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TOPIC HIGHLIGHT

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Avian hepatitis B viruses: Molecular and cellular biology, phylogenesis, and host tropism

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Abstract

The human hepatitis B virus (HBV) and the duck hepatitis B virus (DHBV) share several fundamental features. Both viruses have a partially double-stranded DNA genome that is replicated via a RNA intermediate and the coding open reading frames (ORFs) overlap extensively. In addition, the genomic and structural organization, as well as replication and biological characteristics, are very similar in both viruses. Most of the key features of hepadnaviral infection were first discovered in the DHBV model system and subsequently confirmed for HBV. There are, however, several differences between human HBV and DHBV. This review will focus on the molecular and cellular biology, evolution, and host adaptation of the avian hepatitis B viruses with particular emphasis on DHBV as a model system.

Key words: Hepadnavirus; Pararetroviruses; Evolution; Host range; Genome; Structure, Virions; Subviral particles; *In vitro* and *in vivo* infection; Transport; Fusion; Endocytosis; Hepatocellular differentiation; cccDNA; Gene expression; Morphogenesis and secretion

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INTRODUCTION

Full understanding of the molecular biology of the human hepatitis B virus (HBV) is hampered by a variety of experimental restrictions. There is no small animal model system available for infection studies and only few aspects of the viral life cycle are accessible to biochemical methods. A complete viral infection cycle mimicking natural HBV infection *in vitro* could only be achieved until recently with primary human hepatocytes. The disadvantages of this system are: (1) restricted accessibility to the cells, (2) infection inefficiency and (3) high variability in infection assays. The recent establishment of the HepaRG cell line is therefore a major breakthrough and allows HBV infection studies under defined conditions for the first time[1].

This review will focus on one of two established animal virus models; i.e., the DHBV model system. The human HBV and DHBV share several fundamental features. Both viruses have a partially double-stranded DNA genome that is replicated via an RNA intermediate and the coding open reading frames (ORFs) overlap extensively. In addition, the genomic and structural organization, as well as replication and biological characteristics, are very similar in both viruses. They both infect hepatocytes preferentially and have a very similar life cycle. Most of the key features of hepadnaviral infection were first discovered in the DHBV model system and subsequently confirmed for HBV. This includes replication of the viral genome by reverse transcription of a RNA intermediate[2], mechanisms of covalently closed circular (ccc) DNA formation and amplification[3], details of reverse transcription[4], and determinants of host tropism[5].

In light of the above mentioned austerities for HBV, the DHBV model of hepatitis B virus infection remains a convenient and reliable system that offers several unique advantages. Most importantly, steady availability and highly reproducible infection of primary duck hepatocytes (PDHs) provide the optimal basis for *in vitro* and *in vivo* studies of the molecular and cellular biology of hepatitis B virus infection under defined and controlled conditions. In addition, the chicken hepatoma cell line LMH produces progeny virus after transfection with cloned, mutant or wt DHBV genomes, which can be used to infect PDHs or ducks[6].

Thus, the DHBV model system is a unique system that allows elucidation of the hepadnaviral life cycle in considerable detail. However, there are several differences

Table 1 Comparison of HBV and DHBV

	HBV	DHBV
Natural host	Human	Pekin duck
Related viruses	WM-HBV, WHV	HHBV, SGHBV
Pathogenicity		
Chronic infection	yes	yes
Liver injury	yes	no
Experimental systems		
Cell transfection	yes	yes
In vitro infection	yes	yes
Transgenic mouse	yes	no
Small animal model	no	yes

WMHBV: woolly monkey HBV; WHV: woodchuck HBV; HHBV: heron HBV; SGHBV: snow goose HBV.

between human HBV and DHBV. First of all, DHBV infection normally results in chronicity since the virus is transmitted from the hen to the egg (see[7] and references therein). This infection usually does not lead to liver injury and the infected duck remains healthy throughout life. When an adult duck is infected, the infection is usually eliminated. When HBV is transmitted from mother to child, it also often results in chronic infection. However, in a large number of cases, this leads to liver injury and development of hepatocellular carcinoma or cirrhosis. When an adult is infected, this can either result in fulminant, acute or chronic hepatitis when the virus is not eliminated. Another difference between DHBV and HBV is expression of the X protein (for further differences see Table 1). This regulatory protein, with not fully understood function, is expressed by HBV from a conventional ORF, but in DHBV, an unusual cryptic ORF is used.

In the last two decades, parts of the hepadnaviral life cycle, especially the replication strategy, could be undeceived in considerable detail. In contrast, there is very little information available on the infectious entry or secretion pathway. The cellular partners involved in cell-virus interactions at these stages of infection remain unidentified and the molecular determinants of host specificity, hepatotropism and the nature of the receptor complex still await discovery.

For more information about human HBV and the other corresponding model systems please see the comprehensive reviews in this issue.

AVIAN HEPATITIS B VIRUSES

The duck hepatitis B virus was discovered in 1980 by William Mason and colleagues[8]. They found a virus very similar to HBV in about 10% of Pekin ducks from two different sources in the USA and they pointed out that no abnormal level of mortality or signs of hepatitis were found in the infected ducks. Naturally occurring DHBV infections have been reported in Pekin ducks and related species from China, USA, Canada, Europe, India, and South Africa[9-11].

Since then, avihepadnaviruses have been detected in various duck species[10] including exotic ducks and geese[10] (DHBV), in snow geese[12] (SGHBV), a Ross' goose

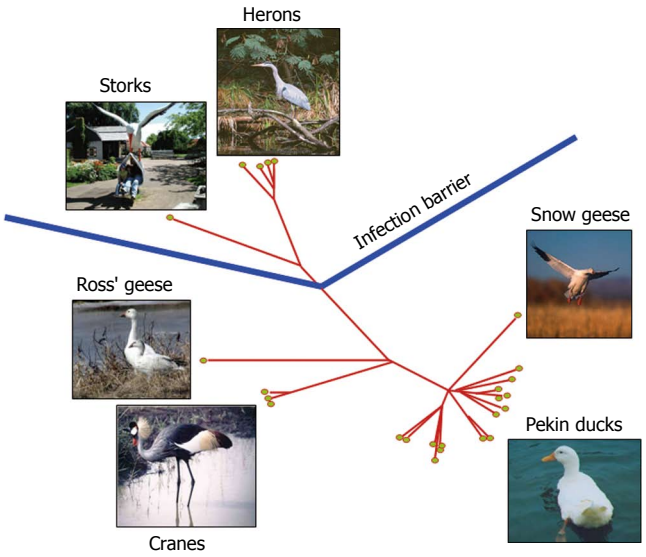

Figure 1 Phylogenetic tree of avian hepadnaviruses and related hosts. Phylogenetic relationship of all known avian hepadnaviruses based on preS/S gene sequence. The corresponding natural hosts are also indicated.

