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Cell and Animal Models for Studying Hepatitis B Virus Infection and Drug Development

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Abstract

Many cell culture and animal models have been used to study hepatitis B virus (HBV) replication and its effects in the liver; these have facilitated development of strategies to control and clear chronic HBV infection. We discuss the advantages and limitations of systems for studying HBV and developing antiviral agents, along with recent advances. New and improved model systems are needed. Cell culture systems should be convenient, support efficient HBV infection, and reproduce responses of hepatocytes in the human body. We also need animals that are fully permissive to HBV infection, convenient for study, and recapitulate human immune responses to HBV and effects in the liver. High-throughput screening technologies could facilitate drug development based on findings from cell and animal models.

Keywords

Treatment; Mouse; Immune Response; Viral Infection

Hepatitis B virus (HBV) is a hepatotropic virus with strict specificity for human hepatocytes.[1,2] This species tropism and the difficulties in culturing human hepatocytes have limited systems for studying HBV. We review the HBV life cycle (Figure 1) and discuss cell culture and animal model systems for studying HBV replication and its mechanisms of pathogenesis. We also discuss strategies for development of anti-HBV agents based on findings from model systems.

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Conflicts of interest

The authors disclose no conflicts.

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Early steps in the HBV replication cycle, from cell binding through formation of covalently closed circular DNA (cccDNA), were difficult to study until the virus's receptor, the solute carrier family 10 member 1 (SLC10A1, also called NTCP),[3] was identified on human hepatocytes. This discovery led to cell culture systems to study these processes. Later steps of the replication cycle, from virus transcription to virion secretion, had been extensively studied in established cell culture systems, although many questions remain.

Because of the strict species specificity of HBV infection, animal models for studying the host response to the virus and disease pathogenesis have been suboptimal. Currently approved antiviral agents target only the DNA synthesis activity of the HBV reverse transcriptase protein, so these block viral DNA synthesis from pregenomic RNA (pgRNA) (step 7, Figure 1),[4,5] with the exception of the cytokine type I interferon, which appears to inhibit multiple steps of the HBV life cycle and promote the antivirus immune response.[5,6] Efforts to develop antiviral agents against viral genome reservoir or cccDNA, or to promote the patient's antivirus response, have been limited by the lack of optimal model systems.

Cell Culture Systems

A number of cell culture systems have been used to study HBV replication (Table 1). What are the advantages and disadvantages of each of these systems? No system is ideal for all studies, but findings from different systems can be complementary.

Human Liver Cell Lines

The hepatoma cell lines HepG2 (derived from a hepatoblastoma) and Huh7 (derived from a hepatocellular carcinoma [HCC]), have been widely used to study HBV replication, especially the later stages, from transcription and virion production. These cells lack the HBV receptor and are therefore not susceptible to HBV infection. However, transfection of these cells with cloned HBV DNA in over-length constructs, which serve as functional mimics of cccDNA to direct virus transcription, leads to expression of all virus gene products, assembly of intracellular empty capsids and nucleocapsids, and formation and secretion of virion particles, including complete (infectious) and empty virions and HB surface antigen (HBsAg) particles (Figure 1).[7–11] Virtually unlimited in supply and relatively stable genetically, the hepatoma cultures are convenient systems for studying these stages of HBV replication. Transient transfection with cloned HBV DNA and stably transfected cell lines derived from these hepatoma cells have been widely used to study HBV replication. Although transient transfection affords the flexibility of testing a large variety of virus mutants, the stable transfection system allows high efficiency of virus replication, including temporally controlled viral gene expression and replication.

