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Hepadnaviruses have a narrow host range – do they?

Kai Dallmeier and Michael Nassal

University Hospital Freiburg, Internal Medicine II / Molecular Biology, Hugstetter Str. 55, 79106 Freiburg, Germany

Abstract

Host range describes the range of species that a virus can infect to productively propagate itself. Productive infection requires compatibility between virus and host molecules. Thus host range may be restricted by lack of appropriate permissivity factors; alternatively, hosts may actively counteract infection using restriction factors. Incompatibility between virus and host can manifest on the level of individual cells, of tissues or organs, and of the entire organism. All hepatitis B viruses are hepatotropic, but individual viruses infect the livers of only selected mammalian (orthohepadnaviruses) and avian (avihepadnaviruses) hosts. Hence a narrow host range is thought to be a salient feature of hepadnaviruses. Here we briefly review general mechanisms of host range restriction, and summarise older as well as recent data pertaining to hepadnaviral host range. Clearly, the term species-specific is inadequate for many hepadnaviruses because they can infect different species from one genus, and even species from different genera. For a few others, only a single species, or genus, has been identified that supports efficient infection; however, this could as well relate to the restricted number of experimentally addressable test species. Together with the uncertainty about quantitative phylogenetic relationships between species, still largely based on morphological rather than molecular criteria, this leaves the term narrow open to interpretation. Finally, few if any of the host molecules enabling productive infection by a hepadnavirus have unambiguously been identified, the role of restriction factors has not yet been assessed, and even on the virus side the so-called host determining regions in the PreS domains of the large envelope proteins appear to be relevant only under specialised experimental conditions. Hence this important aspect of hepadnavirus biology is still far from being understood.

Introduction

While viruses can infect all forms of life, an individual organism cannot be infected by all types of viruses. Though not surprising given the diversity between prokaryotic, plant and animal hosts, even within one kingdom many viruses are selective with respect to what for them constitutes a suitable (susceptible) and a non-suited (non-permissive) host, or host cell. Within metazoan hosts, three types of specificity (tropism) can be distin-

guished: that for specific cells (cell tropism), that for specific organs or tissues (tissue tropism), and that for members of one, but not another species, genus, family, order and so on (host tropism). Host specificity of hepatitis B viruses is the topic of this review; however, all three are closely intertwined such that host range cannot be discussed in isolation.

The common notion is that a narrow cell and tissue specificity, namely for hepatocytes and hepatocyte-derived cells, and a narrow host range are salient features of hepatitis B viruses. Below we summarise the evidence favouring, and, at least in part, questioning this concept; we briefly discuss the viral and host factors that, in general, determine host range in better understood systems and relate them to what is known for hepatitis B viruses. Because several comprehensive reviews pertaining to hepadnavirus biology have recently been published [1–3] we will emphasise unresolved issues from both early and very recent investigations. They reveal that experimental definition of hepadnaviral host range is obscured by insufficient knowledge on the involved viral and host factors as well as host phylogenetic relations, is strongly affected by the test systems used, and thus is much less understood than implicated by prevailing models.

General importance of host range

We are constantly exposed to an enormous variety of viruses of which, fortunately, only few are able to establish a productive infection in humans because of host range restrictions. Hence this species barrier protects us from permanently being infected. The importance of this phenomenon is dramatically highlighted by the occasional crossing of this barrier by a virus and its potentially devastating consequences [4]; notorious examples include human immunodeficiency viruses (HIVs) [5], severe acute respiratory syndrome (SARS) coronavirus [6], or the imminent threat of an influenza pandemic originating from, thus far avian, H5N1 influenza viruses [7]. It should be noted that not every species crossing leads to disease: Simian virus 40 (SV40), possibly introduced into the human population by the poliovirus vaccine, may or may not be associated with cancer [8], and no indication for disease-induction exists for simian foamyvirus infections of humans [9].

A different consequence of relevance for virological and medical research is that host range restrictions can prevent the establishment of animal models for infection with a human viral pathogen, for which HBV represents an eminent example.

Molecular determinants of viral host range

Viral infection, and defense against infection, depend on interactions between viral and host components which, therefore, must be matching.

Two fundamentally distinct mechanisms restrict infectability: Either a lack of factors required for the virus to complete its infectious cycle ('permissivity factors'), or the presence of host factors that actively block that cycle ('restriction factors'); the latter may be divided into factors that are constantly present (innate), and those that are induced in response to the encounter with a specific virus (adaptive). Evidently, cells from different hosts, and from different tissues within one host, differ in their expression profiles; additional diversification comes in by changes related to the cell-cycle and differentiation status. Hence permissivity factors may either completely, or temporarily be absent, or differ between host species in their exact composition such that they cannot productively interact with the corresponding viral component; the same holds, conversely, for restriction factors.

Host range restrictions by lack of host factors required by the virus

Failure to complete any one of the individual infectious cycle steps, for physical lack of, or lack of compatibility with, a cellular factor involved will exclude a cell or organism from being a host for that virus. Related species have a related genetic outfit, and the closer the relation the higher the similarity between individual genes and gene products. Hence susceptibility to a certain virus in one host species will most easily be extendable to closely related hosts. The importance of match between viral and cellular factors is also highlighted by the impact on infectability of individual polymorphisms in some cellular genes; a famous example are humans carrying a non-functional allele of the HIV-1 coreceptor CCR5 gene, CCR5Δ32 [10, 11]. Interestingly, this allele also decreases the likelihood for hepatitis B to become chronic, though probably by an immunomodulatory rather than a direct mechanism [12]; in contrast, an intact CCR5 gene protects against West Nile virus infection [13].

Nonetheless, viruses differ greatly in their host specificities. Vesicular stomatitis virus (VSV) infects cells from a very wide range of species, including vertebrates, invertebrates and even insects; some murine retroviruses, in contrast, are so much restricted that they infect one but not another strain of mice. As outlined below, hepadnavirus host specificity seems to be intermediate between these extremes.

For any virus, including HBV, the events during productive infection can broadly be categorised into attachment, entry, uncoating of the viral genome, genome replication and expression of gene products, and assembly and egress of progeny virions. Most occur at different sites in the cell, hence the importance of intracellular trafficking in virus replication, and consequently the dependence on the involved cell factors, is increasingly recognised [14].

Attachment and entry

Each virus must bind to a suitable host cell, and then must get access to the cytoplasm by crossing either the host cell plasma membrane, or an internal – usually endosomal – membrane; for enveloped viruses this generally involves membrane fusion events. Binding and fusion activities may reside in a single protein, as with VSV G protein, or on separate polypeptides. Obviously, the initial binding requires interactions between molecules that are exposed on the surfaces of the virus and of the cell, commonly named viral attachment proteins, or viral receptors, and cellular receptors. These can be different classes of molecules, including proteins, lipids, and sugars. For many viruses, though not the hepadnaviruses, the host interaction partners of their attachment and fusion proteins are known [15], in several cases even in atomic detail [16]. Examples include HIV-1 gp120 and CD4, influenza virus haemagglutinin (HA) and sialic acid residues. For protein receptors, it is obvious that their amino acid sequences will vary between host species, and hence may act as important host range restriction elements. Notably, even less complex biological structures can exert such a restrictive function, as shown by the differential recognition of α2,3 *versus* α2,6 linkages to galactose in the sialic acid receptors used by avian *versus* human influenza viruses [17]. A second increasingly appreciated feature is that often, perhaps always, more than one host surface molecule is involved. Such secondary molecules, for instance extracellular matrix components such as glycosaminoglycans (GAGs), may generally help concentrating the virus on the cell surface, or be more specific, often sequentially, acting co-receptors that are essential for productive entry. Well known examples include, for non-enveloped viruses, the initial binding of the adenovirus fiber protein to the Coxsackie-Virus Adenovirus receptor (CAR), and the subsequent interaction of the penton base protein with cellular integrins [18], and for enveloped viruses, the HIV-1 receptors and co-receptors CD4 and CXCR4 and CCR5 [19]. Of note, relatively small alterations in a viral protein can lead to the use of alternative host receptors [15] and therefore contribute to extend the virus host range. Finally, virus host cell receptor interactions may be modulated indirectly by auxiliary proteins. For many viruses, fusion activity depends on a balanced proteolytic activation of the fusion protein. The pertinent example is the haemagglutinin (HA) of influenza viruses and its dependence on properly timed cleavage into HA1 and HA2 [17]. Hence, availability of suitable proteases at the right concentration and location can well affect host range.

Uncoating of the viral genome, replication and formation of progeny virions

The steps following entry can as well profoundly affect viral host range. Poxviruses, for instance, using ubiquitously present molecules, possibly GAGs, as receptors can enter many different cells. Their narrow host range (Variola virus infects exclusively humans) must therefore relate mostly to downstream intracellular events [20]. Some of the basic cellular machinery for transcription, translation, and protein folding [21, 22] is probably universal; however, the ongoing search for a mouse model of HIV-1 infection [23] is revealing an ever increasing number of host-specific factors [24] that lack compatibility with components of the human virus. Examples include preintegration complex nuclear import [25] and the Tat protein which together with the host factors cyclin T1 and a cyclin kinase (Cdk9) binds to the TAR element on nascent viral RNA, enhancing the rate of viral RNA transcription by RNA polymerase II; HIV-1 Tat interacts productively with human but not murine cyclin T1 [26]. Also, HIV-1 assembles and buds poorly in mouse cells, suggesting that components of, or associated with, the endosomal sorting complex required for transport (ESCRT) contribute to species tropism [24]. This machinery is probably also involved in HBV morphogenesis [27–29]; however, the single most important species-specific blocks to hepadnavirus infection appear to operate during the initial steps of infection (see below).

Presence of host restriction factors

Cells may actively counteract viral infection by innate restriction factors; adaptive responses, though crucial, are not considered here. Innate restriction factors typically act in a dominant, saturable fashion, i.e., inhibition can be overcome by high doses of virus ('abrogation'), and they can target diverse steps of viral replication, as is best known for retroviruses [30–32]. Classic examples are the endogenous retroviral gene products *Fv4* which restricts Friend murine leukaemia virus by receptor blockade, and *Fv1*, a Gag-like protein that targets the capsid proteins of some retroviruses and interferes with proper functioning of the preintegration complex. Another widely present restriction system is based on the family of tripartite sequence motif (TRIM) proteins [33] which typically contain a RING domain, found in many E3 ubiquitin ligases, one or two B boxes, and a coiled-coil region, plus different C terminal domains. The most famous of the about 70 TRIM family members [34] is TRIM5α, a species-specific restriction factor for various immunodeficiency viruses and also simple retroviruses [35]; for instance, rhesus monkey, but not human, TRIM5α restricts HIV-1 and HIV-2. TRIM5α also targets the capsid protein and prevents proper reverse transcription, probably by premature uncoating, and possibly also through

degradation of Gag polyprotein precursors [36]. This mechanism may be antagonised by the host proline isomerase cyclophilin A (CypA) which augments HIV-1 infection in human cells but inhibits replication in monkey cells [37]. Other TRIM family members are also involved in antiviral resistance [38].

Another restriction system is based on the APOBEC family of cytidine deaminases [39], named after the founding member APOBEC1 which edits the mRNA for apolipoprotein B. APOBEC3G targets instead single-stranded DNA, for instance retrovirus minus-strand DNA, and introduces various C>U mutations, resulting in G>A hypermutation in the plus-strand. The U-residues render the DNA unstable, and hypermutation can prevent formation of functional gene products. The lentiviral Vif gene products protect the viruses by inducing APOBEC3G degradation; species-specificity results mainly from the ability, or inability, of a given APOBEC protein to interact with the respective Vif protein [40]. APOBEC3G and other family members, if overexpressed in hepatic cell lines, also restrict HBV though the deaminase activity is not required [41, 42]. Hepadnaviruses do not seem to encode a Vif-like anti-APOBEC activity; hence potential host range restriction would have to act directly *via* capsid-APOBEC interactions. A role for APOBEC in avihepadnavirus infections is unlikely because APOBEC3 proteins, like APOBEC1, appear to be mammal-specific [43].

Yet another innate factor restriction factor is the zinc-finger antiviral protein (ZAP) which targets viral RNA for destruction by the exosome RNA degradation machinery, as shown for retroviruses and Sindbis virus [44, 45] and recently made likely for filoviruses [46]. Whether ZAP can affect host range is currently not known.