(RGHBV, GenBank Acc.No. M95589), white storks[13] (STHBV), demoiselle and grey crowned cranes[14] (CHBV) as well as grey herons[15] (HHBV). Like their mammalian counterparts, avihepadnaviruses have a rather narrow host range. For instance, DHBV infects only certain duck and goose species but neither infects Muscovy ducks nor chickens[16]. Little is known about the host range of HHBV or STHBV. Despite its substantial sequence homology with DHBV, HHBV does not infect ducks and only very inefficiently primary duck hepatocytes[15]. Recently, we reported that cranes are naturally infected with a hepatitis B virus, designated CHBV[14]. Cranes are phylogenetically very distant from ducks and are more closely related to herons and storks (Figure 1). Interestingly however, CHBV infects primary duck hepatocytes with similar efficiency as DHBV. Collectively this and related data suggest that the host range of hepadnaviruses cannot be simply predicted based on the evolutionary relatedness of their respective hosts. For a comprehensive sequence comparison and a phylogenetic tree of the host birds see[14].

Phylogenetic analysis of the various isolates demonstrated a rather high variability among DHBV strains, whereas genomes from other avihepadnaviruses, such as stork or crane hepatitis B virus, appear less variable. HHBV infection occurs not only in captive grey herons, but also with high prevalence in free-living birds. We have detected HHBV in another heron species (great blue heron) as well as in two of its sub-species (great white heron and Würdemann's heron). Thus, the virus persists in free-living birds and is an endogenous virus of several heron species.

DHBV will certainly remain the most important avihepadnavirus for research purposes since the infection system with PDHs and the important research tools are well established. It is possible to generate mutant viruses after transfection of cloned DHBV genomes into the chicken hepatoma cell line LMH[6]. Thus, different mutations can be studied not only concerning their effects

on replication but also on infection efficiency and events. In addition, *in vivo* infections can be performed in ducks without the need to establish expensive handling facilities and without risk. In these ducks not only the host range of hepadnaviruses can be studied in considerable detail (in addition to *in vitro* studies), but also the activity and toxicity of antiviral substances can be addressed[17].

THE AVIHEPADNAVIRAL LIFE CYCLE: AN OVERVIEW

Avian hepatitis B viruses belong to the family of DNA viruses that replicate their DNA genome by reverse transcription of an RNA intermediate. Thus, they belong, together with the cauliflower mosaic virus, to the group of pararetroviruses that do not integrate their genome into host cell chromosomes.

A schematic view of the DHBV genome and structure of virions is shown in Figure 2. The genome of DHBV has an unusual design. It consists of a ca. 3000 bp partially double-stranded DNA. The circularity of the genome is achieved by overlapping cohesive 5' ends[18]. The coding negative strand is complete and even has a short terminal redundancy but is not covalently closed. Its 5' end is covalently attached to the viral polymerase P[19]. The positive strand is not complete but encompasses between 40% and 85% of the genome[20]. However, the length of the gap varies among different hepadnaviruses and is smallest in DHBV[18]. Attached at the 5' end of the positive strand is a short ribooligonucleotide, which is a remnant of the pregenomic RNA (pgRNA)[21]. Both 5' terminal structures function as primers during viral replication[21]. As another particularity, the viral genome has two direct repeats (DR) with a length of 11 base pairs that have important functions in replication.

The viral genome organization is very compact and economic. All nucleotides have a coding function in at least one of the four open reading frames (ORFs). Regulatory sequences such as enhancers and promoters, as well as several cis-acting elements, overlap with coding regions. The first ORF encodes the surface proteins L and S, the second codes for the capsid protein as well as the e-antigen, the third for the polymerase and a cryptic fourth for the X protein[22].

Two different types of spherical viral particles can be detected in the serum of infected ducks: infectious virus particles (virions) with a diameter between 40 and 60 nm and subviral particles (SVPs) with a diameter between 30 and 60 nm, which lack the 27 nm nucleocapsid, including the viral genome[23]. After transfection of cell lines, a third particle entity can be detected in the cell culture supernatant. These cells secrete so called 'naked capsids' lacking the viral envelope for unknown reasons.

The virus is surrounded by a lipid envelope, which presumably originates from the host cell endoplasmic reticulum (ER) membrane, and contains both viral surface proteins. For HBV, it has been shown that the membrane is rich in phosphatidyl choline (60% of all lipids) as well as cholesterol (30% of all lipids)[24] and thus differs from the lipid composition of the cellular ER membrane,

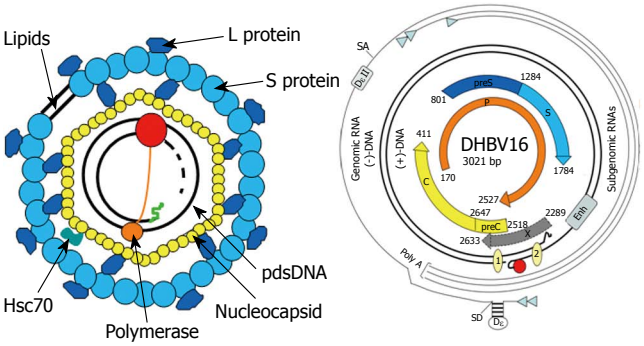

Figure 2 Virion structure and genome organization of avian hepadnaviruses. The viral envelope is derived from hepatocellular membranes and contains the viral surface proteins S and L. It covers the nucleocapsid harbouring the viral genome with the covalently linked terminal protein domain (TP, orange circle) of the polymerase (P, red circle) and cellular proteins like Hsc70. The genome is organized as depicted. The various transcripts are indicated by thin lines with the small arrowhead indicating the start sites. The partially double stranded, viral DNA with the covalently bound TP domain of P (red circle) is symbolized by the thicker lines. The numbered circles 1 and 2 on the viral DNA represent the direct repeats (DR). Enh represents the enhancer domain. The ORFs encoding core (C), polymerase (P), and the surface proteins (preS and S) are symbolized by thick arrows. Epsilon (Dε) is the stem loop structure on the pgRNA which acts as an encapsidation signal and replication origin. The second encapsidation element DεII is unique to avian hepatitis B viruses, since the mammalian counterparts lack this RNA structure. SD and SA represent the major splice donor and acceptor sites, respectively.

implicating that lipids are actively selected for the viral membrane. In total, the amount of lipids in comparison to the amount of protein is very small, in SVPs the weight ratio is about 1:4[25]. Thus, the lipids in the viral envelope are presumably not forming a lipid bilayer but protein aggregates seem to be separated through short lipid regions[26]. This strongly restricts the lateral mobility of the envelope components. The lipid composition of the DHBV envelope remains to be determined.

SVPs largely consist of the viral surface proteins S and L, which are incorporated into the envelope. They do not contain viral DNA and are thus not infectious. This entity is secreted from infected cells in excess compared to virions. It is assumed that per virion 1000 to 10 000 SVPs are secreted[27]. SVPs can compete with viral binding and thus infection[28]. In contrast, it has been shown that SVPs enhance infection when a very low multiplicity of infection (MOI) is used, which indicates an important role in the viral life cycle[29].