Hepatoma cell lines allow not only for constitutive virus expression and replication, but inducible expression and replication of HBV. The HepAD38 and HepDE19 cell lines direct HBV pgRNA expression under control of the tetracycline-repressible promoter instead of the native viral core promoter.[12,13] HepG2 cells, and to a lesser-degree Huh7 cells, also support an intracellular recycling pathway that produces cccDNA following production of intracellular mature nucleocapsids (NCs), which contains relaxed circular DNA (rcDNA), the precursor to cccDNA (Figure 1, steps 8 and 3).[12–15] The heterologous promoter can be

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switched off following virus gene expression, DNA replication, and cccDNA production, so that cccDNA-supported viral gene expression and replication can be monitored, including the use of sensitive reporter systems.[16,17] However, cccDNA-directed transcription in these system is inefficient,[17,18] presumably due to the lack of appropriate transcription factors and/or epigenetic regulation, which promotes high levels of virus transcription in hepatocytes of infected individuals. In addition to not expressing the HBV receptor and providing a suboptimal environment for virus transcription, infected hepatoma cells fail to recapitulate many aspects of the responses in the infected hepatocytes in vivo, including potential innate immune response.[19–21]

The HepaRG cell line is a bi-potent liver progenitor cell line derived from a hepatoma in a patient infected with hepatitis C virus (HCV). These cells differentiate into hepatocyte-like and bile duct epithelium-like cells in culture. In contrast to all other human hepatoma cell lines tested, HepaRG cells, on differentiation, support HBV infection and the subsequent steps of HBV replication.[22,23] Furthermore, HBV-infected cells appear to mount an innate immune response that is able to suppress HBV replication.[20] Their response to interferons, including the suppression of HBV, is similar to that of primary human hepatocytes and better than that of HepG2 cells.[21]

However, there is controversy over the innate immune response to HBV infection. HBV does not induce a strong innate response, such as that mediated by type I interferon in humans or chimpanzees.[6,24–27] However, under certain circumstances, such as on exposure of virus DNA to cytosolic DNA-sensing mechanisms, due to enhanced or accelerated uncoating of viral nucleocapsids,[19] HBV can induce an innate immune response. A shortcoming of the HepaRG cells is that HBV infection is inefficient (even with high multiplicity of infection and differentiation conditions required for cells to become susceptible to infection), complex, and time-consuming (requires 4 weeks). Also, there is little to no amplification of cccDNA (Figure 1, steps 8 and 9) after HBV infection of HepaRG cells,[23] which is also true for HepG2 cells engineered to express NTCP (see later in this article). This could be due to the low levels of HBV replication in these cells, and consequently, the low levels of rcDNA.

Transgenic expression of NTCP in HepG2 cells[3] allows them to become infected with HBV, so one block to infection in these cells is therefore virus binding and entry. Transgenic expression of NTCP in Huh7 cells results in inefficient infection by HBV[28]; Huh7 cells are less efficient than HepG2 cells in supporting cccDNA formation via intracellular recycling (Figure 1, steps 8 and 3).[15] The lower infection efficiency of Huh7-expressing NTCP (Huh7-NTCP) cells vs. HepG2-NTCP cells could result from the lower capacity of Huh7 cells to support cccDNA formation (Figure 1, steps 2 and 3).

HepG2-NTCP cells are more convenient to carry in culture than HepaRG cells. HBV infection of HepG2-NTCP is sufficiently robust to allow for studies of the early stages of HBV infection, from cell attachment to cccDNA establishment and maintenance,[29–33] as well as for testing antiviral agents.[34,35] The infection may spread among cells cultured in the presence of polyethylene glycol (PEG),[36] which is routinely added during the infection period (overnight) and then removed. It is not clear how PEG, or dimethyl sulfoxide

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(DMSO, also used to reduce cell proliferation), increase cell infection by HBV. NTCP-HepG2 cells are transformed, so they lack many features of human hepatocytes, which affects their ability to support virus infection and replication or mount an immune response.[37,38]