A further, complex innate defense system against viruses and other pathogens consists of the various Toll-like receptors (TLRs). Recognition of non-self 'pathogen-associated molecular patterns' (PAMPs) by these proteins, located in the plasma membrane (TLR4) or in endosomal membranes (most other TLRs), induces antiviral signaling pathways that activate, e.g., NFkB or interferon regulatory factor 3 (IRF3), leading to expression of antiviral cytokines and interferons [47]. The viral PAMPS may be proteins (TLR2, TLR4) or nucleic acids such as dsRNA (TLR3), ssRNA (TLR7, TLR8), or dsDNA (TLR9). Recently discovered cytoplasmic sensing systems include the RNA helicases retinoic acid induced gene I (RIG-I) which appears to recognise uncapped double-stranded viral RNA bearing 5'-triphosphates, e.g., from influenza, Sendai or flaviviruses including HCV [48], and melanoma differentiation associated gene 5 (MDA5) which senses 5'-protected RNA, e.g., from picornaviruses [49]. Both activate, through their caspase recruitment domains (CARD), a mitochondria-bound protein variously known as CARD adaptor inducing IFN-β (CARDIF), interferon-β promoter stimulator 1 (IPS-1), virus-induced signaling adaptor (VISA), or mitochondrial antiviral signaling (MAVS), also leading to NF-κB and IRF3-/IRF7-mediated interferon induction. How much these antiviral fac-

tors contribute to species-tropism is not yet clear; however, the antiviral activity RIG-I is emphasised, for instance, by the much better replication of hepatitis C virus (HCV) in a hepatoma cell line clone lacking a functional RIG-I protein, and in that HCV tries to escape from this mechanism by cleaving CARDIF (as well as the TLR3 adaptor TRIF) using its NS3-4A serine protease [50]. While HBV can counteract the downstream interferon effects [51] it is currently not known whether these or similar defence systems affect hepadnaviral host range.

Finally, RNA interference may contribute to permissiveness of cells to viral infection; while this is proven for invertebrates and plants, a role in mammals is still under debate [52].

Hence cells are equipped with a large variety of defence systems that could also affect hepadnavirus infection and host range; however, currently few if any data are available.

Common features of hepadnaviruses and their infectious cycles

As outlined above, each step of the viral infectious cycle may be subject to negative regulation by lack of positive or presence of negative factors in a specific host cell; this certainly holds also for hepadnaviruses. We next briefly consider the hepadnaviral infectious cycle *in toto* (Fig. 1A); however, based on current knowledge, most of the later steps are controlled on the tissue rather than host species level, leaving the early steps of attachment and entry as the most likely candidates for determining host tropism. One line of evidence supporting this view is that artificial introduction of a transcriptional template equivalent to human HBV covalently closed circular DNA (cccDNA) into mouse liver, as a transgene or by hydrodynamic injection [53], yields virions that are infectious in an authentic host but do not infect the mouse.

All hepadnaviruses are small, enveloped, hepatotropic DNA-containing viruses that share a common genetic organisation and replication strategy [1]. Their genomes (Fig. 1B) are all between 3–3.3 kb in length, are produced by reverse transcription of the pregenomic RNA (pgRNA) intermediate, and are present in virions as partially double-stranded circular but not covalently closed, i.e. relaxed circular (RC) DNA with the reverse transcriptase, P protein, covalently linked to the 5' terminal nucleotide of the (–)-strand DNA. All encode, besides P protein, a single capsid or core protein which forms the icosahedral protein shell of the nucleocapsid, and two (avian HBVs) or three (mammalian HBVs) carboxy-terminally colinear surface proteins which are embedded into the lipid of the envelope. The surface proteins can form empty envelopes, termed subviral or S particles (SVPs), which are secreted in large excess over intact virions [54]. Most abundant is the small (S) protein (about 225 amino acids in ortho- and about 160 amino acids in avihepadnaviruses), constituting the majority of HBsAg in human

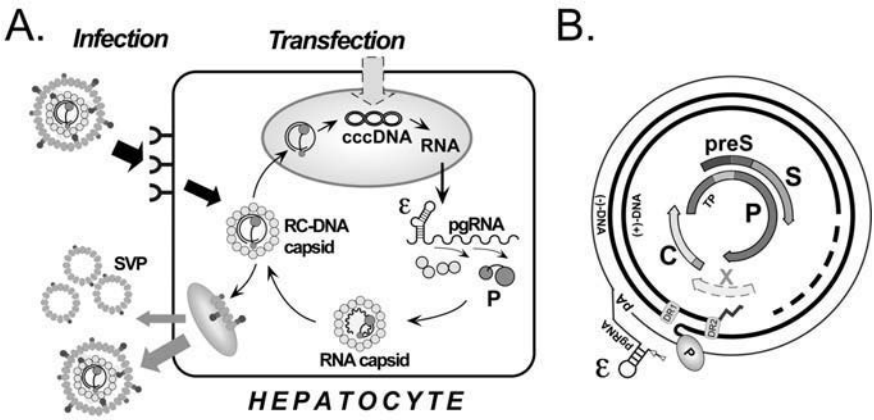


Figure 1. Common aspects of hepadnaviruses. A. Infectious cycle. Enveloped virions enter their host cells, primarily hepatocytes, *via* interaction with largely unknown receptor(s), leading to cytoplasmic release of the nucleocapsid, import of the relaxed circular (RC) DNA genome into the nucleus and conversion into covalently closed circular (ccc) DNA. From this template, subgenomic (not shown) and pregenomic (pg) RNAs are transcribed by cellular RNA polymerase II, exported from the nucleus, and translated. PgRNA acts as mRNA for the capsid and P protein, and via the encapsidation signal e, is copackaged with P into new immature nucleocapsids. Reverse transcription into the RC-DNA form occurs inside the nucleocapsid. Mature progeny capsid can repeat the intracellular amplification cycle, increasing the copy number of cccDNA molecules, or interact at compartments of the secretory pathway (lower left corner of the cell) with the envelope proteins and be secreted. Empty envelopes (subviral particles, SVPs) are secreted in large excess over virions, probably by a distinct mechanism [27, 28]. B. Genome organisation. All hepadnavirus genomes as present in virions are about 3 kb in length, with the (–)-strand DNA covalently linked to P protein and the (+)-strand being incomplete. All encode one capsid protein (C), the reverse transcriptase (P), and completely overlapping with P, a short (S) plus one (avian) or two (mammalian) longer forms (PreS/S) of envelope proteins. Intact genes for X are present in all mammalian, but only in cryptic form in the avian viruses.

HBV infection; the middle (M) protein of mammalian HBVs contains an N-terminal extension of 55 amino acids encoded by the preS2 region, and the L protein an additional extension of around 110–120 amino acids encoded by the preS1 region. Avian HBVs lack a preS2 initiation codon; their preS regions comprise about 160 codons. In all hepadnaviruses, the PreS regions of the L protein are involved in two essential functions, namely nucleocapsid envelopment and attachment to, and entry into, the host cell. This is accounted for by an unusual dual topology with about one half of the PreS domains directed towards the inner, and the other half to the outer face of the virions. An N terminal extension on the core protein, encoded by the preC region, gives rise to the secretory, non-assembling precore protein which, after N and C terminal processing events, is found in the circulation as HBeAg. Finally, mammalian, but most likely not avian, HBVs produce a regulatory protein, HBx, whose function is only poorly understood.

The hepadnaviral infectious cycle (Fig. 1A) is initiated by binding of the virion to as yet unknown cell surface receptor(s), internalisation, release of the nucleocapsids into the cytoplasm, and transport of the RC-DNA genome into the nucleus where conversion into cccDNA occurs.

None of these events is well understood. Whether fusion occurs at the plasma or at an internal membrane, and whether it requires passage through an acidic compartment is controversial, as is the nature of the fusion peptide and the requirement for its exposure, or not, by a proteolytic processing event. Some evidence suggests that artificial cleavage can facilitate entry into non-susceptible cells [55, 56] which would be compatible with the presence of a fusogenic peptide within the first trans-membrane domain (TM1) of the S domain [3]. A recent alternative proposal was that translocation motifs (TLMs), short α-helical peptide sequences predicted to be present in ortho- and avihepadnavirus PreS proteins, would mediate direct translocation [57]. This has not, however, been reproduced, for HBV, by others [58, 59].

Nuclear transport [60] of the relaxed-circular (RC) DNA genome occurs in nucleocapsids along cytoskeletal components, and probably results *via* interactions of the capsid with components of the nuclear pore complex (NPC) in the release of the DNA into the nucleus. NPC components are highly conserved through evolution, and *X. laevis* oocytes appear to behave similarly with respect to hepadnaviral nucleocapsids as human hepatocyte cell lines, suggesting the absence of species-specific effects. cccDNA formation includes completion of the (+)-DNA strand, removal of the covalently bound P protein and the small terminal redundancies, and religation. The virtual absence of cccDNA in HBV-transgenic mice suggested the involvement of species-specific factors; however, in mice with a hepatocyte nuclear factor 1a (HNF1a) null background, cccDNA becomes detectable [61]. Recently initiated efforts to systematically re-evaluate cccDNA formation are certainly worthwhile and may uncover as yet unknown species-specific factors [62].

cccDNA is the pivotal intracellular intermediate of hepadnavirus replication as it serves as template for all viral transcripts. Transcription of cccDNA by RNA polymerase II appears mostly controlled on the tissue – rather than species – specificity level, in that the core promoter driving pregenomic RNA transcription and the preS1 promoter responsible for the preS mRNA require liver enriched factors for high-level activity; for instance, HBV and DHBV replication in mouse cells of nonhepatic origin can be rescued by liver-enriched factors such as hepatocyte nuclear factor 4 (HNF4) [63]. However, WHV replication is not supported in that system and the WHV enhancer I does not work properly in human hepatoma cells that fully support HBV transcription [64]). Attempts to prove species-specific contributions for WHV are hampered by the lack of suitable woodchuck hepatoma cell lines and cloned transcription factors.

pgRNA encapsidation and reverse transcription depend on the specific interaction between the hepadnaviral RT and a stem-loop structure, ε, on

the pregenomic RNA. This interaction is strictly dependent on cellular chaperones [1]; however, there is no known requirement for species-specific chaperones, which is not that surprising given their extreme conservation throughout all kingdoms.

Newly formed mature, RC-DNA containing nucleocapsids can either recycle the genome to the nucleus for cccDNA amplification, or interact with the envelope proteins, bud into the ER or a later compartment of the secretory pathway and be secreted as enveloped virions. New data suggest the involvement of the machinery including ESCRT that generates multivesicular bodies (MVBs), unexpectedly in a distinct fashion regarding virions *versus* subviral particles [27, 28]. Again, a role for species-specific factors is unlikely because DHBV produced in transfected human hepatoma cell lines is infectious in ducks [65–67]; however, *in vivo* infection with DHBV is highly efficient [68], hence even a drastic reduction in the efficiency of virion production between avian and mammalian cells could have gone undetected.

Experimentally assessing viral host range

There are several approaches for exploring viral host range, each with its own benefits and limitations. One is to screen different animal species for the presence of a common or related virus; chances for success are sometimes increased if the virus causes a typical pathology, as is the case for some [69, 70], though not all hepadnaviruses. However, given the limited range of known hepadnavirus hosts, specimen collection is not trivial and certainly cannot be comprehensive; hence some, or many, potential host species may be missed.

A second approach is experimental transmission of a natural virus into a new host; this may be extended to viruses that have genetically been modified in a defined way, yet again ethical and other constraints apply for species that are not commonly used as experimental animals. Furthermore, even if a species is principally susceptible, infection may be difficult to detect if innate and adaptive immune responses limit replication to low levels or short periods of time. For instance, infection of 3-week old *versus* 3-day old ducklings with DHBV leads to the extremely rapid (within 1–2 days) induction of neutralising antibodies which drastically reduce viral spread in the liver and prevent persistent infection [71].

Adaptive immune restrictions can be overcome by using cultured cells or cell lines as targets. For hepadnaviruses, however, only a single human hepatoma cell line, HepaRG, has been identified that, under specialised conditions, can be infected by HBV [3, 72]; hence the nearly only source for infectable cultured cells are primary hepatocytes. Notably, however, we have recently identified a chimeric DHBV which is clearly restricted in

cultured hepatocytes yet *in vivo* replicates as well as wild-type DHBV (K. Dallmeier and M. Nassal; see below). Hence *in vitro* and *in vivo* infection may lead to different conclusions on viral host range.

Natural hepadnavirus hosts

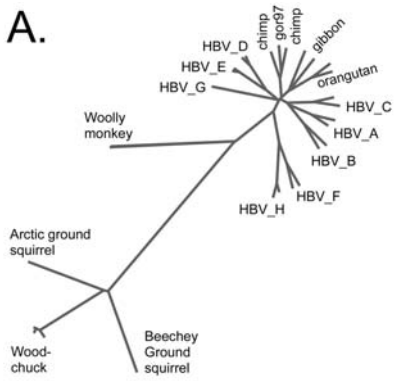
Shortly after human HBV genetically related yet distinct viruses have been found in several mammals (orthohepadnaviruses) and in selected bird species (avihepadnaviruses). Though a single cloned virus genome may not always represent an infectious clone [73], a wild-caught animal from which the corresponding virus has been isolated is a strong candidate for being a genuine host; this is less certain for viruses from captive individuals [74], yet it indicates, at any rate, that the virus can use that species as a host.