Complete virions contain an icosahedral capsid inside the envelope, which is about 35 nm in diameter as seen in cryo-electron microscopy or 27 nm in negative stain pictures and consists of 240 subunits of core protein[30]. The nucleocapsid holds the viral DNA genome with the covalently attached polymerase.

Cellular components can additionally be packed into viral particles. This is the case for Hsc70, which was detected in large amounts[31]. The identity and function of other proteins of cellular origin are still unknown.

A schematic view of the viral life cycle is shown in Figure 3. The life cycle of DHBV starts with attachment of the viral particles to their receptor/receptor complex on the surface of the target host cell. This is mediated by the preS domain of the viral L protein that binds to

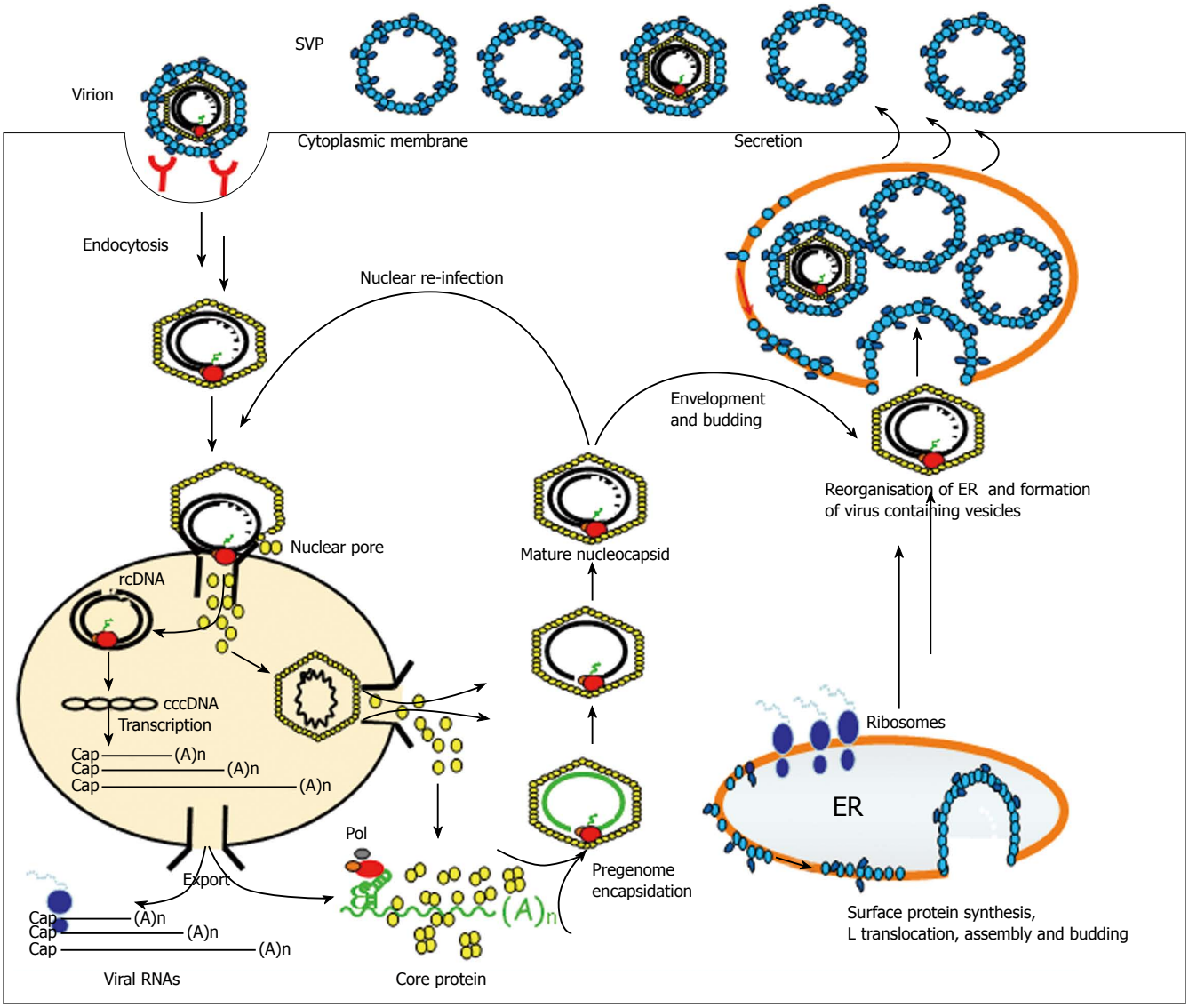

Figure 3 Model of the hepadnaviral life cycle. The hepadnaviral particle binds the hepatocyte via a specific receptor complex/molecule. It then enters the cell via endocytosis and is transported intracellularly in a MT-dependent fashion. After release out of the endosomal compartment, the nucleocapsid containing the viral DNA binds the nuclear pore complex and the viral genome is released into the nucleus. It is also possible that the whole nucleocapsid enters the nucleus and releases the viral DNA inside. Afterwards, the viral rcDNA is converted into cccDNA and viral transcripts are made. These are exported into the cytosol where the pgRNA is packaged together with the viral polymerase into the nucleocapsid. Inside the capsid, the RNA is reverse transcribed into the viral DNA genome. These matured nucleocapsids can then either be transported to the nucleus to add to the cellular cccDNA pool or they interact with viral surface proteins on the cellular ER membrane. There, the nucleocapsid buds into the lumen of the ER and is then transported in a yet undefined fashion to the cell surface where the viral particle is released into the cell exterior.

unknown cellular receptor compounds. After binding, viral particles are taken up into the cell by receptor-mediated endocytosis[32-34]. Then, the nucleocapsid is released from the endosomal compartment into the cytosol. It is currently under discussion whether a low endosomal pH is necessary for this release. The cytoplasmic nucleocapsids are then transported to the nucleus to initiate productive infection. This transport strongly depends on active microtubules (MT) but not on actin[27].

After reaching the nuclear membrane, the core protein is presumably phosphorylated and exposes a nuclear localization signal. This results in binding of nuclear factors; e.g., importins, to the capsid, which leads to uptake of the whole complex into the nucleus[35]. However, disassembly of the capsid near the nuclear membrane and subsequent transport of the viral DNA into the nucleus cannot be excluded.

In the nucleus, the relaxed form of the viral genome, the rcDNA (relaxed circular), is converted into the covalently closed, circular form (cccDNA). This is only possible after removal of the 5'-terminal structures (protein and RNA), repair of the gap and covalent ligation of the strands by cellular proteins. The cccDNA is usually not incorporated into the host genome but is organized in nucleosomes in the form of an episome[36].

