1987). This goes together with the fact that S contains three known hydrophobic domains. Current models, based on theoretical considerations, propose up to four membrane spanning domains, leading to the model shown in fig. 4 (Bruss, 2007).

The first hydrophobic domain at the N terminus, designated signal 1, belongs to a topogenic signal sequence. It initiates chain translocation across the membrane, without however being cleaved by the host signal peptidase. The second hydrophobic domain, signal 2, functions as a stopping signal, which anchors the protein in the bilayer, and acts as a translocation signal for the distal region. This results in a cytoplasmatic loop between signal 1 and 2 (Eble et al., 1987). The sequence between signal 2 and the third hydrophobic domain forms a luminal loop, accommodating the immuno-dominant surface epitope and a glycosylation site, which is N-glycosylated in approx. half of the S proteins. Another cytoplasmic loop is proposed between hydrophobic domains three and four, implying that the C terminus is orientated towards the ER-lumen. Fusion studies, revealing that C-terminally fused foreign domains are located in the ER lumen support this assumption (Eble et al., 1987). After budding, the luminal loop and most likely as well the C terminus, are located on the surface of the virion. M protein has a similar transmembrane topology, exposing the preS2 domain to the ER lumen. In addition to the partial glycosylation site in the S domain, the preS2 domain contains an N-glycosylation site (Stibbe and Gerlich, 1983).

L protein forms two isomers: In the ER membrane, the preS domains are displayed on the cytosolic side (i-preS conformation; see fig. 4). They are not glycosylated at two potential sites, but post-translationally myristoylated at a myristoylation site located in the preS1 region. A post-translational change of the topology in approx. half of the L proteins leads to the exposure of the preS domains on the luminal side (e-preS conformation). As a result, virions carry two isomers of L (Bruss et al., 1994). The mechanism of this process is unknown. However, binding of cytosolic and luminal chaperones to the preS1 domain has been shown, indicating an involvement of these proteins (Prange et al., 1999; Lambert and Prange, 2003; Cho et al., 2003).

Due to the formation of two isoforms, L can fulfil several functions: The i-preS conformation activates different promoter elements and is thought to be important for contacting the nucleocapsid (Hildt et al., 1996; Bruss, 1997; Le Pogam and

Shih, 2002). The e-preS conformation participates in receptor binding on the host-cell surface (Gripon et al., 2005). The myristoylation of the preS1 region has been shown to be essential for infectivity (Gripon et al., 1995).

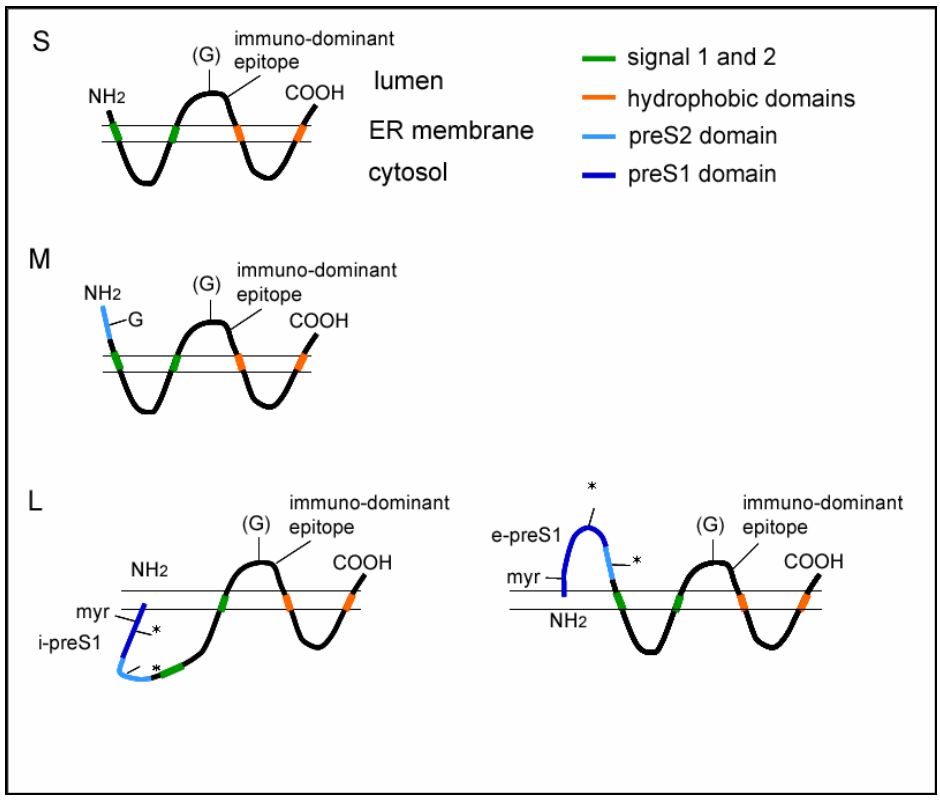

Fig. 4: Transmembrane topology of HBV surface proteins.

S traverses the ER membrane with signal 1 and signal 2 (green boxes) and probably with two additional hydrophobic domains (orange boxes), exposing N- and C terminus to the ER lumen. The luminal loop is partially glycosylated ((G)). M has a similar topology, with an additional glycosylation site (G) at the preS2 domain (light blue). L is further prolonged (preS1 domain; dark blue) and present in two isoforms: The preS1-preS2 domain locates first in the cytosol (i-preS1), in 50% of the L proteins the preS1-preS2 domain is later transferred to the luminal side of the ER (e-preS1). In the virion, 50% of the L proteins carry the preS1-preS2 domain outside. Asterisks indicate potential glycosylation sites, myr indicates the myristoylation site. (Adopted from Bruss, 2007).

During insertion into the ER membrane, the surface proteins obtain asparagine-linked high-mannose oligosaccharide chains. The importance of N-linked glycosylation processes for proper protein folding has been shown for many glycoproteins, as well as for HBV secretion (Helenius, 1994). Metha et al. (1997) demonstrated that M protein accumulates in the lysosome, if α-glycosidase based glycosylation is inhibited. As a result, SVPs containing S and L protein are

secreted, but no virions. This indicates different glycosylation mechanisms for the different surface proteins and emphasises the significance of this process for HBV. The glycosylated protein monomers form homo- and hetero-dimers between S, M and L. Responsible for this are cystein residues in the luminal loop of the S domain, which crosslink the proteins by building disulfide bridges (Wunderlich and Bruss, 1996). Crosslinking into higher oligomers occurs in cisternae of the ER, which belong to the ERGIC (Huovila et al., 1992).

In the oligomerisation process, host proteins are efficiently excluded from the lipid bilayer. Furthermore, a re-organisation of the host lipids in the membrane is assumed. After budding into the intermediate-compartment lumen, the aggregates lack a typical unit membrane structure and exhibit lower lipid contents than conventional lipid membranes (Gavilanes et al., 1982). Since the information required for assembly is located in the S sequence, not only virions bud into the ER cisternae, but also empty envelopes, the SVPs. In comparison to the virions, L protein is underrepresented in spherical SVPs. In fact, the ratio of L to S protein seems to determine the particle form, since a higher proportion of L results in the formation of filaments (Heermann et al., 1984). A high L content of the particles has been shown to inhibit secretion (Persing et al., 1986). This phenomenon can be explained by a retention sequence at the N terminus of the preS1 domain, which can override the secretory elements in the S domain (Kuroki et al., 1989; Prange et al., 1991). In over-expression experiments, it became clear that L inhibits the secretion of S and M in a dose dependent fashion (Chisari et al., 1986; Persing et al., 1986). The over-accumulation of surface proteins in the ER can lead to cell stress and cell death (Chisari and Ferrari, 1995b). Affected cells develop specific cytopathic and morphologic characteristics. These so-called ground glass hepatocytes can be observed in livers of HBV infected mice and are sometimes found in human biopsies of HBV-infected livers (Nakamoto et al., 1997).

Virions and SVPs traverse the secretory pathway before they are exocytosed. In the golgi the carbohydrates are converted to a endoglycosidase H-resistant form (Patzer et al., 1984). The now mature viral and subviral particles are secreted from the cell.

1.1.6 Therapeutic approaches.

Since 1980, prophylaxis by vaccination against HBV is possible and represents an effective method to avoid HBV infection and its consequences. For HBV infection, however, there is no treatment available to date which eradicates the virus. Life-long therapy is therefore necessary, accompanied with an increasing risk of the development of drug-resistant mutants.

Patients with acute hepatitis B are treated symptomatically, if indicated. For chronic hepatitis, there are two different therapeutic approaches, both of which aim at controlling infectivity and progression of liver disease.

Inter**f**ero**n** α (IFN-α), a type 1 interferon, is most currently used. Interferons belong to the cytokine family, are natural mediators of the immune system with regulatory functions, and they are basically synthesised by leucocytes. IFN-α acts on different levels on the viral infection: It displays a direct antiviral effect, has antifibriotic and antiproliferative properties, and stimulates the immune system by activating the T-cell response. Since interferon treatment has constitutional and neuropsychiatric side effects, such as anaemia, thrombocytopenia, neuropenia and depression, its applicability is limited.

An alternative approach is the application of nucleoside- and nucleotide analogues. These so-termed antivirals interfere with viral replication and by that block the formation of new virions (Marcellin et al., 2003; Asmuth et al., 2004). A commonly used antiviral is Lamivudin, a 2`-3`-dideoxy-cytosine analogue. It displays low cytotoxicity and does not interfere with the host DNA synthesis, and is therefore well tolerated.

These treatments help to moderate the disease but cannot not dissolve infection. For that, an elimination of the infected hepatocytes would be necessary, because they contain the cccDNA, which is the template for virion synthesis. For patients with HBV-related hepatocellular carcinoma or liver failure, the only option is to undergo partial hepatectomy or liver transplantation. Simultaneous application of antivirals and anti-HBV surface antigen improves graft re-infection rates and graft survival.

1.1.7 Fluorescent labelling of viruses and viral proteins.

Fluorescent labelling of viral proteins and viruses, or virus-like particles, provides a powerful tool for investigating unexplored aspects in viral life cycles. Direct visualisation enables the real-time microscopic tracking of viral proteins or virions in the context of a living cell. Therefore, it can provide an insight into virus-host cell interactions. Furthermore, it allows co-localisation studies, for instance with cellular compartments or proteins.

Nucleic acids can be labelled with either unspecific intercalating dyes, such as 4',6-**D**i**a**midin-2'-**p**henyl**i**ndol-dihydrochlorid (DAPI), or by incorporation of fluorescent 2'-deoxyuridine-5'-triphosphates. This strategy is advantageous to microscopically follow enveloped virus particles inside the cell, after the envelope has been released. Prerequisite for this approach is a large genome of the virus. Labelling of the outer envelope protein with lipophilic dyes, which laterally diffuse into membranes, has successfully been performed with the influenza virus (Lakadamyali et al., 2003).

A novel technique has currently been reported by Arhel et al. (2006), using the bis-arsenical fluorescein derivate FlAsH. This very small molecule binds with high affinity to a six aa tetracysteine tag and is therefore very specific. By introducing this tag into the protein sequence, theoretically every protein can be targeted.

A common approach is the genetic incorporation of fluorophores, such as **g**reen **f**luorescent **p**rotein (GFP) or **r**ed **f**luorescent **p**rotein (RFP). This method has led to important breakthroughs in understanding of the behaviour of many viruses, such as **ad**enovirus (Ad; Suomalainen et al., 1999), adeno-associated virus (Seisenberger et al., 2001) and simian virus 40 (Pelkmans et al., 2001). Insertion of GFP into the Gag protein of HIV allowed studying the trafficking of this protein, as well as production of infectious virions (Müller, 2004). Campbell et al. (2007) double-labelled HIV-1 with two fluorophores. This permitted the discrimination between unspecific endocytosed virions from those that have entered the host cell via the infection pathway.

GFP is a well characterised, spontaneously fluorescing protein isolated from the pacific jellyfish *Aequoria victoria* (Morin and Hastings, 1971; Ogawa et al., 1995). It is a widely used marker for gene expression monitoring and protein localisation. Another, relatively new fluorophore is the **m**onomeric **RFP** (mRFP), derived from

the red fluorescent protein dsRed isolated from the disc anemone *Discosoma* sp.. dsRed has the disadvantage of forming tetramers and therefore being very large. This problem has been overcome with the development of the monomeric form (Shaner et al., 2004).
Despite many successful studies with labelled viruses, one has to be aware that living systems are sensitive and prone to error if modifications are inserted. Careful analyses are necessary to ensure that the modification is tolerated and does not change the characteristic properties of the natural virus or protein.

Described in this study is the fluorescent labelling of hepatitis B virus surface proteins with GFP and mRFP.

1.2 The immune system.

Creatures are permanently in close contact with their environment and many different micro-organisms. To defend the body from such diverse infectious invaders as viruses, bacteria, fungi or multi-cellular parasites, a highly developed and complex system is necessary. But the seeking and killing of pathogens, together with the ability to differentiate between self and non-self, is not the only function of the immune system. Infected cells have to be recognised and removed, as well as degenerated or damaged cells, which are prone to develop malignant tumours.

Immune cells derive from one common precursor, the haematopoietic stem cells in the bone marrow. They give rise to all types of blood cells. Myeloid progenitors are the precursors of macrophages, granulocytes, mast cells and most **d**endritic **c**ells (DC). Lymphoid progenitors develop into lymphocytes, which further differentiate into two cell types. One population differentiates in the **b**one marrow (B) and is therefore designated as B lymphocytes. The second population differentiates in the **t**hymus (T) into so-called T lymphocytes (Janeway et al., 2004).

The immune system is divided into two systems. The phylogenetic older system is innate and displays the initial, antigen non-specific response of the body to a broad range of antigens. It comprises a number of different cell types, e.g. phagocytic cells, **n**atural **k**iller (NK) and **n**atural **k**iller **T** (NKT) cells, as well as molecules.

The phylogenetic younger, adaptive immune system is acquired and refers to antigen-specific defence mechanisms. The establishment of an adaptive immune response, which takes several days, aims at the specific removal of one particular antigen. Through repeated exposure to the same pathogen, the adaptive immune response improves and in many cases confers lifelong protective immunity. On the one hand, cellular immunity is involved, mediated by T cells, and on the other hand, humoral immunity, mediated by antibodies and cytokines.

Typically, effective immunity to a pathogen requires both the rapid but unspecific innate, as well as the later occurring pathogen-specific adaptive immune response.

1.2.1 Innate immunity.

The innate immune system provides a first line of defence against most micro-organisms. Furthermore, it plays a crucial role in stimulation of the adaptive immune response and control of infections during the first days, until adaptive immunity is established.

The innate immunity relies on the recognition of highly conserved structures, so-called **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMP). These are present in micro-organisms, but not in eukaryotic cells (Kawai and Akira, 2006). Innate immunity includes a cellular compartment and a soluble component, the complement system. The latter comprises a number of different plasma proteins, which activate proteolytic processes on microbial surfaces. This contributes to inflammation and induces pathogen uptake by macrophages and neutrophils (Janeway et al., 2004). This initial inflammation, induced by the innate immune system is crucial for the activation of the adaptive immune system (Janeway et al., 2004).

Pattern **r**ecognition **r**eceptors (PRR) that tag the different PAMPs are expressed by a variety of immune and non-immune cells. PRRs are present mostly on the plasma membrane, but also intracellularly (Medzhitov, 2001). **T**oll-**l**ike **r**eceptors (TLR) represent probably the most important family of PRRs. Their name is traceable to the Toll protein of *drosophila*, which possesses striking functional homologies to the TLRs in mammalians (Rock et al., 1998).

The cellular component of the innate immune system consists of a variety of cell types. If phagocytic cells, e.g. macrophages or DCs, bind a PAMP, a signalling cascade is prompted. The cells get activated and internalisation of the pathogen is triggered. Furthermore, the secretion of cytokines and chemokines is induced. These proteins mediate cellular communication, attract cells from the blood stream to the site of recognition and initiate a local inflammation process (Janeway et al., 2004). Furthermore, cytokines are responsible for the onset of the acute-phase response, a process taking place in the liver. Hepatocytes secrete acute-phase proteins, such as C-reactive protein, which are able to bind pathogens and stimulate the complement system (Janeway et al., 2004).

DCs play a pivotal role in the activation of the adaptive immune system, further described in chapter 1.2.3. Another cell type involved in innate immunity is

NK cells. They are responsible for the recognition of abnormal cells, for example tumour cells or cells containing intracellular pathogens (Trinchieri, 1989; Biron et al., 1999). NKT cells express NK-cell and T-cell markers and mediate direct cytotoxicity (Bendelac et al., 1995). Furthermore, they are producers of high amounts of IFNγ, a type 1 interferon known to inhibit the replication of HBV and other viruses (Guidotti et al., 1999; Pavic et al., 1993; Franco et al., 1997).
The defence mechanism of the innate immune system is rapid and effective. However, it is restricted to invaders that can be recognised by the germ-line encoded PRRs. Since micro-organisms evolve more rapidly then their hosts, some of them developed protective strategies to prevent recognition. This constraint has been overcome by the adaptive immune response.

1.2.2 Adaptive immunity.

The concept of adaptive immunity is to adapt to one specific invader. Key mediators for the humoral and cellular branch of the adaptive immune response are B and T lymphocytes, respectively. They recognise antigen structures present on pathogens, but not on own cells, as foreign. Lymphocytes, carrying the receptor against a specific antigen can promote a specific response against it. This includes cell proliferation and differentiation into specialised effector cells. This adaptation process takes four to seven days and is responsible for the delay between infection and adaptive immune response (Janeway and Medzhitov, 2002). Activation of specialised **a**ntigen **p**resenting **c**ells (APC) is a prerequisite for induction of the adaptive immune response. In this respect, dendritic cells represent the most potent cell population. Their role will be discussed in detail in the next chapter.
The B-cell mediated humoral immune response relies on antibody production and acts on extracellular, circulating antigens, such as pathogens and foreign proteins. Naïve B lymphocytes, circulating in the blood, bear antigen receptors of a particular specificity on their surface, termed **m**embrane-standing **i**mmuno**g**lobulin (IgM) or **B**-**c**ell **r**eceptor (BCR). All individual cells differ in this specificity (Janeway et al., 2004). The body therefore has a broad diverse lymphocyte receptor repertoire at command, enabling it to react to virtually any existing antigen.

However, the number of cells able to bind to a given antigen is very small. A mechanism known as clonal selection ascertains the expansion of lymphocytes, as soon as their specific antigen receptors fit to an invaded pathogen (Janeway et al., 2004). Receptor-antigen interaction is the first signal for the receptor-carrying B cell to get activated. Importantly, a second signal, provided by T cells, is necessary to induce the activation process. A large population of cells with the same receptor specificity is build up through clonal expansion. The original membrane-standing BCR is secreted in the form of antibodies. Their functions are firstly the neutralisation of pathogens or their often toxic products by specific binding. As a result, their access to potential target cells is blocked. Secondly, bound antibodies serve as a marker for phagocytic cells, which recognise the constant region of the antibodies and ingest antibody-covered pathogens. This type of antibody binding is referred to as opsonisation (Janeway et al., 2004). Thirdly, antibodies activate the complement system.

After removal of the antigen, most effector cells undergo apoptosis (Banchereau et al., 1994). However, a small population of effector cells persist. They traverse an immunoglobulin-isotype change from IgM to IgG and develop into memory B cells. These long-living cells circulate within the lymphoid organs and mediate lifelong immunity (Liu et al., 1991).

The high diversity of antigen receptors is based on a genetic mechanism called somatic recombination. BCRs consist of a heavy and a light chain, each containing a constant and a variable region. The genes encoding the variable regions are inherited as sets of gene segments. These segments are joined by DNA recombination, building genes encoding the variable regions of both chains. These genes are unique in every lymphocyte (Janeway et al., 2004). Furthermore, somatic hyper-mutation during the immune response leads to additional variation in the already rearranged genes, often resulting in improved antibody-antigen binding (Weigert et al., 1970; Michael et al., 2002). To ensure that the generated antigen receptors are useful, not directed against the host-self, and that lymphocyte-cell numbers remain relatively constant, further mechanisms are in need. Lymphocyte survival is regulated by signals from their antigen receptors, delivered by cells in the lymphoid organs. A positive selection process guarantees that only those cells survive, which receive survival signals (Linette and Korsmeyer, 1994). A negative selection for cells strongly reacting with

self-antigens prevents autoimmunity by purging off receptors recognising self-peptides (Mannie, 1991; Laufer et al., 1996). In lymphocytes not receiving survival signals, as well as in those lymphocytes strongly reacting with self-antigens, apoptosis is induced.

The cellular component of the adaptive immune response is directed against intracellular antigens. It requires antigen processing and presentation from infected or transformed body cells, the latter presenting tumour-specific antigens. T lymphocytes are the main effectors of the cellular immune response. One differentiates two types of T cells, based on their surface co-receptors, so called **c**lusters of **d**ifferentiation (CD). CD4 and CD8 determine the interaction of the T cell with the antigen presentation complexes (MHC, **m**ajor **h**istocompatibility **c**omplex) MHC I and MHC II on APCs (Woodland and Dutton, 2003). MHC I is expressed on every nuclear cell and presents intracellular antigens. MHC II is present only on professional APCs and carries phagocytosed antigens of extracellular pathogens. An exception displays a mechanism known as cross-presentation. In this case, internalised extracellular antigens are transferred to the MHC I pathway (Bevan, 1976).

CD4 positive ($CD4^+$) T cells bind antigens presented on MHC II molecules. They are divided into the helper-cell and regulatory-cell subsets. T helper cells (T_H) are further differentiated into T_H1 and T_H2 subsets, carrying out different functions, particularly in the defence against bacteria. While T_H1 cells activate macrophages and promote phagocytic activities, thus driving the immune response in the direction of cytotoxicity, T_H2 cells foster the humoral immune response by B-cell activation to produce neutralising antibodies (Ridge et al., 1998; Sigal et al., 1999). The importance for instance of HBV-specific $CD4^+$ T cells in the defence against HBV is due to the induction and maintenance of $CD8^+$ T-cell responses and the activation of B cells (Rehermann, 2003).

CD8 positive ($CD8^+$) T cells are referred to as **c**ytotoxic **T l**ymphocytes (CTL) and recognise antigens in the MHC I context. Their task is to kill infected cells. In case of an HBV infection, CTLs specific for different HBV epitopes remove infected hepatocytes directly by lysis. However, they seem to control infection as well at a non-cytolytic level (Maini et al., 2000).

A small subpopulation of $CD4^+$ and $CD8^+$ T cells, termed **reg**ulatory **T** cells (Treg), exercise control functions of the adaptive immune response, for example in the

regulation of immuno-inflammatory effects during viral infections (Suvas et al., 2004). They are characterised by the expression of the surface markers CD25 and **fo**rkhead bo**x** **p**rotein **3** (FoxP3; Raimondi et al., 2007).

The activation of naïve T lymphocytes takes place in the thymus, basically via the same mechanism as described for B cells. **T** **c**ell **r**eceptors (TCR) differ from BCRs in their structure. Nevertheless, their diversity is also obtained by somatic gene recombination and controlled by positive and negative selection processes. Antigens taken up by APCs are processed, loaded on MHC I or MHC II molecules, and displayed at the cell surface. The clonal selection ensures that only those T cells bearing a specific TCR for the given antigen, and which bind the MHC-antigen complex presented by an APC together with co-stimulatory signals, are activated. A large population of antigen-specific T cells is build up via clonal expansion. After removal of the antigen, the death of most effector T cells is induced by the absence of stimuli from their specific antigen. A small population of memory T cells is retained, which is very sensitive to re-stimulation by its antigen and able to react very fast (Janeway et al., 2004).

1.2.3 Dendritic cells.

Dendritic cells are the key antigen presenting cells. They play a cardinal role in innate and adaptive immunity, especially in T-lymphocyte activation and differentiation (Banchereau and Steinman, 1998). The origin of DCs is elusive, but at least two DC precursor populations are proposed, the myeloid and plasmacytoid progenitors. However, some DC subsets may derive from a third origin (Banchereau et al., 2000). The DC precursors develop into **im**mature **DC**s (imDC) and migrate to peripheral tissues. They persist there for a variable length of time. ImDCs are phagocytic cells continually taking up extracellular material via pinocytosis. In addition to this receptor-independent uptake mechanism, they engulf pathogens via phagocytosis and receptor-mediated endocytosis. ImDCs express many different types of PRRs, such as C-type lectins, mannose receptors and TLRs. Besides the high expression level of PRRs, imDCs are characterised by low expression levels of MHC and co-stimulatory molecules. Small amounts of synthesised MHC II molecules are mainly sequestered in endosomes and

lysosomes (Cella et al., 1997). ImDCs do not interact with lymphocytes or secrete cytokines and chemokines (Janeway et al., 2004). Morphologically, imDCs can be distinguished from **mat**ure **DC**s (matDC) by means of the lack of dendrites. These are typical for matDCs. The main differences between immature and mature DCs are listed in fig.5

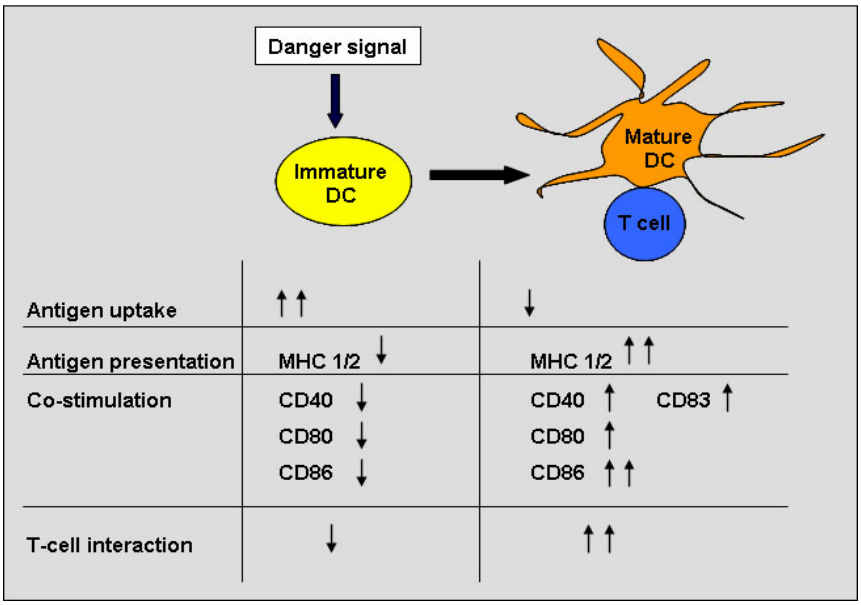

Fig. 5: Main characteristics of immature and mature dendritic cells.

Pathogens ingested by imDCs are intracellular degraded. Activated by pathogen uptake, a signalling cascade is induced, which leads to a change in the protein-expression patterns. The expression of MHC and co-stimulatory molecules is highly up-regulated (Banchereau et al., 2000). Along with the transition, PRRs and antigen uptake are down-regulated (Garret et al., 2000). Functional MHC II peptide-antigen complexes are generated and accumulate at the cell surface, clustered with co-stimulatory molecules (Turley et al., 2000). In this condition, the cells are designated as mature. They are endowed with the ability to effectively present antigens, activate naïve T cells and stimulate the innate and adaptive immune system via cytokine secretion (Banchereau et al., 2000). Remodelling of the chemokine-receptor profile facilitates homing to lymphoid organs (Chan et al., 1999). Subsequently, the matDCs migrate to the lymph nodes, where they provide the naïve T cells with two signals crucial for activation: The first signal is antigen specific and results from binding of the TCR to the antigen, presented by a MHC molecule on the DC surface. The second signal is provided by co-stimulatory molecules, such as CD80 and CD86, also present on the DC surface. The role of

DCs in regulating T-cell responses is well documented (Banchereau et al., 2000): The density of peptides presented at the surface, the types of co-stimulatory molecules expressed, and cytokines secreted by matDCs upon stimulation with the different types of signals, drive naïve T-cell development into a certain direction. $CD4^+$ T-cell differentiation into T_H1 cell is induced by DC IL-12 and IFN type 1 production (Lanzavecchia and Sallusto, 2001; Eisenbarth et al., 2003). In response to extracellular pathogens, DCs mediate $CD4^+$ T-cell development into T_H2 cells (Moser and Murphy, 2000; Sher et al., 2003). $CD8^+$ T cells become effector CTLs or, upon co-stimulation with T_H cells, memory T cells (Janssen et al., 2003; Shedlock and Shen, 2003).

An important characteristic of DCs is the ability to cross-present antigens. The classical MHC I pathway provides for the presentation of endogenous antigens to $CD8^+$ T cells, for instance in virus-infected cells. In contrast, exogenous antigens are presented via the MHC II pathway, activating $CD4^+$ T cells. This phenomenon is known as MHC restriction. Viral proteins expressed in the cytoplasm are subjected to the MHC I pathway and presented to CTLs. However, since not all viruses infect DCs, there must be alternative ways to transfer antigens to the MHC I pathway: DCs can receptor-mediated internalise infected or maligned apoptotic cells and cross-present their endogenous antigens on MHC I molecules to CTLs (Albert et al., 1998). This is likely to occur via a pathway directly transferring the internalised antigens to the ER, where they are loaded on MHC I molecules (Ackermann et al., 2005). Also antigens that are not expected to gain access to the cytoplasm are presented on MHC I molecules via an exogenous pathway. Little is known about this process, but DCs seem to allow the egress of internalised antigens from endocytic organelles into the cytosol (Kovacsovics-Bankowski et al., 1995; Rodriguez et al., 1999). From there, the antigens enter the conventional MHC I pathway and are presented on MHC I instead of MHC II molecules. So far, DCs and to a lesser extend macrophages, are the only cell types known to be capable of breaking MHC restriction (Banchereau et al., 2000).

The main pathway by which DCs get activated, mature and provide all necessary signals to naïve T cells, is PAMP recognition via PRRs. They are therefore regarded as the key cell type coupling PRR-mediated innate immune recognition to the initiation of an adaptive immune response.

DCs play a dual role in the immune system. On the one hand, they respond to foreign antigen stimulation such that T-cell activation occurs. On the other hand, it is presumed that they play a role in induction and regulation of immune-tolerance (Steinman et al., 2003). This is an important feature to avoid immune responses against self, and against antigens permanently present, for instance in the respiratory or digestive tracts (Vermealen et al., 2001). Resident tissue DCs take up self-antigens or permanently present environmental antigens via phagocytosis or macropinocytosis. After a certain time they traverse to lymphoid tissues, although not being activated by infection. Since they lack co-stimulatory molecules on their surface, these cells do not activate T cells, but induce tolerance to the antigen (Steinman et al., 2003). This mechanism is presumably a safeguard against autoimmunity and chronic inflammation (Banchereau and Steinman, 1998). A specific T-cell population, the Tregs, controls the maintenance of self-tolerance (Gershon et al., 1974; Baecher-Allan et al., 2004). This has to be accomplished by a mechanism not interfering with pathogen-specific protective immune response. Pasare and Medzhitov (2003) hypothesised a scenario based on two different control mechanisms: Firstly, upon TLR activation DCs must express co-stimulatory molecules. Secondly, DCs must produce IL-6. The IL-6, in turn, affects T cells in a way that makes them resistant to suppression by Tregs. *In vivo* observations in mice of Yang et al. (2004) also hint in this direction.

Multiple DC subsets have been identified so far. However, their function in immune defence remains to be unravelled. Researches in humans and mice demonstrated that the immune system comprises of many DC subpopulations with distinct gene-expression patterns, PRR profiles and anatomical locations. This suggests a specialisation of the different subsets to interact with different types of pathogens and to induce distinct effector cells (Banchereau et al., 2000; Ito et al., 2005). In the following, the focus lies on DC subsets continuously circulating in peripheral blood, as well as on resident hepatic DCs.

Monocytes make up for a large percentage of the **p**eripheral **b**lood **m**ononuclear **c**ells (PBMC). They differentiate either into tissue macrophages or **m**yeloid **d**endritic **c**ells (mDC). The expression of a variety of TLRs enables them to recognise a wide range of pathogens in the blood and peripheral tissue (Kadowaki et al., 2001a). *In vitro* differentiation of monocytes isolated from peripheral blood is a tool commonly used to obtain dendritic cells. The advantages over the direct

isolation of DCs are on the one hand the straight forward protocol, and on the other hand the high cell number, which can be obtained from a relatively small amount of peripheral blood. Isolated monocytes can be differentiated into immature **mo**nocyte-derived **DC**s (moDC) by addition of **g**ranulocyte-**m**acrophage **c**olony **s**timulating **f**actor (GM-CSF) plus **i**nter**l**eukin-4 (IL-4). By addition of **l**ipo**p**oly**s**accharide (LPS) and **t**umor **n**ecrosis **f**actor α (TNF-α), mature moDCs can be generated (Sallusto and Lanzavecchia, 1994). However, Osugi et al. (2002) demonstrated that *in vitro* generated moDCs differ from blood DCs in several aspects. Therefore their behaviour does not always reflect the situation in peripheral blood DCs.

The major DC precursors *in vivo* are **m**yeloid and **p**lasmacytoid **DC**s (mDC, pDC) (Liu et al., 2001). mDCs are characterised by the absence of surface lineage markers and the presence of **b**lood **d**endritic **c**ell **a**ntigen **1** (BDCA1) and CD11c (Ito et al., 2002). They make up approx. 1 to 2% of PBMCs and are characterised by a strong expression of TLRs 1, 2, 3 and 5, mainly recognising bacterial components, except for TLR 3, which binds viral ds RNA (Kadowaki et al., 2001a). Upon stimulation, they produce pro-inflammatory cytokines, such as TNF-α, IL-6, IL-12 as well as IFN-α and β (Kadowaki et al., 2001a and 2001b; Ito et al., 2002).

pDCs constitute a very small population of 0.5 to 1% of PBMCs. They are characterised by the markers BDCA2, BDCA4 and CD123 (Colonna et al., 2004). A very important feature of pDCs is their role in antiviral defence. During viral infection, pDCs are recruited to the inflamed lymph nodes. They are the main source of type 1 IFNs, which inhibit viral replication and thus have a strong antiviral effect (Cella et al., 1999; Siegal et al., 1999). IFNs are therefore used for the treatment for instance of chronic HBV infection. The ability of pDCs to efficiently recognise viruses is at least partially due to the expression of TLRs 7 and 9, which are specific for ss RNA and ds DNA viruses, respectively (Kadowaki et al., 2001a; Lund et al., 2003; Diebold et al., 2004; Heil et al., 2004). *In vitro* experiments have revealed that pDCs can induce T_H1 differentiation, and activate B cells to secrete antibodies (Cella et al., 2000; Jego et al., 2003). Furthermore, the induction of mDC maturation by HIV-infected pDCs has been demonstrated (Fonteneau et al., 2004). The ability to induce Tregs is suggested (Gilliet and Liu, 2002). Taken together, these data indicate a major function of pDCs in the immune response against viruses.

Resident DCs are present in low numbers in the liver, but expand upon stimulation (Thomson et al., 1999). However, after stimulation the hepatic DC populations still exhibit imDC phenotype, expressing low levels of MHC II, CD80 and CD86. It is suggested that these cells induce tolerance rather then activate naïve T cells (Thomson and Lu, 1999a and 1999b). Recent studies demonstrated that they are less efficient in activating naïve T cells compared to other organ resident DC populations (Goddard et al., 2004; de Creus et al., 2005).