Based on this criterion, currently recognised natural orthohepadnavirus hosts are humans and hominoid primates (chimp/*Pan troglodytes*; gorilla/*Gorilla gorilla*; orangutan/*Pongo pygmaeus*; gibbons/Hylobatidae; [75]); one New World monkey (woolly monkey/*Lagothrix lagotricha*; [73, 76]); and some rodents, namely woodchuck (*Marmota monax*; [70]), California ground squirrel (*Spermophilus beecheyi*; [69]), Richardson's ground squirrel (*Spermophilus richardsoni*; [77]), and arctic ground squirrel (*Spermophilus parryi*; [78]) – but not mice or rats.

A large group of natural hosts for avian hepadnaviruses have been found in the order Anseriformes (waterfowl), but not Galliformes (landfowl), i.e., Pekin ducks (*Anas platyrhynchos* var. *domestica*; [79]) and their wild mallard ancestors; other Anatinae (duck-related birds; [74]) from the genus *Anas* (Puna teal/*A. puna*; Chiloe wigeon/*A. sibilatrix*) as well as other genera such *Aix* (Mandarin duck/*Aix galericulata*), *Neochen* (Orinoco goose/*N. jubata*), *Chloephaga* (Ashy-headed sheldgoose/*C. poliocephala*); and Anserinae (goose-related birds) including snow geese (*Anser caerulescens*; [80]) and Ross' geese (*Anser rossii*; GenBank Accession no.: M95589). Distinct hepadnaviruses have been found in two families of the order Ciconiiformes, namely storks (Ciconiidae), specifically the white stork/*Ciconia ciconia* [81], and herons (Ardeidae), specifically the grey heron/*Ardea cinerea* [82], great white heron/*Ardea alba*, great blue heron/*Ardea herodias*, and Wuerdemann's heron, a hybrid between *A. alba* and *A. herodias* [83], and finally in cranes [84] (order Gruiformes), specifically the crowned crane/*Balearica pavonia*, and demoiselle crane/*Grus virgo* (*synonym*: *Anthropoides virgo*).

Because negative results are usually not published it is unknown how many other species have been tested. Given the limited choices of established hosts, only the Pekin duck [85] and, to some extent, the woodchuck [86], have been developed into feasible animal models for hepadnavirus infection.

A.



B.

virus/ host	HBV (A-H) gen	HBV (A-H) prS	Great Apes gen	Great Apes prS	Woolly monk. gen	Woolly monk. prS	GSHV gen	GSHV prS	ASHV gen	ASHV prS	WHV gen	WHV prS	DHBV gen	DHBV prS
Humans	86	72	86	77	78	65	63	26	64	23	64	24	40	9
Great Apes	86	77	90	85	78	67	64	30	64	27	64	29	41	11
Woolly monk.	76	65	78	67			63	30	63	29	63	29	38	12
Ground sq.	63	26	64	30	63	30			85	77	84	74	38	8
Arctic sq.	63	23	64	27	63	29	85	77			85	76	38	9
Woodchuck	63	24	64	29	63	29	85	74	84	76			38	12
duck	39	9	41	11	38	12	38	8	38	9	38	12		

Figure 2. Phylogeny of orthohepadnaviruses. A. Unrooted full-length genome based tree. For the animal viruses, the host species from which the viruses were isolated are indicated. Human isolates are designated HBV plus the corresponding genotype; where applicable, sequences from two distinct subgenotypes were used. Nodes indicate common ancestors, the lengths of the connections are proportional to sequence distances. Note that divergence among individual human isolates is larger than between human and ape viruses. B. Genome and PreS domain amino acid identities between orthohepadnaviruses. Numbers indicate percent identities for the nucleotide sequences of the genomes (columns labeled gen), and protein sequences of only the PreS1/PreS2 regions (prS). Note the much lower identity scores for the PreS proteins *versus* the genomes, demonstrating the high diversity of PreS between different viruses.

Phylogenetic relationships between hepadnaviruses and between their recognised natural host species

Phylogenetic distances between different hosts are likely reflected in corresponding sequence differences between their respective viruses. The small genome size of hepadnaviruses makes derivation of sequence-based phylogenies rather straightforward; however, this is not the case for determining quantitative distances between hosts.

Due to the medical importance of HBV, thousands of genome sequences have meanwhile been determined; they can be classified into eight distinct genotypes (A to H). A genotype, by definition, must differ in sequence from another by at least 8%, subgenotypes with one genotype by at least 4% [87]. In the unrooted phylogenetic tree shown in Figure 2A, two representatives of each of the eight HBV genotypes (one from each subgenotype if applicable) have been combined with the full-genome sequences of the known animal orthohepdnaviruses; Figure 3A shows a corresponding analysis for the avihepadnaviruses, however based on the protein sequences of only the PreS regions, with three representatives of the Eastern and three of the Western clade of DHBV, including the commonly used laboratory strain DHBV16. Another parameter that illustrates relatedness is 'percent identi-

B.

host genus	Anas gen	Anas prS	Chloephaga gen	Chloephaga prS	Neochen gen	Neochen prS	Aix gen	Aix prS	Anser (Ross´) gen	Anser (Ross´) prS	Anser (snow) gen	Anser (snow) prS	Gruidae (crane) gen	Gruidae (crane) prS	Ardea (heron) gen	Ardea (heron) prS	Ciconia (stork) gen	Ciconia (stork) prS
Anas (various)	90	88	86	86	87	86	82	70	82	71	89	84	84	75	77	53	78	54
Anser (snow)	89	84					81	70	81	71			84	78	78	55	78	55
Anser (Ross´)	82	71					99	98			81	71	85	80	78	56	78	57
Gruidae (crane)	84	73					85	80	84	80	84	78			78	55	79	58
Ardea (heron)	77	53					78	55	78	56	78	55	78	55			86	78
Ciconia (stork)	78	54					78	56	79	57	78	55	79	58	86	78		

Figure 3. Phylogeny of avihepadnaviruses. A. Unrooted tree based on PreS domain protein sequences. Genera are given in italics, examples for specific species from which the viruses were isolated are indicated below. For the cranes, the family designation Gruidae is used to avoid confusion with species designation *Balearica* for the crowned crane. *Anas*, *Neochen*, *Chloephaga*, and also *Aix*, all belong to the family Anatidae. Note the near identity of the viruses from the Mandarin duck and Ross's goose, and the close relation of these viruses with the crane viruses. B. Genome and PreS domain amino acid identities between avihepadnaviruses. Divergence between isolates from different genera of the Anatinae, and even from the snow goose, is not larger than that among isolates from within the genus *Anas*; by contrast, the Mandarin duck/Ross's goose isolates are even less related to the duck isolates than the crane viruses, and their divergence from the other duck isolates is higher than that between heron *versus* the stork isolates.

ty' which, after alignment, indicates the proportion of identical nucleotides, or amino acids, at a given position (Figs 2B and 3B).

The orthohepadnaviruses cluster in three groups: hominoid (i.e., hominid plus gibbon) primate HBVs; WMHBV, i.e., New World monkey virus; and ground squirrel (GSHV) plus woodchuck (WHV), i.e., rodent viruses. For the avihepadnaviruses, three major clades are seen: one encompassing the duck and most goose viruses; one for the Ross's goose/Mandarin duck viruses which clusters with the crane viruses; and one comprising stork and heron viruses. Comparison of the genome sequences gives similar results, except that the Ross's goose/Mandarin duck and crane HBVs are not predicted to have a common ancestor; however, they remain about equidistant from the duck isolates (not shown). These distances are reflected in the percent identity values (Figs 2B and 3B), which for the entire genomes are minimally about 86% within the hominoid group, about 78% for hominoid *versus* WMHBV, 63% for hominoid *versus* rodent, and about 40% for hominoid *versus* avian; minimal identities among the rodent viruses are 84%, among the avian viruses 81–89% for duck, goose and crane viruses, about 75% for these *versus* stork and heron, and about 85% between stork and heron viruses. Percentage values are similar for the S proteins, but significantly lower for the PreS regions; this much higher than average diversity

in PreS is one of the arguments for its role in determining host range (see below). PreS identity scores drop to 72% within the hominoid group, to about 65% for hominoid *versus* WMHBV, and to below 30% for hominoid *versus* rodent viruses; among the rodent viruses, minimal identities are around 75%. Identity between ortho- and avihepadnavirus PreS regions is only around 10%.

For the avian viruses, PreS percent identity scores are around 70% within the duck and goose virus group; again a higher value is observed for these *versus* crane viruses (75–77%), but a significantly lower for all former viruses *versus* heron and stork viruses (53–55%).

There are several noteworthy points. All hominoid ape viruses cluster in between the human isolates, i.e., even the gibbon viruses are as close to the human viruses as these are among each other; WMHBV is the only true outlier. While an initial caveat was that those animals might have acquired their viruses from humans, newer sequences were derived from wild-caught animals that, *bona fide*, had not been in close contact with humans. Hence these viruses appear to be true ape viruses [75]. There is also evidence for recombination between chimp, and notably, gibbon and human HBVs [88], suggesting that primate and human HBVs can share hosts. Hence HBV is certainly not a species-specific virus.

A similar trend is seen among the avihepadnaviruses where Eastern and Western DHBV isolates are as different from each other as they are from various other duck and goose viruses, except for the Ross's goose/Mandarin duck isolates. Notably, the crane virus is not further apart from the duck virus clade than the Ross's goose/Mandarin duck viruses. Thus CHBV is much more a 'DHBV-like' virus than the heron and stork viruses.

Host phylogenies

Establishing phylogenetic relationships between host species is much more complex, and traditional morphology-based *versus* partial sequence-based analyses frequently give discordant results. Quantitative molecular relations between natural hepadnavirus hosts are firmly established only for humans and chimps (about 1% overall diversity on the genome level [89]); the about 7% diversity between the human and the Rhesus macaque genome [90] give at least an indication of the genetic distances between primates, though these monkeys are not hepadnavirus hosts.

For all other hepadnavirus hosts, classification still has to rely on a combination of morphological traits, the sequences of mitochondrial and selected nuclear genes plus for a time-scale, fossile records. Hence the phylogenetic trees shown in Figures 4 and 5 should be regarded as an only approximate indicator of relative kinship between species. This is particularly true for bird phylogeny. While traditional approaches are still continued [91] cross-hybridisation studies revolutionised the prevailing genealogies [92] but now

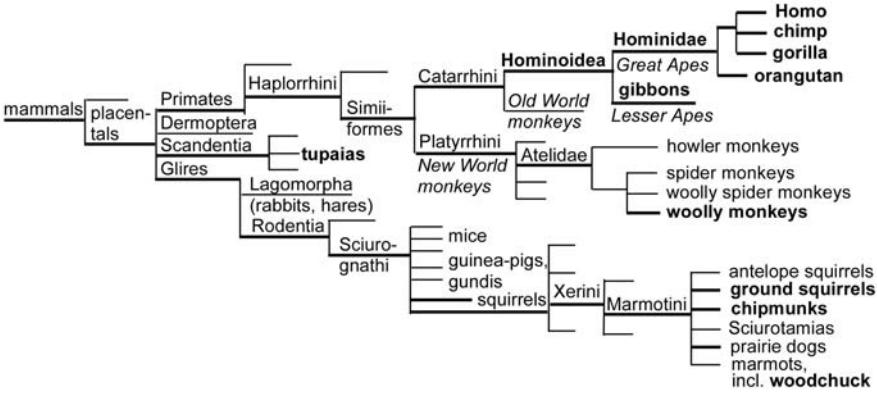


Figure 4. Phylogenetic tree of mammals with respect to recognised orthohepadnavirus host species. The tree is based on the classification used by the NCBI taxonomy data base (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser). Only those branches leading to hepadnavirus infectable host genera (bold face) are explicitly indicated; the lengths of the branches are not proportional to a specific time scale. Note that all known rodent hepadnavirus hosts belong to the tribe Xerini.

are considered more cautiously as more direct sequencing data come in; even then, the choice of DNA (mitochondrial *versus* nuclear) and of representative species can affect the results.

There is no doubt about a close relation among the Hominidae (Great Apes); however, the Hylobatidae (Lesser Apes; gibbons) are clearly distinct yet the HBVs isolated from gibbons cluster within the human isolates (above and [93]). By contrast, baboons, belonging to the Old World monkeys, are not [94], or extremely inefficiently [95], susceptible to HBV infection. Hence human HBV can not infect all primates yet all hominoid apes.

WMHBV, replicating to high levels ($>10^9$ vge/ml) in woolly monkeys, led to only inefficient infection of the supposedly closely related black-handed spider monkey (*Ateles geoffreyi*) [73]. Hence, in this sense, WMHBV appears to have a truly narrow host range. However, the picture is different with primary hepatocytes in culture (see below).