Primary Human Hepatocytes

Primary human hepatocytes are considered to be the most physiologically relevant culture system for HBV infection in vitro, as they most closely represent the human hepatocytes in the liver, the natural target cells that are infected by HBV. Thus, they are best for studying metabolic and innate immune responses to HBV infection. Primary human hepatocytes do support the complete life cycle of HBV.[39–41] However, they are expensive, scarce, do not proliferate in culture, have a limited life span (1 month or shorter), vary among batches, and have low infection efficiency. They support only a limited spread of infection due to their rapid dedifferentiation in culture.[42,43] Human fetal hepatocytes have also been shown to support HBV infection. These cells have greater viability and proliferative capacity than adult hepatocytes.[44,45] In immune-deficient mice, endogenous hepatocytes can be destroyed and replaced with human hepatocytes, which expand following transplantation to generate mice with humanized livers (or chimeric mice).[46] The progeny of these human hepatocytes can be isolated from the chimeric mice and cultured for HBV infection. This approach to carrying primary human hepatocytes could increase reproducibility among studies; however, the procedure is time-consuming and technically challenging.

Efforts have been made constantly to improve systems for culture of primary human hepatocytes. These include methods to promote proliferation and maintain or increase differentiation, using an appropriate extracellular matrix, co-culture with nonparenchymal cells, incubation with DMSO, and 3-dimensional organization.[43,45,47,48] Primary human hepatocytes cultured in 3-dimensions stably express hepatocyte genes and function for a month or longer, and can be infected with HBV at low multiplicity of infection without the need for PEG or DMSO.[27] These improvements, if reproducible and widely adopted, could lead to wider application of primary human hepatocytes for HBV studies.

Induced Human Hepatocyte-like Cells

Developments in cell reprogramming techniques have led to production of induced human hepatocyte-like (iHep) cells. iHep cells or human hepatocyte-like cells are obtained via induced differentiation of human embryonic stem cells at least some aspects of the innate immune response in or induced pluripotent stem cells. They have more physiologic features of hepatocytes in humans than cell lines, and can support the complete life cycle of HBV, including virus spread among cells due to long-term maintenance of hepatic differentiation.[47,49,50] iHep cells are virtually unlimited in supply, amenable to genetic manipulation, and recapitulate infected hepatocytes in humans.[47] Strategies to optimize or transdifferentiate pluripotent stem cells[51–55] might lead to development of a convenient and relevant system for studying HBV infection.

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Nonhepatic and Nonhuman Cells

The human kidney embryonic cell line HEK293 and the chicken hepatoma cell line LMH each support later stages of HBV replication, including cccDNA formation via the intracellular recycling pathway (Figure 1).[14,56] It is therefore clear that no strictly liver- or human-specific factors are required for cccDNA formation. However, nonhepatic cells such as HEK293 are unable to support HBV transcription, due to the lack of appropriate transcription factors in these cells.[57–59] Therefore, strong heterologous promoters such as human cytomegalovirus immediate early enhancer/promoter are used to express HBV genes in these cells.[60] HEK293 cells and LMH cells can be transfected at high efficiency, and along with human hepatocyte-derived cells, they can be useful for comparative studies of the contributions of tissue- and species-specific factors to HBV replication.

Primary hepatocytes from macaques and pigs, but not mice, rats, or dogs, can support HBV infection following ectopic expression of the human NTCP, indicating the existence of host species-specific factors, beyond the receptor, that are required to support HBV infection.[61,62] Interestingly, hepatocytes from tree shrews (*Tupaia*)[63] can be infected with HBV, without the need for transgenic expression of human NTCP. These cells were instrumental in identification of NTCP as the HBV receptor.[3]