1.2.4 HBV-specific immune response.

HBV-infected patients are usually detected only after the onset of clinical symptoms, which occurs two to six months after infection. Therefore, the early events of the HBV-specific immune response are difficult to study in natural human infection. Most data have been obtained in mouse, woodchuck and chimpanzee models (Guidotti et al., 1996; Guidotti et al., 1999; Wang et al., 2003; Wieland et al., 2004). The progression of an acute self-limiting and a chronic HBV infection differ in several aspects. Fig. 6 schematically depicts the characteristics of both.
For approx. four weeks post primary infection, HBV remains quiescent, with low amounts of 10^2 to 10^4 HBV genome equivalents per ml blood in the circulation (Rehermann and Nascimbeni, 2005). HBsAg and HBeAg come up about four weeks after exposure, followed by antibodies against the viral core protein (anti HBc). By this time viremia is established, characterised by viral titers of 10^9 to 10^{10} virus particles per ml blood (Ribeiro et al., 2002). Serum levels of **al**anin-amino**t**ransferases (ALT) rise between weeks eight and twelve. These enzymes are released by apoptotic hepatocytes and serve as markers for liver injury. In 90% of the cases, acute infection is cleared after ten to fifteen weeks. IgG-type antibodies against HBcAg, HBsAg and HBeAg mediate a lifelong protective immunity against HBV (Rehermann and Nascimbeni, 2005). Despite the clearance, low levels of viral replication can be detected for decades in the patients. Trace amounts of persisting virus might contribute to maintaining immunity against HBV in recovered patients, since antigen-specific humoral and cellular immune responses control the virus (Rehermann et al., 1996). Conversely,

the persisting virus may as well be responsible for chronic infection in some individuals (Rehermann and Nascimbeni, 2005).

Compared to many human viruses, such as HIV, CMV and HCV, which display a fast viral spread and subsequent rapid activation of innate immunity, the kinetics of HBV infection appear to be unconventional (Bertoletti and Gehring, 2006). The cause of the delayed appearance of HBV DNA and proteins is unclear. One could speculate that HBV initially infects only a few hepatocytes with a slow doubling time, or that HBV does not reach the liver immediately after infection (Bertoletti and Gehring, 2006).

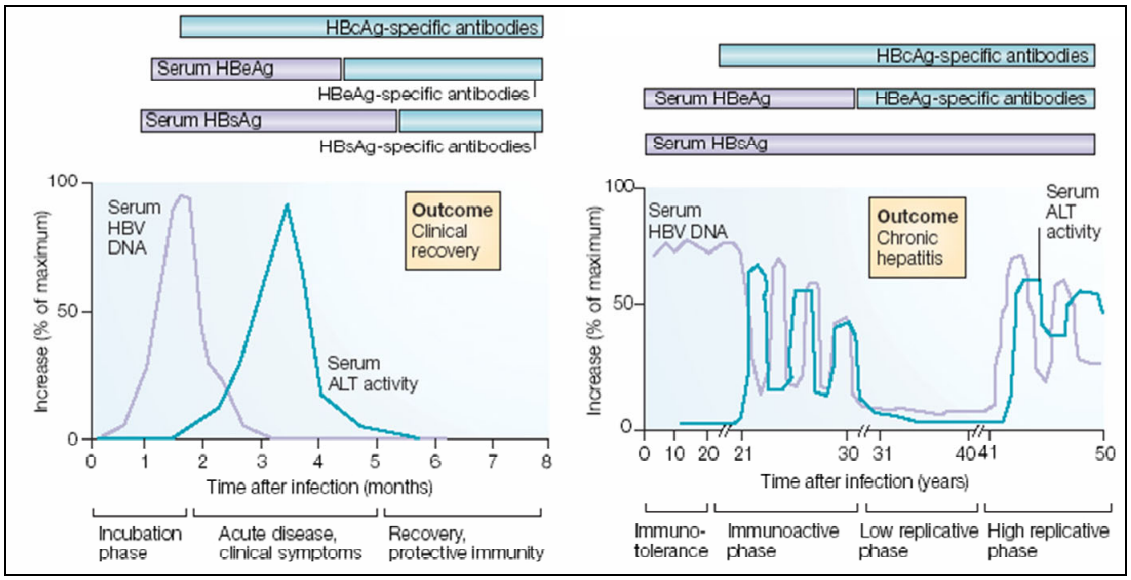

Fig. 6: Course of acute self-limited and chronic HBV infection (from: Rehermann and Nascimbeni, 2005).

Chronic hepatitis B is a heterogeneous disease with variable courses and severity (Ganem and Prince, 2004). In contrast to an acute infection, the chronic progression is characterised by a lack of anti HBs-specific IgGs and a late and weak or missing antibody response to HBeAg. Virus load is variably high but persistent, and often HBeAg is detectable for years after the primary exposure (Ganem and Schneider, 2001). Hepatic flares with irregularly increasing and decreasing ALT levels can be detected in approx. 2% of patients, mostly in correlation with changes in therapy (Mels et al., 1994). Spontaneous recovery of T-cell reactivity during flares, as well as under lamivudine therapy, followed by viral clearance, has been observed (Rossol et al., 1997; Boni et al., 2001).

In general, it has become clear that the course of HBV infection depends mostly on the adaptive T-cell response of the host (Wieland et al., 2004). Cytotoxic T cells are responsible for the clearance of the infection as well as for liver injury (Thimme et al., 2003). In the case of a persistent liver inflammation, which is typical for chronic HBV carriers, DNA damage due to massive regenerative processes promotes HCC formation (Chisari and Ferrari, 1995b). A quantitative difference in virus-specific CTL numbers between chronic and acute infection has been demonstrated, correlating with virus control, but not with liver damage (Maini et al., 2000). Virus-specific T-cell response is weak or undetectable in chronic carriers (Ferrari et al., 1990; Rehermann et al., 1995b). The CTLs infiltrating the liver are, contrary to the acute outcome, virus non-specific or directed against sub-dominant epitopes. They contribute to liver damage (Webster et al., 2004). An HBV-specific T_H-cell response is mostly absent (Ferrari et al., 1990).

Some factors that could explain the impairment of the immune response in chronic HBV infection have been suggested. One candidate escape mechanism is the development of mutated virus. However, HBV mutants are not common in acute infection, as well as in chronic infection, and typically remain in low abundance (Rehermann et al., 1995a; Whalley et al., 2004). Another possibility is the involvement of HBeAg, which is secreted in large amounts during viral replication. Its tolerising effect has been shown in mice (Milich and Liang, 2003; Chen et al., 2005a). This might account for the low levels of core-specific T-cell responses. A role in chronicity has also been proposed for the SVPs produced in up to 10^4-fold excess over virions (Rehermann and Nascimbeni, 2005). The effects of Tregs have not yet been fully elucidated (Maloy and Powrie, 2001). However, a role in chronicity of several virus infections, e.g. with HCV or HIV has been proposed (Sugimoto et al., 2003; Kinter et al., 2007). Therefore, inhibition of HBV-specific T-cell function in chronic HBV by Tregs may be considered. Furthermore, the immunological features of the liver may support immuno-tolerance in chronic HBV infection: The hepatic environment is known to be tolerogenic. Presentation of exogenous antigen to T cells by liver endothelial cells induces specific T-cell tolerance (Limmer et al., 2000). Further on, hepatocytes require a very high antigen concentration to stimulate CTLs, due to a low expression level of MHC I. This implies that pathogens infecting the liver are less likely recognised. However, these data are currently under debate (Janeway et al.,

2004; Chen et al., 2005b). Data concerning CTL priming in the liver in animal models are controversial. Crispe et al. (2000) and Bowen et al. (2004) reported apoptosis of activated $CD8^+$ T cells and tolerance induction in $CD8^+$ T cells in the liver, respectively. Inconsistent with this, in other studies the rapid activation of naïve and effector $CD8^+$ T cells within the liver of mice has been observed (Isogawa et al., 2005; Klein and Crispe, 2006).

The existence of HBV in extra-hepatic sites has been described, possibly contributing to low-level HBV persistence (Ganem and Schneider, 2001). An infection of immune cells by HBV might be the reason for an impaired immune response in chronic hepatitis B carriers. DCs, being crucial for T-cell priming, appear interesting in this context. Functional impairment of these cells could explain the B-cell and T-cell hypo-responsiveness in chronic HBV-infected patients. Furthermore, it is known for many chronic viral infections, such as HCV, HIV and vaccinia virus infection that DC function is impaired (Kanto et al., 1999; Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Knight et al., 1993; Donaghy et al., 2003; Engelmayer et al., 1999). Evidence for DC dysfunction in chronic HBV infected patients also exists, but is controversially discussed. While Beckebaum et al. (2003) found functional deficits in moDCs upon contact with HBV this result was not confirmed in a study by Tavakoli et al. (2004). With regard to the question whether total DC numbers are reduced in chronic HBV infection, the data are contrary as well (Beckebaum et al., 2002; van der Molen et al., 2004; Duan et al., 2004). Some investigators reported the presence of HBV-DNA in DC lysates, detected by **p**olymerase **c**hain **r**eaction (PCR). This finding was generally interpreted as an indication for infection of DCs with HBV (Beckebaum et al., 2002; Tavakoli et al., 2004; Zheng et al., 2004). However, since phagocytosed viruses are also detected with this method, the DNA did not necessarily originate from replicating virus, as shown by Köck et al. (1996). Merely the proof of an established cccDNA pool in these cells would allow the conclusion that DCs are productively infected (Ganem and Schneider, 2001). Thus, the possibility of a direct infection of DCs with HBV in chronic patients remains to be determined.

The following study addressed the question whether immature or mature moDCs can be infected with HBV in cell culture and whether viral antigens are expressed. Advantage was taken from HBV-based vectors, which allow dissecting the early

steps of HBV infection (Protzer et al., 1999). Luciferase expression under the CMV promoter or an HBV-specific promoter, after uptake and nuclear transport, enables a distinction between unspecific viral uptake and infection (Untergasser and Protzer, 2004). Furthermore, the formation of nuclear HBV-cccDNA after incubation with wild-type HBV was investigated in moDCs with an optimised PCR technique. In order to investigate HBV-antigen expression adenoviral vectors carrying a complete HBV genome, as well as a GFP reporter-cassette, were used to efficiently bring the HBV genome into the cells (Sprinzl et al., 2001). To verify the results in the *in vivo* situation, mDCs and pDCs from highly viremic chronic carriers were tested *ex vivo* for HBV infection via PCR.

2. (I) Dendritic cells take up viral antigens but do not support the early steps of Hepatitis B virus infection

2.1 Results I

In chronic HBV carriers, dendritic cells (DC) are functionally impaired. A reason for this might be the infection of DCs by HBV. In this study, it was analysed in an *in vitro* system whether monocyte derived DCs (moDC) support the different steps of HBV infection: Virus uptake, nuclear transport of the genome, and replication. To address the question whether moDCs are capable of HBV antigen production and progeny virus formation, HBV genomes were artificially introduced into the cells with an adenoviral vector system. Taking advantage from HB viral vectors expressing *Renilla* luciferase either under a non–liver-specific promoter or under a HBV promoter, genome delivery into the nucleus was analysed using a luciferase detection assay. Furthermore, moDCs were incubated with wild-type HBV and investigated by quantitative real-time PCR for the presence of the different viral DNA forms: the rcDNA, which is present only in virions in the cytosol, and the cccDNA, which is only present inside the nucleus of an infected cell. To verify the observation in the *in vivo* situation, myeloid (mDC) and plasmacytoid (pDC) DCs from chronic HBV carriers were investigated *ex vivo.* Quantitative real-time PCR was used for the detection of rcDNA and cccDNA in the cells, and with electron microscopy the cells were further analysed for the presence of viral and subviral particles.

2.1.1 HBV antigen expression and replication in moDCs.

Monocyte derived dendritic cells (moDC) are a commonly used model for immature and mature peripheral blood DCs. Monocytes were isolated from buffy coats or fresh peripheral blood and *in vitro* differentiated into immature moDCs using IL-4 and GM-CSF. Upon further stimulation with LPS, TNF-α and PGE_2 mature moDCs were obtained. To determine the maturation status, the cells were examined for the expression levels of HLA-DR, CD80, CD86 and CD83. These proteins are typical surface markers of matured moDCs.

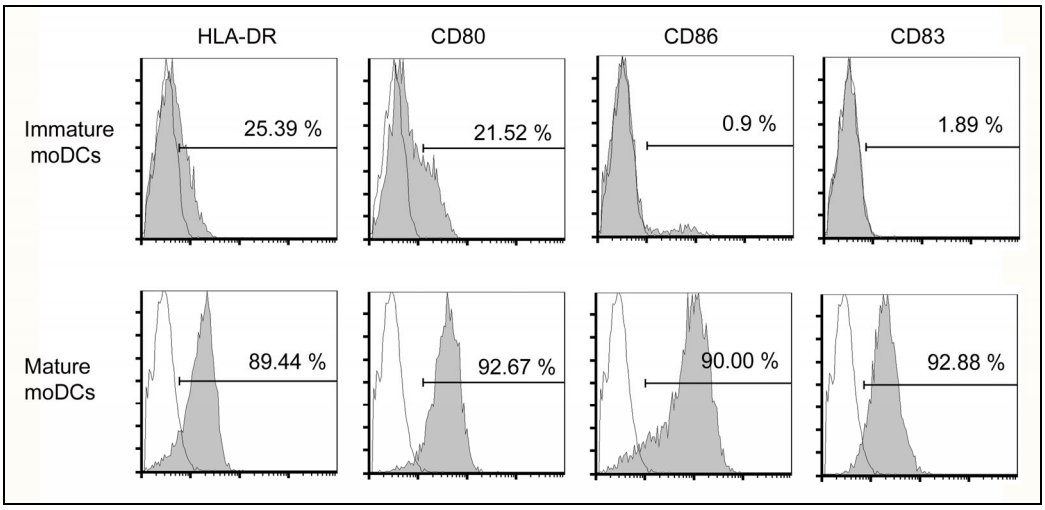

Fig. 7: Flow cytometry analysis of the maturation status of moDCs.

In the upper panel the percentage of moDCs expressing the surface markers HLA-DR, CD80, CD86 and CD83 before *in vitro* differentiation (immature moDCs) are shown. The lower panel depicts the percentages of moDCs expressing the markers after *in vitro* differentiation (mature moDCs).

Fig. 7 shows an example of the flow cytometry data of a moDC preparation before and after *in vitro* maturation. Flow cytometry revealed a significant up-regulation of the surface markers in mature moDCs in comparison to immature moDCs. Only 25.39% of immature moDCs expressed HLA-DR, while 98.44% of mature moDCs were positive for the marker. CD80 was expressed in 21.52% of immature moDCs and 92.67% of mature moDCs. While 0.39% and 1.89% of immature moDCs appeared positive for CD86 and CD83 respectively, these markers were expressed by 90% and 92.88% of cells after differentiation. The results of the flow cytometry analyses for all moDCs are summarised in tab. 1.

Tab. 1: Summary of flow cytometry analyses of moDCs.

The data represent the median percentages of cells, staining positive for the surface markers HLA-DR, CD80, CD86 and CD83 before (immature moDCs) and after (mature moDCs) *in vitro* differentiation.

Marker	Immature moDCs	Mature moDCs
HLA-DR	21.6% ± 5.2%	92.2% ± 3.4%
CD80	32.5% ± 9.9%	90.5% ± 8.4%
CD86	3.1% ± 1.9%	88.0 ± 4.0%
CD83	4.4% ± 4.7%	96.6 ± 2.6%

It demonstrates that the change in the expression pattern of the analysed surface markers after the maturation protocol was generally observed in all experiments. This shows that the *in vitro* differentiation of immature moDCs into mature moDCs was successful.

2.1.1.1 Transduction of moDCs with AdG-HBV1.3.

In order to study whether immature and mature moDCs express hepatitis B viral antigens under HBV-specific promoters, an adenoviral vector system was chosen.

In contrast to the use of wtHBV, this allowed to exclusively analyse the expression of the viral antigens: it is known that DCs are susceptible for adenovirus infection, which includes virus uptake and genome transfer into the nucleus. Therefore, additional effects such as a block in virus entry or genome delivery into the nucleus, which would impair the antigen expression, could be ruled out. The vector AdG-HBV1.3 is based on the adenovirus type 5 and contains a 1.3-fold HBV over-length genome (Fig. 8).

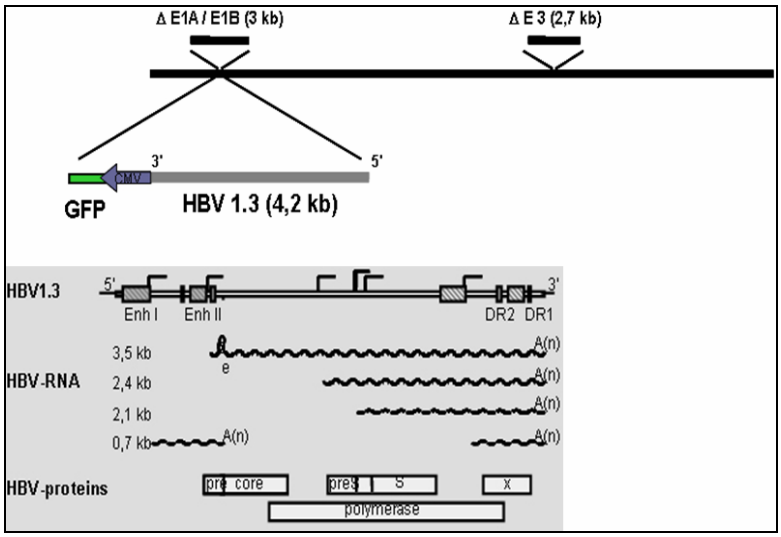

Fig. 8: Schematic depiction of the adenoviral vector AdG-HBV1.3

The insert, consisting of the 1.3-fold HBV over-length genome (grey bar) and the GFP reporter cassette (green bar), is placed in the E1A/E1B region of the adenovirus genome. The E1A/E1B region and the E3 gene of the adenoviral genome are deleted.

The over length is necessary, because the hepatitis B viral circular genome is organised in overlapping ORFs. In addition to the HBV genome, a GFP reporter cassette is integrated in the adenoviral vector under CMV promoter control. AdG-HBV1.3 has been shown to efficiently initiate HBV antigen expression in primary human hepatocytes (PHH; Fig. 10a). Due to the deletions of the E1A/E1B region and the E3 gene, the adenovirus vector is infectious but replication deficient. Since all HBV genes are expressed from the vector, HBV replication is possible and the vector can be used to induce HBV replication. An overview over the transduction of a PHH with adenoviral vectors is provided in fig. 9.

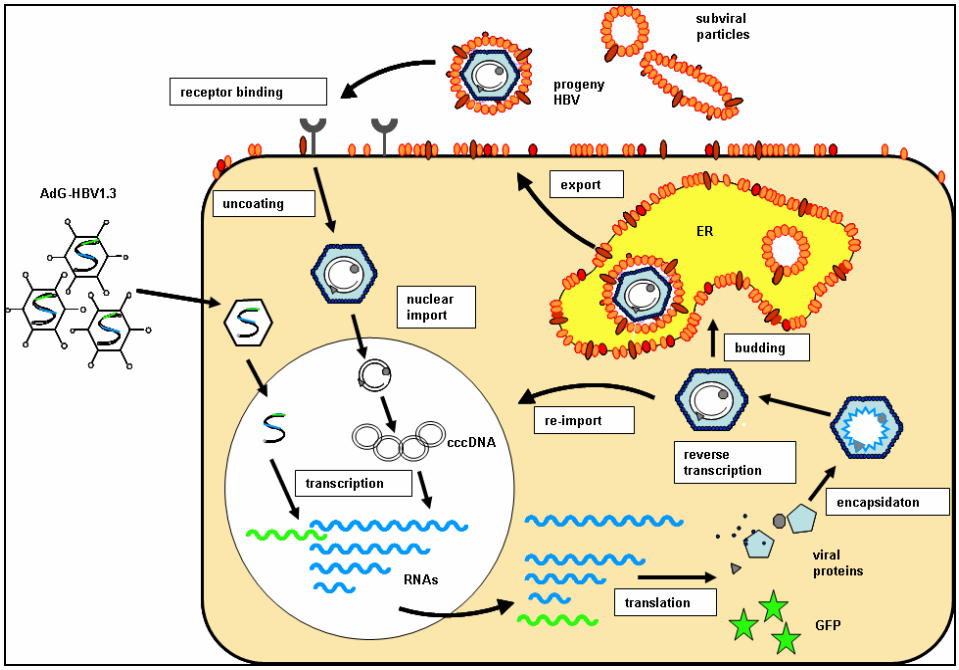

Fig. 9: Transduction of a hepatocyte with AdG-HBV1.3

The adenoviral vector, containing the HBV 1.3 over-length genome (blue) and the GFP reporter cassette (green), enters the cell. The transgenes are delivered to the nucleus and GFP RNA (green serpentine line) and the HBV RNAs (blue serpentine lines) are built. GFP proteins (green stars) accumulate in the cell. HBV capsids assemble and after reverse transcription of the encapsidated pgRNA, they are either exported (progeny HBV) or re-imported into the nucleus. A cccDNA pool can be established. SVPs form and bud out of the cell. The progeny virus can either re-infect the cell or infect other cells.

To investigate the expression of HBV antigens in moDCs, the AdG-HBV1.3 stock was first titrated on a PHH culture (Fig. 10a, left panel). A multiplicity of infection (moi) of 1 virus particle per cell (vp/cell) led to a transduction rate of approx. 65%. With a moi of 3 vp/cell, more than 90% of the PHHs were

transduced, as monitored microscopically by GFP expression. Transduction of the PHHs with a moi of 10 vp/cell did not increase the percentage of GFP-positive cells. In the PHH culture, 10% of the cells remained untransduced. These were most probably non-parenchymal liver cells, which make up for 5-15% in PHH cultures, and are not susceptible for adenovirus type 5 infections. Mock-transduced PHHs served as negative control.

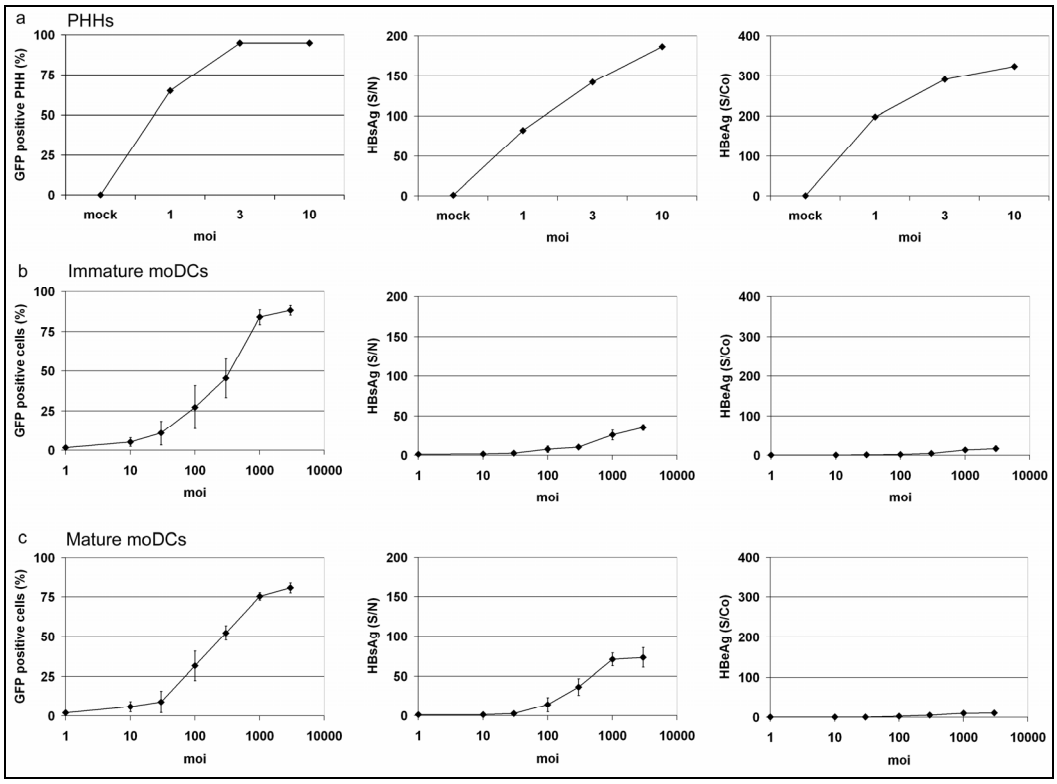

Fig. 10: Transduction of PHHs, immature and mature moDCs with AdG-HBV1.3.

The results for immature and mature moDCs reflect the median values and standard deviations of data obtained from moDCs of three different donors. Left panels: Flow cytometry analysis of GFP positive PHHs (a), immature moDCs (b) or mature moDCs (c) after transduction with the indicated mois of AdG-HBV1.3. Middle panel: ELISA for HBsAg in the supernatant of transduced PHHs (a), immature moDCs (b) or mature moDCs (c) at the indicated mois. Right panel: ELISA for HBeAg in the supernatant of transduced PHH (a), immature moDCs (b) or mature moDCs (c) at the indicated mois. S/Co = Signal/Control; S/N = Signal/Noise.

To obtain similar transduction rates with AdG-HBV1.3 in moDCs, the cells were incubated with mois of 1, 10, 30, 100, 300, 1000 and 3000 vp/cell. The gene transfer efficiency was determined using flow cytometry, sorting for GFP-expressing cells. The results for the titration of AdG-HBV1.3 on immature and mature moDCs are depicted in fig. 10b and c (left panels), respectively. A moi of

1000 vp/cell was required to reach a transduction rate of 80% for both immature and mature moDCs. The amount of secreted HBsAg and HBeAg in the medium of the transduced cells was examined at day four post transduction by ELISA. The results are presented in fig. 10 (middle and right panels). The transduced PHHs secreted high amounts of HBsAg and HBeAg. The secretion correlated with the amount of transduced cells, and reached the highest level after transduction with a moi of 10 vp/cell. For the immature and mature moDCs, low amounts of HBsAg and only trace amounts of HBeAg could be detected, correlating with the amount of transduced cells. Compared to 80% transduced PHHs, the secretion of HBsAg was reduced 4.3-fold in 80% transduced immature moDCs, and 1.6-fold in 80% transduced mature moDCs. HBeAg expression, which is controlled by a liver-specific promoter, was 17-fold lower for immature moDCs and 24.6-fold for mature moDCs. Summarising, moDCs were only to a slight extend able to express HBV antigens, even though the transduction rate with the adenoviral vector was 80%.

The expression level of HBsAg was higher than the expression level of HBeAg, possibly due to the liver specificity of the precore/core promoter. The maturation state of the moDCs seemed to play a minor role in the ability of HBV antigen expression. However, in comparison, HBsAg was higher expressed in moDCs in the matured status, while HBeAg was higher expressed in moDCs with immature status.

The expression of HBsAg and HBeAg is known to depend on the presence of the transcription factors HNF1 and HNF4. The two forms of HNF1, HNF1-α and HNF1-β, enhance the transcription from the preS1 promoter, which gives rise to the HBsAg. HNF4 is necessary for effective transcription from the precore/core promoter, leading to HBeAg expression. In order to investigate whether moDCs express HNF1 and HNF4, Western blotting of cellular lysates with antibodies against HNF1 and HNF4 was performed. The hepatocellular carcinoma cell line HepG2 served as a positive control. To control the amount of blotted protein, the house keeping protein β-actin was stained on the blot. Fig. 11 shows the Western blots for HNF1 and HNF4. Both proteins were clearly detected in HepG2 cells. The 92 kDa band represents HNF1-α, the 61 kDa band results from HNF1-β, which was expressed in lower levels. Neither in immature moDCs nor in mature moDCs could HNF1 be detected. The same held true for HNF4:

The 56 kDa protein was present in HepG2 cells, but not in immature and mature moDCs.

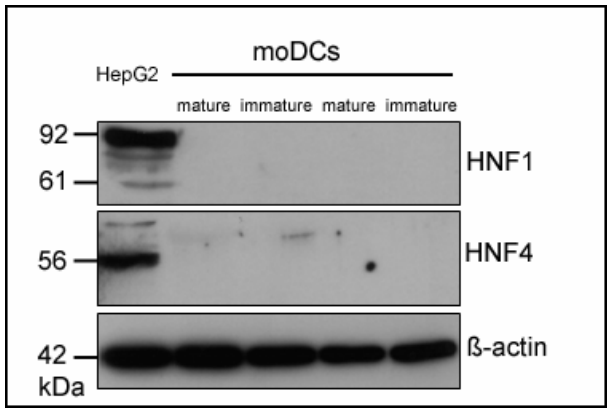

Fig. 11: Western blot analysis of transcription factors required for HBV gene expression. Mature and immature moDCs of two different donors and, as positive control, HepG2 cell lysate were analysed for HNF1 and HNF4 expression. Analysis for β-actin was added to control loading of equal protein amounts.

Although the low expression levels of HBsAg and HBeAg implied that HBV replication did not occur in moDCs, the culture media of immature and mature moDCs transduced with AdG-HBV1.3 were assayed for HBV progeny-virus formation. Therefore, the media were subjected to caesium chloride density gradient centrifugation, followed by dot blot analysis. The density gradient centrifugation allows separating free HBV DNA, DNA-containing capsids and intact enveloped HBV virions, as they sediment in different density fractions. The density fractions were collected and dot blotted on a nylon membrane.

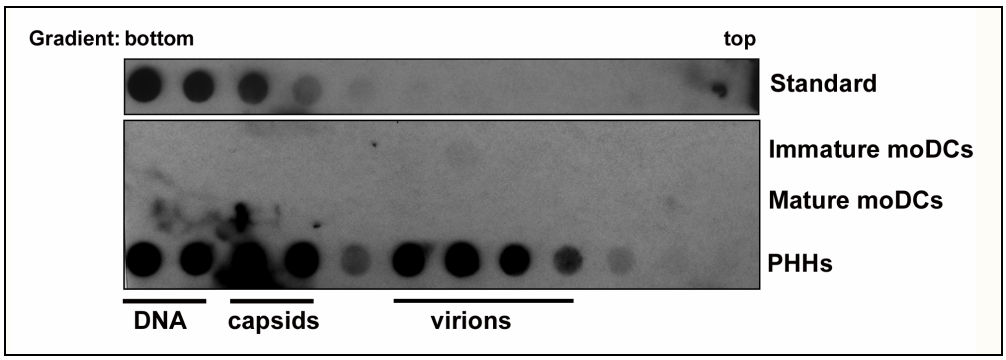

Fig. 12: Dot blot analysis of supernatants of AdG-HBV1.3 transduced PHHs and moDCs. A HBV-DNA standard with concentrations from 1000 pg to 8 pg was added to the blot to be able to quantify the amount of HBV DNA in the samples. The supernatant of AdG-HBV1.3 transduced PHHs served as positive control for progeny virus formation.

The dot blot was probed with a ^{32}P-labelled HBV-specific DNA. In the culture medium of AdG-HBV1.3-transduced PHHs, which was added as positive control, HBV progeny virus was detected in large amounts. Conversely, no progeny virus could be found in the media of immature and mature moDCs after transduction with AdG-HBV1.3 (Fig. 12).

2.1.1.2 Transduction of moDCs with rHBV.

To address the question whether or not the early steps of HBV infection are supported by moDCs, recombinant HBV-based vectors (rHBV) were used. The vectors rHBV-rLuc and rHBV-CMV-rLuc express *renilla* luciferase under the HBV-specific preS2/S promoter or the CMV promoter, respectively. The vectors are schematically depicted in fig. 14a. Because all HBV genes are knocked out by the introduction of stop codons (C, P, L, M, X -), the vectors are replication deficient. Only luciferase can be expressed, as soon as entry and transport of the genome into the nucleus have occurred. Fig. 13 schematically depicts the transduction of a hepatocyte with rHBV.

With this system at hand, it was possible to distinguish between the different steps of early HBV infection:

Firstly, rHBV cannot enter the cells, or is unable to transfer the genome to the nucleus. In this case, no luciferase would be detected after transduction with rHBV-CMV-rLuc or rHBV-rLuc.

Secondly, rHBV enters the cell and delivers the genome into the nucleus, but gene expression from HBV-specific promoters is not supported. In this case, luciferase expression would be detected only after transduction with rHBV-CMV-rLuc, which expresses luciferase under the HBV-independent CMV promoter. Transduction with rHBV-rLuc would not give rise to luciferase expression.

Thirdly, rHBV enters the cell, delivers the genome into the nucleus, and HBV-specific promoters are active. In this case, luciferase would be expressed from both the rHBV-CMV-rLuc and the rHBV-rLuc vector construct.

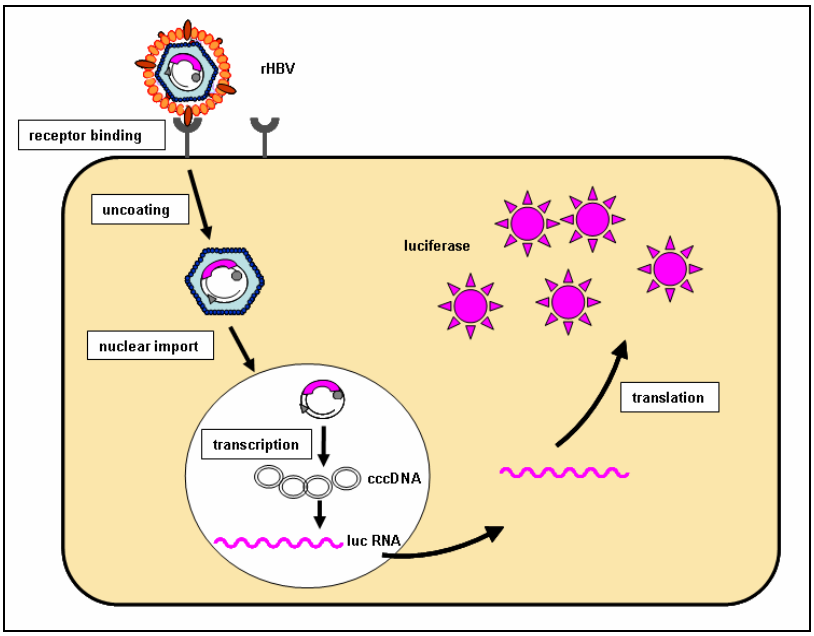

Fig. 13: Transduction of a hepatocyte with rHBV.

HBV-based vectors enter via HBV-specific receptors and uncoat. The HBV rc genome containing the luciferase transgene is transferred to the nucleus. cccDNA is formed and transcription from the respective promoter (the HBV-specific preS/S promoter in case of rHBV-rLuc and the CMV promoter in case of rHBV-CMV-rLuc) leads to luciferase expression and accumulation in the transduced cell. HBV genes cannot be transcribed because of the introduced stop codons.