All other known mammalian HBV hosts belong to the rodents (comprising about 2,000 species), with the lagomorphs (rabbits and hares) as their apparently closest relatives. The rodentia form three major clades: mouse-related, guineapig-/gundi-related, and squirrel-/dormouse-related (Sciuridae). Within the subfamily Xerinae, both the genera *Marmota* (marmots, including woodchucks) and *Spermophilus* (groundsquirrels, rock squirrels) belong to the same tribe (Marmotini). Other genera from this tribe are chipmunks, Chinese rock squirrels, antelope-squirrels and prairie dogs [96, 97]. One special case are tupaias, commonly known as tree shrews. There is no evidence that they are a natural host for a hepadnavirus, yet they

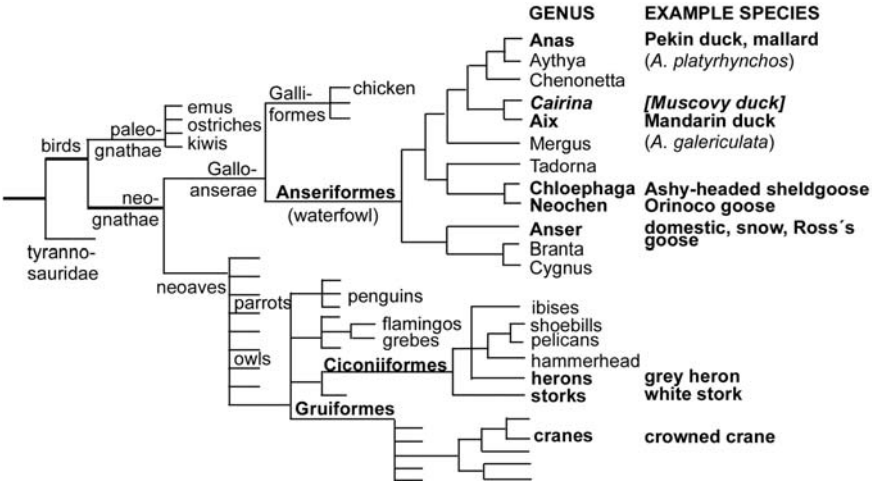


Figure 5. Phylogenetic tree of birds with respect to recognised avihepadnavirus host species. The tree is a composite based on the general Aves classification by Cracraft [105] for the Neoaves, and a mitochondrial DNA based phylogeny [104] for the Anseriformes. Note the close relation between the genera *Aix* and *Cairina*. As in Figure 4, the lengths of the branches do not imply a specific time scale.

may be infectable, at a very low level, by human HBV *in vivo* [98], and isolated tupaia hepatocytes are definitely infectable with HBV and WMHBV [99, 100] but not WHV. The evolutionary aspects of this remain obscure because tupaias are no more considered primates (Fig. 4) but as a distinct sister taxon (Scandentia) to the primates, flying lemurs (Dermoptera), and the rodents plus lagomorphs (Glires) [101].

Avian phylogeny is highly controversial but some basic concepts appear to be generally accepted (e.g., [102]; for additional information see http://animaldiversity.ummz.umich.edu). Accordingly, there are two subclasses, paleognaths (ostriches, emus, kiwis), and neognaths (Fig. 5); these are divided into two large infraclasses, Galloanserae (chicken- plus goose-/duck-related), and Neoaves, which comprise all other birds including hepadnavirus hosts such as herons, storks, and cranes (for a recent phylogenetic analysis of crane-related birds, see [103]). Galloanserae are divided into the orders Galliformes (landfowl), and Anseriformes (waterfowl) which comprise the families Anhimidae (screamers), Anseranatidae with the magpie goose as their only representative, and Anatidae; these are divided into the subclasses Anatinae (ducks), and Anserinae (geese and swans). The phylogeny of the individual genera within the Anatidae as shown in Figure 5 is based on mitochondrial DNA analyses [104], that of the Neoaves branch (see also http://tolweb.org/Neornithes/15834) on reference [105].

None of the known avihepadnavirus hosts belong to the Galliformes but many to the Anseriformes. Perhaps most striking is the wide distribution of infectable genera among the Anatidae, ranging from *Anas* (e.g., Pekin duck/*A. platyrhynchos* v. *domestica*; puna teal/*A. puna*; Chiloe wigeon/*A. sibilatrix*) and *Aix* (e.g., Mandarin duck/*A. galericulata*) to the apparently distant *Chloephaga* (e.g., Ashy-headed sheldgoose/*C. poliocephala*), and beyond to the Anserinae (e.g., snow goose/*A. caerulescens*; Ross's goose/*A. rossii*). Most surprisingly, the genus closest to *Aix* is *Cairina* yet *Cairina moschata*, the Muscovy duck, is not infectable, *in vivo*, by DHBV [106, 107].

Regarding the other bird HBV hosts, classical and molecular phylogenies agree that storks and herons (both Ciconiiformes) are relatively close to each other [102, 108] but not to cranes [103] (Gruiformes). This is consistent with the closer relation between the heron and stork *versus* the crane viruses. It should be noted, though, that even storks and herons have very long independent histories (estimated separation time about 80 Myr ago; [103]), spanning almost the same period of time as that leading to chicken and ducks after separation from their last common ancestors (about 100 Myr; [109–111]); for comparison, ducks and geese are thought to have separated about 35 Myr, rats and mice about 53 Myr ago [109].

Due to its origin from cranes, and ability to infect hepatocytes from the undoubtedly distant Pekin duck, crane HBV has been proposed to be the first, and unexpected, example of a hepadnavirus with a rather broad host range [84]. However, given the close relation of the crane virus isolates to the Ross goose/Mandarin isolates, and the ability of the latter to even infect Pekin ducks *in vivo* (see below), this result is much less surprising. Rather, the question is why the crane and Ross's goose/Mandarin duck viruses are so closely related (Fig. 3). More generally, there is no evidence that the Ross's goose/Mandarin duck virus, or DHBV, have a less broad host range than crane HBV as long no experiments pertaining to this question have been performed.

Cross-species transmission of 'natural' HBVs *in vivo*

One main benefit of experimental transmission is the ability to use a precharacterised, and possibly clonal, virus inoculum; when screening for natural infections the original source of virus is uncertain, particularly for captive animals that are kept in close to other species. Hence the near identity between the Mandarin duck virus isolates and the Ross's goose isolate may be due to that Ross's goose having contracted a hepadnavirus from other birds held in the same facility [74]; this limitation probably holds for most specimens from captive animals.

Cross-species transmission of natural hepadnaviruses, if successful, gives clear hints on host range; negative results, however, cannot be considered absolute. Essentially none of the natural hosts and their relatives is avail-

able in inbred form, and few can be kept under standardised conditions. Hence differences between strains or individuals could contribute to failure of transmission. Similarly, newborn animals which, due to their immature immune system, are more likely to be infectable than adults, are frequently not available. This holds particularly for primates, many of which, including woolly monkeys, are endangered. Even for the avihepadnaviruses, few other species than Pekin ducks are available for routine experiments. Hence, while different avihepadnaviruses have been tested in Pekin ducks, the host-range of DHBV itself has not extensively been explored.

The first cross-species transmissions of a clonal HBV isolate, by intrahepatic injection with cloned virus DNA rather than infection, were performed in chimpanzees and proved that this molecular clone of HBV was principally infectious and could use chimps as host [112, 113]; similar experiments were performed with animal HBVs [114, 115]. Probably, this is the only technique allowing establishment of infection by a single molecular clone; however, transmission is as well achievable by intraperitoneal (i.p.) or intraveneous (i.v.) injection of virions contained in serum from infected animals or supernatants from transfected cells. An alternative test system are primary hepatocytes from ducks [116], humans [117, 118], tupaias [98] and, at selected facilities, from some primates [119], and recently, the human hepatoma cell line HepaRG [3, 72]. However, *in vivo* and *in vitro* assays can give contradictory results (see below).

An additional system likely to reproduce many, though possibly not all, features of orthohepadnavirus infection is the use of hepatitis delta virus (HDV). HDV is an RNA virus that replicates autonomously but for infection and spread depends on the HBV envelope proteins [120]. Though, in contrast to HBV, PreS1 is not required for envelopment of HDV ribonucleoprotein complexes, it is essential for infectivity. Because HDV replicates to very high copy numbers (in the range of 10^5 genomes/cell), infection can very sensitively be monitored. This system was the first to raise doubts on an essential role for the TLM motifs [121], and similar negative data have been forwarded for HBV infection of human hepatocytes [59] and HepaRG cells [58]. Importantly, HDV can be enveloped with surface proteins from different orthohepadnaviruses, allowing to assess their impact on infection of primary hepatocytes from different hosts, in particular primates [119, 122].

Tables 1 to 3 summarise published data on cross-species transmission experiments with primate, rodent, and bird HBVs, indicating the inoculum virus, the recipient species, and the outcome of the experiments *in vivo*, or with cultured hepatocytes. An anecdotal report on HBV transmission into turtles [123] is not incorporated.

Neither for the mammalian nor for the bird HBVs does a uniform picture emerge from these data. HBV-like viruses can certainly infect chimpanzees, and the natural occurrence of highly similar viruses in hominid primates indicates that they can infect a spectrum of related species (Tab. 1).

Table 1. *In vivo* and *in vitro* infectivity of HBV and Woolly monkey HBV (WMHBV) for other primates, tupaias, and their isolated hepatocytes

Virus	Recipient species[a]		Infection[b] in		Remarks[c]	Reference
	Common	Systematic	vivo	vitro		
HBV	chimpanzee	*Pan troglodytes*	+		Intrahepatic HBV DNA transfection	[112, 113]
HBV	chimpanzee			+	HDV with HBV envelope; no PEG required	[119]
HBV	baboon	*Papio* sp.	-		HBV; infection also negative in immuno-suppressed animals	[94]
HBV	baboon	*Papio ursinus orientalis*	+/-		HBV; PCR positive; HBsAg and anti-HBsAg negative	[95]
HBV	baboon	*Papio* sp.		-	HDV with HBV envelope; negative even with PEG	[119]
HBV	tamarin	*Saguinus* sp.		-	HDV with HBV envelope; negative even with PEG	[119]
HBV	spider monkey	*Ateles geoffreyi*		+	HDV with HBV envelope; no PEG required	[119]
HBV	tree shrew	*Tupaia belangeri*	+/-		HBV; transient HBsAg, intrahepatic HBcAg; low HBV DNA, rapid clearance?	[98]
HBV	tree shrew		+		Adeno-HBV vector; HBsAg, HBV viremia; possibly from adenovector	[129]
HBV	tree shrew			+	Adeno-HBV vector; HBsAg, HBV replicative intermediates incl. cccDNA	[129]
HBV	tree shrew			+	HBV; HBsAg + HBeAg + HBV replicative intermediates incl. cccDNA	[99,100]
WMHBV	human			-	WMHBV; negative or below detection limit	Köck, Nassal, unpublished
WMHBV	human			(+)	HDV with WMHBV envelope; only with PEG	[119]
WMHBV	chimpanzee		+/-		WMHBV; negative or at detection limit	[76]
WMHBV	chimpanzee			(+)	HDV with WMHBV envelope; only with PEG	[119]
WMHBV	Rhesus monkey	*Macaca mulatta*	-		No details given	[73]
WMHBV	baboon		-		No details given	[73]
WMHBV	baboon			-	HDV with WMHBV envelope; negative even with PEG	[119]
WMHBV	tamarin		-		No details given	[73]
WMHBV	tamarin			-	HDV with WMHBV envelope; negative even with PEG	[119]
WMHBV	tree shrew			+	WMHBV; HBsAg + HBeAg + replicative intermediates incl. cccDNA	[100]
WMHBV	spider monkey		((+))		WMHBV; 4 log lower viremia than in woolly monkey, despite immuno-suppression	[73]
WMHBV	spider monkey			(+)	HDV with WMHBV envelope; without PEG less efficient than HDV with HBV envelope	[119]

[a]Systematic names are given only at first occurrence of the common name. [b]Infectivity scores are as follows: +, easily detectable viremia and antigenemia; ((+)), viremia and antigenemia clearly demonstrated though at low levels; +/–, some indirect evidence for infection; (+), infection detectable only in the presence of polyethylenglykol (PEG) as facilitator; –, no detectable infection. [c]Comments on specific experimental set-up

WMHBV, by contrast, appears specialised for woolly monkeys because transmission to even spider monkeys, their presumed closest relatives, was very inefficient [73]. Similarly, GSHV (Tab. 2) did not infect mice, guinea-

Table 2. *In vivo* infectivity of ground squirrel (GSHV) and Woodchuck hepatitis virus (WHV) for different rodent species

Virus	Recipient species name		Infection	Remarks	Reference
	Common	Systematic	in vivo		
GSHV	Golden-mantled ground squirrel	*Spermophilus lateralis*	-	GSHV; inoculum virus not sequenced but infectious for *S. beecheyi*	[125]
GSHV	13-lined ground squirrel	*Spermophilus tridecemlineatus*	-	as above	[125]
GSHV	Rat (Sprague-Dawley)	*Rattus norvegicus*	-	as above	[124]
GSHV	Mouse (Balb/c, C57/BL)	*Mus musculus*	-	as above	[124]
GSHV	Syrian hamster	*Mesocricetus auratus*	-	as above	[124]
GSHV	Guinea-pig	*Cavia porcellus*	-	as above	[124]
GSHV	woodchuck	*Marmota monax*	+	GSHV; high level viremia; less oncogenic than WHV in woodchuck	[126,130]
GSHV	chipmunk	*Eutamias* sp.	+	No details on chipmunk species	[125]
WHV	Beechey ground squirrel	*Spermophilus beecheyi*	-	Intrahepatic WHV DNA transfection; DNA infectious for woodchucks	[126]

pigs, and hamsters [124] and, more surprisingly, not two other squirrel species (golden-mantled ground squirrel/*Spermophilus lateralis*; thirteen-lined ground squirrel/*Spermophilus tridecemlineatus*) but could establish infection in the more distant chipmunk (*Eutamias* sp.) [125] and woodchuck [126]; conversely, WHV was not infectious for Beechey ground squirrels [126]. Thus GSHV appears to have a broader host range than WHV but there is currently no rational explanation. One problem is certainly that the true phylogenetic relationships between these species are insufficiently established.