Of particular interest to the issue of species specificity of HBV infection, and the development of a small animal (mouse) model of HBV infection (see below), is the observation that mouse hepatocytes are unable to support HBV cccDNA formation, despite their high levels of viral transcription and subsequent steps through virion production.[64] A human-mouse heterokaryon assay attributed this deficiency to the lack of a factor in mouse cells that is needed to support cccDNA formation.[65] It was reported almost 2 decades ago that livers of mice with disruption of the hepatocyte nuclear factor 1 alpha gene (*Hnf1a*) support low levels of HBV cccDNA formation.[66] Although the underlying mechanism for this observation has remained unclear, this result did suggest that it might be possible to manipulate mouse cells to allow for HBV cccDNA formation. Indeed, the mouse hepatocyte cell line AML12, derived from transgenic mice that express human tumor growth factor alpha, support cccDNA formation as efficiently as human HepG2 cells, when it expresses the HBV pgRNA under control of a tetracycline-repressible promoter (AML12HBV10 cells).[67] Increased formation of cccDNA in AML12HBV10 cells correlates with an increase in uncoating of nucleocapsids, which releases rcDNA into the cell nucleus, a prerequisite for cccDNA formation (Figure 1, steps 2 and 8). Mature nucleocapsids, which contain rcDNA, are normally destabilized in human cells, compared with immature nucleocapsids, which is likely to be a priming event for uncoating[68]; this destabilizing increases in AML12HBV10 cells. AML12 cells that express human NTCP, in contrast to other mouse hepatocyte cell lines or primary mouse hepatocytes, are susceptible to HBV infection.[54,69,70]

These findings indicate that a major block in mouse hepatocytes to HBV infection is at the stage of nucleocapsid uncoating. Interestingly, mutations of the virus core protein increase cccDNA formation in human hepatoma cells and are associated with increased nucleocapsid uncoating.[15] cccDNA formation might therefore be accomplished in mouse hepatocytes either by manipulation of the mouse cells or modification of the viral capsid protein.

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Animal Models

There are many stages to chronic HBV infection.[71–74] Features of chronic HBV infection include formation of cccDNA, assembly of infectious viral particles for dissemination, and persistence. The immune response (innate and adaptive) involves seroconversion to HBsAg and HBe antigen (HBeAg) and eventual clearance of or tolerance to the virus. Chronic infection can lead to hepatitis, liver cirrhosis, and hepatocellular carcinoma. Studies of the mechanisms by which the immune response to HBV infection leads to hepatitis and processes of liver injury and repair require animal models.

Humans have a unique immune response to HBV similar to chimpanzees. Both do not have a conventional innate immune response (interferons, tumor necrosis factor, interleukin 1 beta) to HBV; however, HBV clearance in chimpanzees involves T-cell expression of genes that are regulated by interferon gamma and therefore suggest a nonconventional innate immune response.[24] Furthermore, clearance of HBV infection in humans varies with their age: human newborns exposed to HBV do not clear the virus, but almost all exposed adults eliminate HBV.[75] Animal models are used to systemically study interactions between HBV and the liver microenvironment, the immune response, and interactions between gut and liver.

Some animals are naturally infected with hepadnaviruses that are similar to HBV, such as woodchucks (infected with woodchuck hepatitis virus) and ducks (infected with duck HBV); these animals develop many features of human HBV infection. Chimpanzees are the only nonhuman immune-competent animals that are naturally susceptible to HBV infection. Chimpanzee studies were instrumental in the development of HBV vaccines in the 1970s for evaluation of vaccine safety and efficacy.[76] Natural animal models are also useful in verifying the safety and efficacy of antiviral nucleot(s)ides, small interfering RNAs (siRNAs), or other molecules.[77–83] Limitations to their study include genetic variation, lack of tools for studying the immune response, longer study periods, challenging laboratory techniques, and ethical considerations.[84]

Mice have well-characterized immune systems and are easy to handle and manipulate, but cannot be infected with HBV. Therefore, multiple genetically modified mouse models of HBV infection have been developed, using transgenic, transfection, and humanized liver technologies. Mice are useful and reliable for studying progression of HBV infection and the immune response. What animal models are available and what have we learned from them (see Table 2)?