The experiment was performed in the following way: Every measurement was repeated three times. The cells were transduced with a moi of 100 vp/cell in the absence or presence of neutralising anti-HBs antibodies (nAb; Hepatect, Biotest Pharma). Mock-transduced cells served as negative control. At day four post transduction, 10^6 cells were lysed and assayed for luciferase expression. The measured relative light units (RLU) were normalised to the total protein content of the lysates. As a positive control for the vectors, transduced PHHs were used. A transduction efficiency of approx. 5% of PHHs was usually reached, when using a moi of 100 vp/cell. Despite this low amount of transduced cells, luciferase expression 200-fold above background was measured after transduction of PHHs with rHBV-CMV-rLuc and rHBV-rLuc. As illustrated in fig. 14b, the presence of neutralising antibodies during transduction completely abrogated luciferase expression by blocking the specific uptake of rHBV particles. The RLU measured in rHBV-transduced cells in the presence of neutralising antibodies are comparable to those obtained from mock-transduced PHHs.

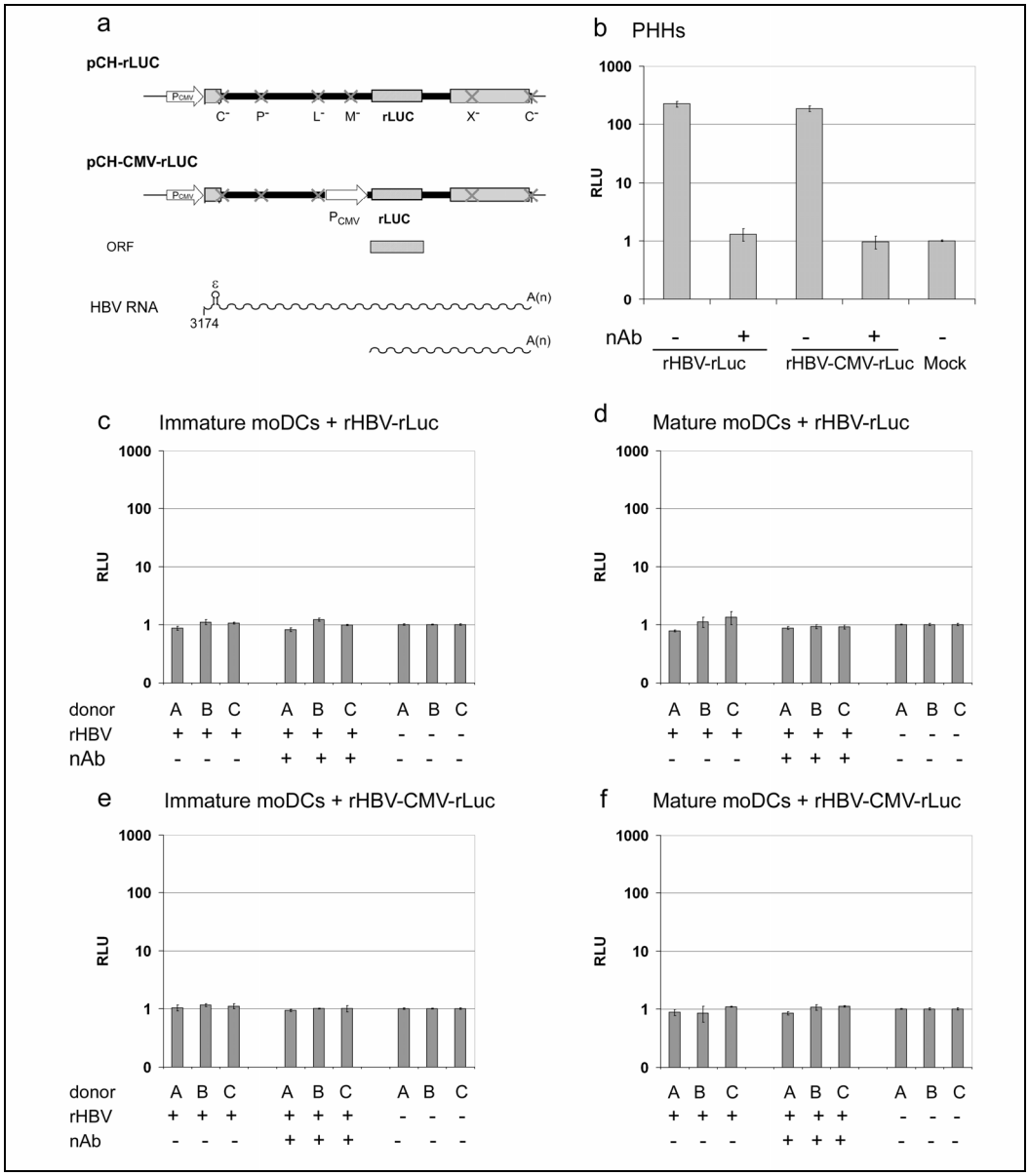

Fig. 14: Transduction of PHHs and moDCs with recombinant HBV expressing luciferase.
(a) Schematic depiction of the constructs rHBV-rLuc and rHBV-CMV-rLuc. (b-f) Transduction of PHHs (b), immature moDCs (c, e) and mature moDCs (d, f) with the indicated rHBV at a moi of 100 vp/cell in the absence or presence of neutralising antibodies (nAb).

To obtain representative results for the transduction of immature and mature moDCs, cells from three different donors A, B and C were used in independent experiments. Fig. 14c and d show the results for immature (c) and mature (d) moDCs, transduced with the vector rHBV-rLuc. The first set of three bars indicates the median values and standard deviations measured with immature (c) and mature (d) moDCs after transduction with rHBV-rLuc. The second set of three bars represents the results obtained after transduction in the presence of

neutralising antibodies. The RLU measured in mock-transduced cells of donor A, B and C (last three bars, Fig. 14c and d) served as negative control. Comparing the results from immature rHBV-rLuc-transduced moDCs, performed in absence or presence of neutralising antibodies with mock-transduced cells (Fig. 14c), it becomes obvious that luciferase was not expressed in the moDCs. Thereby, no difference was observed between the cells from the different donors A, B and C. Transduction of mature moDCs with the vector rHBV-rLuc (Fig. 14d) revealed the same result. No luciferase expression could be detected, independent of the absence or presence of neutralising antibodies.

To examine whether the inability of immature and mature moDCs to express luciferase was a matter of the HBV-specific promoter, moDCs were transduced with the vector rHBV-CMV-rLuc. In this vector, the luciferase gene is controlled by the CMV promoter. Nevertheless, in this experiment luciferase expression was undetectable, independent of the presence or absence of neutralising antibodies and maturation state of the moDCs (Fig. 14e, f). The RLU measured for immature and mature moDCs were at mock-control level for the cells from all three donors.

Tab. 2: Quantitative PCR analysis of HBV DNA forms in DNA extracted from moDCs incubated with rHBV.

Sample		rcDNA copies/10^3cells	cccDNA copies/10^3cells
Immature moDCs	+ rHBV-rLuc	48.3 ± 9.7	-
	+ rHBV-rLuc + nAb	62.0 ± 19.6	-
	mock	< 0.5	-
Mature moDCs	+ rHBV-rLuc	29.3 ± 8.3	-
	+ rHBV-rLuc + nAb	17.8 ± 4.6	-
	mock	< 0.5	-

To dissect between a block in the uptake of rHBV into moDCs and a block in nuclear transfer of the genome, the immature and mature moDCs transduced with rHBV-rLuc were analysed by quantitative real-time PCR, as described in 2.1.1.3. PCR results revealed that rcDNA was present in immature and mature

moDCs, independent of the presence of neutralising antibodies (nAb; Tab. 2). This indicates that rHBV was taken up by moDCs, but was unable to transfer the genome into the nucleus. Furthermore, the internalisation was probably unspecific, e.g. by phagocytosis or macropinocytosis, because anti-HBs antibodies did not block the uptake.

2.1.1.3 Infection of moDCs with wtHBV.

To exclude firstly that wtHBV behaves differently from recombinant HBV and secondly that luciferase expression or detection was problematic, moDCs were incubated with wtHBV at a moi of 100 vp/cell. After 18 h, the inoculum was removed and the cells were extensively washed and trypsinised, in order to remove attached virus. Cells were further cultivated for 24 h, before DNA was prepared from the cell lysates. Using specific quantitative Light Cycler™ real-time PCR, the cellular DNA was assayed for the presence of HBV rcDNA and HBV cccDNA. The binding sites of the primers within the HBV genome are delineated in fig. 15. The primers designed for rcDNA detection ((+) 1745 and (-) 1844) bind a sequence within the S region, giving rise to a 99 bp product. This part is double stranded in both the rc and the cccDNA form of the HBV genome and can therefore be amplified from both DNA forms. Since rcDNA is usually present in more than 100-fold excess in comparison to cccDNA, the amount of amplificates resulting from binding to cccDNA is insignificant. The primers used for cccDNA detection span the single-stranded region, the nick and the gap of the rc genome, and therefore do not amplify the rcDNA form.
Primer (+) 2760 binds close to the direct repeat and primer (-) 156 binds in the region of the gap, which is single stranded in the rcDNA. In the cccDNA form the gap is filled, and the nick and the attached viral polymerase are removed. Only then amplification of the 580 bp product of the cccDNA PCR occurs.
Three independent infection experiments were performed with immature and mature moDCs in the absence or presence of neutralising antibodies (nAb).

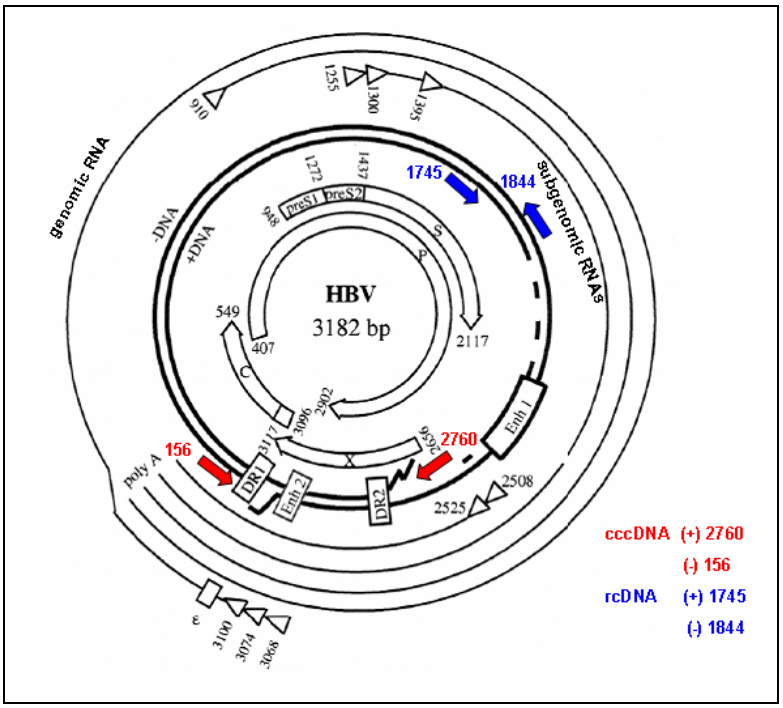

Fig. 15: Location of the primers used for ccc (red arrows) and rcDNA (blue arrows) PCR.

Mock-infected moDCs served as negative control, PHHs infected with wtHBV were used as positive control in the PCR reactions (Tab. 3; Fig. 16). The detection limits were averaged at 0.2 copies/10^3 cells for rcDNA, and 1 copy/10^3 cells for cccDNA. The results are summarised in tab. 3, the PCR products obtained from mature moDCs are shown on an agarose gel in fig. 16.

Tab. 3: Quantitative PCR analysis of HBV DNA forms in DNA extracted from moDCs incubated with wtHBV.

Sample		rcDNA copies/10^3cells	cccDNA copies/10^3cells
Immature moDCs	+ HBV	6.68 ± 9.7	-
	+ HBV + nAb	3.9 ± 3.8	-
	mock	< 0.5	-
Mature moDCs	+ HBV	2.94 ± 2.0	-
	+ HBV + nAb	< 0.5	-
	mock	< 0.5	-

Between 0 and 20 copies/10^3 cells of rcDNA could be found in immature moDCs. The presence of neutralising antibodies during the incubation with wtHBV had no major influence on the uptake.

The lysates of mature moDCs were also positive for rcDNA, with 1 to 6 copies/10^3 cells. The presence of neutralising antibodies (nAb; Hepatect, Biotest Pharma) during the incubation with wtHBV reduced the uptake of viral particles into mature moDCs or directed the particles to rapid degradation. A weak band, observed in the third lane of mature moDCs incubated with HBV plus neutralising antibodies (Fig. 16) was likely due to a minimal contamination, as the crossing point (CP) for this sample was $>$ 40, which is below the detection limit of $<$ 0.2 copies/ 10^3 cells.

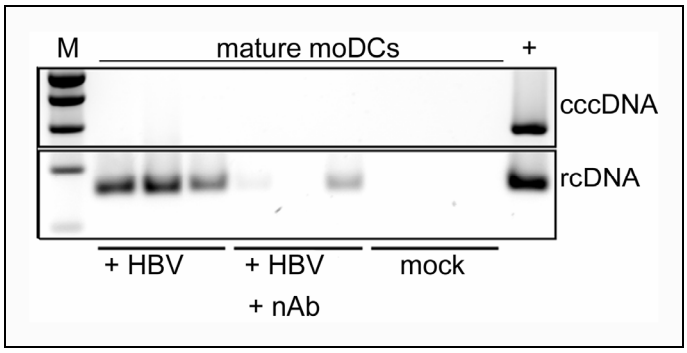

Fig. 16: Agarose gel of the PCR analysis of mature moDCs subjected to wtHBV.
In vitro matured moDCs were incubated with wtHBV (+HBV), or wtHBV plus neutralising antibodies (+HBV +nAb) at mois of 100 vp/cell. Uninfected cells (mock) and HBV-infected PHHs (+) served as controls. M = DNA size marker.

In contrast, the cccDNA PCR was negative in immature and mature moDCs after incubation with wtHBV, irrespective of the presence or absence of neutralising antibodies. This indicates that no HBV genomes were imported into the nucleus.

These results show that low amounts of HBV particles were taken up by moDCs. However, a nuclear transcription template was not established, indicating that either the uncoating or the delivery of the genome into the nucleus was blocked.

2.1.2 Analysis of blood derived dendritic cells from high viremic chronic hepatitis B patients.

In HBe-positive chronic HBV carriers, the blood-circulating DCs are exposed to high amounts of virus. To investigate whether these DCs take up HBV viral or subviral particles, and whether they are infected with HBV, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) were isolated from chronic HBV carriers. Four patients with mild to moderate hepatitis and high titers of HBsAg and HBeAg were selected. The ALT levels of the patients ranged from the upper normal limit to 6-fold elevation. The viremia and the calculated HBV input per cell for each patient are summarised in tab. 4. The DCs were isolated from peripheral blood by MACS in two rounds of positive selection. To control the purity of the obtained cell populations, they were subjected to flow cytometry analyses. The cells were stained for characteristic surface markers. For mDCs, the marker BDCA-1 was used (Fig. 17a), pDCs were stained for CD123 and BDCA-2 (Fig. 17b). Generally, cell purities of 76% to 96% were obtained for both cell populations. Fig. 17 gives an example for a characteristic staining of mDCs and pDCs.

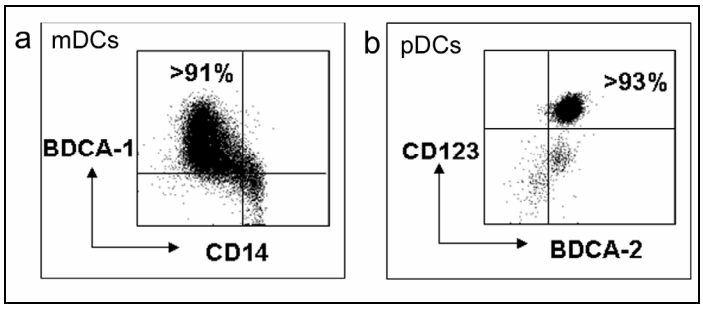

Fig. 17: Characteristic flow cytometry analysis of isolated (a) mDCs and (b) pDCs.

Using quantitative real-time PCR, mDCs and pDCs were investigated for the presence of HBV rcDNA and cccDNA. The presence of cccDNA would indicate nuclear transport and thus infection of the DCs. The exclusive existence of rcDNA would argue for an uptake of virions by the DCs, specific or unspecific, without nuclear transport and thus without infection.

Tab. 4: Quantitative PCR analysis of HBV DNA forms in DNA extracted from blood-circulating mDCs and pDCs of chronic HBV carriers and *in vitro* HBV infected PHHs.

Sample	HBV viremia /ml blood	Cell type	HBV input /cell	rcDNA copies /10^3 cells	cccDNA copies /10^3 cells
1	8.2 x 10^7	mDCs	5460	11.6	-
		pDCs	8200	-	-
2	7.9 x 10^7	mDCs	5260	-	-
		pDCs	7900	-	-
3	2.6 x 10^9	mDCs	173300	-	-
		pDCs	260000	6.2	-
4	4.8 x 10^8	mDCs	32000	-	-
		pDCs	48000	-	-
5	-	PHHs	100	3600	230

DNA from PHHs, *in vitro* infected with HBV at a moi of 100 vp/cell, was used as positive control for rcDNA and cccDNA. As a negative control for cccDNA served DNA isolated from the patients own sera, as well as DNA isolated from the serum of a HBV-infected chimpanzee with a viral titer of 1.25 x 10^8 HBV copies/ml blood. Tab. 4 outlines the PCR results.

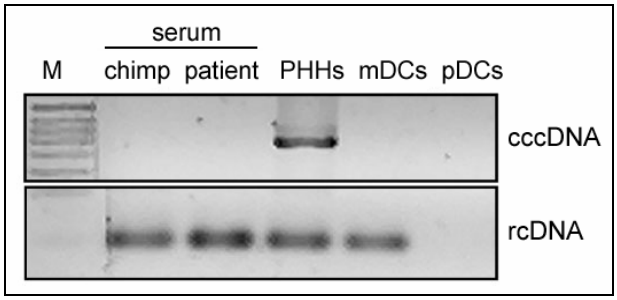

Fig. 18: Agarose gel of the PCR analysis of blood-circulating DCs from patient 1. DNA from sera of an HBV-infected chimpanzee and patient 1 served as negative control for cccDNA, DNA from HBV-infected PHHs served as positive control for cccDNA and rcDNA.

In mDCs and pDCs from only one patient each, low amounts of 11.6 and 6.2 rcDNA copies/10^3 cells were detected, respectively. The PHH DNA, serving as positive control, contained 3600 rcDNA copies/10^3 cells. No cccDNA was present in DCs from the chronic infected patients and the negative controls. In the positive control, 230 cccDNA copies/10^3 PHHs were detected by real-time PCR (Tab. 4; Fig. 18). The PCR products were subjected to agarose gel

electrophoresis. In fig. 18, the PCR products obtained from cells of patient 1 are visualised (mDCs, pDCs). The sera of the HBV-infected chimpanzee and of patient 1 were clearly positive for rcDNA. The cccDNA form is not present in free virions. Accordingly, the sera were negative for cccDNA. The *in vitro* infected PHHs contained both the nuclear ccc form of the HBV genome and the genomic rc form of the virions. This result clearly shows that blood-derived mDCs and pDCs of highly viremic carriers barely take up HBV particles and do not support the HBV replication cycle.

To further analyse whether blood-circulating mDCs and pDCs in chronically HBV infected patients take up viral or subviral particles, transmission electron microscopy was performed with freshly isolated mDCs and pDCs. In fig. 19, an mDCs is depicted. In the cell, endocytic vesicles containing few subviral particles were observed. Neither virions nor free capsids were detected in the cytoplasm or at the nuclear membranes of the cells. pDCs appeared negative for HBV viral or subviral particles.

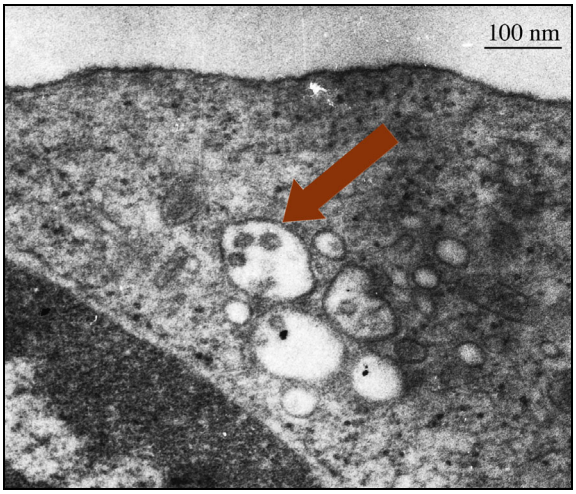

Fig. 19: Transmission electron microscopy of ultra-thin 50 nm sections of an mDC. The red arrow points at an endocytic vesicle containing three SVPs.

Quantitative real-time PCR of other peripheral blood-cell populations revealed that B cells were positive for rcDNA in three of the four analysed patients, with copy numbers per 10^3 cells of 10, 14.6 and 19.3, respectively. Enriched T-lymphocyte fractions of two patients contained 132.8 and 68 copies rcDNA/10^3 cells, respectively, and 4 copies rcDNA/10^3 cells were detected in monocytes of one patient. cccDNA was not detected in any of these cells

(Tab. 5). This indicates that HBV-particle uptake into the cells occurred, but that an HBV infection was not established.

Tab. 5: Quantitative PCR analysis of HBV DNA forms in DNA extracted from B cells (B), enriched T-cell fractions (T) and monocytes (M) of chronic HBV carriers.

Sample	HBV viremia /ml blood	Cell type	rcDNA copies /10^3 cells	cccDNA copies /10^3 cells
1	8.2×10^7	B	19.3	-
		T	132.8	-
		M	-	-
2	7.9×10^7	B	-	-
		T	-	-
		M	-	-
3	2.6×10^9	B	10	-
		T	68	-
		M	-	-
4	4.8×10^8	B	14.6	-
		T	-	-
		M	4	-

To summarise, the PCR data, as well as the EM analyses, reveal that mDCs and pDCs of high viremic chronic HBV carriers were not infected with HBV to a significant proportion; neither were B cells, monocytes and enriched T-lymphocyte fractions of chronic HBV carriers. However, low amounts of VPs and SVPs seemed to be taken up by DCs and may be presented as antigen to T cells.

2.2 Discussion I

Dendritic cells are the most potent antigen-presenting cells, and therefore play an important role in the immune system. They induce antigen-specific cellular and humoral immune responses by presenting internalised antigens to T and B lymphocytes. In chronic HBV carriers, DC dysfunctions and impaired T-cell responses have been reported: Decreased DC numbers, reduced maturation and T-cell stimulatory capacity of mDCs, as well as reduced IFN-α production of pDCs (Beckebaum et al., 2002; van der Molen et al., 2004; Duan et al., 2004). Numbers of virus-specific CTLs are decreased and a virus-specific T-cell response is barely detectable (Rehermann et al., 1995b; Maini et al., 2000).

For various viruses, it has been shown that DC infection, causing an impairment of DC function, is a potent strategy to circumvent a virus-specific immune response. In HIV-infected patients for example, DCs display a reduced capacity to stimulate allogeneic lymphocytes (Knight et al., 1993). A similar correlation was found for vacciniavirus infection (Engelmeyer et al., 1999). Here, the maturation of DCs seems to be inhibited upon infection. A recent study of Wertheimer et al., (2007) provided evidence for an influence of HCV protein expression on DCs. The protein expression profile of the DCs changed, particularly with respect to chemokines, when HCV proteins were expressed in the DCs. Furthermore, DC dysfunction was found to correlate with an impaired HCV-specific $CD4^+$ T-cell response in chronic carriers (Della Bella et al., 2007).

As the impairment of DC and T-cell function in chronic HBV-infected patients might be caused by HBV infection of DCs, the different steps of HBV infection were analysed in blood DCs. The question, at which level HBV might affect DC functionality, was addressed.

Although low-level expression of HBsAg and HBeAg was possible in moDCs, no infection with HBV was detected. The results were confirmed in the *in vivo* situation by *ex vivo* analysis of mDCs and pDCs from high viremic HBV carriers, using quantitative real-time PCR and electron microscopy. Therefore, it can be excluded that HBV infects blood DCs at a frequency sufficient to explain the impairment of the virus-specific T-cell response in chronic HBV carriers.

2.2.1 HBV antigen expression and replication in moDCs.

2.2.1.1 Transduction of moDCs with AdG-HBV1.3.

In order to test whether DCs are able to express HBV antigens, the HBV genome was artificially introduced in moDCs via an adenoviral vector. HBsAg expression, and to a higher extent HBeAg expression, were reduced in moDCs compared to similarly AdG-HBV1.3-transduced PHHs. No progeny virus was formed. A possible explanation for the very low expression level of HBeAg could be the high liver specificity of the precore/core promoter, which is responsible for HBeAg and core expression (Honigwachs et al., 1989; Ganem and Schneider, 2001). Liver-enriched nuclear factors, important for transcription from the precore/core promoter as well as transcription of the pre-genomic RNA, are missing in moDCs (Tong and McLachlan, 2001 and 2002). The reduction of HBsAg expression in moDCs, compared to PHHs, was less strong, presumably because the preS2/S promoter shows only limited liver specificity and is constitutively active in a wide range of cells (Siddiqui et al., 1986; Faktor et al., 1988; Zhou and Yen, 1990). The reduction in HBsAg expression compared to PHHs may therefore be due to differences in the promoter activity. Alternatively, cccDNA, which serves as an additional transcription template in PHHs, might not be present in moDCs (Ganem and Schneider, 2001; Ganem and Prince, 2004). The inability to produce progeny virus may be based on a lack of the nuclear transcription factors HNF1 and HNF4. Western blot analysis of immature and mature moDC lysates showed that these factors were not expressed by moDCs. HNF1 and HNF4 are essential for the transcription from the preS1 promoter, and for the transcription of the HBV pre-genomic RNA, respectively (Courtois et al., 1988; Honigwachs et al., 1989; Raney et al., 1997). The differences in the expression-level reduction between immature and mature moDCs of HBsAg and HBeAg could be explained by a re-modelled protein-expression pattern in moDCs after maturation (Banchereau et al., 2000; Garret et al., 2000). While immature moDCs primarily express proteins necessary for antigen uptake, upon maturation they up-regulate MHC and co-stimulatory molecule expression. This change in the protein expression

pattern during the maturation process may result in an increased expression of HBsAg and a decreased expression of HBeAg.

2.2.1.2 Transduction of moDCs with rHBV.

To investigate whether moDCs can be infected with HBV, HBV-based vectors (rHBV) were used. These vectors express luciferase, either under the HBV-specific preS2/S promoter (rHBV-rLuc) or the non-liver specific CMV promoter (rHBV-CMV-rLuc). They mimic the early steps of HBV infection, these being entry, uncoating, and transport of the genome into the nucleus (Klöcker et al., 2000; Untergasser and Protzer, 2004). rHBV transduction can be completely blocked with neutralising antibodies, as shown in the positive control with PHHs (Schulze-Bergkamen et al., 2003).

The luciferase system is very sensitive, with an estimated detection limit of 1 positive cell/10^4 moDCs. Using this assay, HBV infection of permissive cells can be specifically and sensitively detected. However, luciferase expression was not detected after incubation with rHBV-rLuc or rHBV-CMV-rLuc, neither in immature nor in mature moDC. Both the CMV promoter and the preS2/S promoter are active in moDCs. This was shown in the AdG-HBV1.3 transduced moDCs, which expressed GFP under the CMV promoter and HBsAg under the preS2/S promoter. Therefore, the lack of luciferase expression after transduction with rHBV suggests that the rHBV genome was not delivered to the nucleus. This can either be due to a block of entry into the cells, or to a block of uncoating and nuclear transfer of the genome. Despite the high sensitivity of the luciferase assay, a transduction rate below $1:10^{-4}$ cannot be excluded. However, it is unlikely that infection of DCs with HBV at such low levels can cause DC dysfunction.

To elucidate whether rHBV entered the cells, an optimised quantitative real-time PCR was used, which allowed a distinction between rcDNA and cccDNA. These DNA forms are present in cells after HBV infection as well as after rHBV transduction. The fact that immature and mature moDCs contained rcDNA after transduction with rHBV-rLuc suggests that moDCs internalised rHBV. However, since the cells were extensively washed but not trypsinised, virions which stuck

to the cell surface possibly gave rise at least to some of the rc copies detected in the cell lysates. The nuclear cccDNA form was not present in the cells. The presence of rcDNA but not cccDNA suggests that the block leading to the inability to express luciferase was probably not located in the uptake mechanism, but rather in the uncoating or DNA-delivery step. The internalisation seemed to be an unspecific process, e.g. phagocytosis or macropinocytosis, because anti-HBs antibodies, blocking the specific HBV uptake into hepatocytes, did not have this effect in moDCs. The copy numbers of rcDNA found in immature moDCs were the same when incubated only with rHBV and when incubated with rHBV in the presence of neutralising antibodies. Since immature DCs are specialised for antigen uptake, they phagocytose large amounts of opsonised antigens (Banchereau et al., 2000; Janeway et al., 2004). rHBV uptake was slightly reduced in mature moDCs compared to immature cells, and in the presence of antibodies the amount was further shortened. This was expected, as mature DCs primarily present internalised antigen, whilst uptake activity is down-regulated (Banchereau et al., 2000; Garret et al., 2000).

2.2.1.3 Infection of moDCs with wtHBV.

To exclude that wtHBV behaves differently to rHBV, moDCs were incubated with wtHBV. Quantitative real-time PCR was used to analyse immature and mature moDCs for the presence of rcDNA and cccDNA. cccDNA was found neither in immature nor in mature moDCs, despite an estimated detection limit of 1 copy/10^3 cells. These data coincide with the results obtained with moDCs incubated with the luciferase expressing rHBV (2.2.1.2). In this case, luciferase expression could not be detected with the luciferase assay, and cccDNA could not be found using quantitative real-time PCR analysis. After incubation with wtHBV, moDCs were not infected with HBV, or at the most with a very low frequency below the detection limit of $1:10^{-3}$. However, the presence of minor amounts of rcDNA in immature, and to a lesser extent in mature, moDCs demonstrates that wtHBV uptake was possible. The lack of cccDNA formation, plus the fact that the uptake was independent of the presence of neutralising antibodies, emphasises an unspecific uptake mechanism for wtHBV. This

resembles the PCR data of the aforementioned experiment performed with rHBV. However, the copy numbers of rcDNA per cell differed. moDC lysates of cells incubated with rHBV revealed 5- to 10-fold higher amounts of virus DNA, compared to moDCs incubated with wtHBV. This discrepancy may be due to the extensive trypsinisation and washing performed with moDCs after incubation with wtHBV, but not with cells incubated with rHBV. Therefore, the additional copies may have stemmed from rHBV attached to the cell surface, whereas most of the wtHBV attached to the moDCs have probably been removed by the trypsinisation and washing procedure. Furthermore, differences in the production method of rHBV and wtHBV may be the cause for the differences in the rcDNA amount between moDCs incubated with rHBV, and moDCs incubated with wtHBV: rHBV were produced in serum-free medium via transfection of HuH7 cells. In contrast, wtHBV were produced by the HepG2.2.15 cell line in the presence of FCS. Therefore, rHBV particles did not carry serum proteins on their surface, whilst wtHBV produced by HepG2.2.15 cells were most likely covered with serum proteins bound to the surface. The phenomenon that serum proteins bind to the virus surface *in vivo* and *in vitro* has been described several times (Khelifa and Mo, 1987; Heermann et al., 1988; Krone et al., 1990). These differences in the virus surface characteristics may contribute to variability in the uptake mechanism or efficiency. Furthermore, the two cell lines may differ in their glycosylation machinery, leading to altered glycosylation patterns of the HBV surface proteins. Since it is known that protein glycosylation patterns influence the uptake into APCs by scavenger receptors, this may be an alternative reason, why rHBV was internalised to a higher extent than wtHBV (Vlassara et al., 1985).

2.2.2 Analysis of blood derived dendritic cells from high viremic chronic hepatitis B patients.

To verify the results obtained with moDCs in the physiological situation, blood-circulating DCs of four high viremic chronic HBV carriers were *ex vivo* studied for HBV infection. The main populations of circulating DCs are myeloid

and plasmacytoid DCs (Banchereau, 2000). A very important feature of the latter is their role in antiviral defence. They are the main source of type 1 IFN, which can inhibit viral replication, and thus have a strong antiviral effect (Cella et al., 1999; Siegal et al., 1999). In the four chronic HBV carriers, mDCs and pDCs have been exposed to up to 2.6 x 10^5 virions per cell. Nevertheless, no cccDNA could be detected in mDCs and pDCs of all carriers with quantitative real-time PCR. This argues against an infection of these cells with HBV. Cytoplasmic rcDNA was found in low amounts in mDCs of patient 1 and pDCs of patient 3, indicating that HBV is taken up by mDCs and pDCs to a very low extent, as is the case in moDCs. However, mDCs and pDCs were exposed to a much higher amount of viral particles. Uptake of HBV by mDCs and pDCs appeared to be a rare event in chronic HBV carriers. rcDNA was not detectable in three of the four investigated mDC and pDC samples, respectively, despite a detection limit of 0.2 copies/10^3 cells. In agreement with the molecular analyses, electron microscopy revealed that HBV capsids or complete virions were not present in the cytoplasm, or at the nuclear membrane of mDCs and pDCs. Only SVPs were found in low amounts in endocytic vesicles of mDCs. Once more, this indicates an unspecific uptake mechanism of HBV VPs and SVPs. However, the observation that the internalisation of HBV was such a rare event is unexpected, as blood-circulating mDCs and pDCs display an immature phenotype and usually internalise high amounts of pathogens. One reason could be that HBV VPs and SVPs are covered by serum proteins and therefore might less easily be detected as foreign (Krone et al., 1989).

To conclude, mDCs and pDCs of high viremic chronic HBV carriers were not infected with HBV to a sufficient amount to explain the DC dysfunction in these patients. Furthermore, the DCs rarely internalised HBV VPs and SVPs.

In B cells, monocytes and enriched T-cell fractions, quantitative real-time PCR revealed low amounts of HBV rcDNA, while all cells were negative for cccDNA. This suggests that these cells were also not infected with HBV.

In previous studies, possible HBV infections were mostly analysed using PCR or PCR *in situ* hybridisation, which only detects the rc form of HBV DNA (Beckebaum et al., 2002; Arima et al., 2003; Tavakoli et al., 2004). Thereby, a differentiation between input virus, or unspecifically up-taken virions, and cccDNA formation is hardly possible. Nuclear entry of viral DNA is a

prerequisite for successful HBV infection of a cell and results in the formation of cccDNA, which serves as a transcription template for HBV RNAs (Ganem and Schneider, 2001; Ganem and Prince, 2004). This also holds true for HBV-based vectors, where cccDNA is formed from the rHBV genome to express the transgene. Therefore, only the specific detection of cccDNA is an indication for infection. The detection of rcDNA merely implies the presence of virions, either bound to cells, inside endocytic or lysosomal vesicles, or free in the cytoplasm (Köck et al., 1996).