Transcription of the viral genome is mediated by the cellular RNA polymerase Ⅱ[37]. *In vivo*, three different viral RNA classes could be identified, which are all polyadenylated and posses a cap structure[21]. They all have the same 3' end since only one polyadenylation site is present in the viral genome but have different 5' ends due to different transcription initiation sites. The different viral

RNAs are transported into the cytoplasm and translated. The two longest RNAs (3.5 kb), which stretch the whole genome, have two different functions. They code for the viral proteins core, the polymerase and e-antigen but one also serves as the pregenome[21]. Part of the 3.5 kb mRNA is spliced and serves as mRNA for L protein synthesis. A second class of viral RNAs (2.1 kb) encodes for the large surface protein, and the third class (1.8 kb) codes for the small surface protein, which is the most abundant one. The identity of the X-encoding RNA is unknown.

After export into the cytosol, the viral RNAs are translated and the viral surface proteins are directly inserted into the ER membrane. Once inserted, they can autonomously form subviral particles or interact with capsids to form virions.

In the cytosol, core protein dimers interact with each other and self assemble with the viral polymerase and the pgRNA into ribonucleoprotein complexes[38]. The pgRNA is packaged upon a chaperone-mediated interaction of the polymerase with the stem loop structure epsilon (ε). This structure also serves as the replication origin for the reverse transcription. Prior to packaging, the core protein is phosphorylated[39]. It is currently unclear whether reverse transcription initiates during ribonucleoprotein complex formation or after assembly of the capsid. However, when the pgRNA is inside the capsid, the particles mature, e.g. the RNA is reverse transcribed into the viral DNA genome and the core proteins are dephosphorylated[2,40]. The mature capsids interact with the viral surface proteins at the membrane of the ER and bud into the lumen, thus forming complete virions. The enveloped virions are then presumably transported through the constitutive secretion pathway to the cell surface and are released there. Alternatively, mature capsids can be transported to the nucleus and add to the cccDNA pool. After a successful infection, between one and 20 cccDNA molecules can be detected inside the nucleus[41]. This re-infection cycle preferentially occurs during establishment of an infection when the levels of large surface protein are low[42]. After a successful infection, the levels of L inside the cell rise and capsids preferentially form virions and are secreted.

In the absence of mature capsids and due to the autonomous budding activity of the viral surface proteins, subviral particles are formed. S as well as L proteins seem to accumulate in membrane domains of the ER where they reach quite high densities. When they reach a critical density, they presumably bud into the ER lumen and form subviral particles.

DHBV PROTEINS AND THEIR BASIC FUNCTIONS IN VIRAL ENTRY, REPLICATION, AND MORPHOGENESIS

Core protein and e-antigen

The viral core protein fulfils several opposing functions during the different stages of the viral life cycle; e.g., nucleic acid binding and assembly opposed to disassembly and nucleic acid release. These diverse functions are partly regulated by: (1) subcellular localization, (2) quaternary structure and (3) posttranslational modification by phosphorylation and dephosphorylation of its C-terminus.

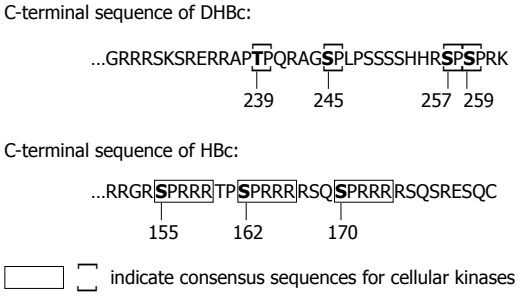

Figure 4 Phosphorylation sites of the viral core protein. The C-terminal sequence of the core proteins from DHBV and HBV are shown. Red brackets indicate the phosphorylation motifs, bold letters indicate the phosphorylated amino acid residue.

The viral nucleocapsid is composed of dimeric subunits of the viral core protein (DHBc) with a molecular weight of 32 kDa. The N-terminal region of the core protein (up to about 144 aa) is acetylated[43] and sufficient for assembly of the capsid shell[44,45]. The carboxyterminal end of the protein, the so called C-terminal domain (CTD), is extremely basic and binds nucleic acids. This is essential for packaging of the pgRNA into the nucleocapsid as well as progression of reverse transcription within[44,46]. In addition, a nuclear localization signal is present between aa 184 and 226 along with a nuclear export signal[47].

The major phosphorylation sites in the core protein are mapped to the arginine-rich C-terminus. This domain contains consensus sequences for different cellular kinases, such as PKC, SRPK1 and SRPK2 (Figure 4). Furthermore, Thr174 is highly conserved and is a cdc2 kinase phosphorylation site. Compared to the extracellular core protein, intracellular core is highly phosphorylated. Mutational analysis of the major phosphorylation sites revealed that individual or combined substitution had no overt effect on pgRNA packaging. However, the S245A mutant was deficient in genome maturation[48]. It has been shown that the CTD contains several phosphosites, which are heterogeneously phosphorylated intracellularly and hypophosphorylated or non-phosphorylated in the secreted virion[40,43]. This dephosphorylation, which occurs as nucleocapsids mature (meaning that the pgRNA is reverse transcribed into the rcDNA genome), is thought to be a maturation signal that results in secretion of only fully matured virions containing the DNA genome[43]. In addition, it has been shown that binding of hepadnaviral capsids to the nuclear pore complex depends on the phosphorylation status of the core protein[35]. The kinases or phosphatases implicated in these steps are not known. This is also true for the fate of the nucleocapsids after nuclear binding; it is not known whether they disassemble at the nuclear pore and thereafter release the viral DNA or if they are transported through the nuclear pore and then disassemble.

DHBc has the autonomous ability to assemble into particulate structures, which is dependent on the DHBc concentration[49]. The nucleocapsid is a dynamic structure and subject to regulated conformational transitions. Formation of progeny virions requires stable

nucleocapsids, whereas during viral entry, the incoming viral structure must disassemble and release the viral genome. Furthermore, reverse transcription of the viral genome occurs within the capsid and it is thought that this is linked with structural rearrangements in the capsid.

The ultrastructural analysis of the HBV core protein revealed that the monomeric form is dominated by a long alpha-helical hairpin structure[45]. The first step of capsid formation is the homodimerization of two core protein monomers that arrange in a way that leads to an antiparallel order, which results in an almost exact twofold symmetry. As a result, the characteristic spike on the capsid surface is formed by a compact four helix bundle. These spikes are the regions that interact in the virion with the viral envelope structures. Preliminary 3D reconstruction of the DHBV capsid suggests that the protein forms T-shaped dimers similar to the human core protein (M. Nassal, personal communication).

The open reading frame that encodes DHBc also codes for a nonstructural viral protein, which is the DHBe or precore. This e-antigen is, compared to the DHBc, truncated at the C-terminus and extended on the N-terminus. In addition it contains a type I signal recognition sequence. It is translocated into the ER during synthesis where the signal recognition sequence is cleaved and the C-terminus removed. After this processing, e-antigen is transported through the Golgi complex and secreted from the infected cell[50,51]. It has been shown that the e-antigen is glycosylated[50]. The glycosylation pattern seems to differ among the different avian hepadnaviruses and even among different isolates of DHBV due to the presence of different numbers of N-glycosylation sites. This is, for instance, obvious in a recent study of HHBV e-antigen that has one glycosylation site. This resulted in two bands in immunoblot analysis (one for glycosylated and for non-glycosylated e-antigen), while CHBV e-antigen with two N-glycosylation sites showed three bands on the immunoblot[14].