Transgenic Mice

HBV transgenic mice that express single virus proteins (envelope, core, precore, or X) or all viral genes have been generated. Infectious HBV virions produced by mouse hepatocytes are morphologically indistinguishable from human-derived virions. Because these mice are transgenic for HBV, they do not have the potential of viral clearance.[64] However, infected mice can be used to study features of the innate immune response, such as HBV inhibition of interferon and tumor necrosis factor.[85,86] Adoptive transfer of immune cells from syngeneic mice immunized against HBV can be used to study the adaptive immune

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response. These mice do not develop liver fibrosis or cirrhosis, and only a few strains of transgenic mice, such as those that express large HBsAg or overexpress HBx protein, develop HCC.[87,88] HBV transgenic mice are used to study the effects of cytokines or drugs that might prevent HBV progression, and for development of strategies for breaking immune tolerance to HBV.[89–96]

NTCP is the receptor not only for HBV, but also for hepatitis D virus (HDV).[3] HDV, but not HBV, can infect transgenic mice that express human NTCP.[70] Similarly, rat or dog hepatocytes that express human NTCP are susceptible to HDV but not HBV infection.[61] This indicates that additional factors are required for HBV replication and for induction of an antiviral immune response.

Transfected Mice

To overcome the limitations of HBV transgenic mice, vectors are used for the introduction of HBV DNA in livers of mice. Hydrodynamic injection is used to transfer genetic material to mouse liver; it involves rapid injection of a large volume of liquid via the tail vein. Hydrodynamic injection of plasmids carrying an overlength HBV 1.3X results in high levels of HBV replication, high blood levels of HBV DNA, and an adaptive immune response that clears the HBV.[97] The system was modified to establish HBV persistence to allow studies of immune tolerance to the virus.[98] Chou et al[99] reported rapid clearance of HBV from BALB/cJ and NOD/ShiLtJ mice, but HBV persisted for longer times in C57BL/6J and C3H/HeN mice. The authors demonstrated the age-dependence of HBV clearance in C3H/HeN mice, which involved age-related establishment of the gut microbiota and the toll-like receptor 4. The mouse immune response to HBV can be studied to identify genes that regulate the innate and adaptive immune response in the liver and the effects of the intestinal microbiota.

HBV DNA can also be delivered into mouse hepatocytes via adenovirus (AdV)- or adeno-associated virus–based vectors. HBV protein production was observed for up to 3 months after intravenous injection of AdV-HBV, followed by HBV clearance by B-cell– and T-cell–mediated responses.[100] Hydrodynamic injections and AdV vectors can induce innate immune responses, so findings from these systems should be interpreted with caution.[101] Finally, these systems can duplicate HBeAg or HBsAg seroconversion without manifestation of liver diseases.

Hepatocytes from transfected mice do not contain HBV cccDNA, but further genetic manipulation of mice might allow this to occur (see above). In addition to its role for studying immune responses during acute HBV infection, the hydrodynamic injection and adeno-associated virus–HBV systems have also been used to evaluate antiviral compounds and strategies to induce an anti-HBV immune response.[94,102–104] Hydrodynamic injection of an siRNA that knocks down expression of a region of HBV DNA that is conserved among genotypes,[105] or an siRNA that knocks down expression of conserved regions of the X gene, reduced levels of HBsAg in mice.[106]

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Humanized Chimera Mice

Mice with humanized livers are used to study HBV infection.[107] SCID mice that express urokinase-type plasminogen activator under control of an albumin promoter (alb-uPA) have subacute liver failure as newborns, but their liver can be reconstituted with human hepatocytes. Although these hepatocytes can reconstitute up to 70% of liver, the alb-uPA SCID mice develop kidney and hematologic disorders and have a low breeding efficiency and narrow time period for liver manipulation. Human hepatocytes can also be transplanted into livers of *Rag2*–/– (immune-deficient) mice with disruption of the fumarylacetoacetate hydrolase gene (*Fah*–/–) and interleukin 2 receptor gamma chain (*Il2rg*–/–) (FRG chimeras). FAH deficiency leads to hepatocellular injury, which can be prevented by injections of 2-(2-nitro-4-trifluoro-methyl-benzoyl) 1,3-cyclohexedione (NTBC); loss of RAG2 and IL2RG deficiencies provides the immune-deficient back-ground required for engraftment of human hepatocytes.