In the study of Beckebaum et al. (2002), HBV DNA was found in pDCs of chronic HBV carriers and complete HBV particles were observed in cytoplasmic vesicles of mDCs by electron microscopy. This result was interpreted as an indication for HBV infection of DCs, which might cause DC dysfunction in chronic HBV carriers. However, the fact that many enveloped virus particles were detected in vesicles, whereas no free capsids were present in the cytoplasm, argues for uptake of these virions by phagocytosis, rather than for infection. The latter would require the release of capsids into the cytoplasm, which transport the viral genome to the nucleus. Köck et al. (1996) used a PCR assay that amplifies cccDNA more efficiently than rcDNA, to test whether HBV infects PBMCs. They found no evidence that PBMCs can be infected with HBV and concluded that adsorbed virus might explain the detection of HBV DNA or RNA by PCR. Contrary to this, in other publications the existence of HBV DNA in PBMCs was interpreted as HBV infection, which could interfere with PBMC function in virus-specific immunity (Stoll-Becker et al., 1997; Trippler et al., 1999). However, the data presented in this project and the investigation of others exclude an infection of blood-circulating DCs as well as monocytes, B-cells and enriched T-cell fractions, to an extent sufficient to cause DC dysfunction and impairment of a virus-specific immune response.

The assumption that an infection of DCs with HBV is not the reason for the impairment of DCs and T-cell function raises the question whether or not the presence of rcDNA in the DCs influences their functionality. Van der Molen et al. (2004) performed functional studies, which did not reveal any correlation between the amount of HBV DNA present in mDCs and their suppressed allostimulatory capacity. Furthermore, they found no link between the amount of HBV DNA and the capacity of mDCs to express CD80 or CD86 during *in vitro*

maturation. Although the amount of HBV DNA, associated with pDCs seemed to be higher than that associated with mDCs, they did not show an altered allostimulatory capacity. Thus, the amount of HBV DNA associated with DCs did not correlate with a certain functional impairment. A similar observation was published by Tavakoli et al. (2004). They investigated immature and mature moDCs of chronic HBV carriers. Contrary to the investigations with HCV of Wertheimer et al. (2007), the amount of HBV DNA and antigen expression detected in the moDCs was not in line with a loss of the allostimulatory capacity of these cells. Thus, there seems to be no correlation between the amounts of rcDNA present in the DCs, and their impaired immune response to HBV.

Considering the data from this study and the literature, one can hypothesise that the contact with HBV antigens, in particular SVPs, which circulate in vast amounts in the blood during infection, may alter the antiviral immune response. Evidence for an influence of high antigen levels on the immune system comes from different studies: In the mouse lymphocytic choriomeningitis virus model, a gradual loss of T-cell function was demonstrated. This so called T-cell exhaustion was caused by a high viral load (Fuller et al., 2003; Wherry et al., 2003). First, the IL-2 production was impaired, followed by a loss of cytotoxicity and a lack in TNF-α secretion. Finally, the INF-γ production was impaired. This functional inactivation then promoted further viral persistence. Whether the exhaustion was induced by the antigen contact itself, e.g. by repeated TCR triggering with presented antigens, or indirect, e.g. by cytokine deprivation, is currently not understood. However, fluxes in the viral load appeared to partially restore at least some T-cell functions (Fuller and Zajac, 2003).

The question whether or not a high HBV load influences cell numbers and functionality on the DC level was recently addressed by Van der Molen et al. (2006). A reduction of the virus load was found to correlate with a substantial increase of mDC but not pDC numbers, to a level comparable to that of uninfected healthy controls. The allostimulatory capacity of isolated and *in vitro* matured mDCs increased significantly. A highly reduced number of DCs could explain the low number of virus-specific T cells, characteristic for chronic HBV infection. Coincidentally, it has been reported that the level of HBV replication in the liver, and thus the amount of circulating VPs and SVPs, influences the profile of the virus-specific cytotoxic T-cell response during chronic infection

(Webster et al., 2004). A study of Maini et al. (2000), points as well in this direction. They proposed that a quantitative difference in virus-specific CTL numbers between chronic and acute infection, correlated with virus control. Furthermore, it was demonstrated by others that the $CD4^+$ and $CD8^+$ T-cell response increased after lamivudine-induced reduction of the virus load (Boni et al., 1998 and 2003). In theory, a mechanism such as the following could explain the spontaneous recovery of chronic patients, sometimes observed after lamivudine therapy: If the immune system is unable to control the virus in the early phase of infection, high viral titers are reached in the blood. Vast antigen exposure leads to an impairment of DCs, followed by T-cell exhaustion. If the virus load can be efficiently reduced by an antiviral therapy, the immune system can recover, and a restored T-cell response might then be strong enough to control the remaining virus (Rossol et al., 1997; Boni et al., 2001).

In the context of the influence of frequent antigen encounter, Stoop et al. (2005 and 2007) highlighted the role of Tregs. There is evidence that these cells contribute to the hypo-responsiveness of the immune response against HCV, HIV and CMV (Sugimoto et al., 2003; Aandahl et al., 2004; Cabrera et al., 2004). In HBV infection, $CD4^+CD25^+FoxP3^+$ Tregs have been shown to impair the immune response in chronic carriers by immuno-suppression of HBV-specific $CD8^+$ T cells (Franzese et al., 2005; Stoop et al., 2005). Furthermore, $CD4^+CD25^+FoxP3^+$ Tregs are present in increased numbers in peripheral blood of chronic HBV carriers, possibly correlating with the virus load (Xu et al., 2006). Frequent antigen encounter for a long period of time, as is the situation in chronic carriers with high viral load, has been suggested to stimulate Treg (Taams et al., 2002). This was confirmed by Stoop et al. (2007), who found a simultaneous decrease in $CD4^+CD25^+FoxP3^+$ Treg numbers and virus load after adevovir treatment of chronic carriers. At the same time, they observed an increased immune response to HBcAg. The authors hypothesised a self-maintaining regulatory loop between Tregs and DCs, which might contribute to HBV persistence: Immature DCs can induce Tregs, and increased Treg numbers can inhibit DC maturation, leading to a decreased stimulatory capacity of T cells (Shevach, 2002; Min et al., 2003; Misra et al., 2004). Alternatively, alterations in the microenvironment of lymphoid organs during HBV infection could contribute to DC and T-cell dysfunction in chronic carriers.

Rehermann et al. (1995b) found that trace quantities of HBV DNA and RNA can persist in the serum and in PBMCs in resolved acute HBV-infected persons. The fact that HBV persistence was not due to an impaired immune response, suggests an immunologically privileged site of persistence, where HBV cannot be reached by CTLs. On the one hand, this persistence may result in the ongoing antigen-specific humoral and cellular immune response, observed after recovered acute infection (Rehermann et al., 1996). On the other hand, the persisting virus may also be responsible for the chronic disease (Rehermann and Nascimbeni, 2005).

Since the liver is the primary site of viral replication and therefore the site where an immune response against the virus is expected to be initiated, possible alterations in the liver must also be considered. First of all, the immunological features of the liver seem to support immuno-tolerance in chronic HBV infection: The hepatic environment is known to be tolerogenic, containing intrahepatic T-cell populations, and high numbers of APCs, such as liver DCs, Kupffer cells and liver sinusoidal endothelial cells (Crispe, 2003). Presentation of exogenous antigens to T cells, by liver sinusoidal endothelial cells, has been shown to induce specific T-cell tolerance (Limmer et al., 2000). This mechanism could also contribute to HBV-specific tolerance. Furthermore, an infection of liver DCs with HBV cannot be excluded, as DC populations are very heterogeneous. The situation in blood DCs may not reflect liver-DC behaviour, with respect to functional impairment and the ability to be infected with HBV. Unfortunately, the isolation of liver DCs is not yet established, and intrahepatic immunological events are difficult to study. The few studies performed in this field revealed differences between intrahepatic and circulating virus-specific $CD8^+$ T cells, concerning phenotype, frequency and activation status in chronic HBV and HCV infections, as well as in acute HBV infection (Bertoletti et al., 1997; Maini et al., 2000; Sprengers et al., 2006).

An interesting feature of the antigen-presenting cells in the liver is the constitutive expression of the **p**rogrammed **d**eath **1** molecule **l**igand **1** (PD1-L1) in Kupffer cells, liver sinusoidal endothelial cells and stellate cells (Iwai et al., 2003; Chen et al., 2006). In hepatocytes, PD1-L1 expression is regulated in an IFN-α- and IFN-γ-dependent manner (Mühlbauer et al., 2006). Its receptor, the PD1 molecule, is an inhibitory receptor of the CD28 co-stimulatory family and is

expressed by myeloid cells, B cells and T cells (Ishida et al., 1992; Chen, 2004; Greenwald et al., 2005). PD1 signalling upon interaction with PD1-L1 after liver infiltration of T cells is a major mechanism to inhibit virus-specific T cells: The antiviral effector functions of the T cells are impaired, and the T cells undergo apoptosis (Iwai et al., 2003; Dong et al., 2004). In a HBV mouse model, Isogawa et al. (2005) already demonstrated that virus-specific $CD8^+$ T cells transferred into HBV transgenic mice, simultaneously up-regulated the PD1 expression and lost their IFN-γ production capacity. In chronically HBV-infected patients, Geng et al. (2006) found that the PD1-L1 expression in $CD14^+$ monocytes was increased in comparison to healthy controls. PD1-L1 expression positively correlated with serum levels of IL-10, which is an immunosuppressive cytokine produced by different types of T cells, and monocytes (Accapezzato et al., 2005; Dikopoulos et al., 2005; Trinchieri 2007). Studies performed in acute and chronic hepatitis C revealed an involvement of the PD1-PD-L1 pathway as well in this disease: Urbani et al. (2006) demonstrated an up-regulation of PD1 levels in HCV-specific $CD8^+$ T cells upon acute HCV infection, followed by a decline as soon as the infection cleared. In contrast, T cells of chronic HCV-infected patients displayed constantly high levels of PD1, together with an exhausted and functionally impaired phenotype. These cells were frequently found in the liver. In an *in vitro* experiment, the proliferative capacity of the exhausted cells increased upon a blockade of the PD1-PD-L1 interaction (Urbani et al., 2006; Radziewicz et al., 2007). Considering these data, together with the notion that IL-10 production correlated with PD1-L1 expression, a role in the development of chronic hepatitis B is possible. IL-10 is known to inhibit IFN-α production of APCs and can induce apoptosis of pDCs (Duramad et al., 2003; Dolganiuc et al., 2006).

In addition to the PD1-PD1-L1 pathway, the immunoproteasome is a factor which might influence the immune response against HBV in the liver. It differs from the constitutive proteasome in quantitative and qualitative aspects of its proteolytic activity (Klötzel et al., 2001; Van den Eynde et al., 2001). In mice, it has been demonstrated that specific subunits of the immunoproteasome were necessary for the efficient processing and presentation of $CD8^+$ T-cell epitopes (Fehling et al., 1994; Van Kaer et al. 1994; Chen et al., 2001). In LCMV-infected mice, replacement of the constitutive proteasomes by *de novo* assembly of

immunoproteasomes has been observed in the infected organ, the liver (Khan et al., 2001). The IFN-inducible catalytic and regulatory subunits of the immunoproteasome, LMP2, LMP7, MECL1 and PA28α/β, seem to play a role in the IFN-induced inhibition of HBV capsid assembly in hepatocytes (Wieland et al., 2000, 2003 and 2005). Furthermore, immunoproteasome activity was shown to be required for IFN-mediated HBV inhibition (Wieland et al., 2000; Robek et al., 2002). In HCV-infected chimpanzees, Shin et al. (2006) differentially analysed the role of the immunoproteasome in innate and adaptive immune response. They found that the antigen-processing machinery of virus-infected hepatocytes is already stimulated by virus-induced type I IFN, prior to T-cell infiltration of the liver. In HBV infection, however, an early IFN response was not detectable in the liver of chimpanzees, consistent with the finding that an increased expression of immunoproteasome subunits was induced only in later phases of infection (Wieland et al., 2004). In a study of Robek et al. (2007), an altered $CD8^+$ T-cell response against the HBV envelope proteins and the polymerase protein was described for mice lacking the immunoproteasome subunit LMP7. Since the T-cell response differs between acute and chronic HBV infection in the multi-specificity of the epitopes, one could hypothesise a differential regulation of antigen processing in acute versus chronic infection. Professional APCs, which constitutively express the immunoproteasome, are responsible for the T-cell priming (Robek et al., 2007). Furthermore, the subunits LMP2 and LMP7, expressed in hepatocytes after IFN induction, might facilitate the recognition of infected hepatocytes (Sallusto and Lanzavecchia, 1999; Morel et al., 2000; Khan et al., 2001).

There is evidence that alteration of DC function in chronic HCV infection might not be caused by the infection of DCs but may be a consequence of liver disease progression (Barth et al., 2005). A similar mechanism is conceivable for chronic HBV infection, because the development of liver cirrhosis and hepatocellular carcinoma, due to persistent inflammation, is very common (WHO, 2007; Chisari and Ferrari, 1995b). However, it has been published in the aforementioned studies of Maini et al. (2000) and Webster et al. (2004) that differences in virus-specific CTL response between chronic and acute infection were independent of the liver disease.

To summarise, the mechanisms underlying the impaired immune response against HBV in chronic carriers remain to be elucidated. In this study it was proven by *in vitro* analyses using moDCs, as well as by *ex vivo* analyses of mDCs and pDCs, isolated from chronic HBV carriers, that an infection of blood-circulating DCs is not the reason for the impairment of DC and T-cell function. The fact that chronic HBV patients do not exhibit a general immunological hypo-responsiveness speaks for a virus-specific defect, rather than a general allostimulatory deficit (Tavakoli et al., 2004). Furthermore, the recovery of chronic HBV patients, whether self-generated or after antiviral therapy, indicates that the mechanisms responsible for clearance can be spontaneously induced. To define the critical event, that allows HBV to affect the host immune system in a way that it can persist, would provide insight into the question as to why some patients recover from an acute hepatitis B, while others develop a chronic disease. Furthermore, it would help to establish an efficient treatment against chronic hepatitis B.

The difficulties of these studies are, on the one hand, the heterogeneity of course and severity of the chronic disease in different patients, as well as the heterogeneity of the different DC and T-cell subsets. Furthermore, these cells are difficult to isolate in a sufficient purity. On the other hand the different compartments of the immune system are interconnected, so that the failure of one of them will have an impact on the whole system. Therefore, it will be difficult to define the determinant or determinants, which allow HBV persistence.

3. (II) Generation and characterization of hepatitis B virus surface proteins fused to GFP and RFP

3.1 Results II

Many aspects of the HBV life cycle still remain unclear. To elucidate the different steps of virus entry and egress, the visualisation of viral (VP) and subviral (SVP) HBV particles is desired. Therefore, plasmids encoding the HBV small (S), middle (M) or large (L) surface protein fused to a green (GFP) or red (RFP) fluorescent protein were constructed. These fluorescent proteins could be used to observe not only the surface protein behaviour in cells, but also particle formation and release from the cells. Furthermore, if infectious fluorescent virus is built, the early steps of infection could be monitored. Prerequisite for using the fluorescent fusion proteins is that they display the same properties as their parental counterparts. Otherwise, results would hardly be conferrable on the natural situation in HBV infection. Therefore, the fusion proteins were analysed for their fluorescence and stability properties, and compared to the parental proteins with regard to their localisation in cellular compartments and the ability to form SVPs and VPs.

3.1.1 Cloning of GFP- and RFP-fused HBV surface proteins.

To generate fluorescent HBV surface proteins, the plasmid pCH S An-b-glob, encoding S under CMV promoter control was used as basis. The GFP and RFP genes were gained from the plasmids pCH GFP An-b-glob and RFP1_pRSETB, respectively. The fusion constructs and the resulting proteins are schematically depicted in fig.20. The plasmid shown in fig. 20a encodes the GFP-S fusion protein, with GFP N-terminally fused to S. Since current HBV surface-protein models propose a localisation of the N terminus in the ER lumen, upon budding of SVPs and VPs into the ER lumen the fluorescent domain should be located at the particle outside. In order to remove possible sterical problems which could influence the folding of GFP or S, an additional fusion protein based on GFP-S was designed. It carries a glycine spacer (sp) consisting of 12 nucleotides between GFP and S. This fusion protein is designated GFP-sp-S (Fig. 20b).

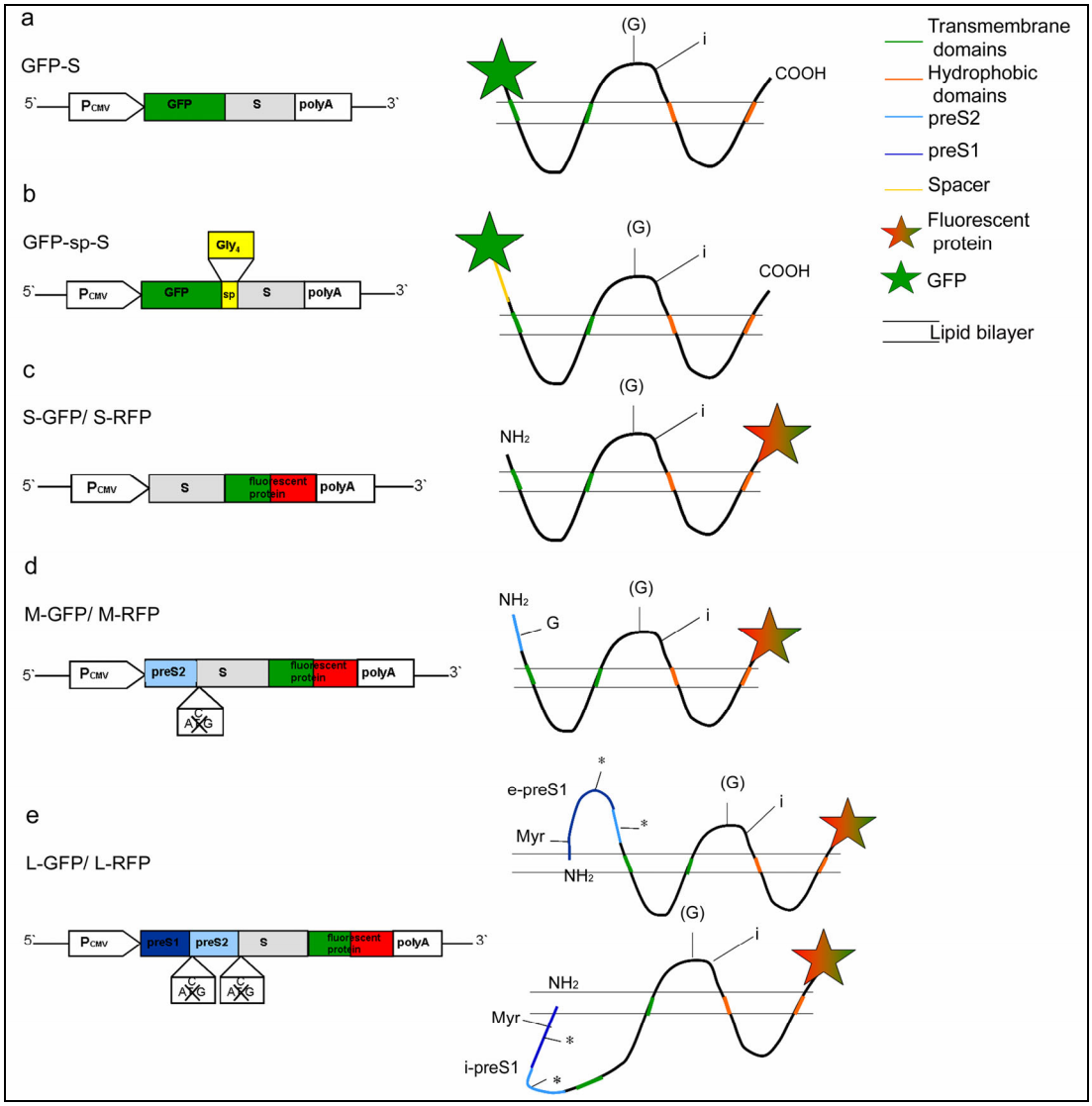

Fig. 20: Generated DNA constructs and the resulting fusion proteins.

The HBV envelope proteins traverse the ER membrane with two transmembrane domains (green boxes) and probably with two additional hydrophobic domains (orange boxes), exposing N- and C terminus to the ER lumen. After budding, the termini are located at the outer surface of VPs and SVPs. All fusion proteins are under control of a CMV promoter (P_{CMV}). a) GFP-S carries the GFP at the N terminus of S. b) GFP-sp-S is based on GFP-S with a spacer (sp; yellow box) consisting of four glycins integrated between GFP and S. c) S-GFP and S-RFP carry the fluorescent part at the C terminus of S. d) M-GFP and M-RFP base on S-GFP and S-RFP, respectively. They were extended by the preS2 domain (light blue). The ATG of S was exchanged to ACG. e) L-GFP and L-RFP are based on M-GFP and M-RFP, and were further extended by the preS1 domain (dark blue). Both ATGs from S and M were exchanged to ACG. (G) = partial glycosylation site; G = glycosylation site; i = immunodominant epitope; asterisks = potential glycosylation sites, Myr = myristoylation site.

Alternatively, the fluorescent protein was fused to the C terminus of S. Like the N-terminus, the C terminus of S is supposed to be located in the ER lumen, which is topologically equivalent to the VP and SVP surface. Therefore, the fused fluorescent protein, either GFP or RFP, is as well suggested to be placed at the surface of VPs and SVPs (Fig. 20c; S-GFP, S-RFP). For the generation of fluorescent M proteins carrying GFP or RFP at the C terminus (M-GFP; M-RFP), the preS2 region was introduced between CMV promoter and S of the plasmids S-GFP and S-RFP, respectively. The location of the fluorescent part of the fusion proteins was not changed by this modification. To abolish expression of S-GFP or S-RFP from these plasmids, the start codons of S were exchanged from ATG to ACG (Fig. 20d). The fusion proteins L-GFP and L-RFP were generated by introducing the preS1 sequence in front of the preS2 region of the plasmids M-GFP and M-RFP, respectively (Fig. 20e). Thereby, the translation initiation codons of M and S were replaced by ACGs. Therefore, only the L fusion proteins should be expressed from these plasmids. The correct introduction of the ACGs was controlled by sequencing of the generated plasmids.

3.1.2 Fluorescence and stability of GFP- and RFP-fused HBV surface proteins.

In a first step it was elucidated whether the fusion of GFP or RFP to the HBV surface proteins resulted in a fluorescent fusion protein. Therefore, S-GFP, GFP-S and GFP-sp-S were transiently transfected into HuH7 cells and fixed with 4% PFA 24 h later. After Dapi staining of the nuclei the cells were analysed using confocal fluorescence microscopy (FluoView1000, Olympus). Comparing S-GFP (Fig. 21a) with GFP-S (Fig. 21b), it becomes obvious that the fusion site had an impact on the protein formation. In case of S-GFP, the GFP was fused to the C terminus of S. This gave rise to a higher fluorescence compared to GFP-S, which carries the GFP at the N terminus of S. The introduction of the glycine spacer between GFP and S in the fusion protein GFP-S, which led to the new protein GFP-sp-S, did only slightly improve the fluorescence of the fusion protein (Fig. 21c).

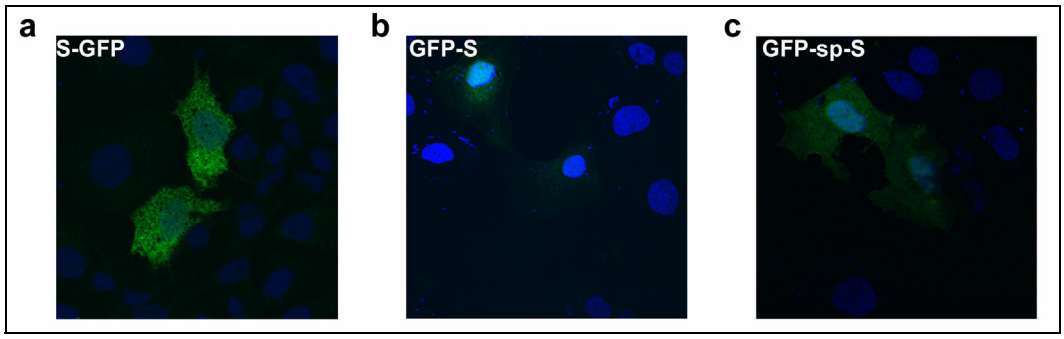

Fig. 21: Confocal fluorescence microscopy of HuH7 cells transfected with a) S-GFP, b) GFP-S or c) GFP-sp-S.

Western blotting and detection with a polyclonal anti-GFP antibody (rαcoreGFP, in-house production) of cellular lysates reveals that S-GFP transfected HuH7 cells expressed an approx. 52 kDa protein which likely displays the fusion protein (Fig. 22). The 30 kDa band is most probably a fusion protein fragment, because solitary GFP, which could theoretically be expressed in low amount from the internal translation initiation site of the GFP gene, has a size of 28 kDa.

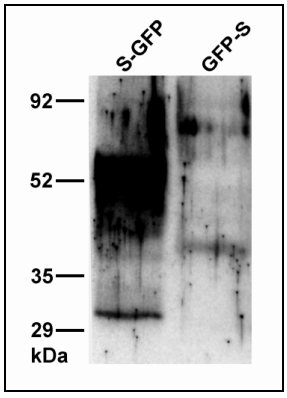

Fig. 22: Western blotting of lysates of S-GFP or GFP-S transfected HuH7 cells.

Conversely, in cells transfected with GFP-S, only two weak bands of approx. 40 kDa and 80 kDa were detected, although the same amount of plasmid was transfected and similar protein amounts were loaded on the gel. Both protein bands neither corresponded to the intact fusion protein nor to solitary GFP. This implies rapid degradation of the GFP-S fusion protein, which would also explain the low fluorescence of the fusion protein in the fluorescence microscopic analysis. Another possibility is that translation of GFP-S was incomplete due to misfolding of the protein during the translation process. The 80 kDa band may indicate the formation of aggregates.

Because the N-terminal fusion of GFP to S seemed not suitable for the formation of a fluorescent fusion protein, the fusion of GFP to the middle (M) and large (L) surface proteins was executed only at the C terminus of the surface proteins. Protein expression of M-GFP and L-GFP was examined using confocal fluorescence microscopy as described. The results are presented in fig. 23. Comparable to S-GFP, M-GFP and L-GFP were brightly fluorescent, with the fluorescence distributed in cytosol and nuclei.

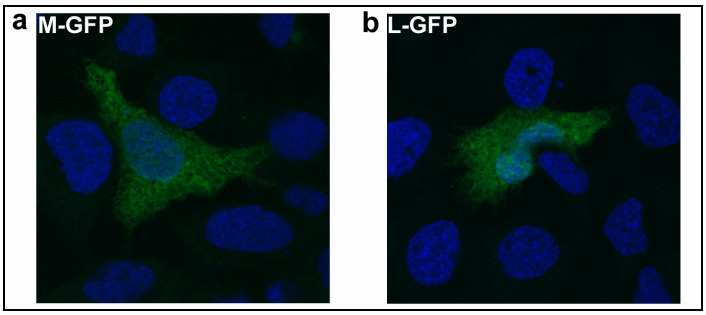

Fig. 23: Confocal fluorescence microscopy of HuH7 transfected with a) M-GFP or b) L-GFP.

The localisation of the fluorescence indicates either that GFP was expressed not only in the context of the fusion protein but also from its own internal translation initiation site, or that the fusion protein was instable.

In order to further investigate the stability of the GFP fusion proteins, Western blotting of cellular lysates with a polyclonal antibody against GFP (rαcoreGFP, in-house production) and a polyclonal antibody recognising all HBV surface proteins (gαHBsAg, Abbott) was performed (Fig. 24). GFP transfected cells (GFP) and S transfected cells (S) served as the respective positive controls, untreated HuH7 cells (HuH-) were added as negative control. To be able to examine whether the presence of parental protein has an impact on the stability of the fusion proteins, these were transfected either alone or together with S. The same blot was first stained with the antibody against the HBV surface proteins, and after stripping analysed with the antibody against GFP.

The faint 55 kDa band visible in the negative control (HuH-) is unspecific and present in all samples independent of the detection antibody. The GFP control sample (GFP) was negative after using the gαHBsAg antibody, but after incubation with the antibody directed against GFP, two protein bands were detected. The 28 kDa band stemmed from monomeric GFP. The presence of

the additional, approx. 55 kDa protein speaks for dimerisation of GFP, leading to 56 kDa GFP dimers. These are formed by wild type GFP in addition to the monomers. The S transfected control cells (S) expressed a 17 kDa band detected with gαHBsAg antibody. This band is a product of an internal translation initiation ATG within the S gene, and present in all samples co-transfected with S (S-GFP/S, M-GFP/S, L-GFP/S).

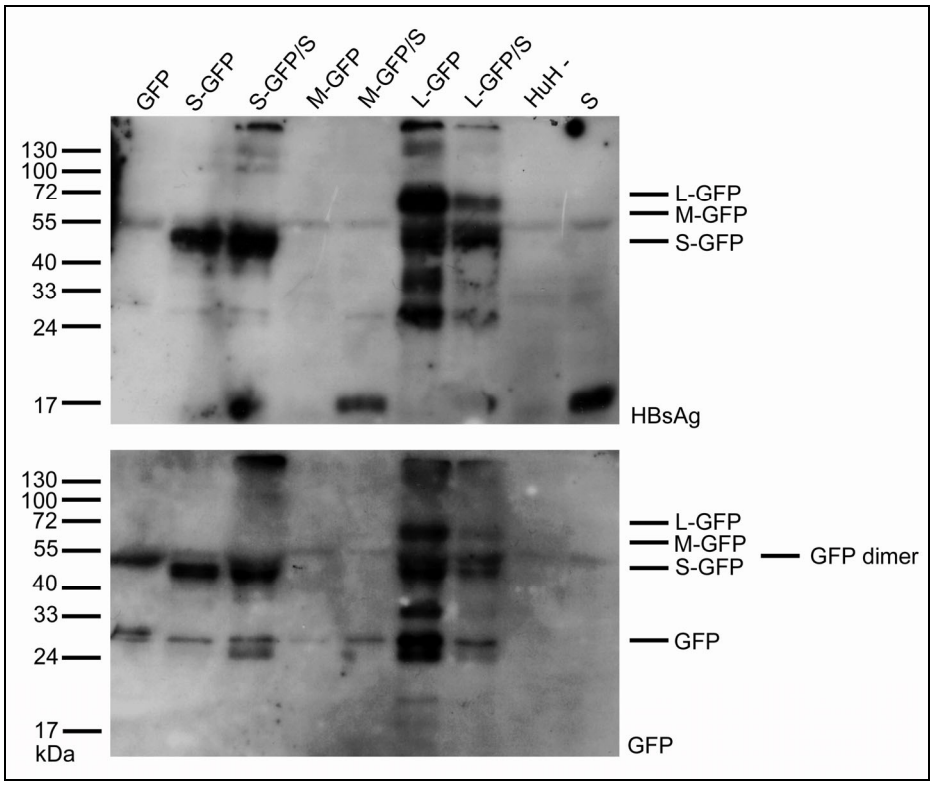

Fig. 24: Western blot analysis of HuH7 cell lysates after transfection with GFP fusion proteins.

In S-GFP transfected and S-GFP/S co-transfected cells a band of approx. 52 kDa was detected with both the αHBs and the αGFP antibody and therefore most probably represents the fusion protein. A glycosylated form was not detected. The approx. 28 kDa band visible in the GFP blot represents solitary GFP. Bands of approx. 100 kDa and 130 kDa and a band of unknown size larger than 130 kDa were detected after co-transfection of SGFP and S. These bands might be protein oligomers. In the GFP blot of the S-GFP/S co-transfected cells an approx. 24 kDa was detected in addition to the 28 kDa band present in both the S-GFP transfected cells and the S-GFP co-transfected

cells. The same band is visible in L-GFP and L-GFP/S transfected cells and presumably represents incomplete translated GFP or GFP fragment.
Despite the fact that fluorescence microscopy analysis clearly demonstrated the expression of a fluorescent protein (Fig. 23a), the M-GFP fusion protein could not be detected by Western blotting. This was independent of co-transfection of S (M-GFP/S). As is the situation for the S-GFP and SGFP/S samples, the faint band of approx. 27 kDa hints at the low expression of solitary GFP.
The approx. 72 kDa protein present in the cells after transfection of L-GFP and L-GFP/S was detected with both the αHBsAg and the αGFP antibody and corresponds to the fusion protein. The 52 kDa band present in both blots might be due to S-GFP expression from the L-GFP plasmid, or a degradation product. With the anti GFP antibody an additional 24 kDa protein was detected. This is possibly incompletely translated GFP, as well as the two bands of approx. 17 kDa and 20 kDa detected only with the anti GFP antibody. The bands of approx. 100 kDa and 130 kDa and the large band of unknown size present in the S-GFP/S, L-GFP and L-GFP/S samples indicated the formation of oligomers.

In order to obtain a red variant of the fluorescent surface proteins, RFP was fused to the C terminus of S, M and L. The expression of the fusion proteins was analysed as described before. All proteins were brightly fluorescent, with the fluorescence signal detectable predominantly in the cytosol, and little or no nuclear localisation (Fig. 25).

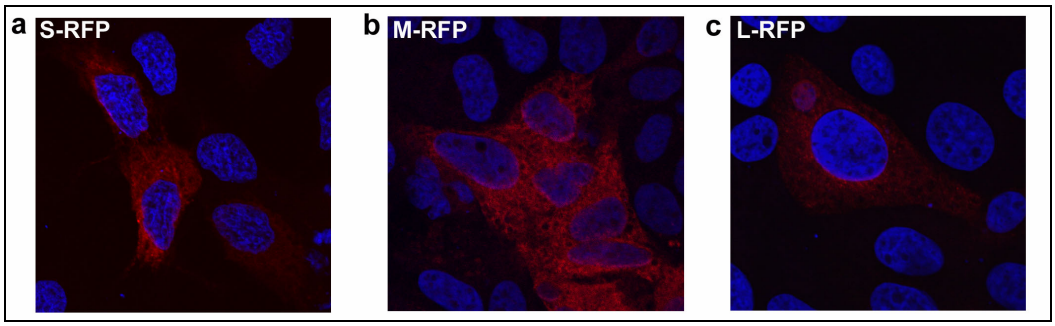

Fig. 25: Confocal fluorescence microscopy of HuH7 cells transfected with RFP fusion plasmids.

To confirm that the RFP fusion proteins are stable, a Western blot analysis was performed using a polyclonal antibody against all HBV surface proteins

(gαHBsAg, Abbott; Fig. 26). Due to the lack of an anti-RFP antibody, a parallel control for RFP expression could not be performed.

As described also for the Western blot of the GFP fusion proteins, an unspecific 55 kDa protein is visible in all samples. In addition, a second unspecific protein of approx. 33 kDa was detected. In cells expressing only S, the approx. 50 kDa band corresponded to S dimers. The disulphide bridges, which cross-link the dimers are often not completely destroyed by the denaturing conditions of the Western blot procedure. The unglycosylated and the glycosylated monomers were detected as 24 kDa and 27 kDa bands. Transfection of S-RFP led to the expression of a 52 kDa protein, likely representing S-RFP. Additionally, a double band of 24 kDa and 27 kDa indicates that solitary S was also expressed in both the unglycosylated and the glycosylated form. Alternatively, these bands may represent N-terminal proteolytic fragments of the fusion protein.