DHBe can be detected in the serum of infected ducks[52] and serves as a marker for efficient viral replication. Its exact function is unknown, but it has been shown that it plays no essential role in viral replication, morphogenesis or infectivity[53]. However, it seems to play a role in the establishment of chronic infections[54] and its absence may confer a growth advantage of precore-minus mutants over wildtype virus[55].

Polymerase

The viral polymerase is a multifunctional protein of about 90 kDa in size. It has a DNA-dependent DNA polymerase activity/domain to fill the gap in the viral DNA during replication[56] and an RNaseH activity/domain to selectively digest RNA from an RNA-DNA-hybrid molecule[57], as it has been shown for HBV. It also has reverse transcriptase (RT) activity to transcribe the RNA pregenome into the DNA genome during replication[2]. The polymerase homology domain is centrally located, whereas the RNaseH homology domain is located near the C-terminus of the protein. Viruses with point mutations in these regions are either defective for viral DNA synthesis or only allow negative-strand but not positive-strand DNA synthesis[56]. Most N-terminal sections of the polymerase domain are spacer regions without any other apparent function[58]. Most N-terminal sections are the region implicated in the covalent linkage of the P protein to the viral DNA often referred to as terminal protein.

In the process of viral genome replication, during which the pregenomic RNA is transcribed into the genomic DNA, the different domains can assert their different functions. The pgRNA is transcribed from the viral cccDNA by cellular polymerase Ⅱ. This RNA is then transported into the cytosol and binds the viral polymerase and the core proteins. The polymerase recognizes the epsilon and another downstream region on the viral RNA and binds there with the help of cellular proteins like Hsp90[59]. Inside the particle, the RNA is transcribed into the negative strand DNA by the RT domain of the polymerase. This process is primed by the protein itself and tyrosine 96 of the P protein serves as a primer[60]. This results in covalent attachment of the nascent DNA strand onto the terminal protein part of the polymerase. After attachment of about 4 nucleotides, this DNA product is transferred to the DR1 sequence on the viral pgRNA. This is possible since the epsilon signal and the DR1 share a 4 nucleotide identity. From this position, the DNA negative strand is elongated by the conventional mechanism[61,62]. As this elongation proceeds, the template RNA is degraded by the RNaseH activity of the viral polymerase[2,63]. The end product of this reaction is a negative-stranded DNA, which is terminally redundant by about 8 nucleotides.

Positive strand synthesis is initiated at DR2 and primed by a short oligoribonucleotide, which is a remnant from the pgRNA[64]. This primer is transferred to a complementary region at the 5' end of the negative strand DNA for positive strand synthesis. From there, synthesis of the positive strand proceeds.

Sometimes (in about 1% to 5% of cases) the primer fails to translocate. This results in a process called *in situ* priming, where a fully duplex linear DNA is the end product[65]. This is a dead end for viral replication but may be the cause for integration of some viral genomes into the chromosomal DNA of the host cell.

Elongation of the positive strand proceeds until the 5' end of the negative strand is reached. Then, an intramolecular strand transfer is needed to complete positive strand synthesis. This happens after circularization of the genome, which is possible because of the short redundancies at each strand end. After the circularization, positive strand synthesis can proceed. Usually elongation does not proceed until the end of the template is reached. In the case of DHBV, normally about 80% of the positive strands complete elongation[20].

Envelope proteins

The multiple functions of the viral envelope proteins are reflected by the domain organization and unique biochemical features. The DHBV envelope proteins are encoded by a single ORF consisting of the preS and S domain. The viral envelope proteins are inserted into the membrane of the virus that originated from intracellular membranes (presumably the ER) of the infected cell. In the case of DHBV, these envelope proteins are the small

protein S, which constitutes about 80% of total envelope proteins, and the large protein L, which constitutes about 20%[66,67]. S protein is thus the most abundant protein in the viral envelope. It determines the envelope curvature and is indispensable for both budding and secretion of viral particles. Both viral surface proteins are unique compared to other viral surface proteins in their relatively complex structure and topology[68]. They have a molecular weight of 18 and 36 kDa, respectively, and are anchored in the membrane by several transmembrane domains. The C-terminus of both proteins are identical, while L is N-terminally extended by 161 aa compared to S. The length of this extension varies with the isolate. This unusual organisation results from differential transcription of a single ORF[67] (Figure 2).

The L protein is modified with myristic acid at its N-terminal glycine after removal of the first amino acid methionine, which presumably anchors the protein in the membrane in addition to the transmembrane domains[69]. This myristoylation is required for infectivity of the virus but not for assembly[69]. Unlike the envelope protein of HBV, DHBV L and S are not modified by N-glycosylation although they contain consensus glycosylation sites. In contrast, it has been shown that the DHBV L protein (p36) is the only surface protein that is phosphorylated[70,71]. This phosphorylation occurs at serine 118 by an ERK-type MAP kinase and is not essential for viral replication, particle formation or infectivity[71].

As transmembrane proteins, L and S are incorporated cotranslationally into the ER membrane. The proteins have four transmembrane domains (TM1 to TM4) that anchor them in the membrane and consist of membrane-spanning hydrophobic alpha-helices (Figure 5). In addition, the preS domain of the L protein contains two translocation motifs (TLM), which are 12 amino acid encompassing domains that form an amphipathic alpha helix[32]. TLMs mediate an energy- and receptor-independent transfer of peptides, nucleic acids and proteins when fused to them across membranes without affecting their integrity[72]. The DHBV-TLMs are located between amino acids 20-31 (TLM1) and 42-53 (TLM2) and required for viral infectivity.

Worth mentioning is the dual topology of the L protein[73]. After cotranslational insertion of the protein into the cellular membrane, part of the proteins changes the topology (Figure 5). This leads to exposure of the N-terminus to the cytosolic side of the membrane in about half of the L proteins while the other half has the N-terminus directed to the luminal side[74]. In this way, the protein can fulfil two different functions: it can interact with the cellular receptor on the outside of the virus and it can interact with the nucleocapsid on the inside. Whether S also adopts a dual topology remains to be determined.