Nearly all of the avihepadnaviruses isolated from Anatidae (Fig. 5) are infectious (Tab. 3) for Pekin ducks *in vivo*, and DHBV is transmittable to domestic geese, though not chicken [106] and Muscovy ducks (*Cairina moscata*; [106, 107]). The Orinoco sheldgoose (genus *Neochen*) isolate was infectious for Paradise shelduck (genus *Tadorna*) and Pekin duck (genus *Anas*), and even the Ross' goose virus-like Mandarin duck isolate efficiently infected Pekin ducks [74], clearly demonstrating cross-genera transmission. Thus many of these viruses have an expanded host range. However, there is also an exception in that the ashy-headed sheldgoose isolate, though closely related to DHBV (Fig. 5), did not infect Pekin ducklings (six out of six inoculated animals remained negative; [74]); whether this relates to a clone-specific problem (the full-length genome had to be restored using a separately amplified core gene) or a genuine restriction is unclear. Less surprisingly, the heron virus was not detectably infectious for Pekin ducks [82, 127].

The *in vitro* infection experiments with cultured hepatocytes gave often, though not always, consistent results; in general, *in vitro* infection was less

Table 3. *In vivo* and *in vitro* infectivity of natural avihepadnaviruses for other bird species and their hepatocytes

Virus from	Recipient species		Infection in		Remarks	Reference
	Common	Systematic	vivo	vitro		
Pekin duck	Wild mallard	*Anas platy-rhynchos*	+	n.d.	I.v. injection of DHBV-positive serum	[131]
Wild mallard	Pekin duck	*A.p. var. domestica*	+	n.d.	Possibly higher viremia than with DHBV from Pekin duck	[131]
Pekin duck	Domestic goose	*Anser anser domesticus*	+	n.d	High level viremia; increased pathogenicity (?)	[106]
Pekin duck	chicken	*Gallus gallus*	-	-	Same inoculum infectious in Pekin ducks	[106,107]
Pekin duck	Muscovy duck	*Cairina moscata*	-	n.d.	Same inoculum infectious in Pekin ducks	[106]
Pekin duck	Muscovy duck		n.d.	((+))	At most 1% as efficient as DHBV in Pekin duck hepatocytes	[107]
Puna teal	Mallard + Pekin duck		+	+	Apparently similarly efficient as DHBV	[74]
Orinoco goose	Paradise shelduck	*Tadorna variegata*	+	n.d.	Used to expand inoculum for cloning and Pekin duck infections	[74]
Orinoco goose	Mallard + Pekin duck		+	+	Similarly efficient as DHBV in vivo; reduced in Pekin duck hepatocytes	[74]
Chiloe wigeon	Pekin duck		+	+	Similarly efficient as DHBV in vivo; reduced in Pekin duck hepatocytes	[74]
Mandarin duck	Pekin duck		+	+	Efficient infection although virus most similar to Ross´ goose virus	[74]
Ashy-headed sheldgoose	Pekin duck		-	+	No in vivo infection detected in 6/6 birds, reduced in duck hepatocytes	[74]
Ross´ goose	Pekin duck		n.d.	+	~ 15% as efficient as DHBV	K. Dallmeier, unpublished
Snow goose	Pekin duck		n.d.	+	Efficient infection	[80]
White stork	Pekin duck		n.d.	((+))	Very inefficient infection	[81]
Crowned crane	Pekin duck		n.d.	+	Efficient infection	[84]
Grey heron	Pekin duck		-	n.d.	Negative with virus, and intra-hepatic HHBV DNA injection	[82,127]
Grey heron	Pekin duck			((+))	At most 1% as efficient as DHBV in Pekin duck hepatocytes	[127]

See footnotes to Table 1 for explanations; n.d., not determined.

restrictive. Examples include the ability of both HBV and WMHBV to infect primary tupaia hepatocytes [100, 128], or the infectivity, though at low efficiency, of the ashy-headed sheldgoose isolate to infect duck hepatocytes [74]. They also revealed in several cases that the apparent block to *in vivo* infection was not absolute; Muscovy duck hepatocytes were susceptible to DHBV infection [107], and Pekin duck hepatocytes to heron [127] and stork virus [81] infection, though all at 100- to 1,000-fold reduced efficiency. The crane virus infected Pekin duck hepatocytes with DHBV-like efficiency [84]; however, its sequence is, as mentioned, highly similar to the Ross's goose/ Mandarin duck isolates that are able to infect Pekin duck hepatocytes ([74] and K. Dallmeier, unpublished results) and Pekin ducks *in vivo* [74]. There are, however, also examples which are not easily reconciled with the *in vivo* data. HDV with an HBV envelope efficiently infected, in the absence of the facilitating agent polyethylenglykol (PEG), hepatocytes from humans

and chimps, but also from spider monkey yet not baboons. HDV with a WMHBV envelope was, as expected, poorly infectious for hepatocytes from humans, chimps, and baboons but the efficiency of spider monkey hepatocyte infection was not higher [119].

Hence no simple unifying concept on hepadnaviral host range can be inferred from these data.

Molecular basis of hepadnaviral host range

The genome-wide distribution of nucleotide exchanges between isolates in cross-species transmission of natural hepadnaviruses prevents an accurate assignment of infectivity phenotypes to specific genes or gene products. This problem can be overcome by using one characterised virus with defined mutations.

Attachment and entry represent major barriers for successful hepadnavirus infection (see above) yet the responsible cellular factors are only partially known for the avian HBVs, and not at all for the mammalian HBVs; hence identifying the host receptor(s) remains one of the holy grails in hepatitis B virology. For the human virus, many different candidate receptors have been proposed [3], including annexin V, IgA receptor, squamous cell carcinoma antigen 1, different forms of the asialoglycoprotein receptor, transferrin receptor, apolipoprotein H, fibronectin, or most recently, lipoprotein lipase as one of several proteins containing a peptide motif that binds to HBV PreS [132]. None, however, confers susceptibility to HBV infection of cells that, like Huh7 or HepG2, support downstream events in the HBV replication cycle. For DHBV, a 180 kDa glycoprotein (gp180, now known as carboxypeptidase D [133, 134]) and p120, a subunit of mitochondrial glycine decarboxylase [135, 136], have been implicated in the early steps of infection, yet again, neither of the two fulfils all criteria for a functional virus receptor; also, carboxypeptidase D is highly conserved between different bird species and not liver-specifically expressed, hence the PreS–carboxypeptidase D interaction can explain neither host nor tissue tropism of avihepadnaviruses [3].

In contrast, there is no doubt that the PreS domains of the hepadnaviral large envelope proteins (Fig. 6) are crucial for binding, and probably entry into, host cells [3], and thus are prime candidates for being involved in host specificity. This view is supported by the much higher sequence divergence of the PreS regions compared to other parts of the genome (Figs 2B and 3B). Hence most efforts on understanding the molecular basis of hepadnaviral host range have concentrated on mutating PreS and monitoring the consequences in one of the infection systems. While such data led to a seemingly precise definition of rather simple ‘host range determining regions’ within PreS (Fig. 6), the outcome of these experiments can drastically be affected by the specific test system used (see below).

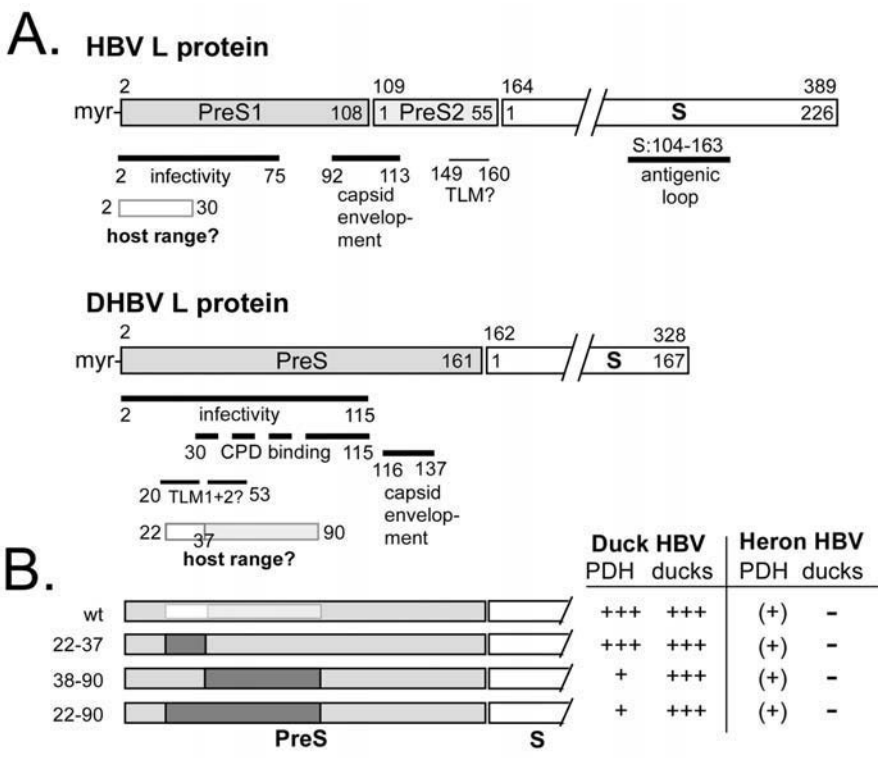


Figure 6. A. Hepadnaviral large envelope proteins and supposed functions of individual PreS regions. Numbers represent amino acid positions, numbers inside the bars indicate the lengths of the individual domains; myr refers to the myristoylation at the Gly-2 residues of all L proteins. Functional subregions are indicated similarly as described in reference [3], except that question marks have been added to the translocation motifs (TLM) and the so-called host range determining regions to indicate that their roles are, at the least, debatable. For the DHBV L protein, the carboxypeptidase D (CPD) binding region (essential, amino acids 86-115; stabilising, amino acids 30-86) and the positions of the short (PreS amino acids 22-37) and long (amino acids 22-90) forms of the proposed host determining region [127, 146] are indicated. B. Infectivities of chimeric DHBV and HHBV viruses for primary duck hepatocytes (PDH) and ducklings *in vivo.* Duck HBV refers to DHBV genomes carrying, in *cis*, the indicated regions of the heron HBV preS gene, and HHBV to heron HBV genomes carrying the corresponding regions of the DHBV preS gene. Infectivities are scored as follows: +++, wild-type DHBV-like; +, at least 10-fold reduced; (+), at least 100-fold reduced; –, no detectable infection.

Infection experiments with hepadnaviruses carrying defined envelope protein mutations

The two major experimental approaches to study the effects of PreS mutations on infectivity and host range in use are pseudotyping [127, 137] and the creation of chimeric viruses [126]; a third alternative, not discussed here in

detail, is infection inhibition by PreS-derived peptides [3]. Pseudotypes are viruses that carry an envelope which they do not encode themselves, usually provided in *trans* from a suitable expression vector; pseudotyped as well as chimeric virions can be generated by transfection of suitable cell lines, such as Huh7 or HepG2 for the primate, and LMH for the bird HBVs. An advantage is that only minimal changes in the genome are required to knockout the open reading frames for the large and, if desired, also small envelope proteins; this prevents problems potentially arising from more complex manipulations that might have unforeseen consequences on the overlapping P gene (Fig. 1B) and the numerous cis-elements distributed over the hepadnaviral genome [138]. However, because pseudotyped viruses do not encode their envelope proteins, infection is restricted to a single round and therefore can only be monitored in cultured cells, not *in vivo*. The reverse is true for chimeric viruses; however, principal replication competence and proper envelopment [54] can be tested beforehand by transfection.