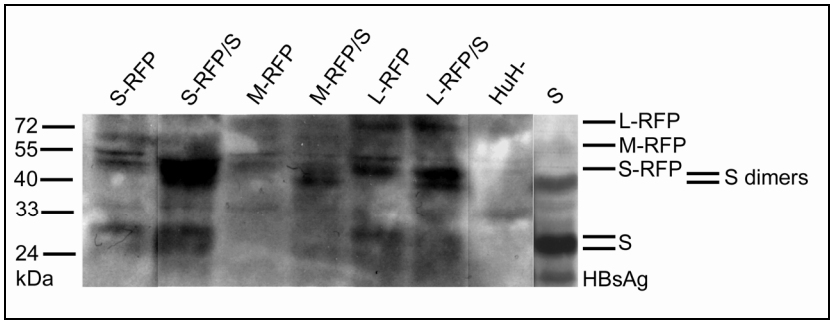

Fig. 26: Western blot analysis of HuH7 cell lysates after transfection with RFP fusion proteins.

Upon co-transfection of S-RFP and S, more S was built, as indicated by the strong 24 kDa and 27 kDa bands. Furthermore, an approx. 50 kDa band corresponding to the size of S dimers was present. As described before for M-GFP, in cells transfected with M-RFP the protein detection with an antibody against HBs was not possible, although a bright fluorescent protein was previously seen in fluorescence microscopy. This fluorescent protein did not exhibit the characteristic distribution of RFP, implying that the fluorescence was not due to expression of solitary RFP. However, only a faint 52 kDa protein was detected, which likely resulted from S-RFP expression from the M-RFP plasmid. If S was co-transfected, an approx. 50 kDa band indicated the presence of S-RFP or S dimers. However, S monomers were not present in a detectable amount in M-RFP/S transfected cells.

After transfection with L-RFP or L-RFP/S, the cells expressed a 72 kDa protein which likely corresponded to the fusion protein. Furthermore, a 52 kDa band was found, which might display S-RFP. Weak 24 and 27 kDa bands likely corresponded to the unglycosylated and glycosylated forms of S. If S was co-transfected to L-RFP, a double band of approx. 50 kDa was detected, which most likely stemmed from S dimers.

To gain information on whether the fluorescence signal in cytosol and nucleus derives from the fusion proteins or from solitary GFP or RFP, cells transiently transfected with S-GFP or S-RFP were stained with a polyclonal antibody (rαHBvax, in-house production) against S and a secondary antibody coupled to Alexa 564 (gαr Alexa Fluor 564, Invitrogen) in case of S-GFP transfected cells.

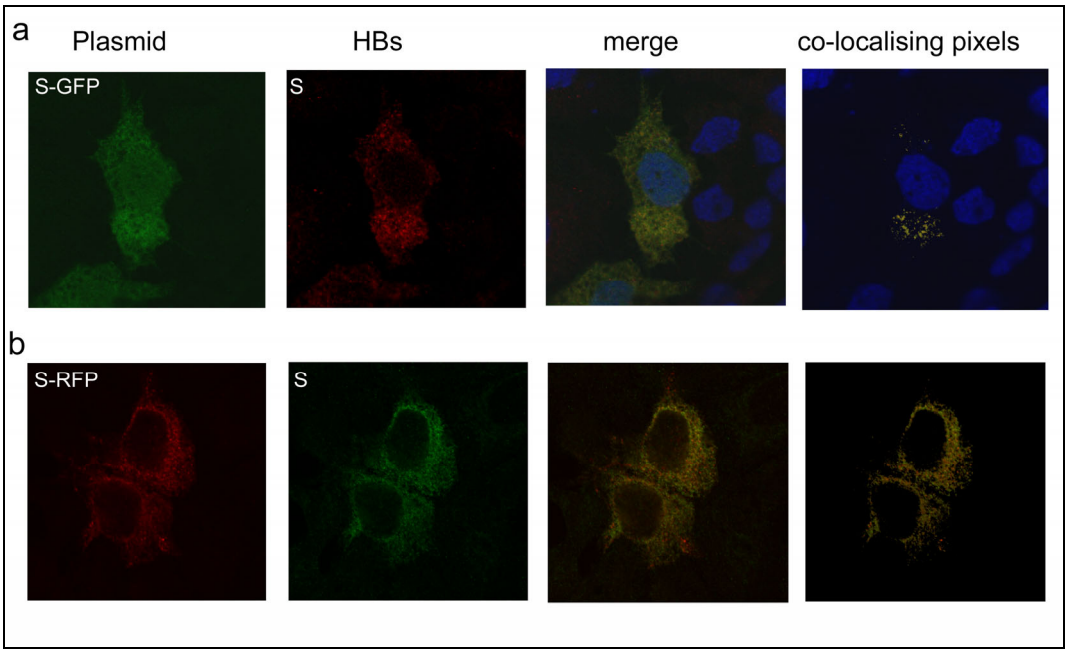

Fig. 27: Co-localisation analysis of S-GFP and S-RFP with an anti-S antibody.
Confocal fluorescence microscopy of HuH7 cells transfected with a) S-GFP or b) S-RFP and stained for S protein.

To investigate co-localisation of RFP with S, a secondary antibody coupled to Alexa 488 (gαr Alexa Fluor 488, Invitrogen) was chosen to visualise S. The staining should co-localise with the fluorescence derived from the fusion proteins, if these are intact. Analyses were performed using a laser scanning confocal microscope (FluoView1000, Olympus). With help of the co-localisation tool of the microscope software (CellP, Analysis GmbH), areas not positive for

both red and green signals at the same spot were extracted from the co-localising pixels. The latter display the areas in which the analysed proteins co-localise. The results are provided in fig. 27. Visualisation of the co-localising pixels revealed that only little green fluorescent signal in the cytosol was positive for both GFP and S (Fig. 27a, co-localising pixels). This implies that the green fluorescence in the nucleus and in parts as well in the cytosol of the S-GFP transfected cells was not due to the presence of intact fusion protein, but mostly caused by solitary GFP or fusion-protein fragments. This is consistent with the fact that the observed distribution of the fluorescence is characteristic for GFP but not for trans-membrane proteins such as the HBV surface proteins.
On the contrary, most of the red fluorescence signals seen after S-RFP transfection appeared to stem from intact fusion protein. Most pixels were positive for red and green fluorescence, indicating the presence of both RFP and S at the same locus (Fig. 27b, co-localising pixels).
In summary, the C-terminal fusion of GFP and RFP to the HBV surface proteins resulted in fluorescent proteins. In contrast to the RFP fusion proteins, most of the fluorescence of the GFP fusion proteins did not originate from the fusion proteins and was located in cytosol and nuclei. Western blotting of the S and L fusion proteins revealed that not only the fusion protein was present in the cells but also smaller protein fragments.

3.1.3 Localisation of GFP- and RFP-fused HBV surface proteins.

To gain information on the distribution of the fusion proteins in cells, their localisation in cellular compartments was investigated. Since the GFP fusion proteins appeared at least partially unstable in Western blot analysis and immuno-fluorescence data revealed that the fluorescence did mostly not originate from the fusion protein but rather from solitary GFP or fusion protein fragments, the following experiment concentrated on the RFP fusion proteins. The HBV surface proteins traverse from the ER into the golgi. Therefore, a localisation in these compartments could as well be expected for the fusion proteins, presuming that they still behave as their HBV parental counterparts. Co-localisation analyses were performed using a laser scanning confocal

microscope (FluoView1000, Olympus) as described in 3.1.2. HuH7 cells transfected with a plasmid encoding S (pCH S An-b-glob) were used as positive control. Due to the lack of anti-M and anti-L antibodies suitable for immunofluorescence staining, these wild type controls could not be performed. To exclude that RFP per se is located in the ER, RFP (pCH RFP An-b-glob) transfected cells were analysed. The cells were fixed with 4% PFA and stained 24 h post transfection with a monoclonal antibody for the ER membrane protein calnexin (mαcalnexin, Santa Cruz Biotech). A secondary antibody coupled to Alexa Fluor 488 (gαm Alexa Fluor 488, Invitrogen) visualised the ER.

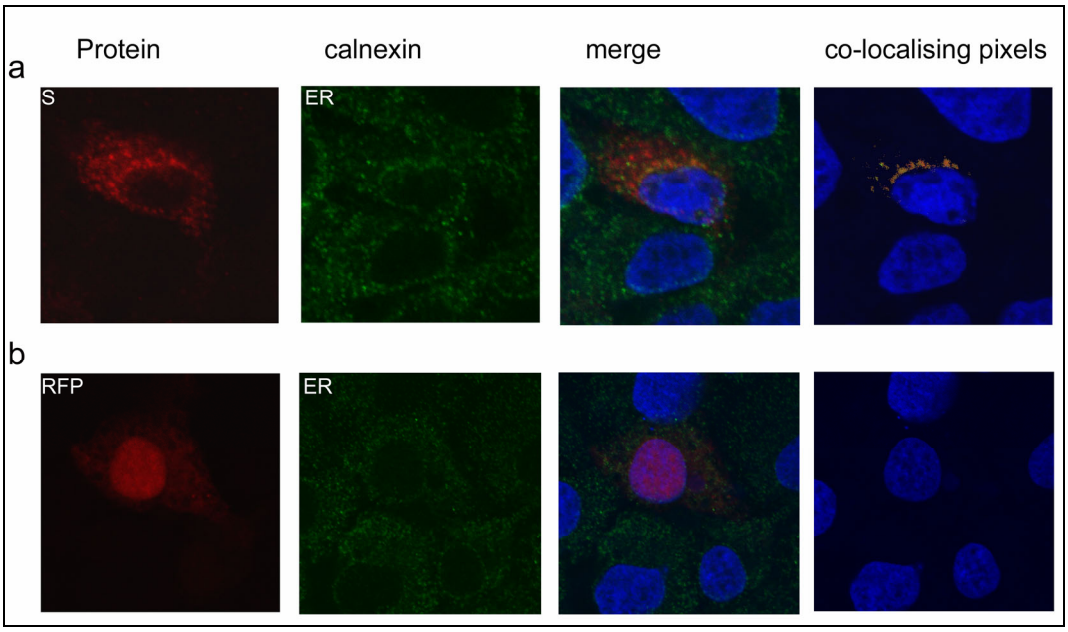

Fig. 28: Co-localisation analysis of S and RFP with the ER marker calnexin.
Confocal fluorescence microscopy of HuH7 cells transfected with a) S or b) RFP. The right panel (co-localising pixels) shows the pixels positive for green and red fluorescence.

S was stained with a polyclonal antibody against the HBV vaccine (rαHBvax, in-house production) and an Alexa Fluor 568 coupled secondary antibody (gαr Alexa Fluor 568, Invitrogen). S appeared to partially localise in the perinuclear region of the ER (Fig. 28a). On the contrary, RFP was mostly present in the nuclei with only low amounts in the cytosol. Fig. 28b demonstrates that RFP was not distributed in the ER, since no co-localising areas were detected.

The next step was to clarify whether the fusion proteins were partially distributed in the ER and whether the presence of other HBV proteins

influenced their localisation. The S-RFP encoding plasmid was transfected into HuH7 cells either alone (S-RFP, Fig. 29a) or together with equal amounts of pCH S An-b-glob encoding S (S-RFP/S, Fig. 29b).

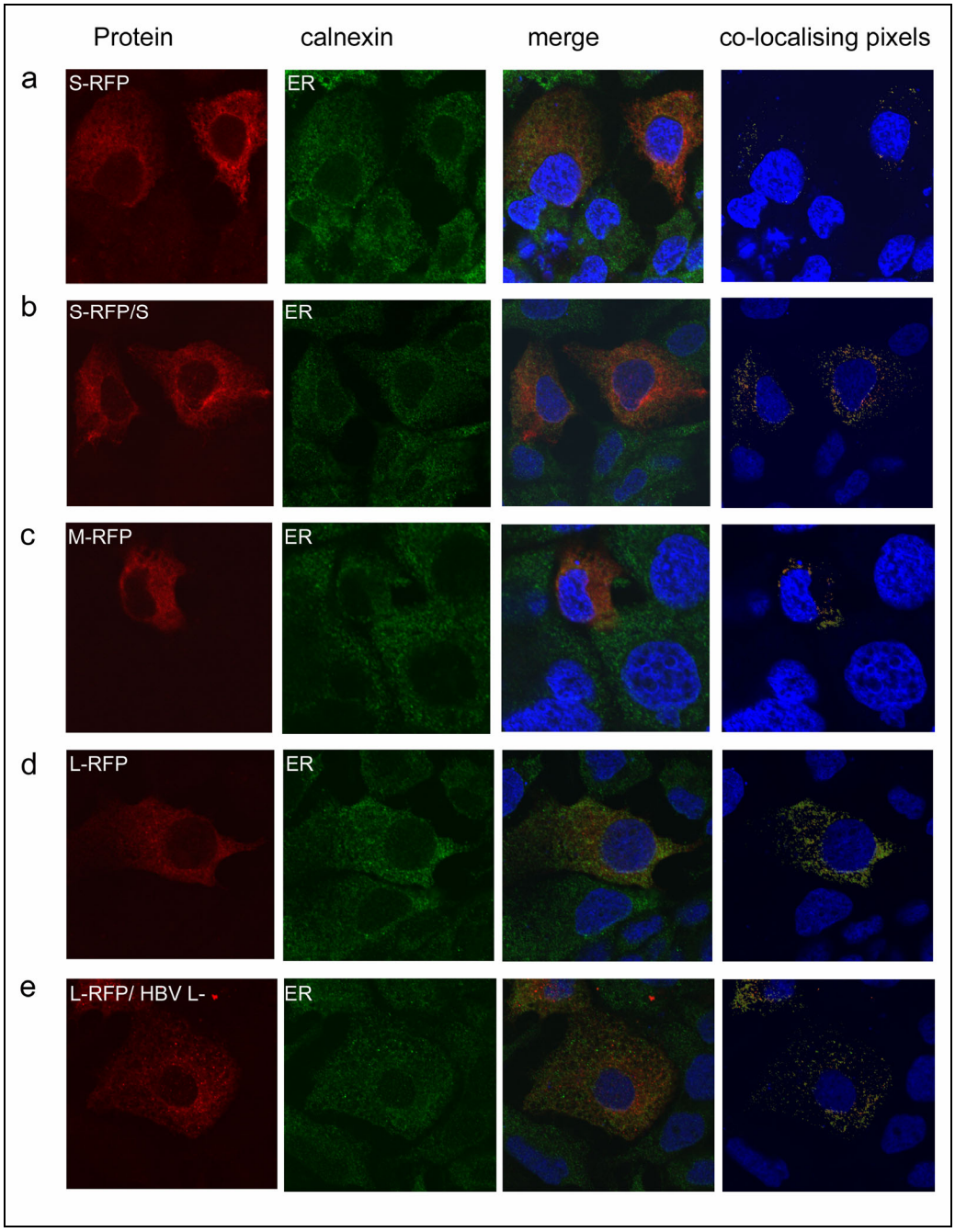

Fig. 29: Co-localisation analysis of RFP fusion proteins with the ER marker calnexin.
Confocal fluorescence microscopy of HuH7 cells transfected with a) S-RFP, b) S-RFP plus S, c) M-RFP, d) L-RFP or e) L-RFP plus HBV L-. The right panel (co-localising pixels) shows the pixels positive for both green and red fluorescence.

In both cases pixels with co-localising red and green fluorescence could be detected, demonstrating a partial localisation of the S-RFP fusion protein in the

ER. The presence of S did not influence this distribution. The investigation of M-RFP proves that this protein was as well partially distributed in the ER (Fig. 29c). L-RFP also showed a partial co-localisation with the ER, independent of the presence or absence of the other HBV proteins, provided by the co-transfected plasmid HBV L-. This plasmid encodes all HBV proteins except for L (Fig. 29d, 29e).

The HBV surface proteins are known to oligomerise and bud into the ER as spherical or filamentous SVPs, and, in presence of the other HBV proteins, as well as VPs. From the ER the particles are transported to the golgi, before they are exocytosed. Therefore, in the following co-localisation with the golgi marker **g**olgi **m**atrix protein **130** (GM130) was investigated. A monoclonal antibody against GM130 (mαGM130, BD Pharmingen) was used together with an Alexa Fluor 488 coupled secondary antibody (gαm Alexa Fluor 488, Invitrogen) to stain the golgi apparatus.

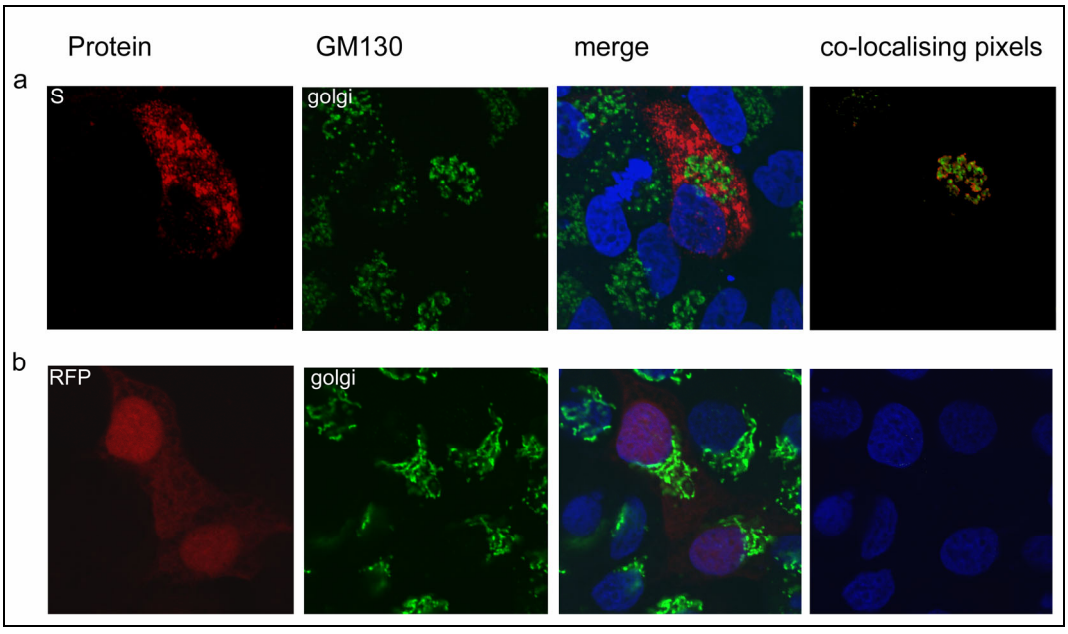

Fig. 30: Co-localisation analysis of S and RFP with the golgi marker GM130.
Confocal fluorescence microscopy of HuH7 cells transfected with a) S or b) RFP. The right panel (co-localising pixels) shows the pixels positive for green and red fluorescence.

As positive control, S was transfected into HuH7 cells and stained after 48 h with the aforementioned antibodies directed against S and GM130, respectively. To exclude that RFP per se localises within the golgi, RFP transfected cells were analysed in parallel.

Fig. 30 depicts the results of the control stains. S was present in the whole cell, with a clear distribution in the golgi. RFP could be seen predominantly in the nucleus, but as well in the cytosol. It did not co-localise with the golgi marker GM130.

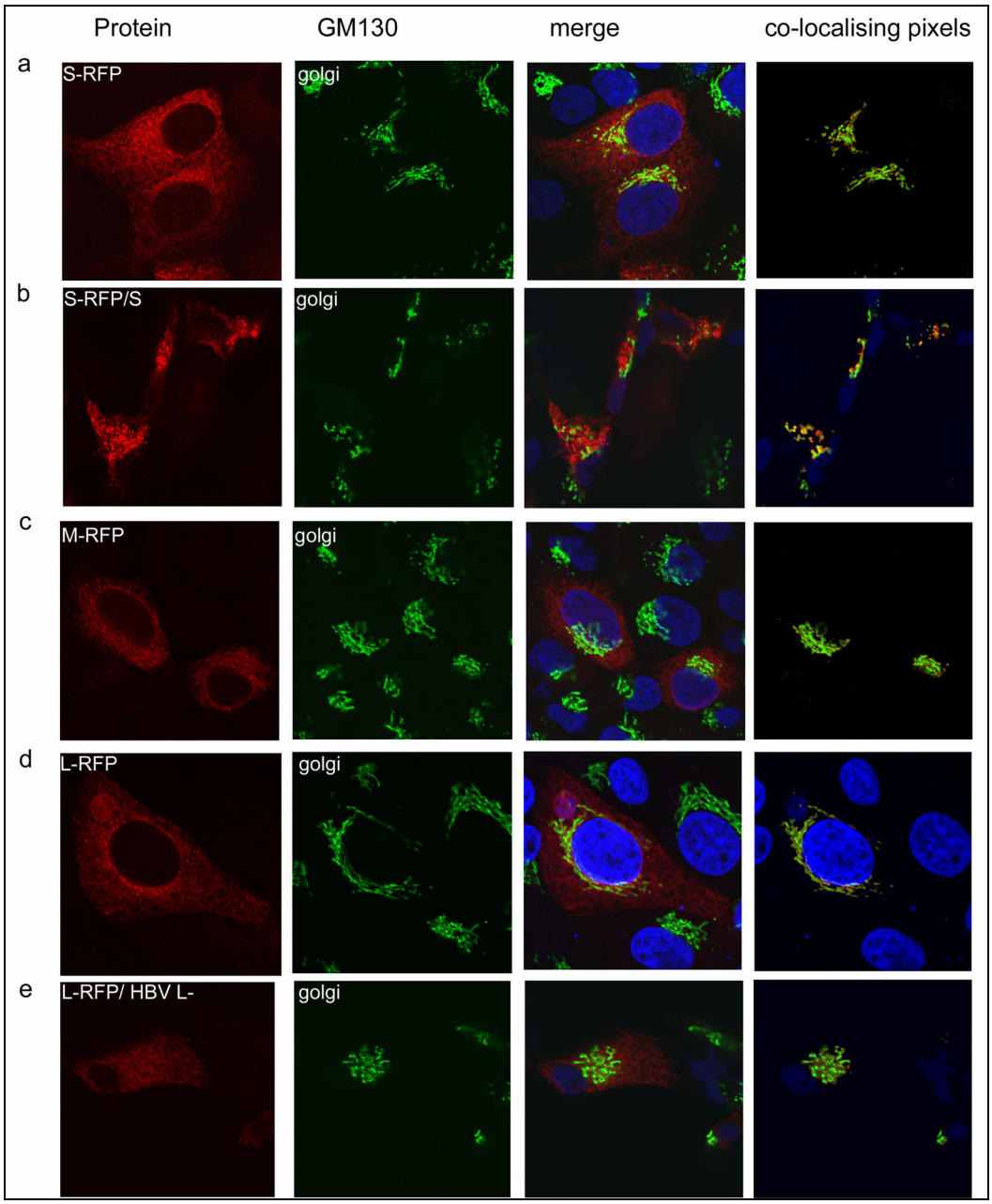

Fig. 31: Co-localisation analysis of RFP fusion proteins with the golgi marker GM130.
Confocal fluorescence microscopy of HuH7 cells transfected with a) S-RFP, b) S-RFP plus S, c) M-RFP, d) L-RFP or e) L-RFP plus HBV L-. The right panel (co-localising pixels) shows the pixels positive for green and red fluorescence.

For S-RFP and S-RFP co-transfected with S, a localisation in the golgi could be demonstrated (Fig. 31a and b). A difference in the distribution due to the

presence of S was not observed. M-RFP also exhibited a clear localisation in the golgi (Fig. 31c). The analysis of L-RFP and L-RFP co-transfected with the plasmid pCH HBV L- revealed that the presence of the other HBV proteins did not influence the partial localisation of L-RFP in the golgi (Fig. 31d and e).
In summary, all RFP-fused HBV envelope proteins partially co-localised with the ER marker calnexin and with the golgi marker GM130, resembling the behaviour of the wild type S protein. The distribution was unaffected by co-transfection of wt S in case of S-RFP or HBV L- in case of L-RFP.

3.1.4 Formation of SVP containing GFP- or RFP-fused HBV surface proteins.

SVPs are present in vast amount in the blood of HBV infected persons. In cell culture infection or transfection experiments, SVPs are secreted in 100- to 10000-fold excess to VPs. They can be produced solely from S, which harbours the self assembly properties necessary for SVP formation. To investigate whether the S fusion proteins still exhibit this characteristic, supernatants of cells transfected either with S-RFP or S-GFP alone or together with equal vector amounts for S expression were collected after four days. Fluorescence microscopy analysis previously confirmed that the transfected cells expressed the fluorescent proteins. The supernatants of untransfected cells served as negative control (Fig. 32; HuH). As positive controls, the supernatant of cells transfected only with the parental S protein (Fig. 32, S) or with a plasmid encoding all HBV proteins and able to initiate HBV replication (Fig. 32, HBV1.1) was used. To isolate the SVPs from the supernatants, an **i**mmuno-**p**recipitation (IP) was performed using sepharose G beads and a polyclonal antibody (rαHBvax, in-house production) directed against S. The precipitate was analysed by Western blot using another polyclonal antibody (gαHBsAg, Abbott) directly coupled to horseradish peroxidase. As depicted in fig. 32, cells transfected with HBV1.1 (left panel) secreted a high amount of SVPs containing all types of HBV envelope proteins in their different glycosylation forms. Cells transfected with S (right panel) secreted SVPs consisting of both unglycosylated

and glycosylated S. The transfection of S-GFP and S-RFP did not give rise to a detectable amount of SVPs in the supernatant.

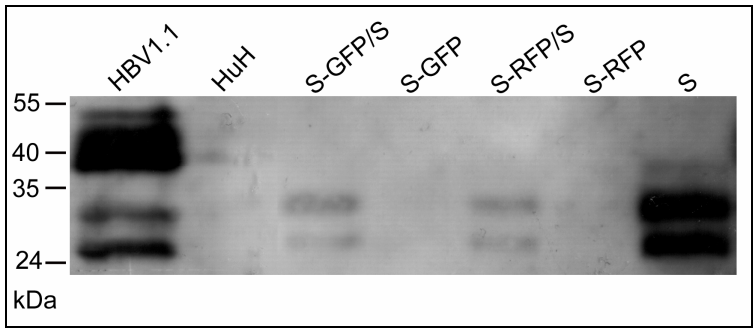

Fig. 32: Western blot analysis of cell supernatant immuno-precipitates for the presence of SVPs after transfection with S-GFP or S-RFP.

Only if the parental S protein was co-transfected with S-GFP and S-RFP, weak bands revealed that small amounts of SVPs were present in the supernatants. These SVPs did not contain any S-GFP or S-RFP, as the detected protein bands had the size of the wild type S.
This experiment shows that the S fusion proteins were not capable of SVP formation.

3.1.5 Formation of VPs containing RFP-fused HBV surface proteins.

Although the inability to form SVPs implies that particle formation in general was impaired, it was investigated whether the fluorescent HBV envelope fusion proteins were capable of progeny virus production. Therefore, HuH7 cells were transiently co-transfected with HBV1.1 and the fusion proteins in different ratios. Four days post transfection, the supernatant was concentrated using centrifugal devices, and transferred to caesium chloride density ultracentrifugation. This allows the separation of free HBV DNA, DNA-containing capsids and intact enveloped virions by their sedimentation in different density fractions. The collected density fractions were dot blotted on a nylon membrane and hybridised with a ^{32}P-labelled HBV DNA probe. Fig. 33 depicts an example for the virus production in cells transfected with 1 part S-RFP DNA plus 1, 10 or 100 parts HBV1.1 DNA. In the supernatant of the positive control, HuH7 cells transfected with plasmid HBV1.1, viral DNA was detected, as well as low amounts of DNA-containing unenveloped capsids, and virions (Fig. 33,

HBV1.1). Conversely, if fusion construct plasmids were co-transfected with HBV1.1, the capability of virus formation appeared to be impaired. Only HBV DNA, but no DNA-containing capsids and intact virions were detected. The same held true, if the DNA ratios for the transfection were changed, for example to a 10:1 or 100:1 ratio of fusion construct DNA to wtHBV DNA or if M-RFP or L-RFP were co-transfected to wtHBV DNA. Only in rare cases traces of DNA-containing capsids and virions could be detected. If the supernatant of cells co-transfected with fusion construct DNA and wtHBV DNA was concentrated and transferred to PHHs, no infection of PHHs could be detected. This indicates that even if small amounts of virus were produced in the HuH7 cells after transfection, this virus was not infectious or the amount was too minor to initiate infection in PHHs. Possibly, the fusion proteins even impair virus maturation.

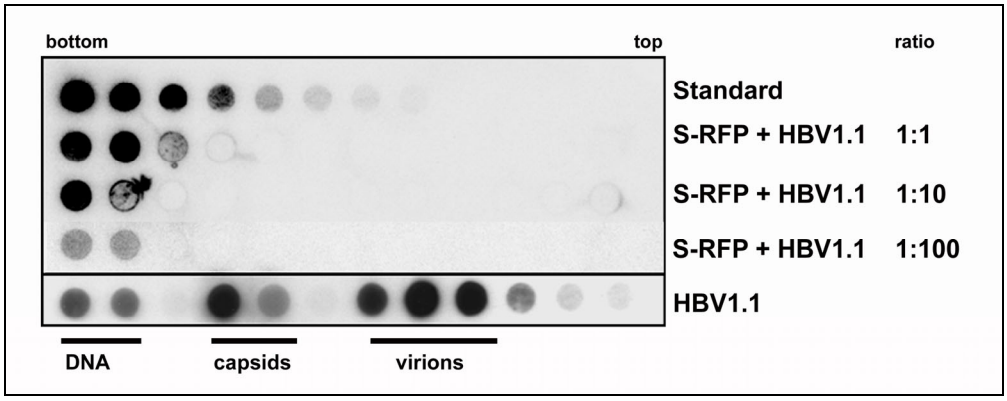

Fig. 33: Dot Blot analysis of supernatants of HuH7 cells co-transfected with fusion protein DNA and wtHBV DNA. S-RFP and wt HBV were co-transfected into HuH7 cells in a ratio of 1 part fusion protein DNA and 1 (1:1), 10 (1:10) or 100 (1:100) parts HBV DNA. HuH7 cells transfected with the HBV encoding plasmid (HBV) served as positive control.

In summary, different GFP and RFP fused HBV surface proteins were generated. The N-terminal fusion of GFP did not give rise to a strong fluorescent protein. The C-terminal fusion of GFP and RFP resulted in fluorescent proteins well detectable in fluorescence microscopy. In contrast to the RFP fusion protein however, most of the fluorescence detected in GFP fusion protein transfected cells did not originate from the fusion proteins but rather from solitary GFP or protein fragments. RFP fusion proteins partially localised in the ER and golgi, as observed for the parental HBV surface proteins. However, they were not capable of SVP or VP formation, and even impaired the capacity of the parental surface proteins co-expressed.

3.2 Discussion II

Fluorescent labelling of viral proteins and viruses, or virus-like particles, has been shown to provide a powerful tool for investigating unexplored aspects in viral life cycles. A commonly used technique is the genetic incorporation of fluorophores into virus proteins. The insertion of GFP into the Gag protein of HIV for instance allowed studying the trafficking of this protein, as well as production of infectious virions (Müller et al., 2004). In case of adeno-associated virus, Lux et al. (2005) were able to directly monitor the cytosolic and nuclear trafficking with GFP-tagged virus particles.

Since many aspects of the HBV life cycle still remain unclear, the visualisation of viral (VP) and subviral (SVP) HBV particles is a promising approach to elucidate different steps of virus entry and egress. It has already been reported by Lambert et al. (2004) that the fusion of GFP at the N but not the C terminus of S gave rise to a fluorescent fusion protein, which was incorporated into envelopes and secreted as fluorescent subviral and viral particles. It was described that the fluorescent virions were able to attach to liver cells, indicating that the fusion of GFP to the HBV surface protein is a promising approach. Therefore, GFP or RFP fused HBV surface proteins were constructed and analysed for their fluorescence and stability properties. To assay whether the fusion altered the characteristic properties of the HBV surface proteins, they were compared to the parental proteins with regard to their localisation in cellular compartments and the ability to form subviral and viral particles. In contrast however to the above mentioned study (Lambert et al., 2004), the GFP fused proteins did not co-localise with the parental proteins within the cell, suggesting a change in the protein characteristics. The RFP fusion proteins clearly co-localised with the parental proteins. But although they behaved similar to the parental proteins with regard to their distribution in the ER and golgi compartment, neither of them contributed to the formation of SVP or VP.

Thus, the GFP and RFP fusion to HBV surface proteins characterised in this work were not suitable to generate fluorescent HBV particles. The main impediment was most probably misfolding of the fusion proteins. The bulky GFP and RFP domains seem to have topological and sterical effects, affecting stability, correct integration into the ER membrane or assembly and secretion of

properly formed particles. Labelling with small fluorescent molecules can minimise or overcome these problems and therefore perhaps provides a better strategy.

3.2.1 N-terminal fusion of GFP to the small HBV surface protein.

In order to test whether the constructed GFP and RFP fusion proteins were fluorescent, they were transiently transfected into HuH7 cells and the fluorescence was monitored by confocal fluorescence microscopy. S-GFP could clearly be detected in the cells as a bright fluorescence distributed in the cytosol and nucleus. In contrast, constructs carrying the GFP at the N terminus of S, designated GFP-S and GFP-sp-S, were hardly detectable. To elucidate whether this was due to protein instability or whether the fusion protein was stably expressed but only weakly fluorescent, the constructs were compared in Western blot analysis. While S-GFP was expressed, GFP-S was not detected as intact protein. Although comparable amounts of DNA and total protein were used for transfection and Western blotting, respectively, only very low amounts of a protein fragment reacting with the anti-HBs antibody were detectable. From this, it can be inferred that the N-terminal fusion of GFP to S led to an unstable protein, which was degraded in the cells. It has already been shown that the N terminus of S is very sensitive to alterations (Eble et al., 1986 and 1987; Bruss and Ganem, 1991): Upon addition of a 100 aa sequence derived from alpha globin, the correct translocation of the N terminus into the ER was impaired. However, this mutant was still integrated in the ER membrane and entered the secretory pathway, as shown by the detection of a glycosylated and an unglycosylated form of this mutant (Bruss and Ganem, 1991). In case of GFP-S, not only 100 aa but rather 238 aa of a bulky protein were fused to the N terminus of S. This seemed to have even more deleterious effects: Neither could the intact fusion protein be detected in Western blotting, nor was a bright fluorescence visible after transfection into HuH7 cells. This hints at rapid degradation. Translocation of the N terminus of the surface proteins across the ER membrane occurs co-translational and is mediated by a topogenic signalling sequence, designated signal 1, at the N terminus of the proteins (Eble et al.,

1987). Upon N-terminal fusion of GFP, this signal sequence is now internal and might not any longer be accessible to the interactions with the **s**ignal **r**ecognition **p**article (SRP) mediating translocation of the ribosome to the ER (Rothman and Lodish, 1977; Walter and Blöbel, 1981, Keenan et al., 2001). Alternatively, later steps of the translocation process might be impaired, such as the binding of the SRP-ribosome complex to the ER translocon. The latter is a channel which conducts secretory proteins across the ER membrane and is also used by membrane proteins (Rapoport et al., 2004; van den Berg et al., 2004; von Heijne, 2006). It has been evidenced that the second hydrophobic domain of the HBV surface proteins, designated signal 2, is sufficient to translocate the C terminus to the ER lumen and anchor the protein in the ER membrane. However, without a functional signal 1, the N terminus cannot be translocated and remains in the cytosol. This conformation hampers the formation of SVPs (Eble et al., 1987; Bruss and Ganem, 1991). In contrast to GFP-S, the mutants lacking a functional signal 1 analysed by Bruss and Ganem (1991) were stable. This indicates that GFP-S was not integrated into the ER membrane. Possibly, the folding of the GFP prevented the membrane integration. It has been shown that the folding of sequences N terminal to a signal sequence can sterically prevent this (Denzer et al., 1995). Lambert et al. (2004) reported the functional incorporation of GFP N-terminally fused to S into subviral particles. However, since they did not add an N-terminal signal sequence, their data are in contrast to the results presented here as well as by others (Bruss and Ganem, 1991). Although it has been published that the N terminus of S tolerates the addition of 84 foreign residues, it can be doubted that the addition of 238 aa of a bulky protein leads to correct integration into the ER membrane (Michel et al., 1988; Prange et al., 1995).