The viral surface proteins are able to autonomously form subviral particles without the help of an interacting nucleocapsid. The exact mechanism of this phenomenon is not known, yet it is assumed that the proteins interact with each other to form microdomains in the ER membrane from which they bud when they reach a critical density. A prerequisite for this budding activity would be a tight interaction of the viral surface proteins with the membrane lipids and a membrane bending activity. When

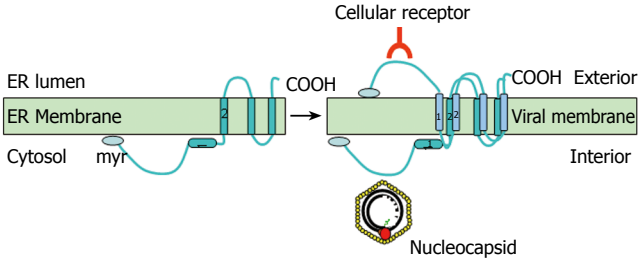

Figure 5 Dual topology of the viral surface protein L. The L protein is inserted into the ER membrane during synthesis with transmembrane domain 2 being inserted into the membrane. Half of the proteins then change their topology and insert the transmembrane domain 1 into the ER membrane. After this change and formation of the virus, L can exert its two basic functions; interaction with the nucleocapsid on the cytosolic preS domain and interaction with the host cell receptor on the surface of the viral particle.

L protein is expressed without the S component, particles are formed inside the cells, but are not secreted. This retention and secretion defect can be overcome by co-expression of the S protein[75]. This implies an important function for the S protein in secretion of viral particles.

Another function of the L protein is regulation of cccDNA amplification. As mentioned above, cccDNA is the replication template for all hepadnaviruses. It is a prerequisite for maintaining chronic infection of hepatocytes and is the main obstruction during antiviral therapy since it is mostly not eliminated from the cells. The amount of cccDNA inside the host cell nucleus is thus of great importance for the virus. To maintain its replication template in the nucleus, it re-infects and this process is regulated in an elaborate manner by the large viral surface protein[76]. During the early stage of infection when the amount of L protein is low inside the cell, mature core particles preferentially infect the nucleus to add to the cccDNA pool. Concomitant with the increase in cccDNA, the levels of L increase. This leads to redirection of the mature capsids into the secretory pathway and reduces cccDNA amplification.

The L protein is also responsible for superinfection exclusion, which prevents hepadnaviral infection of already infected hepatocytes[77]. This phenomenon was first ascribed to downregulation of the putative viral receptor protein gp180. This has not been confirmed and it has been suggested that superinfection exclusion may result from a role of L as a regulator of intracellular trafficking[77].

The L protein not only plays a role in viral morphogenesis or cccDNA amplification, but it is also implicated in additional regulatory functions such as signalling[71]. The cytosolically exposed preS domain of the L protein has the potential to activate gene expression from cellular promoter elements in *trans*. It is also phosphorylated by ERK2 at serine 118, which is induced by various stimuli and may play a role in intracellular virus-host crosstalk[71].

X protein

One major difference between the avian and mammalian hepatitis B viruses is the presence of an ORF called X in the latter. The function of this regulatory protein is still far from being understood. Recently it has been reported

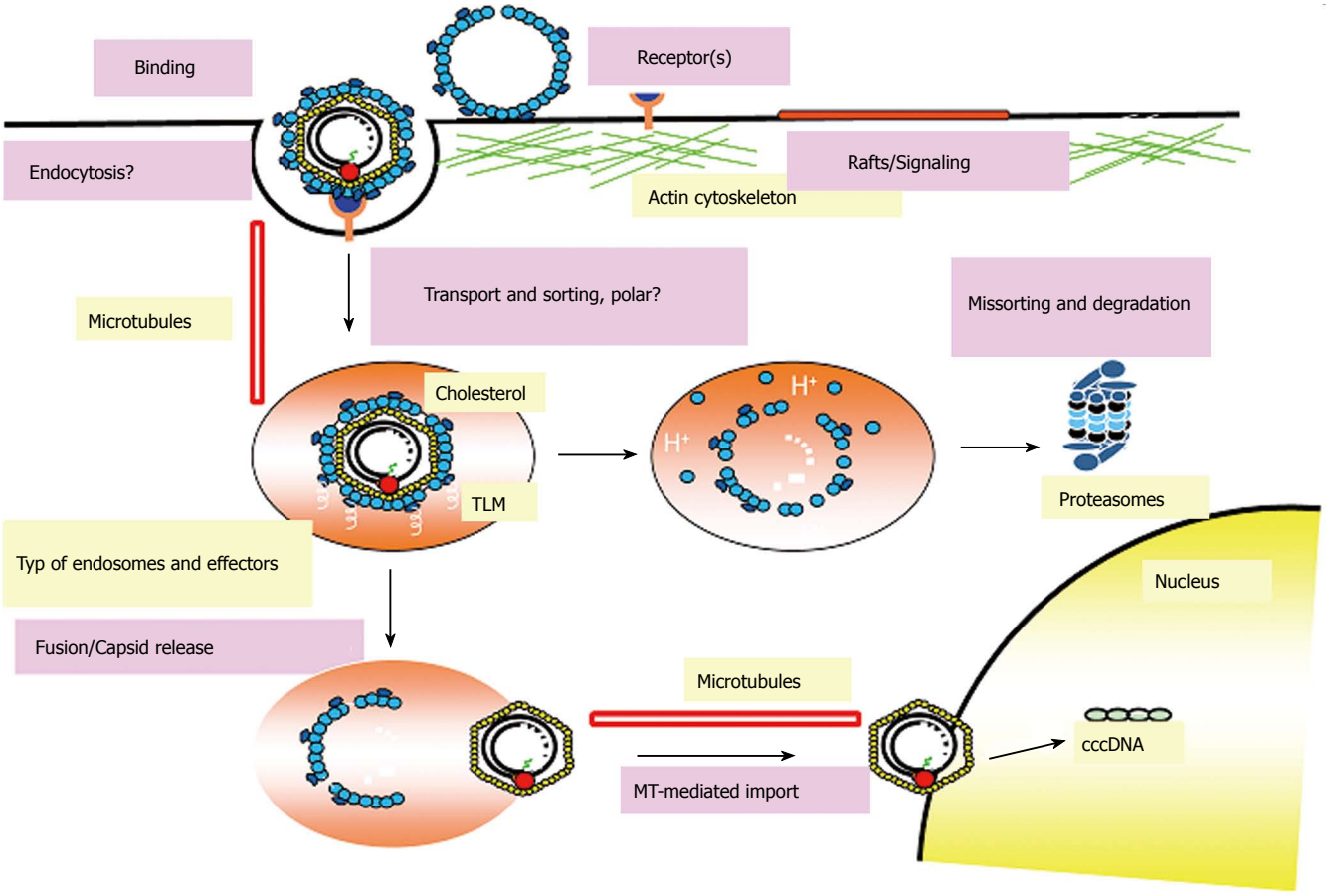

Figure 6 Current model for entry and intracellular trafficking of DHBV. The hepadnaviral life cycle starts with the attachment of virions to specific binding sites on the surface of hepatocytes mediated by the preS region of the large viral envelope protein L. Afterwards the virus enters the cell via endocytosis and resides in an endocytic vesicle which is transported in a MT-dependent and probably polar fashion. The endosomal release of the incoming particle requires the activity of the vacuolar H^+-ATPase and presumably the cholesterol of the viral envelope. After release, the particle is transported in a MT-dependent fashion mediated by dynein/dynactin to the nucleus and initiates infection after release of the viral genome at the nuclear membrane or within the nucleus. However, the majority of viral particles is missorted and degraded by the proteasome and other cellular proteases.

that X protein enhances the replication of transfected HBV genomes[78]. DHBV lacks an apparent X-ORF. But a cryptic X-like ORF has recently been reported[22]. *In vivo* experiments have suggested no functional role for this gene product in DHBV short term infection[79].