Because of the lack of suitable host animals for the primate HBVs all studies so far have used the pseudotyping approach, sometimes with HDV instead HBV [119, 139–143], and primary human or primate hepatocytes or recently, the HepaRG cell line [58]. Mutations in the envelope proteins included systematic deletions in PreS, or in S [143, 144], or the replacement of segments with the homologous segments from another hepadnavirus, usually WMHBV [119, 145]. Collectively, the most recent, comprehensive data indicate that all infectivity determinants within HBV PreS1 are confined to the first 75 amino acids [58], with a possible role for the antigenic loop in S [143] but not the TLM motif in PreS2. HBV pseudotyped with a WMHBV-derived envelope displayed reduced infectivity for primary human hepatocytes, yet infectivity was restored when the N terminal 30 amino acids were derived from HBV PreS1 [145]; conversely, replacement of this segment by that of WMHBV in the context of the HBV L protein reduced infectivity. Partly congruent data have been obtained using the HDV pseudotype system [119] in that the first 40 WMHBV PreS1 amino acids implanted into the HBV L protein reduced infectivity for human hepatocytes; however, in the reverse setting the first 40 HBV PreS1 amino acids in WMHBV L only partly increased infectivity for the human cells; most surprisingly, however, this chimeric L protein had a strongly enhanced infectivity for spider monkey hepatocytes, to a level higher than that provided by a complete WMHBV L protein. Other than suggesting that PreS contains separate domains that interact with distinct cell receptors, interpretation of these results remains enigmatic, and farther-reaching *in vivo* experiments are currently not feasible with the primate HBVs.

Also the rodent HBVs are not ideal for such studies because no cell lines exist that, upon transfection, support efficient production of recombinant viruses, or pseudotypes. Hence there is only one early study on chimeric GSHVs in which about 30%, or about 80% of the S gene were replaced by WHV sequence; these viruses were infectious for ground squirrels upon

intrahepatic DNA inoculation [126], suggesting that S does not determine host range.

Due to these limitations, most studies on hepadnaviral host range have been performed with avihepadnaviruses although, until recently (see below), the potential of this system for true *in vivo* studies has not been exploited.

Most influential for the entire field has been a pseudotype study by Ishikawa and Ganem [127]. Based on the high infectivity of DHBV but about 100-fold lower infectivity of heron HBV (HHBV) for primary Pekin duck hepatocytes, a HHBV genome deficient for surface protein production was pseudotyped with chimeric HHBV envelope proteins in which various parts of the PreS region were replaced by the corresponding DHBV PreS sequences. All constructs yielded enveloped virions after transfection into LMH cells, which were then used to inoculate primary duck hepatocytes; formation of intracellular replicative intermediates indicated that the entire DHBV PreS sequence, and segments 1–108, 22–108, 1–90, but not 43–161, rescued infectivity; from this it was concluded that residues 22 to 90 determine the host range of avian HBVs. An additional chimera bearing only DHBV amino acids 22–37 was apparently also infectious for duck hepatocytes (T. Ishikawa, pers. communication), and PreS-derived peptides spanning slightly more than this region (DHBV PreS2-41), particularly if myristoylated like natural PreS, inhibited DHBV infection of duck hepatocytes [146]. Therefore, the prevailing view is that PreS amino acids 22–37, or possibly 22–90, constitute the host determining region of the avian HBVs [2, 3, 84, 146]. Notably, this region is not involved in carboxypeptidase D binding (Fig. 6), and a myristoylated peptide corresponding to the heron HBV PreS sequence 1–44 blocked DHBV infection of duck hepatocytes as efficiently as the autologous DHBV PreS peptide [146]. Evidently, this is not easily reconciled with a specific role of this PreS segment in determining host range.

In order to shed more light on the relevance of these data for a true *in vivo* infection, we generated two series of chimeric duck and heron HBVs, bearing, in cis, the short and long forms of the proposed host determining PreS regions from the other virus (Fig. 6, bottom); being able to inherit the chimeric envelope proteins to their progeny, these viruses are suitable for *in vivo* studies ([147] and Dallmeier and Nassal, submitted). Based on the published pseudotype data we expected that implantation of the heron sequence into DHBV would reduce, and conversely, implantation of the supposed DHBV PreS host determining region into heron HBV would increase infectivity for duck hepatocytes and ducklings. All the chimeric viruses, upon transfection, were fully replication-competent and formed comparable amounts of enveloped virions as wild-type DHBV. However, none of the heron HBV chimeras containing DHBV PreS segments 22–37, 22–90, 1–90 and 22–108, and or even the entire PreS region, displayed significantly increased infectivity for primary duck hepatocytes over wild-type heron HBV, or was able to establish *in vivo* infections in ducklings. Repeating the original pseudotype experiments, the complete DHBV PreS

region, as well as segments 22–108 and 22–37, though not 1–90 as reported, and 22–90 as inferred, clearly increased infectivity for hepatocytes. Thus in our hands substantial parts of, though not all, the previously published data were reproduced with the pseudotype system – but not with the chimeric viruses. There is currently no trivial explanation for this difference.

Even more disturbing are the data obtained with DHBV genomes carrying the corresponding heron HBV PreS sequences in their L proteins. As anticipated, a reduction in infectivity for duck hepatocytes was seen with chimeras in which the DHBV PreS segments 22–90, and also its subsegment 38–90, were replaced by heron HBV sequence; however, replacement of residues 22–37 had no negative impact on infectivity for duck hepatocytes. Hence in this setting, the short form of the supposed host determining region did not affect host range. Most surprising, however, were the *in vivo* infection data. All three viruses, including those with the large heterologous PreS segments and poor infectivity for cultured hepatocytes, were able to infect ducklings *in vivo*. The chimera with the entire originally proposed duck specificity determining region 22–90 replaced by heron virus sequence was further characterised and shown to establish high-titered, chronic infections in ducklings, and to be both horizontally and vertically transmittable. Quantitative monitoring during the early infection phase also showed kinetics of viral spread that were equal to, or even faster than those for wild-type DHBV. Thus, although seriously handicapped in cell culture, this virus without the proper host determining region for ducks was as infectious *in vivo* as its authentic DHBV parent. This defies the view that cultured cells are less restrictive than an intact host organisms, and it seriously questions the current concept of a short amino acid sequence in PreS as the crucial factor in host range determination of avian, and possibly also mammalian, HBVs. Unless the chimeric virus has switched to a different receptor, the data strongly suggest that, *in vivo*, the heron HBV PreS sequence interacts as well with the duck receptor(s) as the authentic DHBV PreS sequence. Notably, this is in accord with the lack of species-specific restrictions suggested by the comparably efficient inhibition of DHBV infection by both duck HBV and heron HBV PreS peptides [146], and the strong binding of the downstream PreS regions from both viruses to duck carboxypeptidase D [148]. Evidently cultured hepatocytes are not the ultimate system for definite statements on what determines hepadnaviral host range; moreover, the entire current concept of a short PreS segment as host determining region may need reevaluation.

Cellular restriction factors as determinants for hepadnaviral host range?

Another unresolved question is the suspicious non-susceptibility of Muscovy ducks for *in vivo* DHBV infection [106, 107]; in cell culture, infection is

not completely blocked, as is it with chicken hepatocytes, but is similarly inefficient as infection of Pekin duck hepatocytes with heron HBV [127]. Phylogeny provides no clue as to why the genus *Cairina* should not be infectable while Mandarin ducks can host a DHBV-like virus (see above). Hence Muscovy ducks may fortuitously lack a functional permissivity factor, or possess (a) restriction factor(s) that is/are absent from Pekin and other DHBV-infectable duck species. That non-permissiveness can, at least in cultured hepatocytes, be partially overcome by high doses of virus would be compatible with out-titration (abrogation) of such a negative factor but also with the absence of, or incompatibility with, host permissivity factors. As a first step towards distinguishing between these possibilities, we resorted to a classic genetic approach. Muscovy and Pekin ducks can interbreed, attesting to their close phylogenetic relation; such mule ducks are produced in large numbers in the food industry. A Muscovy drake was crossed with chronically DHBV infected Pekin duck females, and the offspring embryos were analysed for DHBV infection (K. Dallmeier, U. Schultz, M. Nassal; unpublished data). Four of four embryos tested showed clearly detectable, though slightly varying, levels of typical DHBV replicative intermediates in their livers. As F1-hybrids, these embryos carried one copy each of the paternal and of the maternal alleles. Hence their gain of susceptibility for infection is incompatible with the presence of a dominantly acting paternally inherited restriction factor yet compatible with the presence of a functional permissivity factor encoded by a maternal gene. The nature of this factor is currently unclear but differential cloning or similar techniques might be suited for its identification. Also, these results do not rule out that restriction factors play an important role in other hepadnavirus-host systems.

Conclusions and outlook

Many aspects of hepadnaviral replication have been elucidated in considerable detail; however our understanding of hepadnavirus host range and its molecular basis lags far behind. Certainly, hepadnaviruses do not have a broad host range yet whether their host range is truly narrow remains a matter of interpretation as long as the phylogenetic relations between the proven hepadnavirus host species are not rigidly defined. Unfortunately, hepadnaviral host range is sufficiently restricted so as to limit *in vivo* studies to few experimentally accessible species; even for DHBV the full spectrum of infectable hosts is unknown. *In vitro* infection assays allow access to cells from a wider range of hosts yet recent data both for orthohepadnaviruses, using human and Woolly monkey HBV, and for avihepadnaviruses, using duck and heron HBV, cast doubts on the transferability of *in vitro* data on statements on *in vivo* host range. One of the major challenges, as mentioned years ago [149], remains the unambiguous identification of the cellular receptor(s) mediating productive hepadnavirus infection; carboxypeptidase

D, though likely involved in DHBV infection, does not explain tissue- and host tropism, and does not at all seem to play a role for human HBV infection. Given the limited success of conventional approaches it is hoped that application of new techniques, such as expression profiling and gene knock-down by RNA interference, to permissive *versus* non-permissive cells will help answering this question; however, the potentially crucial roles of non-proteinaceous molecules as well as of host restriction factors must not be disregarded, and similarly, viral factors other than PreS could importantly contribute to hepadnaviral host range. Hence both systematic work and imaginative thinking will be required to solve this enigmatic aspect of hepadnavirus biology and realise the medically highly relevant issue of a small animal model for human HBV infection.

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References

1 Beck J, Nassal M (2007) Hepatitis B virus replication. *World J Gastroenterol* 13: 48–64

2 Funk A, Mhamdi M, Will H, Sirma H (2007) Avian hepatitis B viruses: molecular and cellular biology, phylogenesis, and host tropism. *World J Gastroenterol* 13: 91–103

3 Glebe D, Urban S (2007) Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol* 13: 22–38

4 Louz D, Bergmans HE, Loos BP, Hoeben RC (2005) Cross-species transfer of viruses: implications for the use of viral vectors in biomedical research, gene therapy and as live-virus vaccines. *J Gene Med* 7: 1263–1274

5 Heeney JL, Dalgleish AG, Weiss RA (2006) Origins of HIV and the evolution of resistance to AIDS. *Science* 313: 462–466

6 Zhao GP (2007) SARS epidemiology – From descriptive to mechanistic analyses. *Virus Res* doi: 10.1016/j.virusres.2007.01.010

7 Peiris JS, de Jong MD, Guan Y (2007) Avian influenza virus (H5N1): a threat to human health. *Clin Microbiol Rev* 20: 243–267

8 Vilchez RA, Kusne S (2006) Molecular and clinical perspectives of polyomaviruses: emerging evidence of importance in non-kidney transplant populations. *Liver Transpl* 12: 1457–1463