Assuming that GFP-S was not targeted to the ER membrane, it was translated in the cytosol. This would result in a misfolded protein, which is degraded most probably by the ubiquitin-proteasome system, as described for cytosolic and ER import-incompetent proteins (Kostova and Wolf, 2003; Park et al., 2007).

The introduction of a four aa glycin spacer between GFP and S slightly improved the fluorescence of GFP. This may be explained by a facilitative folding of the protein, which might have an impact on the degradation time. It has recently been reported that one of the multiple types of proteasomes, the

26S proteasome, is not capable of unfolding the compactly folded GFP moiety of some fusion proteins (Liu et al., 2003).
One possibility to overcome the wrong localisation of GFP-S might be the fusion of an additional ER localisation sequence at the very N terminus, in front of the GFP. This would probably restore the membrane topology, as described by Bruss and Ganem (1991) for the above mentioned S fusion protein carrying sequences from alpha globin at its N terminus. After fusion of a signal sequence to the N terminus this protein was not only synthesised into the ER membrane in the correct topology, but was also competent to form secreted SVPs.

3.2.2 C-terminal fusion of GFP and RFP to the HBV surface proteins.

3.2.2.1 The GFP fusion proteins S-GFP, M-GFP and L-GFP.

The C-terminal fusion of GFP or RFP to the HBV surface proteins S, M and L resulted in brightly fluorescent proteins, as shown by confocal immuno-fluorescence analyses. However, in case of the GFP fusion proteins the fluorescence was distributed in the cytosol as well as nucleus. The co-localisation study performed with S-GFP and S demonstrated that most of the cytosolic fluorescence and none of the nuclear fluorescence stemmed from the intact fusion proteins. Due to the high signal to noise ratio caused by the strong green auto-fluorescence of the HuH7 cells, the degree of co-localisation might be higher as the pictures indicate. Nevertheless, the fluorescence in the nucleus can only be explained by solitary GFP, which is known to be distributed in the cytoplasm and diffuses also into the nucleus (Cubitt et al., 1995; Yang et al., 1996). It is unlikely that the fusion of GFP changes the original trans-membranous localisation pattern of the HBV surface proteins towards a cytoplasmic and nuclear distribution (Kahaner et al., 1995; Marshall et al., 1995; Yang et al., 1996). Because the translation initiation ATG of GFP has not been changed in the GFP fusion constructs, the expression of GFP from these plasmids is theoretically possible. However, since the promoter is 226, 281 or 400 aa distal from the ATG in S-GFP, M-GFP and L-GFP, respectively, and is not embedded in a Kozak sequence, this should be a rare event occurring by

leaky scanning of the ribosome (Kozak, 1987, 1989 and 1991). That solitary GFP was expressed in low amounts from the fusion protein constructs was shown by the Western blotting of cellular lysates. However, in case of S-GFP, the expression level of the fusion protein was higher than that of GFP, as indicated by the difference in band strength. This does not fit together with the co-localisation study, which revealed a major presence of solitary GFP. A brighter fluorescence of GFP in comparison to S-GFP may contribute to the contradictory results: The fluorescence of the fusion protein would be masked by the GFP fluorescence and fall under the threshold limit set for the co-localisation. In this case, S-GFP would only be stained red, while the green fluorescence of the GFP domain would be extracted together with the background in the co-localisation analysis. This would explain why, despite the low rate of co-localisation, the S staining revealed a well detectable amount of S in the cells. Furthermore, solitary S was rarely detected in Western blot analysis of lysates of S-GFP transfected cells. This emphasises the hypothesis that S-GFP might not be brightly fluorescent.

It is known that the fluorescence of GFP depends on the correct folding of the fluorophore (Ward and Bokman, 1982; Cody et al., 1993). The mechanism underlying the cyclisation of the three C-terminal aa involved in fluorophore formation is yet unknown (Zacharias and Tsien, 2006). Although many examples for brightly fluorescent GFP fusion proteins have been published, among them also membrane proteins, it cannot be ruled out that an interaction with the HBV surface proteins impairs the proper folding of the imidazolone ring which forms the fluorophor (Cubitt et al., 1995; Olsen et al., 1995; Marshall et al., 1995; Moores et al., 1996; Yang et al., 1996). Alternatively, the incomplete synthesis or degradation of the fusion protein, leading to proteins consisting only of S or S carrying a small amount of GFP could be the reason for the minimal co-localisation. However, the Western blot results, which did not show expression of solitary S or protein fragments, argue against this hypothesis.

M-GFP, which appeared as a brightly fluorescent protein in the microscopic analysis, was not detectable by Western blot under denaturing conditions. One can speculate that incomplete denaturation led to a folding which masked the epitopes of M and GFP. However, as both the anti HBsAg and the anti GFP antibody are polyclonal, this is unlikely, especially because S-GFP and L-GFP,

being treated equally and carrying partially the same epitopes, were recognised by both antibodies. Only a faint band of 28 kDa revealed low level GFP expression from the M-GFP plasmid. Upon sequencing it was ascertained that the translation initiation codon of S, was ATG instead of ACG as expected. One would therefore assume that S-GFP was also expressed from the M-GFP plasmid in low level. However, this was not confirmed by Western blotting. The translation initiation site of M is weak in the context of wild type HBV. However, the transfer of the translation site into the optimal surrounding of a Kozak sequence leads to the strong expression of M and a minor expression of S (Sheu and Lo, 1992). As this was performed by construction of the fusion protein plasmids, a weak translation initiation could be ruled out. Although it is known that M behaves like S with respect to trans-membrane topology and SVP formation, differences might exist contributing to the controverse picture obtained by Western blotting. Metha et al. (1997) found evidence for different glycosylation mechanisms for the different surface proteins, indicating that structural differences exist. In spite of many attempts to detect M-GFP in Western blot by using higher DNA amounts for transfection or different antibodies, the result could not be improved.

Western blot analysis of L-GFP gave rise to a number of bands, indicating that not only the fusion protein was expressed from the plasmid. Sequencing of the plasmid revealed that the translation initiation ATG of S was present instead of ACG. The exchanged translation initiation site of M was unaltered. This explained the 52 kDa band detected with the anti HBsAg and anti GFP antibodies, which was due to S-GFP expression. A slightly smaller protein, which was also detected with both antibodies, might be explained to the expression of a smaller transcript from a translation start within S, contributing to a protein of approx. 45 kDa. Another possibility is the partial glycosylation of S-GFP, which would also explain the double band. However, since S-GFP appeared only in one form when expressed from the S-GFP plasmid, this was unlikely. The 33 kDa band may have been due to incomplete translation of L-GFP or represents a product of incomplete proteolytic degradation. Whether the 28 kDa band found in both blots represents the same protein fragment or expression of solitary S and solitary GFP cannot be concluded due to comparable protein sizes of GFP and S. However, the small proteins in the GFP

blot indicate degradation. The presence of the wt S protein did not stabilise the GFP fusion proteins, as the same protein fragments were present in the cells after co-transfection.

The results of immuno-fluorescence and Western blot analyses implicate that the GFP fusion proteins were at least initially incorporated into a membrane. Otherwise, they should rapidly degrade. Many different approaches were used in order to confirm this hypothesis. However, the data obtained were contradictory, and left this question open.

Nevertheless, it was tested whether the fusion proteins were capable of subviral particle formation. Immuno-precipitation of SVPs from supernatants of transfected cells revealed that particle formation was inhibited. The fact that low amounts of SVP were built from wild type S upon co-transfection indicated that the insertion of GFP resulted in an inablility of the S domain to assemble to SVPs. The secretion of wild type SVPs, however, was reduced but not blocked by S-GFP. The possible reasons for this effect, which has also been observed with S-RFP, are discussed in detail in the next chapter (3.2.2).

To summarise, the GFP fusion proteins were unstable and dysfunctional with regard to SVP formation. This resulted most probably from misfolding or incorrect membrane topology. It has been published for different viral proteins that the incorporation of GFP can lead to a disruption of viral functions and has an impact on replication (Heinzinger et al., 1994; Engelmann et al., 1995; Petit et al., 2001). The formation of GFP and possibly also GFP fusion protein dimers was indicated by the Western blot results of GFP, S-GFP and L-GFP. It is a general problem of the type 1 GFP used for generating the fusion proteins. Three N-terminal aa are responsible for dimerisation of *Aequorea* derived GFP, which can occur in living cell at relatively low concentrations (Zacharias and Tsien, 2006). Changing the hydrophobic aa to positively charged residues inhibits dimerisation by preventing the formation of the hydrophobic contacts and hydrophilic interactions responsible for dimer formation (Zacharias et al., 2002). It is obvious that dimerisation of the GFP part of the fusion proteins, which occurs in a head to tail fashion, interferes with the formation of the correct topology of the surface proteins and their dimerisation properties necessary for particle formation (Wunderlich and Bruss, 1996; Zacharias and Tsien, 2006).

Due to the instable expression, the high fluorescence background and the inability to secrete SVPs, the GFP fusion proteins were not suitable to trace the HBV surface proteins and were therefore not further analysed.

3.2.2.2 The RFP fusion proteins S-RFP, M-RFP and L-RFP.

The C-terminal fusion of RFP to the HBV surface proteins S, M and L resulted in brightly fluorescent stable proteins with a cytosolic distribution, as shown by confocal fluorescence microscopy. This was emphasised by the high degree of co-localsation between the RFP and S, proving that RFP was mostly expressed fused to S as an intact hybrid protein. However, Western blot analysis revealed that unglycosylated and glycosylated S was also expressed from plasmids S-RFP and L-RFP. Although M-RFP transfected cells displayed a bright fluorescent protein, M-RFP was undetectable by Western blotting. Because the fluorescent protein was not distributed in the nucleus, it can be excluded that the observed fluorescence derived from RFP expression. Like for M-GFP, sequencing of the M-RFP plasmid also revealed that the ATG of S was not mutated and gave rise to an additional translation start codon. This explains the expression of S-RFP observed in the Western blot of the M-RFP samples. The co-transfection of S did not stabilise M-RFP steady state levels. Only S dimers were found, monomers were probably present in an amount not detected with the anti HBsAg antibody. The reason why M-RFP and also M-GFP could not be detected by Western blotting although a fluorescent protein was clearly expressed in the cells remained obscure. In case of L-RFP, the corresponding band of 72 kDa was present in the transfected cells. Furthermore, they also built a protein of approx. 52 kDa. Because the introduced mutations of the M and S start codons were still intact, as controlled by sequencing, this might be due to protein expression from an internal start codon located within the S gene. The internal translation initiation can be observed with wild type S and was hypothesised also for L-GFP. Whether the RFP fusion proteins are expressed in the different glycosylation forms known for S, M and L cannot be concluded from this blot. Due to the lack of an anti RFP antibody recognising the fusion proteins, a differentiation between the expression of S-RFP fusion proteins

(52 and 55 kDa) and S dimers (48 and 54 kDa), was impossible by this method (Lawenza et al., 2006; Sole et al., 2007).

The HBV surface proteins are synthesised directly at the ER membrane and further transported to the golgi, before they are secreted (Ganem and Schneider, 2001). Therefore, it was investigated whether the RFP fusion proteins co-localised with ER and golgi markers. Confocal fluorescence microscopy clearly showed that the RFP fusion proteins were partially localised in the ER and golgi, and displayed a pattern comparable to wt S. In contrast, RFP was not located in the respective compartments. This strongly supports the assumption that the RFP fusion proteins were integrated into the ER membrane and further transported to the golgi. A difference in the distribution was not observed upon co-transfection of S or HBV L-. However, since the Western blot showed the expression of S from the fusion protein plasmids, S was probably present also in the cells transfected only with the fusion protein plasmid. Thus, an influence of the wt proteins on the fusion protein distribution cannot be ruled out.

The arising question was now whether the RFP fusion proteins, which were obviously localised in the ER and further transported to the golgi, were capable of particle formation. Immuno-precipitation experiments performed with supernatants of S-RFP and S-GFP transfected cells demonstrated that the fusion proteins were incapable of SVP formation, independent on the presence of wt S: Only moderate amounts of SVPs were secreted after co-transfection of S. These SVPs contained only the wild type protein, as shown by Western blot. Limitations of the approach due to improper antibody binding to the fusion proteins can be excluded. The capture antibody used for precipitation had proven to recognise non-denatured S-RFP in the immuno-fluorescence experiments. The detection antibody was previously used for the Western blot analysis of the fusion proteins. This leads to the conclusion that the S fusion proteins lost the self assembly properties of S or that assembled particles could not be secreted. The dysfunction of S-RFP and S-GFP did not inhibit the assembly and secretion of wild type SVP, which were detected at least in moderate amount.

A reason for the inability to form SVPs is probably an interaction of the bulky RFP or GFP domain with the second hydrophilic loop of S, which is located in

the ER lumen. This could interfere with the different processing steps necessary for assembly and secretion: Firstly, glycosylation might be inhibited for instance due to sterical hindrances or general changes in the topology of S. It has been shown that the N-linked glycosylation processes, which take place in the ER and golgi, are important for proper membrane protein folding and HBV particle secretion (Helenius, 1994; Metha et al., 1997). Therefore, an impact on the fusion protein folding and the ability to enter the secretory pathway can be suggested. Lu et al. (1997) described that in glucosidase-inhibited cells the HBV surface proteins were dysfunctional. In contrast however to other glycoproteins, they were not rapidly degraded but were found in ER, golgi and lysosomes more than 20 h after synthesis (Moore and Spiro, 1993; Kearse et al., 1994). The authors suggested that the membrane proteins trafficked in the cells as lipoprotein particles rather than as single proteins. This scenario would go together with the fact that the fusion proteins co-localised with ER and golgi markers. Secondly, dimerisation could be impaired by topological changes or sterically masking the cystein residues in the luminal loop of the S domain, which crosslink the proteins by building disulfide bridges (Wunderlich and Bruss, 1996). Thirdly, the interaction with the chaperones BiP and calnexin in the ER lumen, which are known to bind to S and support its maturation, might be disturbed by the presence of the large RFP or GFP domain (Xu et al., 1997; Cho et al., 2003; Bruss, 2007). Fourthly, the cystein residues located in the cytosolic loop between signal 1 and signal 2 seem to be required for subviral particle formation, as shown by Wunderlich and Bruss (1996). Although it is unlikely that the topology of the cytoplasmic loop was changed upon the fusion, an effect on the overall protein structure which could concern also the cytoplasmic domain must be considered. A sterical hindrance caused by the RFP or GFP domain may also be responsible for the reduced capacity of S to form particles. The SVPs are very densely packed structures with a re-organised bilayer structure and low lipid content (Gavilanes et al., 1982; Ganem and Schneider, 2001). The mechanisms involved in membrane re-organisation and budding are unclear, but one can speculate that a changed topology of a certain percentage of S or the presence of large protein domains in the ER lumen interfere with these processes (Bruss, 2007). Impairment of the different steps of S maturation would result in the retention of the proteins in the

membrane. The stuck proteins would most probably be subjected to the lysosomal degradation pathway, which is in agreement with the co-localisation observed with the ER and golgi markers (Lu et al.1997, Mehta et al., 1997). Another possibility is that not particle formation but rather the secretion is impaired. Budded SVPs traverse the golgi, where the carbohydrates are converted to an endoglycosidase H-resistant form (Patzer et al., 1984). The now mature viral and subviral particles are secreted from the cell. As mutational analyses performed by Bruss and Ganem (1991) hint at a direct or indirect involvement of the C terminus of S in budding or intracellular vesicle transport, an impact of the RFP fusion on these later steps of SVP production has to be considered.

It has already been described for other modified S proteins that assembly or transport of these proteins was impaired (Delpeyroux et al., 1987; Michel et al., 1988; Bruss and Ganem, 1991; Prange et al., 1992 and 1995; Netter et al., 2001; Lambert et al., 2004). The attempts to generate HBV surface protein chimera mostly derived from the idea to use the highly immunogenic SVPs as carriers for foreign antigens, so as to develop new potent vaccines. The foreign sequences were usually introduced into the first or second hydrophilic loop rather than at the termini. Delpeyroux et al. (1986) generated SVPs presenting the poliovirus neutralisation epitope, which elicited a strong immune response against poliovirus in mice. The immune response against HBsAg was only minor. Other examples are the creation of an HBsAg vaccine vector presenting HCV epitopes or the modification of M and S in order to induce a stronger immune response against the preS regions (Prange et al., 1995; Netter et al., 2001). In the latter case the preS domains were fused to the N- or the C terminus of S and M. The fusion of 42 aa to the termini allowed secretion of SVPs, whereas the extension of the insert sequence was tolerated only at the N terminus. Immune responses were induced by some constructs against the introduced sequences, while production of antibodies against S, which represents the major immunogenic epitope, was negligible. This demonstrates that already the introduction of short sequences derived from the protein itself has an impact on the formation and secretion of the SVPs.

Lambert et al. (2004) reported that the C-terminal fusion of GFP to S resulted in a dysfunctional fusion protein. They observed that the fusion protein, which was

secretion deficient, acted in a trans-dominant negative manner, inhibiting also the wild type S particle export. Inconsistent with this, the data presented here showed that the S-GFP fusion protein allowed the secretion of S. Since the ratio of fusion construct and S DNA used for co-transfection were the same in both experiments, this contradiction is difficult to explain. Lambert et al. (2004) suggested that misfolding of the S-GFP fusion protein was the reason for the inhibitory effect on the secretion of SVPs containing S-GFP, as well as the secretion of wild type SVPs. However, misfolding does not necessarily result in an inhibition of S particle formation, as observed in multiple repetitions of this experiment with the C-terminal fusion proteins S-GFP and S-RFP.

Despite the inability to form SVPs, the fusion proteins were also examined for the formation of virions. Surprisingly, only very low amounts if any virions were secreted after co-transfection of the fusion proteins with wild type HBV. Unexpectedly, the presence of the fusion proteins had an inhibitory effect on the formation of wild type HBV particles. Since moderate amounts of wild type SVPs were secreted after co-transfection of the S fusion proteins with S, the secretion of VPs was also expected. Fluorescence of the cells confirmed successful transfection. Although the experiment was repeated several times with the different fusion proteins and different DNA ratios of fusion construct and wild type HBV, the results remained the same. This implies very drastic effects of the fused RFP domain on the process of VP formation. The aforementioned processes important for SVP formation also play a role in VP formation. In contrast to SVP formation however, which only requires S, the virion formation is more complex. At least two proteins, S and L have to be integrated in a certain ratio and order. M has been reported to be not required (Bruss and Ganem, 1991; Fernholz et al., 1991; Ueda et al., 1991). Furthermore, a correct topology of the surface proteins might be prerequisite for capsid binding to L. It can be hypothesised that the RFP fusion domain interferes with the correct arrangement of the surface proteins and the cellular lipids. Whether the lack of virion secretion was due to a block in the assembly steps, such as surface protein dimerisation and the interaction between surface proteins and core protein, or secretion has to be further analysed. However, since the constructed fusion proteins obviously were not capable of SVP and VP formation and the function could not be restored by the addition of wild type proteins, this was not

a successful strategy to generate fluorescent surface proteins. Firstly, the trafficking of these fusion proteins most probably does not mirror the behaviour of the wild type counterparts, and can therefore not be transferred to the situation of an HBV infection. Secondly, fluorescent SVPs and virions, which are desired to follow virus assembly and egress, were not formed.

3.2.3 Perspective

GFP or RFP fused HBV surface proteins were constructed with the aim to visualise trafficking of HBV surface proteins and assembly of viral particles in cells. Furthermore, the generation of fluorescent HBV subviral and viral particles was intended. This was hampered by the instability of the fusion proteins and the inability to form SVPs and VPs. This may be due to misfolding or sterical hindrance of protein-protein interactions by GFP or RFP, affecting correct integration into the ER membrane or the capability to build properly formed particles. The difference between GFP and RFP fusion proteins with regard to protein stability is surprising, because both are globular proteins of similar size. Possibly differences in their overall folding can explain this phenomenon. However, since the three dimensional structure of the monomeric form of RFP has not yet been elucidated, this remains speculative.

The results presented in this study implicate that the addition of bulky sequences impairs the HBV surface protein structure and functionality. Therefore, this approach is not useful to visualise the different steps of HBV infection. The introduction of GFP or RFP changed the characteristics of the surface proteins in a way that they did not resemble the wild type proteins any more. Thus, the results obtained with the fusion surface proteins could not be transferred on the wild type surface proteins.

The labelling with small fluorescent molecules may represent an alternative strategy. The recently developed tetracystein tag technology is especially promising. In this approach advantage is taken from the features of a non-fluorescent, bisarsenic derivate of fluorescein, named FlAsH (Griffin et al., 1998; Martin et al., 2005). Besides the green fluorescent FlAsH, a red variant is available, designated ReAsH. The membrane-permeable reagent specifically

binds a tetracystein tag consisting of six aa integrated into the protein of choice. Since the tag is very small, the risc of an interference with wild type protein structures or activity is minimised. Upon covalent binding to the tag, the FlAsH is converted into a highly fluorescent form. First publications already provided promising results in elucidating the different steps of HIV trafficking in cells (Arhel et al., 2006; Turville et al., 2008). The incorporation of the tetracystein tag into the HBV surface proteins would most probably not influence the proteins, as it has been shown for the termini as well as for the hydrophilic loop, that small insertions are tolerated (Delpeyroux, 1986; Michel et al., 1988; Prange et al., 1995). This would allow tracking *de novo*-produced proteins, SVPs and VPs and probably as well virus production. Furthermore, labelling of the core or polymerase protein might also be possible, permitting for the specific monitoring of virions.

The covalent binding of amino-reactive dyes represents an alternative method of virus labelling. However, as the dyes unspecifically incorporate into membranes by binding to amines, this approach is only useful for the labelling of purified SVPs and VPs. These can then be used to study the early steps of infection, such as virus uptake.

4. Material and Methods

4.1 Material

All solutions were prepared with deionised water from the Ultra Pure Water System Easy Pure UV/UF (Werner Reinstwassersysteme, Wilhelm Werner GmbH, Leverkusen, Germany).

4.1.1 Chemicals

Acidic acid	Roth, Karlsruhe, Germany
Acryl amide	Sigma, Deisenhofen, Germany
Agarose SeaKem LE	Cambrex Bio Science, Rockland, USA
Ammonium acetate	Merck, Darmstadt, Germany
Bromphenol blue	Merck, Darmstadt, Germany
Caesium chloride	Roth, Karlsruhe, Germany
Collagen	Serva, Heidelberg, Germany
Developer G153 A + B	Agfa Geveart NV, Mortsel, Belgium
1,4- Diazabicyclo[2,2,2]octane 98% (DABCO)	Sigma Aldrich Chemie GmbH, Steinheim, Germany
4',6-Diamidino-2-phenylindol (DAPI)	Molecular Probes, Inc, Eugene, USA
Ethylenedinitrilotetraacedic acid (EDTA)	Roth, Karlsruhe, Germany
Ethidium bromide	Merck, Darmstadt, Germany
Ethanol	Roth, Karlsruhe, Germany
Ficoll	Pan Biotech, Aidenbach, Germany
Glycerol	Roth, Karlsruhe, Germany
Hydrochloric acid	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
Methanol	Roth, Karlsruhe, Germany
Milk powder, low fat	Sigma, Deisenhofen, Germany
Mowiol 4-88 reagent	Calbiochem, La Jolla, CA, USA
Paraformaldehyde	Merck, Darmstadt, Germany
Polybed 812 and epoxy resin	Polysciences, Warrington, PA, USA

Ponceau S	Roth, Karlsruhe, Germany
Potassium	Roth, Karlsruhe, Germany
Potassium acetate	Merck, Darmstadt, Germany
Proteinase Inhibitor tablets	Roche Diagnostics, Mannheim, Germany
Rapid Fixer G354	Agfa Geveart NV, Mortsel, Belgium
Sodium acetate	Merck, Darmstadt, Germany
Sodium chloride	Roth, Karlsruhe, Germany
Sodium dihydrogenphosphate	Roth, Karlsruhe, Germany
Sodium hydroxide	Roth, Karlsruhe, Germany
Sucrose	Sigma, Deisenhofen, Germany
Tetramethylethylendiamine (TEMED)	Sigma, Deisenhofen, Germany
Tris base	Roth, Karlsruhe, Germany
Triton X-100	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany

4.1.2 Cell culture

4.1.2.1 Cell lines and primary cells

HuH7	Human hepatoma cell line
HepG2.2.15	Stably HBV producing hepatoma cell line (Sells et al., 1987)
293	Human embryonic kidney cell line, stably producing adenovirus E1 Protein, ATCC no. CRL-1573 (Graham et al., 1977)
PHH	Primary human hepatocytes, isolated from fresh liver specimen
moDC	Monocyte derived dendritic cells, differentiated from blood monocytes
mDC	myeloid dendritic cells, isolated from fresh peripheral blood
pDC	plasmacytoid dendritic cells, isolated from fresh peripheral blood

4.1.2.2 Media and supplements

Ampicillin	Sigma, Deisenhofen, Germany
Collagenase	Worthington Biochemical Corporation, Lakewood, NJ, USA
Dulbeccos MEM	Gibco, BRL, Eggenstein, Germany
Dimethylsulfoxid (DMSO)	Merck, Darmstadt, Germany
Ethyleneglycolbis (2-aminoethyl) -tetraacetic acid (EGTA)	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	Biochrom AG, Berlin, Germany
Gentamycin	Gibco BRL, Eggenstein, Germany
Glutamine	Gibco, BRL, Eggenstein, Germany
Granulocyte-macrophage colony -stimulating factor (GM-CSF)	R&D Systems, Wiesbaden, Germany
HBSS	Gibco BRL, Eggenstein, Germany
Heparin Liquemin N 25000	Roche, Mannheim, Germany
HEPES	Gibco BRL, Eggenstein, Germany
Hydrocortison	Sigma, Deisenhofen, Germany
Inosine	Serva, Darmstadt, Germany
Insulin	Serva, Darmstadt, Germany
Interleukin-4 (IL-4)	R&D Systems, Wiesbaden, Germany
Lipopolysaccharid (LPS) *E.coli* O26:B6	Sigma, Deisenhofen, Germany
Penicillin/ Streptomycin (P/S)	Biochrom AG, Berlin, Germany
Sodium pyruvate	Gibco BRL, Eggenstein, Germany
Non essential amino acids (NEAA)	Biochrom AG, Berlin, Germany
Polyethylenglycol (PEG) 6000	Serva Electrophoresis, Heidelberg, Germany
Prostaglandin E_2 (PGE_2)	Sigma, Deisenhofen, Germany
RPMI 1640	Gibco, BRL, Eggenstein, Germany
Tumor necrosis factor-α (TNF-α)	R&D Systems, Wiesbaden, Germany
Williams Medium E	Gibco, BRL, Eggenstein, Germany

4.1.2.2.1 Primary human hepatocytes

Preperfusion medium	HBSS, Ca/Mg-free	500 ml
	EGTA, 100 mM	2.5 ml
	Heparin, 5000 U/ml	1 ml
Collagenase medium	Williams Medium E	250 ml
	Calcium chloride, 1 M	0.9 ml
	Gentamycin, 10 mg/ml	2.5 ml
	Collagenase type IV	200 mg
Wash medium	Williams Med E	500 ml
	*Glutamine, 200 mM	5.6 ml
	*Glucose, 5%	6 ml
	*Hepes, 1 M, pH 7.4	11.5 ml
	*P/S, 5000 U/ml	5.6 ml

(*Solutions were mixed and stored as premix at –20°C)

PHH medium	Wash medium	500 ml
	Gentamycin, 10 mg/ml	5 ml
	Hydrocortison	0.5 ml
	Insulin	0.45 mg
	DMSO	8.7 ml
	Inosine, 82.5 mg/ml	2 ml

4.1.2.2.2 Monocytes

Monocyte medium	VLE RPMI	500 ml
	FCS	10%

4.1.2.2.3 moDC

Immature moDC medium	VLE RPMI	500 ml
	FCS	10%
	IL-4	10 ng/ml
	GM-CSF	10 ng/ml

Mature moDC medium	VLE RPMI	500 ml
	FCS	10%
	IL-4	10 ng/ml
	GM-CSF	10 ng/ml
	LPS	1 µg/ml
	TNF-α	50 ng/ml
	PGE_2	50 ng/ml

4.1.2.2.4 HuH7

Cultivation medium	Dulbeccos MEM	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml
	NEAA, 100 x	5.5 ml
Freezing medium	Dulbeccos MEM	500 ml
	FCS	20%
	DMSO	10%

4.1.2.2.5 HepG2.2.15

Cultivation medium	Dulbeccos MEM	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml
	NEAA, 100 x	5.5 ml
Virus production medium	PHH medium	250 ml
	Williams E medium	250 ml
	FCS	25 ml
	Glutamine, 200 mM	2.5 ml
	P/S, 5000 U/ml	2.5 ml
	NEAA, 100 x	2.5 ml

4.1.2.2.6 293 cells

Cultivation medium	Dulbeccos MEM	500 ml
	FCS	50 ml
	Glutamine, 200 mM	5.5 ml
	P/S, 5000 U/ml	5.5 ml

4.1.3 Bacteria culture

4.1.3.1 Bacteria strains

DH5α *E. coli*	Promega, Mannheim, Germany
XL1blue *E. coli*	Stratagene, La Jolla, USA

4.1.3.2 Media and supplements

Agar	Becton Dickinson Microbiology Systems, Sparks, USA
Ampicillin	Sigma, Deisenhofen, Germany
Geniticin (Neomycin; G418)	Invitrogen, Karlsruhe, Germany
Sodium chloride	Roth, Karlsruhe, Germany
Trypton oxide	Hampshire, England
Yeast extracts	Becton Dickinson Microbiology Systems, Sparks, USA

LB medium, pH 7.0	Antibiotics	50 mg/ml
	Natrium chloride	5 g/l
	Yeast extracts	5 g/l
	Trypton oxide	10 g/l

LB agar	LB medium	
	Agar	15 g/l

4.1.4 Buffers and solutions

4.1.4.1 Dot blot

Denaturation solution	Natrium hydroxid	0.5 M
	Natrium chloride	1 M
Neutralisation solution	Tris, pH 7.4	0.5 M
	Natrium chloride	3 M
Hybridization buffer	SSC	6 x
	Denhardts	5 x
	Salmon sperm DNA	10 µg/ml
Wash buffer	SSC	1 x
	SDS	0.5%
Denhardts solution	Ficoll 400	1% (w/v)
	Polyviynlpyrrolidon	1% (w/v)
	BSA	1% (w/v)
SSC	Natrium chloride	3 M
	Na_3citrat x $2H_2O$	0.3 M

4.1.4.2 Immunofluorescence

Fixation solution	Paraformaldehyde	3.7%
	PBS	1 x
Saturation solution	Ammonium chloride	50 mM
Permeabilisation buffer	Saponin	0.5%
	PBS	1 x

Blocking solution	FCS	10%
	Saponin	0.1%
	PBS	1 x
Antibody incubation buffer	Saponin	0.1%
	PBS	1 x
Mounting medium	Mowiol	2.4 g
	Glycerol	12 g
	Tris, pH 8.5	0.2 M
	Dabco	50 mg

4.1.4.3 Magnetic cell sorting

MACS buffer	PBS	1 x
	HSA	0.5%
	EDTA	2 mM
	(Degas and store at 4°C)	

4.1.4.4 Plasmid preparation, *rapid boiling* method

STET buffer	Sucrose	8% (w/v)
	Triton X-100	0.1% (v/v)
	EDTA, pH 8.0	50 mM
	TrisCl, pH 8.0	50 mM
Isopropanol/ Ammonium acetate	Isopropanol, 100%	75 ml
	Ammonuim acetate	19.27 g
	(Fill with ddH_2O to 100 ml)	

4.1.4.5 Western blot

2 x Sample buffer	Tris HCl, pH 8.8	200 mM
	EDTA	5 mM
	SDS	3%
	Bromphenol blue	0.1%
	Sucrose	10%
	β-Mercaptoethanol	1.7%
10 x Running buffer, 1l	Glycine	144 g
	Tris Base	30 g
	SDS	10 g
5 x Blotting buffer, 1l	Tris Base	15.15 g
	Gycine	72 g
	(Fill with ddH_2O)	
1 x Usage buffer	Blot buffer	1 x
	Methanol	1 x
	H_2O	3 x
Blocking solution	Milk powder, non fat	0.5%
	PBS	1x
	Tween 20	0.05%
Resolving gel, 12.5%	Acryl amide, 40%	6.25 ml
	Tris, 1.5 M, pH 8.8	5 ml
	H_20	8.55 ml
	SDS, 10%	230 µl
	TEMED	20 µl
	APS, 10%	100 µl

Stacking gel, 5%	Acryl amide, 40%	0.375 ml
	Tris, 0.5 M, pH 6.8	1.2 ml
	H_20	3.325 ml
	SDS, 10%	50 µl
	TEMED	4 µl
	APS, 10%	50 µl
1. Antibody incubation buffer	Tween20	0.05%
	PBS	1 x
2. Antibody incubation buffer	Tween20	0.05%
	PBS	1 x
	Milk powder, non fat	0.1%
Wash buffer	Tween20	0.05 %
	PBS	1 x
Stripping buffer	Sodium hydroxide	0.2 M

4.1.5 Antibodies

4.1.5.1 Flow cytometry

Mouse anti BDCA-2 (FITC)	Miltenyi, Bergisch Gladbach, Germany
Mouse anti CD80 PE	BD Pharmingen, San Diego, CA, USA
Mouse anti CD83	Immunotech, Marseille, France
Mouse anti CD86	BD Pharmingen, San Diego, CA, USA
Mouse anti CD123 FITC	Miltenyi, Bergisch Gladbach, Germany
HLA-DR	BD Pharmingen, San Diego, CA, USA
Streptavidin (PE)	BD Pharmingen, San Diego, CA, USA