DHBV INFECTION

In vitro infection

Hepadnaviruses can only efficiently infect primary hepatocytes with the exception of the recently published HepaRG cell line, which can be infected with the human HBV[1]. Thus, primary hepatocyte cultures have to be established for use with hepadnaviruses. In the case of DHBV, either primary fetal or adult hepatocytes can be used. Fetal hepatocytes can be obtained easily from the livers of non-hatched duck embryos. These are digested with collagenase, washed and plated. This results in a mixed culture containing hepatocytes as well as other cells of the liver, which are the non-parenchymal cells, such as macrophages, sinusoidal endothelial cells, and fibroblasts. Alternatively, primary duck hepatocyte cultures can be obtained from adult ducks by liver perfusion. This results in a rather homogenous suspension of cells containing high amounts of hepatocytes (up to 90%) compared to non-parenchymal cells. Compared to primary human hepatocytes, PDHs are about 20 times more permissive to hepadnaviral infection.

The cells are cultured in a standard medium containing hydrocortisone, insulin as well as DMSO. The DMSO is important for maintaining differentiation and thus infectability of the cells[80]. Under these culture conditions, the cells remain infectable for up to 2 weeks in culture and viral spread occurs. The DMSO as well as omitting serum from the cell culture medium are essential conditions for maintaining cellular infectability since addition of serum to the cultures decreases the amount of cellular receptor proteins on the cell surface[81].

Research with the *in vitro* model of DHBV infection has lead to the discovery of many different features of the hepadnaviral life cycle. However, while factors involved in the early and most vulnerable steps of the viral life cycle (Figure 6) have been identified for a variety of viruses, little is known for hepadnaviruses (for a review see[7] and references therein).

Hepatoma cell lines, which can replicate the viral DNA and produce progeny virus after transfection of the viral genome into the nucleus, are not permissive for infection with the virus itself[82]. This phenomenon led to the assumption that the absence of a viral receptor or

receptor complex on the cell surface of these cells is the major determinant for infection. Great effort was invested to identify these molecules. But to date, the molecules responsible for the viral uptake that leads to productive infection are still unknown, although carboxypeptidase D (gp180) has been shown to specifically interact with the viral L protein and leads to internalization of viral particles after heterologous expression[83-85]. However, this did not lead to productive infection of these cells. In addition, the protein has been shown to not only bind DHBV, but also the viral surface protein from heron HBV, which does not infect PDHs, and this protein is not liver specific. It thus cannot be the determinant for viral host range or tissue specificity. This shows that either additional factors are required for the infection or that gp180 plays no role in the productive uptake and binding of the virus.

Initial binding of hepadnaviral particles seems to involve a component with low affinity without saturation and a component with high affinity and saturation[28]. This indicates that the binding involves at least two determinants and thus components. Several competition experiments with recombinant preS peptides, neutralizing antibodies and SVPs showed that the preS region of the viral L protein is essential for viral binding and the establishment of an infection[26,28,86,87]. In addition, it has been shown that preS peptides that were myristoylated were much more efficient in preventing DHBV infection than non-myristoylated peptides, which indicates that the myristoylation of L plays an important role during infection[86].

To gain more insight into the enigmatical early steps of hepadnaviral infection, we recently characterized the early attachment and entry events of DHBV infection in PDHs[27]. To do so, we established a sensitive, PCR-based assay that allowed us to investigate viral binding and entry. This binding and entry assay showed that only a small proportion of the inoculum binds to the cell surface of hepatocytes. Also the overall number of particles that bind to the cell surface is quiet low, after 2 h at 4℃, only up to 10 virions and 10 000 SVPs per cell were detectable. This indicates that the number of hepatocellular surface binding sites is about 10 000 per cell. Binding was prevented by the use of neutralizing antibodies as well as suramin, which also prevented viral infection showing that the detected binding sites are relevant for productive infection.

The steps after viral binding also remain quite elusive. It was shown years ago that the kinetics of DHBV uptake is unusual since binding and entry seem to be very slow. For a maximal infection efficiency, cells have to be incubated with the virus up to 16 h[80]. We have recently shown that viral uptake indeed needs an unusually long time period[27]. 1 h after attachment only about 70% of bound DHBV was taken up and internalization was complete within 3 h. This shows that virus uptake itself takes a relatively long time period, but since after 3 h all bound virions were internalized, uptake is very efficient. In contrast, viral trafficking inside the cell does not seem to be efficient since a high proportion of viral particles are degraded after viral entry into the cell and thus cannot establish infection[34].

It has been shown previously that DHBV entry into PDHs requires energy, which indicates that cellular and/or viral processes actively take place and that DHBV is presumably entering the cell via endocytosis[33,34]. Studies addressing the pH-dependency of DHBV infection by the use of chemical substances that increase endosomal pH led to contradicting results[33,88-90]. However, the weight of data favours pH-independent entry as well as an endocytic mechanism and shows that the virus does not require passage through a highly acidic compartment. The effect of the vATPase inhibitors seem to be due more to effects on viral trafficking inside the cell than on the endosomal pH alone[34].

After the virus is taken up by the cell via endocytosis, it has to be transported to the nucleus to establish infection. In the nucleus, the viral rcDNA is converted into the cccDNA. This conversion is detectable within the first 24 h after virus inoculation[27,91]. Thus, after efficient viral uptake there is an unusually long gap of 13 to 17 h before the appearance of nuclear viral cccDNA. This gap suggests that there is a rate-limiting post entry step that preceeds cccDNA formation, which involves viral uncoating and nuclear genome transport, or is required for rc- to cccDNA conversion. The intracellular transport has been shown to be independent of the actin skeleton, which in contrast seems to restrict entry, and is strictly dependent on the microtubule (MT) network of the cell[27]. Overexpression of dynamitin, a cofactor subunit of the motor protein complex dynactin-dynein, which mediates transport along microtubules, also reduced DHBV infection (our unpublished data). To date it remains unknown whether the MT-dependent transport of the virus (or the nucleocapsid alone) occurs only at the stage of the endosome or if the virus alone also interacts with microtubules.

To deliver the viral DNA into the nucleus, it has to be released from the viral particle and, prior to that, the viral particle has to be released from the endosomal compartment it resides in. It has been shown that this involves an unusual mechanism that depends on the integrity of a so called TLM[32]. The TLM thus mediates release of the viral particle out of the endosome into which it initially entered.