9 Falcone V, Schweizer M, Neumann-Haefelin D (2003) Replication of primate

foamy viruses in natural and experimental hosts. *Curr Top Microbiol Immunol* 277: 161–180
10 Brumme ZL, Harrigan PR (2006) The impact of human genetic variation on HIV disease in the era of HAART. *AIDS Rev* 8: 78–87
11 Reiche EM, Bonametti AM, Voltarelli JC, Morimoto HK, Watanabe MA (2007) Genetic polymorphisms in the chemokine and chemokine receptors: impact on clinical course and therapy of the human immunodeficiency virus type 1 infection (HIV-1). *Curr Med Chem* 14: 1325–1334
12 Thio CL, Astemborski J, Bashirova A, Mosbruger T, Greer S, Witt MD, Goedert JJ, Hilgartner M, Majeske A, O'Brien SJ et al (2007) Genetic protection against hepatitis B virus conferred by CCR5Delta32: Evidence that CCR5 contributes to viral persistence. *J Virol* 81: 441–445
13 Lim JK, Glass WG, McDermott DH, Murphy PM (2006) CCR5: no longer a ‚good for nothing' gene–chemokine control of West Nile virus infection. *Trends Immunol* 27: 308–312
14 Marsh M, Helenius A (2006) Virus entry: open sesame. *Cell* 124: 729–740
15 Baranowski E, Ruiz-Jarabo CM, Domingo E (2001) Evolution of cell recognition by viruses. *Science* 292: 1102–1105
16 Weissenhorn W, Hinz A, Gaudin Y (2007) Virus membrane fusion. *FEBS Lett* 581: 2150–2155
17 Neumann G, Kawaoka Y (2006) Host range restriction and pathogenicity in the context of influenza pandemic. *Emerg Infect Dis* 12: 881–886
18 Law LK, Davidson BL (2005) What does it take to bind CAR? *Mol Ther* 12: 599–609
19 Briz V, Poveda E, Soriano V (2006) HIV entry inhibitors: mechanisms of action and resistance pathways. *J Antimicrob Chemother* 57: 619–627
20 McFadden G (2005) Poxvirus tropism. *Nat Rev Microbiol* 3: 201–213
21 Mayer MP (2005) Recruitment of Hsp70 chaperones: a crucial part of viral survival strategies. *Rev Physiol Biochem Pharmacol* 153: 1–46
22 Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* 62: 670–684
23 Ambrose Z, Kewalramani VN, Bieniasz PD, Hatziioannou T (2007) HIV/AIDS: in search of an animal model. *Trends Biotechnol* 25: 333–337
24 Goff SP (2007) Host factors exploited by retroviruses. *Nat Rev Microbiol* 5: 253–263
25 Tsurutani N, Yasuda J, Yamamoto N, Choi BI, Kadoki M, Iwakura Y (2007) Nuclear import of the preintegration complex is blocked upon infection by human immunodeficiency virus type 1 in mouse cells. *J Virol* 81: 677–688
26 Sun J, Soos T, Kewalramani VN, Osiecki K, Zheng JH, Falkin L, Santambrogio L, Littman DR, Goldstein H (2006) CD4-specific transgenic expression of human cyclin T1 markedly increases human immunodeficiency virus type 1 (HIV-1) production by CD4+ T lymphocytes and myeloid cells in mice transgenic for a provirus encoding a monocyte-tropic HIV-1 isolate. *J Virol* 80: 1850–1862
27 Lambert C, Doring T, Prange R (2007) Hepatitis B Virus Maturation is Sensitive to Functional Inhibition of ESCRT-III, Vps4, and {gamma}2-Adaptin. *J Virol* 81: 9050–9060

28 Watanabe T, Sorensen EM, Naito A, Schott M, Kim S, Ahlquist P (2007) Involvement of host cellular multivesicular body functions in hepatitis B virus budding. *Proc Natl Acad Sci USA* 104: 10205–10210
29 Mhamdi M, Funk A, Hohenberg H, Will H, Sirma H (2007) Assembly and budding of a hepatitis B virus is mediated by a novel type of intracellular vesicles. *Hepatology* 46: 95–106
30 Goff SP (2004) Retrovirus restriction factors. *Mol Cell* 16: 849–859
31 Goff SP (2004) Genetic control of retrovirus susceptibility in mammalian cells. *Annu Rev Genet* 38: 61–85
32 Perez O, Hope TJ (2006) Cellular restriction factors affecting the early stages of HIV replication. *Curr HIV/AIDS Rep* 3: 20–25
33 Nisole S, Stoye JP, Saib A (2005) TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 3: 799–808
34 James LC, Keeble AH, Khan Z, Rhodes DA, Trowsdale J (2007) Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc Natl Acad Sci USA* 104: 6200–6205
35 Sebastian S, Luban J (2007) The Retroviral Restriction Factor TRIM5alpha. *Curr Infect Dis Rep* 9: 167–173
36 Sakuma R, Noser JA, Ohmine S, Ikeda Y (2007) Rhesus monkey TRIM5alpha restricts HIV-1 production through rapid degradation of viral Gag polyproteins. *Nat Med* 13: 631–635
37 Towers GJ, Hatziioannou T, Cowan S, Goff SP, Luban J, Bieniasz PD (2003) Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. *Nat Med* 9: 1138–1143
38 Gack MU, Shin YC, Joo CH, Urano T, Liang C, Sun L, Takeuchi O, Akira S, Chen Z, Inoue S et al (2007) TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446: 916–920
39 Cullen BR (2006) Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors. *J Virol* 80: 1067–1076
40 Mariani R, Chen D, Schrofelbauer B, Navarro F, Konig R, Bollman B, Munk C, Nymark-McMahon H, Landau NR (2003) Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114: 21–31
41 Nguyen DH, Gummuluru S, Hu J (2007) Deamination-independent inhibition of hepatitis B virus reverse transcription by APOBEC3G. *J Virol* 81: 4465–4472
42 Rösler C, Köck J, Kann M, Malim MH, Blum HE, Baumert TF, von Weizsäcker F (2005) APOBEC-mediated interference with hepadnavirus production. *Hepatology* 42: 301–309
43 Conticello SG, Thomas CJ, Petersen-Mahrt SK, Neuberger MS (2005) Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Mol Biol Evol* 22: 367–377
44 Gao G, Guo X, Goff SP (2002) Inhibition of retroviral RNA production by ZAP, a CCCH-type zinc finger protein. *Science* 297: 1703–1706
45 Guo X, Ma J, Sun J, Gao G (2007) The zinc-finger antiviral protein recruits the RNA processing exosome to degrade the target mRNA. *Proc Natl Acad Sci USA* 104: 151–156
46 Müller S, Möller P, Bick MJ, Wurr S, Becker S, Günther S, Kümmerer BM

(2007) Inhibition of filovirus replication by the zinc finger antiviral protein. *J Virol* 81: 2391–2400
47 Uematsu S, Akira S (2007) Toll-like Receptors and Type I Interferons. *J Biol Chem* 282: 15319–15323
48 Bowie AG, Fitzgerald KA (2007) RIG-I: tri-ing to discriminate between self and non-self RNA. *Trends Immunol* 28: 147–150
49 Vitour D, Meurs EF (2007) Regulation of interferon production by RIG-I and LGP2: a lesson in self-control. *Sci STKE* 2007: pe20
50 Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J (2005) Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 437: 1167–1172
51 Christen V, Duong F, Bernsmeier C, Sun D, Nassal M, Heim MH (2007) Inhibition of alpha interferon signaling by hepatitis B virus. *J Virol* 81: 159–165
52 Cullen BR (2006) Is RNA interference involved in intrinsic antiviral immunity in mammals? *Nat Immunol* 7: 563–567
53 Yang PL, Althage A, Chung J, Chisari FV (2002) Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci USA* 99: 13825–13830
54 Bruss V (2007) Hepatitis B virus morphogenesis. *World J Gastroenterol* 13: 65–73
55 Lu X, Block TM, Gerlich WH (1996) Protease-induced infectivity of hepatitis B virus for a human hepatoblastoma cell line. *J Virol* 70: 2277–2285
56 Maenz C, Chang SF, Iwanski A, Bruns M (2007) Entry of duck hepatitis B virus into primary duck liver and kidney cells after discovery of a fusogenic region within the large surface protein. *J Virol* 81: 5014–5023
57 Stoeckl L, Funk A, Kopitzki A, Brandenburg B, Oess S, Will H, Sirma H, Hildt E (2006) Identification of a structural motif crucial for infectivity of hepatitis B viruses. *Proc Natl Acad Sci USA* 103: 6730–6734
58 Blanchet M, Sureau C (2007) Infectivity determinants of the hepatitis B virus pre-S domain are confined to the N-terminal 75 amino acid residues. *J Virol* 81: 5841–5849
59 Lepere C, Regeard M, Le Seyec J, Gripon P (2007) The Translocation Motif of Hepatitis B Virus Envelope Proteins is Dispensable for Infectivity. *J Virol* 81: 7816–7818
60 Kann M, Schmitz A, Rabe B (2007) Intracellular transport of hepatitis B virus. *World J Gastroenterol* 13: 39–47
61 Raney AK, Eggers CM, Kline EF, Guidotti LG, Pontoglio M, Yaniv M, McLachlan A (2001) Nuclear covalently closed circular viral genomic DNA in the liver of hepatocyte nuclear factor 1 alpha-null hepatitis B virus transgenic mice. *J Virol* 75: 2900–2911
62 Gao W, Hu J (2007) Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. *J Virol* 81: 6164–6174
63 Tang H, McLachlan A (2002) Avian and Mammalian hepadnaviruses have distinct transcription factor requirements for viral replication. *J Virol* 76: 7468–7472

64 Di Q, Summers J, Burch JB, Mason WS (1997) Major differences between WHV and HBV in the regulation of transcription. *Virology* 229: 25–35
65 Galle PR, Schlicht HJ, Fischer M, Schaller H (1988) Production of infectious duck hepatitis B virus in a human hepatoma cell line. *J Virol* 62: 1736–1740
66 Hirsch R, Colgrove R, Ganem D (1988) Replication of duck hepatitis B virus in two differentiated human hepatoma cell lines after transfection with cloned viral DNA. *Virology* 167: 136–142
67 Pugh JC, Yaginuma K, Koike K, Summers J (1988) Duck hepatitis B virus (DHBV) particles produced by transient expression of DHBV DNA in a human hepatoma cell line are infectious *in vitro*. *J Virol* 62: 3513–3516
68 Jilbert AR, Miller DS, Scougall CA, Turnbull H, Burrell CJ (1996) Kinetics of duck hepatitis B virus infection following low dose virus inoculation: one virus DNA genome is infectious in neonatal ducks. *Virology* 226: 338–345
69 Marion PL, Oshiro LS, Regnery DC, Scullard GH, Robinson WS (1980) A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. *Proc Natl Acad Sci USA* 77: 2941–2945
70 Summers J, Smolec JM, Snyder R (1978) A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc Natl Acad Sci USA* 75: 4533–4537
71 Zhang YY, Summers J (2004) Rapid production of neutralizing antibody leads to transient hepadnavirus infection. *J Virol* 78: 1195–1201
72 Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C (2002) Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA* 99: 15655–15660
73 Lanford RE, Chavez D, Barrera A, Brasky KM (2003) An infectious clone of woolly monkey hepatitis B virus. *J Virol* 77: 7814–7819
74 Guo H, Mason WS, Aldrich CE, Saputelli JR, Miller DS, Jilbert AR, Newbold JE (2005) Identification and characterization of avihepadnaviruses isolated from exotic anseriformes maintained in captivity. *J Virol* 79: 2729–2742
75 Robertson BH, Margolis HS (2002) Primate hepatitis B viruses – genetic diversity, geography and evolution. *Rev Med Virol* 12: 133–141
76 Lanford RE, Chavez D, Brasky KM, Burns RB 3rd, Rico-Hesse R (1998) Isolation of a hepadnavirus from the woolly monkey, a New World primate. *Proc Natl Acad Sci USA* 95: 5757–5761
77 Minuk GY, Shaffer EA, Hoar DI, Kelly J (1986) Ground squirrel hepatitis virus (GSHV) infection and hepatocellular carcinoma in the Canadian Richardson ground squirrel (*Spermophilus richardsonii*). *Liver* 6: 350–356
78 Testut P, Renard CA, Terradillos O, Vitvitski-Trepo L, Tekaia F, Degott C, Blake J, Boyer B, Buendia MA (1996) A new hepadnavirus endemic in arctic ground squirrels in Alaska. *J Virol* 70: 4210–4219
79 Mason WS, Seal G, Summers J (1980) Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J Virol* 36: 829–836
80 Chang SF, Netter HJ, Bruns M, Schneider R, Frolich K, Will H (1999) A new avian hepadnavirus infecting snow geese (*Anser caerulescens*) produces a significant fraction of virions containing single-stranded DNA. *Virology* 262: 39–54
81 Pult I, Netter HJ, Bruns M, Prassolov A, Sirma H, Hohenberg H, Chang SF,