4.1.5.2 Immunofluorescence

4.1.5.2.1 Primary antibodies

Mouse anti Calnexin	Santa Cruz Biotech., Santa Cruz, CA, USA
Mouse anti Golgi matrix protein 130 (GM130)	BD Pharmingen, San Diego, CA, USA
Goat anti Ras-like GTPase 6 (Rab6)	Santa Cruz Biotech., Santa Cruz, CA, USA
Rabbit anti HBvax	in-house production; polyclonal antibody against the HBV vaccine
Sheep anti preS1	gift from M. v. Roosmalen, BioMerieux, Rotterdam; polyclonal antibody against the preS1 region of the large surface protein

4.1.5.2.2 Secondary antibodies

Alexa Fluor 488 F(ab)2 fragment goat anti mouse IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 488 F(ab)2 fragment goat anti rabbit IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 488 F(ab)2 fragment rabbit anti goat IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 568 goat anti mouse IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 568 F(ab)2 fragment goat anti rabbit IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 568 rabbit anti goat IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 568 donkey anti sheep IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 594 F(ab)2 fragment goat anti mouse IgG (H+L)	Invitrogen, Karlsruhe, Germany
Alexa Fluor 594 F(ab)2 fragment goat anti rabbit IgG (H+L)	Invitrogen, Karlsruhe, Germany

4.1.5.3 Infection studies

Hepatect®	human hepatitis B immunoglobulin, Biotest Pharma GmbH, Dreieich, Germany

4.1.5.4 Western blot

4.1.5.4.1 Primary antibodies

Goat anti HBsAg	Antibody from Murex HBsAg, Version 3, Abbott, Wiesbaden, Germany
Mouse anti multidrug resistant protein 2 (MRP2)	BD Pharmingen, San Diego, CA, USA
Mouse anti heatshock protein 90 (HSP90)	BD Pharmingen, San Diego, CA, USA
Mouse anti golgi matrix protein (GM130)	BD Pharmingen, San Diego, CA, USA
Rabbit anti β-actin	Sigma, St. Louis, MO, USA
Rabbit anti hepatocyte nuclear factor (HNF) 1	Santa Cruz Biotech., Santa Cruz, CA, USA
Rabbit anti hepatocyte nuclear factor (HNF) 4α	Santa Cruz Biotech., Santa Cruz, CA, USA
Rabbit anti *core*/GFP	Combination of 2 mAbs from rabbit (Boehringer Mannheim); mAbs 10E11 and 10F10 against epitopes aa8/20 and aa135/144 of the denatured *core*-protein

4.1.5.4.2 Secondary antibodies

Goat anti rabbit, HRP-conjugated	Sigma, Deisenhofen, Germany
Goat anti mouse, HRP-conjugated	Sigma, Deisenhofen, Germany

4.1.6 Enzymes

Restriction endonucleases	Fermentas, St. Leon Rot, Germany
T4-DNA Ligase	Roche Diagnostics, Mannheim, Germany
Klenow Enzym	Roche Diagnostics, Mannheim, Germany
Alkaline phosphatase	Roche Diagnostics, Mannheim, Germany
Lysozyme 50 mg/ml	Novagen, Madison, USA
RNaseA 10 mg/ml	Roche Diagnostics, Mannheim, Germany

4.1.7 Kits

4.1.7.1 Cell isolation

BCAProtein™ Assay Kit	Pierce, Rockford, IL, USA
BDCA-4 cell isolation kit	Miltenyi, Bergisch Gladbach, Germany
CD1c cell isolation kit	Miltenyi, Bergisch Gladbach, Germany
CD14 cell isolation kit	Miltenyi, Bergisch Gladbach, Germany
CD19 cell isolation kit	Miltenyi, Bergisch Gladbach, Germany

4.1.7.2 DNA amplification

LC FastStart DNA Master^{Plus} SYBR Green1 mix	Roche Diagnostics, Mannheim, Germany
PCR DNA Amplification Kit	Roche Diagnostics, Mannheim, Germany
PCR Purification Kit	Qiagen, Hilden, Germany

4.1.7.3 DNA isolation

MinElute PCR Purification Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAprep Maxiprep Kit	Qiagen, Hilden, Germany
QIAprep Miniprep Kit	Qiagen, Hilden, Germany

4.1.7.4 DNA labelling

Rediprime DNA Labeling System	Amersham, Buckinghamshire, England

4.1.7.5 ELISA

Murex HBsAg Version 3	Abbott, Wiesbaden, Germany

4.1.7.6 Luciferase detection

Renilla Luciferase Assay System	Promega GmbH, Mannheim, Germany

4.1.7.7 Transfection

FuGENE 6®	Roche Diagnostics, Mannheim, Germany

4.1.7.8 Western blot detection

ECL Western Blotting Detection Reagents	Amersham, Buckinghamshire, England
Enhanced Chemiluminescence	Pierce, Rockford, USA

4.1.8 Nucleic acids

4.1.8.1 Vectors

pAdGH1.3

Adenoviral vector for transduction of eukaryotic cell lines, containing a 1.3-fold HBV over length genome and a GFP reporter cassette under CMV promoter control (Sprinzl et al., 2001).

rHBV-rLuc

Recombinant HBV vector containing *renilla* luciferase as transgene under control of the HBV preS2/S promoter. All viral ORFs are knocked out, the gene encoding for the viral small envelope protein is replaced by a cDNA encoding *renilla* luciferase. The development of this type of recombinant HBV-based vectors is published in Protzer et al. (1999).

rHBV-CMV-rLuc

Based on rHBV-rluc, the HBV preS2/S-promoter is replaced by a CMV immediate early promoter/enhancer.

4.1.8.2 Plasmids

000HBV

Contains the wtHBV circularised genome.

pCH -9/3091

Contains a 1.3 over length HBV construct under the control of the CMV promoter (HBV nucleotides 3091-3182, 1-3182, 1-84). It was used to produce wtHBV (from R. Bartenschlager; Nassal et al., 1990).

pCH 3142

Helper plasmid for the rHBV production. The encapsidation signal is deleted (from R. Bartenschlager; Bartenschlager et al., 1990).

pCH 1452 rLuc CPLX

Transfer plasmid for the production of luciferase expressing rHBV vectors, with the luciferase gene under the control of the CMV promoter. It contains the complete HBV genome. The HBV S gene is replaced by the *renilla* luciferase gene under CMV promoter control. All HBV ORFs are deleted (from A. Untergasser).

pCH luc

Transfer plasmid for the production of luciferase expressing rHBV vectors, with the luciferase gene under the control of an HBV specific promoter. It contains the complete HBV genome. The HBV S gene is replaced by the *renilla* luciferase gene under S promoter control. All other HBV ORFs are deleted (from A. Untergasser).

pCH GFP An-b-glob

Contains the GFP gene under CMV promoter control, with poly-A side from the β-globin gene.

mRFP1_pRSETB

Encodes the monomeric RFP (mRFP) under the T7 promoter (kindly provided by R. Tsien)

pCH S-Ag An-b-glob

This plasmid contains the HBV S gene under the control of a CMV promoter. The poly-A side stems from the β-globin gene (from A. Untergasser).

pCH GFP-S An-b-glob

Generated from the plasmids pCH S-Ag An-b-glob and pCH GFP An-b-glob. It encodes the GFP gene N-terminally fused to the S gene.

pCH GFP-sp-S An-b-glob

Generated from the plasmid pCH GFP-S An-b-glob. It encodes the GFP gene N-terminally fused to the S gene with a glycin spacer sequence of 12 aa inserted between GFP and S gene.

pCH S-GFP An-b-glob

Generated from the plasmids pCH S-Ag An-b-glob and pCH GFP An-b-glob. It encodes the GFP gene C-terminally fused to the S gene.

pCH M-GFP An-b-glob

Generated from the plasmids 000HBV and pCH S-GFP An-b-glob. It encodes the GFP gene C-terminally fused to the M gene. The ATG start codon for the S gene, which is present within the ORF of the M gene, was replaced by ACG.

pCH L-GFP An-b-glob

Generated from the plasmids 000HBV and pCH S-GFP An-b-glob. It encodes the GFP gene C-terminally fused to the L gene. The ATG start codons for the M and S genes, which are present within the ORF of the L gene, were replaced by ACG.

pCH S-RFP An-b-glob

It encodes the RFP gene C-terminally fused to the S gene. The mRFP gene was obtained from the mRFP1_pRSETB plasmid. After restriction of the mRFP PCR fragment and the plasmid pCH S-GFP An-b-glob with AccI and PaeI, the RFP was inserted into plasmid backbone of the pCH SGFP An-b-glob plasmid. The GFP was removed by the restriction.

pCH M-RFP An-b-glob

It encodes the RFP gene C-terminally fused to the M gene. The mRFP gene was obtained from the mRFP1_pRSETB plasmid. After restriction of the mRFP PCR fragment and the plasmid pCH M-GFP An-b-glob with AccI and PaeI, the RFP was inserted into plasmid backbone of the pCH MGFP An-b-glob plasmid. The GFP was removed by the restriction.

pCH L-RFP An-b-glob

It encodes the RFP gene C-terminally fused to the L gene. The mRFP gene was obtained from the mRFP1_pRSETB plasmid. After restriction of the mRFP PCR fragment and the plasmid pCH L-GFP An-b-glob with AccI and PaeI, the RFP was inserted into plasmid backbone of the pCH L-GFP An-b-glob plasmid. The GFP was removed by the restriction.

4.1.8.3 Oligonucleotides

Amplification Primers

S-GFP C-term fw	5´ TCTTTGGGTATACATTATGAGCAAGGGCGAG 3´
S-GFP C-term rev	5´ GCAAGCTGCATGCTCACTTGTACAGCTCG 3´
GFP-S N-term fw	5´ ACGATTAGTCGACATGAGCAAGGGCGAG 3´
GFP-S N-term rev	5´ GAATCGAGGTACCCTTGTACAGCTCGTCC 3´
GFP-S N-term sp fw	5´ TTCTGGAGGCGGAGGGTCCGGTAC 3´
GFP-S N-term sp rev	5´ CGGACCCTCCGCCTCCAGAAGGTAC 3´
L-GFP fw	5´ AGAACGGTACCATGGGGCAGAATCTTTCCAC 3´
L-GFP rev	5´ AGAACGGTACCATGCAGTGGAATTCCACAACC 3´
M-GFP fw	5´ CTCAGGCCACGCAGTGGAATTCCACAAC 3´
M-GFP rev	5´ GTTGTGGAATTCCACTGCGTGGCCTGAG 3´
RFP fw	5´ GCAAGCTGCATGCTTAGGCGCCGGTGGAGT 3´
RFP rev	5´ GCAAGCTGCATGCTTAGGCGCCGGTGGAGT 3´

Primers for quantitative real time PCR

HBV1745 fw	5´ GGAGGGATACATAGAGGTTCCTTGA 3´
HBV1844 rev	5´ GTTGCCCGTTTGTCCTCTAATTC 3´

HBVccc2760 fw	5´ GACTCTCTCGTCCCCTTCTC 3´
HBVccc156 rev	5´ ATGGTGAGGTGAACAATGCT 3´

4.1.9 Ladders

4.1.9.1 DNA standards

SmartLadder, 0.2-10 kb	Eurogentec, Liege, Belgium
SmartLadder, 0.1-2 kb	Eurogentec, Liege, Belgium

4.1.9.2 Protein standards

Pre-stained Protein ladder	Invitrogen, Karlsruhe, Germany

4.1.10 Expendable items

Cell culture flasks	Nunc, Wiesbaden, Germany
Cell culture dishes	Nunc, Wiesbaden, Germany
Cell culture well plates	Nunc, Wiesbaden, Germany
Centricon Plus-70, Biomax 100	Millipore Corp., Billerica, MA, USA
Centrifuge vials, Falcon	Becton Dickinson GmbH, Heidelberg, Germany
Chamber Slides LabTekII, RS Glass	Nunc, Wiesbaden, Germany
Cryo vials	Nunc, Wiesbaden, Germany
Cuvettes	Sarstedt, Nümbrecht, Germany
Freezing Container Nalgene™	Nunc, Wiesbaden, Germany
Hyperfilm ECL	GE Healthcare, Buckinghamshire, UK
Pipette tipps	Starlab GmbH, Ahrensburg, Germany
Pipetboy Swift Pet®	Abimed, Langenfeld, Germany
Pipetts, single use	Sarstedt, Nümbrecht, Germany
Reaction vials	Eppendorf, Hamburg, Germany
Tissue paste Histoacryl®	Braun, Melsung, Germany
Whatman 3MM	Biometra, Göttingen, Germany
Ultra centrifuge-tubes, polyallomer	Beranek Laborgeräte, Weinheim, Germany

4.1.11 Equipment

Centrifuges:	
Centrifuge 5417C / 5417R	Eppendorf, Hamburg, Germany
Megafuge 1.0 / 1.0 R	Heraeus Holding GmbH, Hanau, Germany
Fraction recovery system	Beckman, München, Germany
Biocycler Thermocycler T3	Biometra, Göttingen, Germany
Blot chamber MiniProtean®3 Cell	BIO-RAD Laboratories, Hercules, USA
Dot blot apparatus Minifold I	Schleicher & Schuell, Dassel, Germany
ELISA Reader MRX Revelation	Dynex, Gaithersburg, USA
Film processor Curix 60	Agfa Geveart NV, Mortsel, Belgium
Flow cytometer FACSCanto™	BD Biosciences, Heidelberg, Germany
Gel chambers	BIO-RAD Laboratories, Hercules, USA
Heating block Thermomixer comfort	Eppendorf, Hamburg, Germany
Incubator	Heraeus Holding GmbH, Hanau, Germany
Light Cycler System	Roche Diagnostics, Mannheim, Germany
Luminometer	Berthold Technologies, Bad Wildbad, Germany
Microscopes	
Fluorescence microscope IX81	Olympus, Hamburg, Germany
Confocal microscope FluoView1000	Olympus, Hamburg, Germany
Nylon membrane, positively charged	Roche Diagnostics, Mannheim, Germany
PVDF membrane	Amersham, Buckinghamshire, England
pH-Meter	WTW, wissenschaftlich technische Werkstätten
Pipetboy Swift Pet®	Abimed, Langenfeld, Germany
Phosphoimager, Molecular Imager FX	BIO-RAD Laboratories, Hercules, USA
Photometer Smart Spec 3000	BIO-RAD Laboratories, Hercules, USA
Photo system for agarose gels Gel-doc 2000	BIO-RAD Laboratories, Hercules, USA
Power Supplies Pack300	BIO-RAD Laboratories, Hercules, USA
Refrigerators and freezer	Liebherr, Lientz, Germany
Rocking platform WT12	Biometra, Göttingen, Germany

Shaker	Innova 4230, New Brunswick Scientific, USA
Sterile hood (cell cultur)	Heraeus Holding GmbH, Hanau, Germany
UV-Oven GS Gene Linker™	BIO-RAD Laboratories, Hercules, USA
Ultra centrifuges:	
Sorvall RC 50 Plus	Kendro, Langenselbold, Germany
XL 70	Beckman, München, Germany
Scales Kern 440-47	Sartorius AG, Göttingen, Germany

4.1.12 Software

Autoradiography	Quantity One, 4.2.1, BIO-RAD Laboratories, Hercules, USA
Data processing	Windows 2000, MS Office 2000, Word and Excel, Microsoft, Redmont, USA
Flow cytometry	FACSDiva™, BD Biosciences, Heidelberg, Germany
Fluorescence microscopy	Cell P, AnalySIS, Soft Imaging System GmbH, Münster, Germany
	FV10-ASW, Version 1.6a
Graphic programmes	Adobe Photoshop 5.5, Adobe, San Jose, USA
	Power Point 2000, Microsoft, Redmont, USA
Light Cycler	Probe Design Analysis and Rel Quant, Roche Diagnostics, Mannheim, Germany
Luminometer	Magellan Software, Tecan, Grödig, Austria

4.2 Methods

All procedures were performed at room temperature, if not indicated otherwise.

4.2.1. Cell culture

All procedures were carried out under sterile conditions, using sterile solutions and equipments. All cells were cultivated in a humidified incubator at 37°C containing 5% CO_2.

4.2.1.1 Calculation of cell numbers and cell viability

To determine cell numbers, a Neubauer hemacytometer was used. The hemacytometer consists of a glass chamber, which is divided into four large squares, each consisting of 16 small squares. It holds a quartz coverslip exactly 0.1 mm above the chamber floor. A homogenous cell suspension was filled in the hemacytometer by capillary action. In all four large squares the cells were counted and the cell number was calculated as follows:

$$\text{Cell number/ml} = \frac{\text{total cell number} \times \text{dilution factor} \times 10^4}{4}$$

The cell viability was determined with trypan blue. Therefore, the cell suspension was mixed with an equal volume of trypan blue prior to counting. Trypan blue diffuses into cells. While living cells continuously remove the dye via pumps, it remains in the cytosol of dead cells. Therefore, dead cells appear blue and can be distinguished from living cells using light microscopy.

4.2.1.2 Freezing and thawing of cells

Cell pellets were re-suspended in freezing medium. 1 ml of cell solution with a density of approx. 1 to 3 x 10^6 cells was transferred to one cryo vial. The vials were slowly cooled to -80°C in a freezing container and then stored in liquid nitrogen. To re-cultivate frozen cells, they were quickly thawed and carefully

re-suspended in 10 ml cultivation medium. After centrifugation for 5 min at 1200 rpm, the cells were re-suspended in cultivation medium.

4.2.1.3 Cell lines

The human hepatoma cell line HuH7 was maintained in complete DMEM medium. Passage was performed at a ratio of 1:5 when the cells were 100% confluent. HepG2.2.15 cell were cultivated in complete DMEM or, for virus production, in a 1:1 mixture of PHH medium and complete Williams E medium. 293 cells were kept in complete DMEM and passaged at a ratio of 1:5 when 100% confluent.

4.2.1.4 Primary cells

Primary human hepatocytes

PHH were isolated from fresh surgical liver specimen from patients undergoing partial hepatectomy. The procedure was approved by the local Ethics Committee, with obtained informed consent of the patients.

The protocol based on a collagen perfusion established by Barry and Friend (1969), which was further developed into a two-step perfusion by Seglen (1976). The collagen perfusion with the additional pre-perfusion step using Ca^{2+}- and Mg^{2+}-free medium, followed by differential centrifugation, is standard for the isolation of primary human hepatocytes (Hsu, 1985; Berry, 1997; Schulze-Bergkamen et al., 2003).

The pre-perfusion, perfusion and the PHH medium were warmed to 37°C prior to the perfusion, and the wash medium was cooled to 4°C. Cell culture dishes were coated with collagen. For that purpose, the dishes were incubated with a 1:10 diluted collagen solution (Serva, Heidelberg) for 30 min at 37°C. Afterwards the dishes were washed twice with ddH_2O and air dried.

The healthy liver tissue piece was placed on a kidney dish. A large branch of the port vein was canulated, and the canula was fixed with tissue paste (Histoacryl®, Braun, Melsung). The two-step collagenase perfusion started with 500 ml pre-perfusion medium, with a flow rate between 20 and 40 ml/min. At cut surfaces with high medium passage, the large vessels were occluded with tissue paste. The

medium was discarded after traversing the liver tissue. After 15 to 20 min, perfusion was continued with 250 ml perfusion medium containing freshly added collagenase type IV (Worthington, Lakewood). 100 ml of the collagenase perfusion medium were kept and perfusion was continued with this remaining amount of medium. Collagenase treatment was performed for 15 to 20 min, depending on liver-section size. As soon as the tissue softened and liver cells appeared in the medium, the liver was cut into small peaces and the tissue was scratched off with a scalpel. If collagenase digestion was incomplete, the suspension was transferred to a sterile beaker and stirred for 10 min at 37°C. The cell suspension was filtrated through double-layered gaze and a 70 µm cell strainer. After centrifugation in 50 ml Falcon tubes for 5 min at 50 x g at 10°C, the pellet was re-suspended in 40 ml wash medium. The wash step was repeated three times. The cells were re-suspended in PHH medium and the cell number and viability was determined. The cells were seeded on collagenised cell culture dishes at a density of 8 x 10^5 cells/ml of PHH medium supplemented with 10% FCS. After 3 h the medium was exchanged to remove non-adherent cells. One day post seeding, the cells were kept in medium containing 5% FCS. From day two post seeding the cells were cultivated in FCS-free medium and used for experiments. The obtained cell culture consisted of 85-95% PHHs and 5-15% non-parenchymal liver cells. The latter are not susceptible for adenovirus type 5 infections (Hegenbarth et al., 2000; Schulze-Bergkamen et al., 2003)

Peripheral blood mononuclear cells

PBMCs were obtained from standard buffy coat preparations of healthy blood donors or fresh peripheral blood by Ficoll Paque (Pan Biotech, Aidenbach) density gradient centrifugation. The blood was diluted with PBS to a final volume of 120 ml. 30 ml of the diluted buffy coat were carefully layered on top of 13 ml Ficoll solution (1.077 g/ml, Pan Biotech). Gradient centrifugation was performed for 20 min at 320 x g without brake. Within the density gradient, the blood cells sediment in the Ficoll according to their density. Granulocytes and erythrocytes, which posses high densities, sediment at the bottom. PBMCs are located in the interface between Ficoll and the supernatant, which contains the plasma and thrombocytes. The interfaces were collected, combining interfaces of two tubes into one new tube. Tubes were filled up to 50 ml with PBS, centrifuged for 15 min

at 1500 rpm and the supernatant was completely removed. If the cell pellet contained many erythrocytes, an erythrocyte lysis was performed. Therefore, cells were incubated with 0.15 M NH_4Cl at 37°C for 5 min. After another centrifugation step at 1000 rpm for 10 min, the cells were re-suspended in PBS, counted and the viability was checked with trypan blue. In case of a contamination with thrombocytes, cell were washed twice in 50 ml PBS and centrifuged at 1200 rpm for 10 min. PBMCs were seeded in RPMI medium containing 10% FCS at a density of 5 x 10^6 cells/ml.

Monocytes

Monocytes represent approx. 8% of PBMCs. Two methods were used to isolate monocytes from PBMCs. The plastic adherence method relies on the characteristic of $CD14^+$ monocytes to adhere to plastic surfaces. Other cell types within the PBMCs culture do not posses this feature and can therefore be separated from the monocytes by washing steps. The PBMCs were seeded at a density of 5 x 10^6 cells/ml of RPMI 1640 medium supplemented with 10% FCS for 2 h. Subsequently, non-adherent cells were removed by washing 4-fold with PBS. Plates were examined using a phase-contrast microscope, to determine the level of contamination with lymphocytes. These can be distinguished from monocytes by their very small size and round shape. If lymphocytes were present in the monocyte culture, additional washes were performed.

Alternatively, monocytes were isolated by immuno-selection with CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach). This technique is referred to as **ma**gnetic **c**ell **s**orting (MACS). The principle of this method is the labelling of cells with specific antibodies coupled to magnetic beads. All cells are incubated with the antibody-bead complexes and subsequently transferred to a column placed in a magnetic field in a MACS separator (Miltenyi Biotec, Bergisch Gladbach). Magnetically labelled cells are retained in the column, while unlabeled cells flow through. By removal of the magnetic field the labelled cells can be eluted from the column.

The CD14 antigen is expressed on most monocytes and on macrophages. The method was performed according to the manufacturers' instructions. The PBMCs were counted, washed in MACS buffer and centrifuged at 12000 rpm for 10 min at 4°C. For more then 4 x 10^8 cells, the cells were split to perform two separate

stainings and separation in two columns. The cells were re-suspended in 400 µl MACS buffer per 1x 10^8 cells, and 50 µl CD14 MicroBeads were added per 1 x 10^8 cells. After 15 min incubation at 4°C, 10 ml MACS buffer were added and cells were centrifuged at 1200 rpm for 10 min at 4°C. The pellet was re-suspended in 3 ml MACS buffer and applied to one or two equilibrated LS columns (Miltenyi Biotec, Bergisch Gladbach), located in the magnetic field of the MACS separator. The column was washed 3 times by adding 3 ml buffer. Then, the column was removed from the separator and the $CD14^+$ cells were eluted in 5 ml MACS buffer. After a washing step in MACS buffer and centrifugation at 4°C and 1200 rpm, the cells were re-suspended in RPMI medium and cell number and viability were determined. The $CD14^+$ cells were cultured in complete RPMI medium, with a density of 3 to 4 x 10^5 cells/ml.

Monocyte-derived dendritic cells (moDC)

Dendritic cells can directly be derived from $CD14^+$ blood monocytes (Zhou and Tedder, 1996). Upon cultivation with cytokines, monocytes differentiate into a fairly homogenous cell population with dendritic cell morphology and functionality.

Monocytes were prepared as described and cultured in complete RPMI medium supplemented with 10 ng/ml IL-4 and 10 ng/ml GM-CSF (R&D Systems, Wiesbaden) for three to five days to allow differentiation into immature DCs. For further maturation, immature moDCs were stimulated by addition of 1mg/ml LPS, 50 ng/ml PGE_2 (Sigma, Deisenhofen) and 50 ng/ml TNF-α (R&D Systems, Wiesbaden) for 24 to 48 h. Cell differentiation was monitored by light microscopy. After IL-4 and GM-CSF treatment, the monocytes built small semi-adherent clusters, and a small number of cells with dendrites could be seen. After cultivation with LPS, TNF-α and PGE_2, the cells formed large clusters and exhibited dendritic-cell morphology.

Plasmacytoid and myeloid dendritic cells

Circulating pDCs and mDCs were gained from PBMCs of patients with chronic HBV infection and one healthy volunteer after informed consent.

The isolation protocol is based on the MACS technique. In the first step, pDCs were separated by a positive selection using the BDCA-4 cell isolation kit (Miltenyi Biotec, Bergisch Gladbach) following the manufactures' instructions. The BDCA-4

antigen is specifically expressed on pDCs, which make up approx. 0.5% of PBMCs.

The PBMCs were obtained as outlined above and re-suspended in 300 µl MACS buffer per 1 x 10^8 cells. Cells were incubated with 100 µl blocking reagent and 100 µl BDCA-4 MicroBeads per 1 x 10^8 cells for 15 min at 4°C. Cells were washed with 10 to 20 volumes of MACS buffer and centrifuged at 1200 rpm for 10 min at 4°C. The supernatant was discarded and the cells were re-suspended in 500 µl buffer per 1x 10^8 cells. A MS column (Miltenyi Biotec, Bergisch Gladbach) was equilibrated with 0.5 ml buffer, before the cells were applied. The flow through containing the BDCA-4$^-$ cells was collected. Three washing steps with 0.5 ml buffer were performed and the flow through was combined with the BDCA-4$^-$ cells. pDCs were eluted from the column by addition of 1 ml buffer after removal of the magnetic field. To increase the purity of the pDC fractions, the separation procedure was repeated. Then the cells were counted and a small aliquot was subjected to FACS analysis (see below). The remaining cells were pelleted, frozen in liquid nitrogen and stored at -80°C.

The flow through was used for the isolation of mDCs with the CD1c (BDCA-1) Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach). The CD1c antigen is expressed on myeloid dendritic cells, but also on a subpopulation of CD19$^+$ B cells. Therefore, a two-step magnetic separation was necessary to obtain a pure population of mDCs.

In the first step CD1c-expressing CD19$^+$ B cells were depleted using anti CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach). This step is a so-called negative selection, with the target cell type, in this case mDCs, being in the flow through. In the second step, mDCs were separated in a positive selection step with anti CD1c MicroBeads.

The pDC-depleted flow through was washed with MACS buffer and re-suspended in 200 µl buffer per 1 x 10^8 cells. Blocking reagent, CD19 MicroBeads and biotinylated CD1c MicroBeads, 100 µl each, were added to the cells. Incubation was performed at 4°C for 15 min, followed by a washing step with 10 to 20 volumes of buffer. Cells were centrifuged and re-suspended in 500 µl buffer per 1 x 10^8 cells. A LD column was equilibrated prior to the application of the cells. The flow through containing the CD19$^-$ cells was collected in the same tube as the flow through of the following two washing steps. The CD19$^+$ cells were removed

from the column with 2 ml of buffer, counted and frozen as described above. A small aliquot was subjected to FACS analysis.

The B-cell depleted flow through was centrifuged and re-suspended in 400 µl buffer per 1 x 10^8 cells. 100 µl of Anti-Biotin MicroBeads were added per 1 x 10^8 cells. Following 15 min incubation at 4°C, the cells were pelleted and re-suspended in 500 µl buffer per 1 x 10^8 cells. The separation of the mDCs was performed with a MS column as previously described. The flow through was kept and subjected to further isolation steps (see Enriched lymphocyte fraction). After counting the cells, a small aliquot was kept for FACS analysis, and the remaining cells were frozen and stored at -80°C.

Enriched lymphocytes fractions

The flow through, which remained after the pDC and mDC isolation protocols, was used to obtain enriched lymphocyte fractions. The cells, which were already depleted for mDCs, B cells and pDCs, were further depleted for $CD14^+$ monocytes. Isolation with the CD14 MicroBeads was performed as described, using a MS column for separation. The collected flow through, as well as the separated monocytes, were counted and frozen at -80°C. A small aliquot of both fractions was used for FACS analysis.

4.2.1.5 Transfection of cells

4.2.1.5.1 Calcium phosphate method

Fifteen micrograms of plasmid DNA were re-suspended in 450 µl ddH_2O and 50 µl of a 2.5 M calcium phosphate solution was carefully added. The mixture was dropped into a new tube containing 500 µl 2 x HBS while shaking on a vortexer. After 20 min of incubation, the DNA solution was added to the cells. The inoculum was removed on the next day. The cells were washed twice with PBS, and further cultivated in fresh medium.

4.2.1.5.2 FuGene6®

HuH7 cell were transfected with indicated plasmids using FuGene6® (Roche Diagnostics, Mannheim) according to the manufacturers' protocol: Cells were plated one day prior to transfection to obtain a 40 to 60% confluent monolayer at the following day. The medium was exchanged 1 to 3 h before transfection. For transfection, 1 µg plasmid per 1.2 x 10^6 cells was used. The DNA was mixed with serum free medium and 2 µl FuGene per µg DNA. After 20 min incubation the solution was added to the cells. Inoculums were left in the cell culture over night. Fresh medium was applied at the next day.

4.2.1.6 Production of wild type HBV

For the production of wtHBV, the HepG2.2.15 cell line was used. This HepG2-based cell line contains four dimeric HBV genomes stably integrated into the cellular genome (Sells et al., 1987). Therefore, this cell line permanently produces HBV, which can be purified from cell culture supernatants.

For virus preparation, the cells were cultivated in complete DMEM medium until 100% confluent. Then, the medium was exchanged to 50% PHH medium and 50% complete Williams E medium. Every three days, the virus-containing medium was collected. Cell debris was removed by centrifugation at 1000 rpm for 5 min. The supernatant was transferred to centrifugal filter devices (Centricon Plus-70, Biomax 100, Millipore Corp., Billerica) The first centrifugation was performed at 3500 x g for 1 h at 4°C, to capture the virus particles in a filter. Due to the exclusion limit of 100 kDa, serum proteins flow through, while proteins larger than 100 kDa remained in the filter. In a second, invert centrifugation step with the filter system turned upside down, performed at 2600 x g for 10 min, the virus was eluted. The virus concentrate was supplemented with 10% glycerol and stored at -80°C. The titer of the produced wtHBV was determined with Caesium chloride density-gradient, followed by dot blot analysis, as outlined below.

4.2.1.7 Production of recombinant HBV-based vectors

HBV-based vectors (rHBV) were produced in HuH7 cells via transfection. The cells were seeded one day prior to transfection in 10 cm² cell culture dishes. At the next day, the cells were transfected with equal amounts of helper plasmid and transfer plamid using FuGene6® (Roche Diagnostics, Mannheim) as described.

The transfer plasmid encodes the transgene, inserted in the HBV genome at the site of the S protein. All viral ORFs are knocked out. Therefore, only pregenomic RNA is transcribed from the transfer plasmid. The helper plasmid consists of an HBV genome with a deletion in the encapsidation signal. It provides all viral RNAs except the pregenomic RNA. This guarantees that only recombinant virions emerge, but no wtHBV.

At the following day the HuH7 medium was exchanged with a 1:1 mixture of HuH7 medium and PHH medium. At day two post transfection, PHH medium was applied. Another four days later, the medium was collected and the recombinant HBV particles were concentrated using centrifugal filter devices as described in 4.2.1.6. Quantitative analysis of the produced rHBV stocks was performed with a caesium chloride density-gradient centrifugation and dot blot.

4.2.1.8 Production of adenoviral vectors

Adenoviruses (Ad) are known to infect a broad range of cells and are therefore widely used as vectors. The adenoviral vectors used in this study were based on Ad type 5 and produced as described in Sprinzl et al. (2001 and 2004). The plasmids were linearised with Pac1 before transfection of 293 cells using the calcium phosphate method. The 293 cells provide the adenoviral E1 genes in *trans*, which are necessary for virus production (Graham et al., 1977). After three days the cells died, due to the apoptosis induction of the Ad. At this time point, virions were present in the apoptotic cells and in the medium. The medium, containing dead cells and virions, was collected and subjected to three freeze and thaw cycles in liquid nitrogen and a 37°C water bath. This breaks the cells and sets free the intracellular virions. Following a centrifugation step for 5 min at 2000 rpm to remove the cell debris, the virus containing supernatant was supplemented with 10% glycerol and stored at -80°C. To determine the titer of the produced Ad vectors, 293 cells were plated on a 12 well cell-culture dish. Serial

dilutions of the vector stock from 1:10 to 1:1000 were added to the confluent cells. The cytopathic effect as well as GFP fluorescence was monitored 48 h after transduction. Statistically, one GFP expressing or cytopathic cell has been transduced with three Ad particles. A moi of 1 was calculated for the dilution, which led to 80% GFP expressing cells or cytopathic cells.

4.2.1.9 Caesium chloride (CsCl) density-gradient centrifugation

During virus preparation, different types of viral particles are obtained: naked DNA, unenveloped DNA-containing capsids, and enveloped virions. Their different densities enable to separate them in a density gradient. DNA is centrifuged down to the bottom, due to the very small size. DNA-containing capsids sediment at a density of 1.3 g/l, while intact virions can be found in the 1.22 g/l fraction.

The CsCl (Roth, Karlsruhe) density-gradient ultra centrifugation was performed with the SW-60 swing bucket rotor. In SW-60 polyallomer vials (Beranek Laborgeräte) 500 µl of CsCl solutions with the following densities were carefully layered one upon the other: 1.4 g/l, 1.3 g/l and 1.15 g/l. On top of the CsCl solutions, 500 µl of a 20% sucrose solution was layered, and the sample was applied. The vials were filled up with PBS and tared on micro scales. Ultra-centrifugation was performed at 55000 rpm at 20°C for 4 h to over night. The density fractions were collected with a Fraction recovery system (Beckman). Each fraction contained 6 drops, which approximates a volume of 175 µl. The fractions were subjected to quantitative dot blot analysis.

4.2.1.10 Dot blot

For quantitative and qualitative analysis of the produced wtHBV or rHBV, a DNA dot blot was performed. The DNA fractions, obtained by CsCl density centrifugation, were dotted to a nylon membrane (Roche Diagnostics, Mannheim) in a dot blot aperture (Schleicher & Schuell, Dassel). A HBV plasmid standard ranging from 8 pg to 1000 pg was added. The dotted samples and standards were washed with 200 µl PBS, before the membrane was transferred to a 3 mm Whatman paper soaked with denaturation solution. Subsequently the membrane was transferred to a 3 mm Whatman paper soaked with neutralisation solution.