The infectious entry pathway of hepadnaviruses appears to involve a series of highly coordinated and directional steps leading to the nuclear delivery of viral genomes essential for the establishment of a productive infection. These steps may, alone or in combination, determine the species and host cell tropism common to all hepadnaviruses.

In vivo infection

DHBV-infected ducks exhibit age-related outcomes of infection, which is similar to HBV-infected humans. In principle, hepadnaviruses have the ability to cause either a transient or chronic infection. When infected with DHBV, young ducks develop persistent infection whereas adult ducks become transiently infected and eliminate the virus[92]. These different outcomes are viral dose dependent; persistent infection in young ducks develops more frequently with higher doses of virus[92]. In some cases, dependent on age and infection with a given mutant,

experimental infections can cause symptoms of a liver inflammation[93]. It has been shown that one genome equivalent is sufficient to infect a duckling and that spread of the virus within the liver is very efficient: 14 d after inoculation, virtually all hepatocytes were infected[94]. In addition, it has been shown that the difference between the infection outcome in older and neonatal ducks depends on the production of neutralizing antibodies against the virus[95]. The rapid production of neutralizing antibodies in older ducks led to an efficient inhibition of viral spread in the liver.

The duck hepatitis B virus infects Chinese domestic and American pekin ducks as well as geese. Normally, infection takes place through vertical transmission from the infected hen to the eggs and results in a chronic infection that is without symptoms and is tolerated by the immune system[23]. The virus then replicates in the egg yolk sack and is transferred to the embryonic hepatocytes by day 6 of development[96]. Thus, all offspring from an infected hen are DHBV-positive.

When DHBV infection is persistent in the duck, viral replication mainly occurs in the hepatocytes of the liver. Usually, the level of viral replication is then very high, with most hepatocytes infected and expressing the viral antigens. This is also reflected in the amount of viral antigens circulating in the blood stream. Up to 10^{10} virions and 10^{13} SVPs can be detected per ml of serum[97].

In vivo infections with DHBV are often used to study the growth kinetics of viral mutants[79]. This allows elucidation of specific mutations in the viral genome on the behaviour of this respective mutant in their natural host. In contrast to the *in vitro* situation, the role of the immune system and the spread of the virus within the infected liver can be assessed.

It has been shown that the addition of lipopolysaccharides (LPS, endotoxin) to PDH cultures inhibited DHBV replication efficiently[98]. This was due to the release of interferon alpha and gamma from non-parenchymal cells (i.e. Kupffer cells, resident macrophages of the liver). The exact mechanism behind this phenomenon is not known.

HOST SPECIFICITY

All known hepadnaviruses are strongly, but not exclusively, cell type specific and have a narrow host range restricting them to their natural host and a few closely related species (Figure 1). DHBV, for example, infects only certain duck and goose species, but either does not or very inefficiently infects chicken or Muscovy ducks, respectively. Despite its substantial sequence homology with DHBV, the heron HBV (HHBV) does not infect PDHs. Although ducks and duck-derived primary hepatocytes are virtually non-permissive for HHBV, substitution of a region of the HHBV-specific preS domain of the L protein by the corresponding sequence from DHBV overcomes this species barrier. As a consequence, the pseudotyped HHBV virions can efficiently infect primary duck hepatocytes[5]. The same is true for mammalian hepadnaviruses, as shown for woolly monkey hepatitis B virus pseudotyped with a small stretch of preS1 sequence from HBV, which was

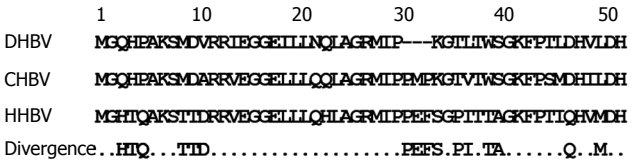

Figure 7 Sequence comparison of the viral L protein from different hepadnaviruses. The first 50 amino acids of the preS domain of hepadnaviral L from duck, crane, and heron HBV are shown. The divergent amino acids are shown in the lowest lane.

then infectious for human hepatocytes. Thus, although the sequence of this region is very divergent among the different hepadnaviruses, the biological functions seem to be conserved. The so called host-determining region (HDR) in the preS part of the avian L protein was mapped to amino acids 22 to 90, and an exchange of this small region also changed the species specificity[5]. These studies clearly indicate that the block to cross-species infection by hepadnaviruses is destined at the level of infectious viral entry. A small domain within the preS region of the L protein plays a pivotal role in host discrimination.

We showed that cranes are naturally infected with a novel hepadnavirus, designated crane HBV (CHBV)[14]. Phylogenetically, cranes are very distant from ducks and are closely related to herons and storks. However, we found that CHBV infects PDHs with similar efficiency as DHBV, indicating a rather broad host range of this virus at least *in vitro*. Whether CHBV can establish chronic infection in ducks *in vivo* and is as non-pathogenic as DHBV remains to be elucidated. Interestingly, comparison between the HDR of DHBV and the HDR of CHBV reveals a short insert of 3 amino acids (PMP) in the CHBV L protein, a sequence similar but not identical to the analogous region of HHBV and STHBV, whereas all other known duck and goose hepadnaviruses have no such insert (Figure 7). It remains to be shown which sequence features of the L protein are responsible for the unusual broad host range of CHBV and at which level of infection the block in cross-species infection is determined.

Accordingly, comparative genomic and subgenomic sequence alignment from different avihepadnaviruses facilitates the prediction of specific properties of each virus and helps to gain insight into the mechanisms controlling species specificity and host adaptation.

CHEMOTHERAPY AND VACCINATION

Antiviral drugs currently in use for therapy of chronic hepatitis B are nucleoside analogues and interferon. These therapies are unsatisfactory since the virus is usually not eliminated from the infected patient and resistant viruses frequently appear after treatment with nucleoside analogues. These data show the need for additional therapies and therapeutic strategies. The therapeutic effect of new vaccination strategies as well as chemotherapeutic agents can be assessed with the DHBV model system.

A long-term study showed that treatment of persistently infected ducks with 0.1 mg/kg per day of entecavir resulted in a rapid 4-log drop in serum DHBV

surface antigen[99]. However, a rapid rebound of levels of DHBV DNA and antigens in serum and liver was observed when entecavir was discontinued. When entecavir was administered at the time of DHBV inoculation, it was not effective to prevent infection but it led to a significant suppression of viral spread even after withdrawal of the drug[100]. Thus, short term suppression of DHBV infection shortly after infection provides the opportunity for the immune response to successfully control the infection.

Immunotherapy using DNA vaccines has been proposed as a way to improve viral clearance via the induction of an effective immune response. It has been shown that a DNA vaccine expressing DHBV surface antigens induces high levels of antibodies directed against these antigens, which protected or partially protected the animals against a challenge with DHBV[101]. This suggests that DNA vaccines may be an alternative to conventional vaccines for inducing immune response and protection against infection.

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