Frolich K, Krone O, Kaleta EF et al (2001) Identification and analysis of a new hepadnavirus in white storks. *Virology* 289: 114–128
82 Sprengel R, Kaleta EF, Will H (1988) Isolation and characterization of a hepatitis B virus endemic in herons. *J Virol* 62: 3832–3839
83 Lin L, Prassolov A, Funk A, Quinn L, Hohenberg H, Frolich K, Newbold J, Ludwig A, Will H, Sirma H et al (2005) Evidence from nature: interspecies spread of heron hepatitis B viruses. *J Gen Virol* 86: 1335–1342
84 Prassolov A, Hohenberg H, Kalinina T, Schneider C, Cova L, Krone O, Frolich K, Will H, Sirma H (2003) New hepatitis B virus of cranes that has an unexpected broad host range. *J Virol* 77: 1964–1976
85 Schultz U, Grgacic E, Nassal M (2004) Duck hepatitis B virus: an invaluable model system for HBV infection. *Adv Virus Res* 63: 1–70
86 Menne S, Cote PJ (2007) The woodchuck as an animal model for pathogenesis and therapy of chronic hepatitis B virus infection. *World J Gastroenterol* 13: 104–124
87 Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO (2004) Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 47: 289–309
88 Simmonds P, Midgley S (2005) Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol* 79: 15467–15476
89 Chimpanzee Sequencing and Analysis Consortium (2005) Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature* 437: 69–87
90 Gibbs RA, Rogers J, Katze MG, Bumgarner R, Weinstock GM, Mardis ER, Remington KA, Strausberg RL, Venter JC, Wilson RK et al (2007) Evolutionary and biomedical insights from the rhesus macaque genome. *Science* 316: 222–234
91 Livezey BC, Zusi RL (2007) Higher-order phylogeny of modern birds (Theropoda, Aves: Neornithes) based on comparative anatomy. II. Analysis and discussion. *Zoo J Linnean Soc* 149: 1–95
92 Sibley CG, Ahlquist JE (1990) *Phylogeny and classification of birds: A study in molecular evolution.* Yale University Press, New Haven, CT
93 Lanford RE, Chavez D, Rico-Hesse R, Mootnick A (2000) Hepadnavirus infection in captive gibbons. *J Virol* 74: 2955–2959
94 Michaels MG, Lanford R, Demetris AJ, Chavez D, Brasky K, Fung J, Starzl TE (1996) Lack of susceptibility of baboons to infection with hepatitis B virus. *Transplantation* 61: 350–351
95 Kedda MA, Kramvis A, Kew MC, Lecatsas G, Paterson AC, Aspinall S, Stark JH, De Klerk WA, Gridelli B (2000) Susceptibility of chacma baboons (Papio ursinus orientalis) to infection by hepatitis B virus. *Transplantation* 69: 1429–1434
96 Herron MD, Castoe TA, Parkinson CL (2004) Sciurid phylogeny and the paraphyly of Holarctic ground squirrels (*Spermophilus*). *Mol Phylogenet Evol* 31: 1015–1030
97 Huchon D, Madsen O, Sibbald MJ, Ament K, Stanhope MJ, Catzeflis F, de Jong WW, Douzery EJ (2002) Rodent phylogeny and a timescale for the evolution

of Glires: evidence from an extensive taxon sampling using three nuclear genes. *Mol Biol Evol* 19: 1053–1065

98 Walter E, Keist R, Niederost B, Pult I, Blum HE (1996) Hepatitis B virus infection of tupaia hepatocytes *in vitro* and *in vivo. Hepatology* 24: 1–5

99 Glebe D, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH (2003) Pre-S1 antigen-dependent infection of *Tupaia hepatocyte* cultures with human hepatitis B virus. *J Virol* 77: 9511–9521

100 Köck J, Nassal M, MacNelly S, Baumert TF, Blum HE, von Weizsäcker F (2001) Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. *J Virol* 75: 5084–5089

101 Murphy WJ, Eizirik E, Johnson WE, Zhang YP, Ryder OA, O'Brien SJ (2001) Molecular phylogenetics and the origins of placental mammals. *Nature* 409: 614–618

102 Dyke GJ, van Tuinen M (2004) The evolutionary radiation of modern birds (Neornithes): reconciling molecules, morphology and the fossil record. *Zool J Linnean Soc* 141: 153–177

103 Fain MG, Krajewski C, Houde P (2007) Phylogeny of "core Gruiformes" (Aves: Grues) and resolution of the Limpkin-Sungrebe problem. *Mol Phylogenet Evol* 43: 515–529

104 Donne-Goussé C, Laudet V, Hänni C (2002) A molecular phylogeny of anseriformes based on mitochondrial DNA analysis. *Mol Phylogenet Evol* 23: 339–356

105 Cracraft J, Keith Barker F, Braun MJ, Harshman J, Dyke GJ, Feinstein J, Stanley S, Cibois A, Schikler P, Beresford P et al (2004) *Phylogenetic relationships among modern birds (Neornithes): Toward an avian tree of life*. Oxford University Press, New York

106 Marion PL, Cullen JM, Azcarraga RR, Van Davelaar MJ, Robinson WS (1987) Experimental transmission of duck hepatitis B virus to Pekin ducks and to domestic geese. *Hepatology* 7: 724–731

107 Pugh JC, Simmons H (1994) Duck hepatitis B virus infection of Muscovy duck hepatocytes and nature of virus resistance *in vivo*. *J Virol* 68: 2487–2494

108 Gibb GC, Kardailsky O, Kimball RT, Braun EL, Penny D (2007) Mitochondrial genomes and avian phylogeny: complex characters and resolvability without explosive radiations. *Mol Biol Evol* 24: 269–280

109 Pereira SL, Baker AJ (2006) A mitogenomic timescale for birds detects variable phylogenetic rates of molecular evolution and refutes the standard molecular clock. *Mol Biol Evol* 23: 1731–1740

110 Slack KE, Jones CM, Ando T, Harrison GL, Fordyce RE, Arnason U, Penny D (2006) Early penguin fossils, plus mitochondrial genomes, calibrate avian evolution. *Mol Biol Evol* 23: 1144–1155

111 van Tuinen M, Dyke GJ (2004) Calibration of galliform molecular clocks using multiple fossils and genetic partitions. *Mol Phylogenet Evol* 30: 74–86

112 Will H, Cattaneo R, Darai G, Deinhardt F, Schellekens H, Schaller H (1985) Infectious hepatitis B virus from cloned DNA of known nucleotide sequence. *Proc Natl Acad Sci USA* 82: 891–895

113 Will H, Cattaneo R, Koch HG, Darai G, Schaller H, Schellekens H, van Eerd

PM, Deinhardt F (1982) Cloned HBV DNA causes hepatitis in chimpanzees. *Nature* 299: 740–742
114 Seeger C, Ganem D, Varmus HE (1984) The cloned genome of ground squirrel hepatitis virus is infectious in the animal. *Proc Natl Acad Sci USA* 81: 5849–5852
115 Sprengel R, Kuhn C, Manso C, Will H (1984) Cloned duck hepatitis B virus DNA is infectious in Pekin ducks. *J Virol* 52: 932–937
116 Tuttleman JS, Pugh JC, Summers JW (1986) *In vitro* experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. *J Virol* 58: 17–25
117 Gripon P, Diot C, Theze N, Fourel I, Loreal O, Brechot C, Guguen-Guillouzo C (1988) Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *J Virol* 62: 4136–4143
118 Shimizu Y, Nambu S, Kojima T, Sasaki H (1986) Replication of hepatitis B virus in culture systems with adult human hepatocytes. *J Med Virol* 20: 313–327
119 Barrera A, Guerra B, Lee H, Lanford RE (2004) Analysis of host range phenotypes of primate hepadnaviruses by *in vitro* infections of hepatitis D virus pseudotypes. *J Virol* 78: 5233–5243
120 Sureau C (2006) The role of the HBV envelope proteins in the HDV replication cycle. *Curr Top Microbiol Immunol* 307: 113–131
121 Gudima S, Meier A, Dunbrack R, Taylor J, Bruss V (2007) Two potentially important elements of the hepatitis B virus large envelope protein are dispensable for the infectivity of hepatitis delta virus. *J Virol* 81: 4343–4347
122 Barrera A, Lanford RE (2004) Infection of primary chimpanzee hepatocytes with recombinant hepatitis D virus particles: a surrogate model for hepatitis B virus. *Methods Mol Med* 96: 131–142
123 Lee WY, Yoo NC (1989) Hepatitis B virus (HBV) infections in turtles. *Yonsei Med J* 30: 144–150
124 Ganem D, Weiser B, Barchuk A, Brown RJ, Varmus HE (1982) Biological characterization of acute infection with ground squirrel hepatitis virus. *J Virol* 44: 366–373
125 Trueba D, Phelan M, Nelson J, Beck F, Pecha BS, Brown RJ, Varmus HE, Ganem D (1985) Transmission of ground squirrel hepatitis virus to homologous and heterologous hosts. *Hepatology* 5: 435–439
126 Seeger C, Marion PL, Ganem D, Varmus HE (1987) *In vitro* recombinants of ground squirrel and woodchuck hepatitis viral DNAs produce infectious virus in squirrels. *J Virol* 61: 3241–3247
127 Ishikawa T, Ganem D (1995) The pre-S domain of the large viral envelope protein determines host range in avian hepatitis B viruses. *Proc Natl Acad Sci USA* 92: 6259–6263
128 Glebe D, Urban S, Knoop EV, Cag N, Krass P, Grun S, Bulavaite A, Sasnauskas K, Gerlich WH (2005) Mapping of the hepatitis B virus attachment site by use of infection-inhibiting preS1 lipopeptides and tupaia hepatocytes. *Gastroenterology* 129: 234–245
129 Ren S, Nassal M (2001) Hepatitis B virus (HBV) virion and covalently closed circular DNA formation in primary tupaia hepatocytes and human hepatoma cell lines upon HBV genome transduction with replication-defective adenovirus vectors. *J Virol* 75: 1104–1116

130 Seeger C, Baldwin B, Hornbuckle WE, Yeager AE, Tennant BC, Cote P, Ferrell L, Ganem D, Varmus HE (1991) Woodchuck hepatitis virus is a more efficient oncogenic agent than ground squirrel hepatitis virus in a common host. *J Virol* 65: 1673–1679
131 Lambert V, Cova L, Chevallier P, Mehrotra R, Trepo C (1991) Natural and experimental infection of wild mallard ducks with duck hepatitis B virus. *J Gen Virol* 72: 417–420
132 Deng Q, Zhai JW, Michel ML, Zhang J, Qin J, Kong YY, Zhang XX, Budkowska A, Tiollais P, Wang Y et al (2007) Identification and characterization of peptides that interact with hepatitis B virus via the putative receptor binding site. *J Virol* 81: 4244–4254
133 Kuroki K, Cheung R, Marion PL, Ganem D (1994) A cell surface protein that binds avian hepatitis B virus particles. *J Virol* 68: 2091–2096
134 Kuroki K, Eng F, Ishikawa T, Turck C, Harada F, Ganem D (1995) gp180, a host cell glycoprotein that binds duck hepatitis B virus particles, is encoded by a member of the carboxypeptidase gene family. *J Biol Chem* 270: 15022–15028
135 Li J, Tong S, Lee HB, Perdigoto AL, Spangenberg HC, Wands JR (2004) Glycine decarboxylase mediates a postbinding step in duck hepatitis B virus infection. *J Virol* 78: 1873–1881
136 Li JS, Tong SP, Wands JR (1996) Characterization of a 120-Kilodalton pre-S-binding protein as a candidate duck hepatitis B virus receptor. *J Virol* 70: 6029–6035
137 Horwich AL, Furtak K, Pugh J, Summers J (1990) Synthesis of hepadnavirus particles that contain replication-defective duck hepatitis B virus genomes in cultured HuH7 cells. *J Virol* 64: 642–650
138 Lewellyn EB, Loeb DD (2007) Base pairing between cis-acting sequences contributes to template switching during plus-strand DNA synthesis in human hepatitis B virus. *J Virol* 81: 6207–6215
139 Blanchet M, Sureau C (2006) Analysis of the cytosolic domains of the hepatitis B virus envelope proteins for their function in viral particle assembly and infectivity. *J Virol* 80: 11935–11945
140 Barrera A, Guerra B, Notvall L, Lanford RE (2005) Mapping of the hepatitis B virus pre-S1 domain involved in receptor recognition. *J Virol* 79: 9786–9798
141 Gudima S, He Y, Meier A, Chang J, Chen R, Jarnik M, Nicolas E, Bruss V, Taylor J (2007) Assembly of hepatitis delta virus: particle characterization, including the ability to infect primary human hepatocytes. *J Virol* 81: 3608–3617
142 Engelke M, Mills K, Seitz S, Simon P, Gripon P, Schnolzer M, Urban S (2006) Characterization of a hepatitis B and hepatitis delta virus receptor binding site. *Hepatology* 43: 750–760
143 Jaoude GA, Sureau C (2005) Role of the antigenic loop of the hepatitis B virus envelope proteins in infectivity of hepatitis delta virus. *J Virol* 79: 10460–10466
144 Le Seyec J, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P (1999) Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. *J Virol* 73: 2052–2057
145 Chouteau P, Le Seyec J, Cannie I, Nassal M, Guguen-Guillouzo C, Gripon P (2001) A short N-proximal region in the large envelope protein harbors a

determinant that contributes to the species specificity of human hepatitis B virus. *J Virol* 75: 11565–11572

146 Urban S, Gripon P (2002) Inhibition of duck hepatitis B virus infection by a myristoylated pre-S peptide of the large viral surface protein. *J Virol* 76: 1986–1990

147 Nassal M, Dallmeier K, Schultz U, Sun D (2005) Phenotyping hepatitis B virus variants: from transfection towards a small animal *in vivo* infection model. *J Clin Virol* 34 Suppl 1: S89–95

148 Urban S, Schwarz C, Marx UC, Zentgraf H, Schaller H, Multhaup G (2000) Receptor recognition by a hepatitis B virus reveals a novel mode of high affinity virus-receptor interaction. *EMBO J* 19: 1217–1227

149 Nassal M, Schaller H (1993) Hepatitis B virus replication. *Trends Microbiol* 1: 221–228