Then the DNA was cross linked on the membrane in a UV oven. The membrane was hybridised with a ^{32}P-labelled HBV DNA probe at 68°C over night. At the next day, the membrane was washed 3-fold 20 min each at 68°C with wash solution. After wrapping the radioactive membrane in seran wrap, the DNA was exposed to a phosphor-screen and quantified with a phosphoimager (BIO-RAD, Hercules).

4.2.1.11 HBV infection of cells

Infection of cells with HBV was performed in medium containing 5% PEG 8000 (Gripon et al., 1993). The cells were incubated over night with HBV at a moi of 100 vp/cell. If indicated, neutralising antibodies (1 IU/10^6 cells; Hepatect, Biotest Pharma, Dreieich) were added simultaneously (Schulze-Bergkamen et al., 2003). After over night inoculation, cells were washed 3-fold with PBS. Cells were further cultivated in fresh medium, and cell culture medium was collected at indicated time points. To monitor infection, HBsAg and HBeAg were determined using commercial immuno-assays (Axsym®, HBeAg 2.0, HBsAg V2, Abbott Laboratories, Wiesbaden) in the Institute of Virology at the University of Cologne.

4.2.1.12 Transduction with HBV-based vectors

The transduction of cells with HBV-based vectors was performed by the same protocol as HBV infection experiments (see 4.2.1.4).

4.2.1.13 Transduction with adenoviral vectors

Prior to transduction of cells with adenoviral vectors the medium was exchanged. The indicated moi of vector was added to the cells. After 3 h, the inoculum was removed and fresh medium was applied.

4.2.1.14 Luciferase detection

Renilla lucifase is an enzyme that converts its substrate coelenterazin to coelenteraid. The luciferase system is very sensitive. Detection is possible in a broad linear range, with the input not giving a background signal. Furthermore, background luminescence of cells is very weak and does not interfere with the

measurement (Matthews et al., 1977). In the enzyme reaction, light is emitted proportional to the enzyme concentration. To quantify the light emission the Renilla Luciferase Assay system (Promega, Mannheim) was used. 1 x 10^6 cells were lysed in 200 µl lysis buffer per well at day 4 post transduction. The samples were centrifuged for 1 min at 12000 rpm and 10 µl of the lysates were pipetted in a reaction vial. After mixing the samples with 100 µl substrate, the samples were transferred to a luminometer (Berthold Technologies, Bad Wildbad). After 2 sec, the light emission was measured for 3 sec. The relative light units (RLU) were normalised to the total protein content of the respective samples. Mock transduced cells served as negative controls. The RLU determined in these samples were defined as 1, the other values were adapted proportionally.

4.2.2. DNA techniques

4.2.2.1 Transformation

Competent *E.coli* strains DH5α (Promega, Mannheim) and XL1blue (Stratagene, La Jolla) were produced as described by Inoue et al. (1990) and stored at -80°C.
Bacteria aliquots of 25 µl per reaction were thawed on ice. 50 to 200 ng plasmid DNA were added to the bacteria and incubated for 30 min on ice to allow the attachment of the DNA to the bacteria. A 60 sec long heat shock at 42°C led to DNA uptake. The bacteria were directly incubated on ice for 5 min and re-suspended in 500 µl LB medium without antibiotics. After 1h incubation at 37°C, the antibiotic resistance encoded on the transferred plasmid was established, and 100 µl of the bacteria suspension were plated on LB agar plates containing the antibiotic corresponding to the resistance. Agar plates were incubated at 37°C over night.

4.2.2.2 Plasmid preparation

Plasmid preparation was either performed with the rapid boiling method for small DNA amounts or with the QiaQuick Maxiprep Kit (Qiagen, Hilden) for large amounts.

To obtain plasmid DNA with the rapid boiling method, 1.5 ml bacteria suspension were centrifuged for 5 min at 12000 rpm and the bacteria pellet was re-suspended in 300 µl STET buffer. Bacteria were lysed for 10 min and boiled for to 2 min at 100°C. After centrifugation for 10 min at 12000 rpm, the pellet, consisting of protein and protein-associated genomic DNA, was removed. The plasmid DNA in the supernatant was precipitated with 500 µl of 75% isopropanol/ 2.5 M sodium acetate. After 20 min incubation and centrifugation for 10 min at 12000 rpm, the supernatant was discarded and the DNA pellet was washed with 80% ethanol. This step is necessary to remove salt and purify the DNA. Following an additional centrifugation step for 10 min at 12000 rpm, the pellet was dried and re-suspended in 30 µl ddH_2O.

The plasmid preparation using the QiaQuick Maxiprep Kit (Qiagen, Hilden) was performed according to the manufacturers' instructions. The method is based on alkaline lysis of the bacteria. Proteins and protein-associated DNA are precipitated by neutralization. The following purification of plasmid DNA relies on anion exchange chromatography. After several washing steps the plasmid DNA was precipitated with 100% isopropanol and cleaned with 70% ethanol. The pellet was re-suspended in 500 µl ddH_2O.

4.2.2.3 Calculation of DNA concentrations

To calculate the concentration of a DNA preparation, the absorption at 260 nm and 280 nm was determined with a photometer (Smart Spec 3000, BIO-RAD, Hercules). This technique relies on the characteristic of nucleic acids to absorb UV light with a wave length of 260 nm. Absorption at 260 nm (OD_{260}) of one equals a DNA concentration of 50 µg/ml. The ratio of the absorption at 260 nm and 280 nm allows estimating the purity of the DNA and should range between 1.8 and 2.0. The absorption was always normalised to ddH_2O.

4.2.2.4 Restriction

Restriction endonucleases cut dsDNA at specific sequences. The ends generated in this process can either be blunt without ss overhangs, or sticky with small ss overhangs. Depending on the parental organism of the restriction

endonucleases, they differ in the optimal temperature and chemical conditions. The enzyme activity is defined in units, with 1 unit enzyme cutting 1 mg DNA in 1 h. Restrictions were always performed following the manufactures' (Fermentas, St. Leon Roth) instructions.

4.2.2.5 Ligation

For a ligation reaction, 50 ng linearised vector DNA was mixed with 3 to 5-fold the amount of the insertion fragment in 1 x ligase buffer in a reaction volume of 20 µl. After addition of 1 unit T4 ligase (Roche Diagnostics, Mannheim) the DNA-enzyme mix was incubated for 1 to 2 h.

4.2.2.6 Gel electrophoresis

DNA molecules are negatively charged, with the charge being proportional to the molecular weight. Therefore, they can be separated according to their size in an electric field.

Electrophoresis was performed in 0.8 to 2% horizontal agarose gels. The agarose (Cambrex Bioscience, Rockland) was dissolved in 1 x TAE buffer, and ethidium bromide was added to a final concentration of 0.5 µg/ml after the agarose was cooled. Ethidium bromide intercalates into dsDNA. This results in a complex, which fluoresces when exposed to UV light (254 nm to 366 nm). Emission of 590 nm light allows the visualisation of the DNA, with a detection limit of approx. 20 ng dsDNA. The polymerised gel was covered with TAE buffer and electrophoresis was performed with a constant voltage of 50 to 120 V. The samples were mixed with 1x DNA sample buffer to firstly ballast the DNA with glycerol, and to secondly mark the separation front with bromphenol blue. In addition to the samples a DNA standard with defined DNA sizes was loaded on the gel.

4.2.2.7 Gel elution

To isolate a DNA fragment which was previously separated on a gel, the DNA band was excised as precisely as possible from the gel. The purification was

performed with the QiaQuick Gel Elution Kit (Qiagen, Hilden) according to the instructions. The DNA was eluted in 30 µl ddH_2O.

4.2.2.8 Cloning of RFP and GFP fusion proteins with HBV envelope proteins

pCH S-GFP An-b-glob

The plasmid pCH S-GFP An-b-glob encodes a fusion protein consisting of the HBV S protein and the C-terminally fused GFP. The protein is under CMV promoter control and contains the polyA chain from the β-globin gene. The GFP gene was obtained from the plasmid pCH GFP An-b-glob via PCR. The primer S-GFP C-term fw contained a restriction site for AccI, the primer S-GFP C-term rev contained a restriction site for SpHI. The PCR product contained the GFP gene and the newly generated restrictions sites. Both the plasmid pCH SAg An-b-glob and the GFP PCR product were cleaved with AccI and SpHI. After ligation, the GFP gene was inserted in the plasmid at the C-terminus of the S gene.

pCH GFP-S An-b-glob

The plasmid pCH GFP-S An-b-glob encodes a fusion protein consisting of the HBV S protein and the N-terminally fused GFP. The protein is under CMV promoter control and contains the polyA chain from the β-globin gene. The GFP gene was obtained from the plasmid pCH GFP An-b-glob via PCR. The primer GFP-S N-term fw contained a restriction site for SalI, the primer GFP-S N-term rev contained a restriction site for KpnI. The PCR product contained the GFP gene and the newly generated restrictions sites. Both the plasmid pCH SAg An-b-glob and the GFP PCR product were cleaved with SalI and KpnI. After ligation the GFP gene was inserted in the plasmid at the N-terminus of the S gene.

pCH GFP-sp-S An-b-glob

This plasmid is based on pCH GFP-S An-b-glob. To introduce a spacer of 9 aa between the GFP gene and the S gene, a small spacer of 24 bp was generated (GFP-S N-term sp fw and GFP-S N-term sp rev). The spacer sequence contains a KpnI restriction site on both ends. The vector pCH GFP-S An-b-glob as well as the spacer sequence were cleaved with KpnI and ligated. The orientation of the insert

in the plasmid was controlled via restriction patterns after cleavage with different enzymes.

pCH M-GFP An-b-glob

To obtain a plasmid encoding the M gene with the GFP C-terminally fused to it, the plasmids 000HBV and pCH S-GFP An-b-glob were used. Via PCR using the primers M-GFP fw and M-GFP rev a sequence containing the preS1 region and the beginning of the S region, was amplified. In front of the sequence a KpnI restriction site was generated, at the 5`end of the minus strand a AvrII cleavage site was introduced. The ATG of the S region, which is the start codon of the S protein, was exchanged to ACG. The plasmid pCH S-GFP An-b-glob and the PCR product were cleaved with KpnI and AvrII and ligated.

pCH L-GFP An-b-glob

To obtain a plasmid encoding the L gene with the GFP C-terminally fused to it, the plasmids 000HBV and pCH S-GFP An-b-glob were used. Via PCR using the primers L-GFP fw and L-GFP rev a sequence containing the preS1 region and the beginning of the preS2 region was amplified. At the 5`end of the plus strand a KpnI restriction site was generated, at the 5`end of the minus strand a AvrII cleavage site was introduced. The ATG of the preS2 region, which is the start codon of the M protein, was exchanged to ACG. The plasmid pCH S-GFP An-b-glob and the PCR product were cleaved with KpnI and AvrII and ligated.

pCH S-RFP An-b-glob

The plasmid encodes the RFP gene C-terminally fused to the S gene. The mRFP gene was obtained from the mRFP1_pRSETB plasmid via PCR with the primers RFP fw and RFP rev. With the PCR restrictions sites for AccI and PeaI were generated at the 5`end of the plus strand and the 5`end of the minus strand, respectively. After restriction of the mRFP PCR fragment and the plasmid pCH S-GFP An-b-glob with AccI and PaeI, the RFP was ligated into the plasmid pCH S-GFP An-b-glob. The GFP was removed by the restriction.

pCH M-RFP An-b-glob

The plasmid encodes the RFP gene C-terminally fused to the M gene. The mRFP gene was obtained from the mRFP1_pRSETB plasmid as described. After restriction of the mRFP PCR fragment and the plasmid pCH M-GFP An-b-glob with AccI and PaeI, the RFP was ligated into the plasmid pCH M-GFP An-b-glob. The GFP was removed by the restriction.

pCH L-RFP An-b-glob

The plasmid encodes the RFP gene C-terminally fused to the L gene. The mRFP gene was obtained from the mRFP1_pRSETB plasmid as described. After restriction of the mRFP PCR fragment and the plasmid pCH L-GFP An-b-glob with AccI and PaeI, the RFP was ligated into the plasmid pCH L-GFP An-b-glob. The GFP was removed by the restriction.

4.2.2.9 Polymerase chain reaction

PCR is a method to amplify specific DNA sequences located between two primers. These are complementary to the 5` and 3` ends of the sequence. The polymerase specifically amplifies the sequence in repeated amplification rounds.
A master-mix was prepared with the following contends per reaction:

Reagent	Concentration
Polymerase buffer	4 µl
Pfu polymerase	2 µl
Primer fw	5 pmol
Primer rev	5 pmol
ddH_2O	12 µl

150 to 300 ng template DNA or ddH_2O were added per reaction. The Thermocycler was programmed as follows:

Step	Temperature	Time
1. Initial denaturation	94°C	2 min
2. Denaturation	94°C	30 sec
3. Annealing	60°C	30 sec
4. Elongation	72°C	90 sec
5. Final elongation	72°C	5 min

Steps two to four were repeated 30 times. The final elongation step is necessary to allow the complete amplification of the sequence fragments.

4.2.2.10 Real-time quantitative polymerase chain reaction

Real-time PCR detection techniques make a kinetic quantification possible. PCR amplification depends mostly on the template concentration. However, reaction efficiency is as well important: In high efficient reactions with low template concentrations the same plateau can be reached as in reactions with high template concentrations but low reaction efficiency. Therefore, end point quantification analyses are sometimes not accurate. The advantage of real time PCR is the measurement in the log-linear phase of constant amplification. This allows the precise quantification of the amount of starting material.

The Light Cycler© instrument from Roche Diagnostics, Mannheim is a thermocycler, combined with a fluormeter. For fluorescence detection the SYBR Green I Dye was used. This fluorescent dye intercalates only in dsDNA. The fluorescence intensity of this DNA-SYBR green complex is much higher than the intensity of the dye alone. Therefore, during annealing and elongation phases the fluorescent signal increases. The signal intensity is directly proportional to the DNA amount, and the highest signal is obtained at the end of the elongation phase. Due to the glass capillary system, in which the PCR reaction takes place, a rapid thermal transfer is guaranteed. The glass capillaries are located in a continuously rotating carousel in a single thermal chamber. The fluorescence intensity is measured at the end of each elongation phase at a certain temperature in a single optical unit. The LightCycler PCR is very sensitive with a detection limit of one to ten copies for plasmid DNA per sample. For genomic DNA the detection of a single-copy gene in 3 pg DNA is estimated.

Different quantification methods are available with the LightCycler© System. Absolute quantification is performed with an external standard curve of known concentrations of the target DNA. The relative quantification expresses the target gene concentration in relation to a reference gene, usually a house keeping gene or mitochondrial DNA. Standard curves of both target and reference gene are used to obtain the concentrations. The template concentration is determined with so-called crossing points. These points are defined as the cycle numbers, in which all amplifications exhibit the same fluorescence intensity. The identification of specific DNA products is possible using melting curve profiles. The melting temperature of dsDNA depends on length, GC contend and sequence. Thus, every sequence has a specific melting curve profile. Within the PCR reaction, the melting curve is obtained by steadily increasing temperatures while the fluorescence is monitored.

This sensitive PCR technique was used to detect low amounts of HBV rcDNA and cccDNA and to be able to clearly distinguish between the two DNA forms. Quantification was performed relative to an external plasmid HBV DNA standard on a Light Cycler© instrument with the LightCycler FastStart DNA Masterplus SYBR Green I (Roche Diagnostics, Mannheim). This reaction mix contains the Taq-DNA-polymerase, reaction buffer, magnesium chloride and dNTPs. The following master-mix was prepared per reaction:

Reagent	Vol.
LightCycler FastStart DNA Masterplus SYBR Green I	4 µl
Primer fw	1 µl
Primer rev	1 µl
ddH_2O	12 µl

2 µl template DNA or ddH_2O were added to the mix. The light cycler instrument was programmed as shown:

Step	Temperature	Time
1. Initial denaturation	95°C	5 min
2. Denaturation	95°C	15 sec
3. Annealing	60°C	4 sec
4. Elongation	72°C	25 sec
5. Detection	88°C	2 sec

4.2.3. Protein analysis

4.2.3.1 Protein precipitation

4.2.3.1.1 Whole cell protein

For whole cell proteins the cells were lysed in sample buffer.

4.2.3.1.2 Sub-cellular fractionation

To separate the membranous, cytosolic and nuclear fractions of cellular proteins, the cells were washed twice with PBS and scraped off the dishes. The cells were re-suspended in a minimal volume of pre-cooled NP40 buffer and incubated on ice for 20 min.. Afterwards, cells were aspirated with a syringe, and destroyed by passaging 10 times through a needle (24 gauge). Nuclei were pelleted at 800 x g for 5 min at 4°C. The supernatants were ultra-centrifuged at 100000 x g for 30 min at 4°C. The pelleted membrane proteins were re-suspended in 50 µl ddH_20 or protein sample buffer. The cytosolic proteins in the supernatant were precipitated as follows: The supernatant was mixed with 4 vol. 100% aceton (pre-cooled at -20°C) and incubated for 2 to 3 h at -80°C. After centrifugation at 8000 x g for 10 min, the protein pellet was air dried and re-suspended in 200 µl ddH_20 or protein sample buffer.

4.2.3.1.3 Immunoprecipitation

To isolate proteins from supernatants of cells, 2 ml supernatant was mixed with 50 µl sepharose, which has previously been washed once with PBS and centrifuged for 5 min. at 1000 x g. Hepatect (Biotest Pharma, Dreieich) was added in a concentration of 0.5 IE/ml. If rαHBvax (in-house production) was used, 2 µl/ml

were added. Supernatants slowly rotated o.n. at 4°C. After centrifugation at 1000 x g for 5 min., the supernatant was discarded and the sepharose beads were washed twice with PBS. Protein sample buffer was added and the beads were incubated at 99°C for 10 min. After centrifugation the supernatant was transferred to SDS page and Western blot analysis.

4.2.3.2 Calculation of protein concentrations

To determine total protein concentrations, the BCA™ Protein Assay Kit (Pierce, Rockford) was used. This assay is based on the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium, combined with the colorimetric detection of Cu^{+} with a reagent containing **bic**inchonic **a**cid (BCA). The complex formed by the chelation of 2 BCA molecules with one Cu^{+} molecule exhibits a strong absorbance at 562 nm. The absorbance is linear with increasing protein concentrations over a working range of 20 to 2000 µg/ml. The reaction was performed according to the manufacturers' introductions, and an albumin protein dilution series served as protein standard.

4.2.3.3 SDS-page gel electrophoresis

Protein samples were mixed with Laemmli buffer and boiled for 10 min at 99°C. 10 to 30 µg of protein were transferred to a 12.5% SDS gel. As a standard for protein size a pre-stained marker (Fermentas) was added. The proteins were electrophoresed in a blot chamber (MiniProtean®3 Cell, BIO-RAD, Hercules) with constant amperage (30 mA per gel).

4.2.3.4 Western blot

The proteins were transferred from the SDS gel onto a methanol-activated PVDF membrane (Amersham, Buckinghamshire) using a semi-dry transfer cell (BIORAD, Hercules). The amperage was calculated with the following formula:

1.2 mA/cm² of the gel for 1 h transfer.

After protein transfer the membrane was blocked in blocking solution for 1 h and probed for 1 h at RT or over night at 4°C with the indicated antibody. After three

washing steps 10 min each, the secondary antibody was added at in a 1: 2000 dilution for 1 h. The detection was performed with the Enhanced Chemiluminescence detection kit (Amersham, Buckinghamshire).
If re-probing of the membrane with another antibody was necessary, the membrane was previously stripped with 0.2 M NaOH for 10 min. PDVF membranes were re-activated in methanol, and blocking and antibody incubation were performed as described.

4.2.3.5 ELISA

The HBsAg ELISA (Murex, Abbott, Wiesbaden) detects different epitopes of the HBsAg. It was performed according to the manufacturers' instructions. In short, 75 µl of each sample was pipetted into the microtiter wells containing 25 µl sample dilution buffer. Additionally, a provided negative and positive control was applied. 60 min incubation at 37°C allowed the binding reaction between the monoclonal anti HBsAg antibodies, which are immobilised on the well, and the antigen in the samples. 50 µl of conjugate were added to the samples. It consists of polyclonal antibodies against HBsAg, which are conjugated to HRP. If HBsAg was captured by the coated antibodies, a complex of antibody-antigen-antibody-HRP would form. The plate was carefully moved to ensure mixing prior to 30 min incubation at 37°C. Following several washes, 100 µl of the substrate for the HRP was added. After 30 min incubation at 37°C the colour reaction of HRP and substrate had preceded, and a stop solution, containing 0.5 m sulphuric acid, was added to stop the reaction. The readout was in dual frequencies at 450 nm and, as a reference wave length, 620 nm in a plate reader.
The measurement of HBsAg and HBeAg in cell supernatants was performed in the diagnostic laboratory of the Institute for Virology, University of Cologne, with the commercial immuno-assays from Abbott, Wiesbaden (Axsym HBeAg 2.0 and HBsAg V2).

4.2.3.6 Flow cytometry

Flow cytometry is a technique used to separate cells by different parameters. A single cell suspension flows through multiple detectors, passing a laser light beam.

The suspended cells scatter the light beam in a specific way, depending on size and density. Fluorescently labelled cells emit light in specific wave lengths, which are picked up by the detectors. The data obtained by the flow cytometer are plotted 2-dimensional, and regions within the plot are subsequently separated by fluorescence intensity.

This method was used to determine the purity of isolated cell populations. The cells were labelled with fluorescent antibodies specific for the targeted cell population. The maturation state of moDCs was examined by staining immature and mature moDCs with monoclonal antibodies against HLA-DR (BD Pharmingen, Dan Diego), CD80 (BD Pharmingen, Dan Diego), CD83 (Immunotech, Marseille) and CD86 (BD Pharmingen, Dan Diego) in the concentrations recommended by the manufacturers. To assay the purity of isolated mDCs, they were stained with monoclonal FITC-conjugated antibodies against the mDC markers BDCA-2 and CD123 (Miltenyi, Bergisch Gladbach). For pDCs, streptavidin-PE (BD Pharmingen, Dan Diego) was used to stain the biotinylated BDCA-1 antibody previously used for isolation. The cells were incubated in PBS with the indicated antibodies for 30 min on ice, with occasional shaking. After two wash steps, the cells were re-suspended in 400 µl PBS supplemented with propidium iodide, to stain for dead cells. The analysis was performed in a flow cytometer (FACSCanto^{TM}, BD Biosciences, Heidelberg).

4.2.3.7 Immuno-fluorescence staining

HuH7 cells were plated on 8-well glass slides (Lab Tec, Nunc, Wiesbaden). At 60% confluency, they were transfected with 0.1 µg of plasmid per well, using FuGene6®. 1 or 2 days post transfection, cells were washed 3-fold with PBS and fixed in 3.7% PFA for 10 min.. Immediately after fixation, the cells were incubated with 50 mM ammonium acetate to saturate the free PFA residues. Following three washes with PBS, the cells were permeabilised with 0.5% saponin/PBS for 10 min.. Unspecific antibody binding sites were blocked with 10% serum in 0.1% saponin/PBS for 1 h. The primary antibody was added in a dilution of 1 : 500. Three washing steps with 0.1% saponin/PBS for 10 min each followed, before the cells were incubated for 1 h with the secondary antibody, diluted 1 : 2000 and supplemented with Dapi (0.5 µg/ml). The cells were washed 3-fold with

0.1% saponin/PBS. All incubation and washing steps were performed on a slowly rocking platform. After the staining procedure, the 8-well chambers were removed from the slide and the cells were mounted with Mowiol/Dabco. A cover slip was sealed on the slide with nail polish. The slides were stored at 4°C. Immunofluorescence analysis was performed on an Olympus IX81 microscope or on an Olympus FluoView1000 confocal microscope (Olympus, Hamburg).

4.2.3.8 Co-localisation studies

In the cell biological context, co-localisation is defined as the presence of two molecules in the same physiological location within a specimen, for example in the same organelle or cellular compartment or bound to the same receptor. Regarding digital imaging, co-localisation means that the same pixel of an image is shared by different colours, which are emitted by fluorophores.

To be able to obtain trustable and reproducible results from co-localisation studies, a few points have to be considered. First of all, the sample preparation and staining should be optimised in a way, that unspecific labelling and auto-fluorescence are reduced to a minimum. Furthermore, selected fluorophore combinations should have widely separated emission spectra to avoid bleed-through artefacts. Another important consideration for choice of fluorophores is the matching to the power spectrum of the laser lines.

Each antibody and fluorophore needs to be controlled in separate labelling experiments to determine the bleed-through under the actual experimental conditions. Auto-fluorescence of the specimen can be monitored in an unlabeled control. On the basis of these data, parameters such as photomultiplier voltage, offset and gain can be adjusted separately for each channel. It is important to find the conditions in which all fluorophores are displayed in the full 8-bit range. Differences in the intensities of the different fluorophores that are to be analysed for co-localisation can lead to false negative or positive results.

The co-localisation studies were performed using the Olympus FluoView1000 confocal microscope (Olympus, Hamburg). Single-labelled samples and negative controls were examined in both wavelengths to determine the experimental settings and to exclude bleed-through. Images were obtained using sequential excitation in the confocal microscope scanning configuration. At least 6 images

were examined in each experiment and analysed using the co-localisation tool of the FV10-ASW software (Version 1.6a, AnalySIS, Soft Imaging System GmbH, Münster).

4.2.3.9 Electron microscopy

The cells were pelleted in a 1.5 ml microfuge tube at 1000 x g. The supernatant was removed, and the cell pellet was fixed in 2.5% glutaraldehyde for 12 h at 4°C. After 3-fold washing in PBS, the cells were incubated in 2% OsO_4 and 1% $K_3Fe(CN)_6$ for 1 h. After three further washes, the pellets were dehydrated and infiltrated in a 1:1 mixture of propylene oxide (Polybed 812 and epoxy resin; Polysciences) and embedded in resin. Ultra thin (50-nm) sections were collected on 200-mesh copper grids and stained with 6% uranyl acetate in ddH_2O for 10 min followed by 1% lead citrate for 15 min. Sections were viewed using a Zeiss 902 transmission electron microscope at 50 kV (Carl Zeiss AG, Jena).

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6. Abbreviations

aa	Amino acid
Amp	Ampicillin
Approx.	Approximately
APS	Ammonium persulphate
α	Anti
BDCA	Blood dendritic cell antigen
bp	Base pairs
cccDNA	Covalently closed circular DNA
CD	Cluster of differntiation
CMV	Cytomegalovirus
CMV-promoter	CMV-IE-Promoter
CsCl	Cesium chloride
C-terminal	Carboxy-terminal
DABCO	1,4- Diazabicyclo[2,2,2]octane 98%)
DAPI	4',6-Diamidino-2-phenylindol
DC	Dendritic cell
ddH_2O	Double distilled water
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
Ds	Double-stranded
EDTA	Ethylenedinitrilotetraacedic acid
EEA1	Early endosomal antigen 1
e.g.	exempli gratia
EGTA	Ethylene glycol bis2-aminoethyl)-tetraacetic acid
EtBr	Ethidiumbromide
ELISA	Enzyme linked immunosorbent assay
Enh	Enhancer
FCS	Fetal Calf serum
GFP	Green fluorescent protein
GM130	Golgi matrix protein 130
GMCSF	Granulocyte-makrophage colony stimulating factor

h	Hour
HBsAg	S antigen of HBV
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HNF	Hepatic nuclear factor
HRP	Horse radish peroxidase
HSP90	Heatshock protein 90
l	Liter
lamp1	Lysosome associated membrane protein 1
Ig	Immunglobulin
IL-4	Interleucin-4
kb	Kilo base
kDa	Kilo dalton
LPS	Lipopolysaccharid
mA	Milli-Ampere
mDC	Myeloid dendritic cell
µg	Microgram
µl	Microliter
µM	Micromolar
mg	Milligram
MHC	Major histocompatibility complex
Min	Minute
ml	Milliliter
mM	Millimolar
moDC	Monocyte derived dendritic cell
MOI	Multiplicity of infection
mRNA	Messenger RNA
MRP2	Multidrug resistant protein 2
NEAA	Non-essential amino acids
nM	Nanomolar
N-terminal	Amino-terminal
nt	Nucleotide

OD	Optical density
ori	Origin of replication
ORF	Open reading frame
PAA	Polyacrylamid
PAGE	Polyacrylamid gel electrophorese
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
Pg	Pregenomic
PGE_2	Prostaglandin E_2
PHH	Primary human hepatocytes
P/S	Penicillin/Streptomycin
Rab6	Ras-like GTPase 6
Rc	Relaxed circular
RFP	Red fluorescent protein
RLU	Relative light units
Sec	Second
Ss	Single-stranded
SVP	Subviral particle
TAE	Tris-Acetat-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylendiamin
TGN	*trans*-Golgi network
TNFα	Tumor necrosis factor α
Tween 20	Polyoxyethylensorbitanmonolaurat
V	Volt
VP	Virus particle
w/v	Weight per volume

7. Sequences

pCH -9/3091

(HBV sequence underlined)

ATGGACATCGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTG
ACTTCTTTCCTTCAGTACGAGATCTTCTAGATACCGCCTCAGCTCTGTATCGGGAAGCCTTAGA
GTCTCCTGAGCATTGTTCACCTCACCATACTGCACTCAGGCAAGCAATTCTTTGCTGGGGGGAA
CTAATGACTCTAGCTACCTGGGTGGGTGTTAATTTGGAAGATCCAGCGTCTAGAGACCTAGTAG
TCAGTTATGTCAACACTAATATGGGCCTAAAGTTCAGGCAACTCTTGTGGTTTCACATTTCTTG
TCTCACTTTTGGAAGAGAAACAGTTATAGAGTATTTGGTGTCTTTCGGAGTGTGGATTCGCACT
CCTCCAGCTTATAGACCACCAAATGCCCCTATCCTATCAACACTTCCGGAGACTACTGTTGTTA
GACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAGGTCTCAATCGCC
GCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAGTATTCCTTGGACTCATAAGGTGGG
GAACTTTACTGGGCTTTATTCTTCTACTGTACCTGTCTTTAATCCTCATTGGAAAACACCATCT
TTTCCTAATATACATTTACACCAAGACATTATCAAAAAATGTGAACAGTTTGTAGGCCCACTCA
CAGTTAATGAGAAAAGAAGATTGCAATTGATTATGCCTGCCAGGTTTTATCCAAAGGTTACCAA
ATATTTACCATTGGATAAGGGTATTAAACCTTATTATCCAGAACATCTAGTTAATCATTACTTC
CAAACTAGACACTATTTACACACTCTATGGAAGGCGGGTATATTATATAAGAGAGAAACAACAC
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TTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACCACCAGTTGGATCCAGCCTTCAGAGCAAA
CACCGCAAATCCAGATTGGGACTTCAATCCCAACAAGGACACCTGGCCAGACGCCAACAAGGTA
GGAGCTGGAGCATTCGGGCTGGGTTTCACCCCACCGCACGGAGGCCTTTTGGGGTGGAGCCCTC
AGGCTCAGGGCATACTACAAACTTTGCCAGCAAATCCGCCTCCTGCCTCCACCAATCGCCAGTC
AGGAAGGCAGCCTACCCCGCTGTCTCCACCTTTGAGAAACACTCATCCTCAGGCCATGCAGTGG
AATTCCACAACCTTCCACCAAACTCTGCAAGATCCCAGAGTGAGAGGCCTGTATTTCCCTGCTG
GTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCTGACTACTGCCTCTCCCTTATCGTCAATCTT
CTCGAGGATTGGGGACCCTGCGCTGAACATGGAGAACATCACATCAGGATTCCTAGGACCCCTT
CTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACT
CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGCCAAAATTCGCAGTC
CCCAACCTCCAATCACTCACCAACCTCTTGTCCTCCAACTTGTCCTGGTTATCGCTGGATGTGT
CTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTC
TGGACTATCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCCTCAACAACCAGCACGGG
ACCATGCCGGACCTGCATGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACC
AAACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATTCC
TATGGGAGTGGGCCTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTT
CGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGT
CTGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACA
TTTAAACCCTAACAAAACAAAGAGATGGGGTTACTCTCTAAATTTTATGGGTTATGTCATTGGA
TGTTATGGGTCCTTGCCACAAGAACACATCATACAAAAAATCAAAGAATGTTTTAGAAAACTTC
CTATTAACAGGCCTATTGATTGGAAAGTATGTCAACGAATTGTGGGTCTTTTGGGTTTTGCTGC
CCCTTTTACACAATGTGGTTATCCTGCGTTGATGCCTTTGTATGCATGTATTCAATCTAAGCAG
GCTTTCACTTTCTCGCCAACTTACAAGGCCTTTCTGTGTAAACAATACCTGAACCTTTACCCCG
TTGCCCGGCAACGGCCAGGTCTGTGCCAAGTGTTTGCTGACGCAACCCCCACTGGCTGGGGCTT
GGTCATGGGCCATCAGCGCATGCGTGGAACCTTTTCGGCTCCTCTGCCGATCCATACTGCGGAA
CTCCTAGCCGCTTGTTTTGCTCGCAGCAGGTCTGGAGCAAACATTATCGGGACTGATAACTCTG
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GCGCGGGACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCTGCGGACGACCCTTCTCGGGGT
CGCTTGGGACTCTCTCGTCCCCTTCTCCGTCTGCCGTTCCGACCGACCACGGGGCGCACCTCTC
TTTACGCGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCT
GCACGTCGCATGGAGACCACCGTGAACGCCCACCAAATATTGCCCAAGGTCTTACATAAGAGGA
CTCTTGGACTCTCAGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTTTGTTTAA
AGACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCAT
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GC

pCH GFP An-b-glob

(GFP sequence underlined)

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mRFP1_pRSETB
(RFP sequence underlined)

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pCH mRFP An-b-glob

(RFP sequence underlined)

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pCH S-Ag An-b-glob

(S sequence underlined)

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pCH GFP-S An-b-glob

(GFP-S sequence underlined)

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pCH GFP-sp-S An-b-glob

(GFP-sp-S sequence underlined, spacer in small letters)

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pCH S-GFP An-b-glob

(S-GFP sequence underlined)

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pCH M-GFP An-b-glob

(M-GFP sequence underlined)

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pCH L-GFP An-b-glob

(L-GFP sequence underlined)

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pCH S-RFP An-b-glob

(S-RFP sequence underlined)

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pCH M-RFP An-b-glob

(M-RFP sequence underlined)

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Publikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Thomas Langer und Herrn Prof. Herbert Pfister betreut worden.

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(Uta Zedler)

Teilpublikation:

Dendritic Cells Take Up Viral Antigens But Do Not Support the Early Steps of Hepatitis B Virus Infection.

Andreas Untergasser, Uta Zedler, Anja Langenkamp, Maria Quasdorff, Marianna Hösel, Knud Esser, Hans-Peter Dienes, Barbara Tappertzhofen, Waldemar Kolanus and Ulrike Protzer.

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