FRG mice can receive transplants of human hepatocytes at any age, and the degree of liver damage can be controlled by adjusting NTBC administration; serial transplantations gradually improve the efficiency of engraftment of human hepatocytes. The chimeras can be infected with HBV virions, and its infected grafted human hepatocytes generate functional HBV cccDNA and viral spreading. Because these mice do not have an immune system, they are not used to study the antivirus immune response. However, human T cells can be transferred to the mice to study their response to infection of human hepatocytes with HBV.[108,109] Some drugs cannot be studied in these mice because of the administration of NTBC or other treatments during the process. Chimeric mice therefore provide unique platforms to study the mechanisms of HBV cccDNA formation and direct antiviral agent studies.[110–118] For example, myrcludex B, a synthetic lipopeptide derived from HBV envelope preS1 domain, was found to block de novo infection of human hepatocytes with HBV and virus spread in FRG chimeras.[118,119]

Mice with humanized immune systems were developed by transplantation of human hematopoietic cells or fetal liver cells.[120–123] AFC8-hu HSC/Hep and A2/NSG-hu-HSC/Hep mice have humanized immune systems and livers following co-transplantation of human CD34+ hematopoietic stem cells and human hepatocyte progenitors (or fetal liver cells). A2/NSG-hu-HSC/Hep mice can be infected with HBV, which persists for at least 4 months with limited antiviral innate and adaptive immune responses. Some mice even develop hepatitis and liver fibrosis following transplantation of fetal liver cells.[122] These mice can be used to study the antiviral immune response and its contribution to liver disease. A2/NSG-hu-HSC/Hep mice have T-cell– and B-cell–mediated immune responses to HBV infection, but these differ from those of humans due to the cellular configuration of the liver microenvironment. Chimeric mice contain fewer hepatocytes and have lower levels of virus replication than humans,[107] but are still useful for studying the mechanisms by which HBV causes liver disease.

Nonmouse Models

It has been a challenge to develop other models of HBV infection, because most animals are not infected by HBV or do not develop disease. Although chimpanzees are susceptible to

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HBV infection and develop an antiviral immune response, studies have been restricted mainly due to ethical concerns. The hepadnavirus capuchin monkey HBV was recently reported to infect Brazilian capuchin monkeys, without causing inflammation, and infect human hepatocytes via NTCP.[124] The capuchin monkey system requires further study. WHV is similar to HBV in terms of morphology, genome structure, replication, epidemiology, course of infection, and development of HCC with integrated WHV DNA. Most WHV integrations observed in HCC samples occurred in proximity to N-myc genes; similar HBV insertions are also found near *TERT*, *MLL4*, and *CCNE1* in HCC samples from patients with HBV infection. Studies based on HCC tissues have observed both WHV and HBV to integrate in the genome at specific yet distinct sites,[125–127] as a consequence of simulating clone expansion. However, the viruses cause liver disease via distinct mechanisms and several treatment strategies, such as core protein allosteric modulators, are specific to HBV. Capuchin monkeys are difficult to handle, have limited availability, are of outbred origin, and have insufficiently characterized immune systems.[128–131]

Tupaia is the only nonprimate animal found susceptible to HBV infection. HBV infection of *Tupaia* is transient with low levels of HBV replication. Liver fibrosis and HCC have been observed in some studies, whereas rapid clearance of HBV was reported in others. *Tupaia* hepatocytes were used to discover the first HBV receptor, NTCP.[3,63,132]

Mauritian cynomolgus macaques were reported to have persistent HBV infection.[133] Although rhesus macaques are not naturally susceptible to HBV infection, transgenic expression of human NTCP by their hepatocytes allows for infection by HBV. HBV infection persisted in 2 transgenic macaques, leading to induction of HBV-specific T-cell responses, whereas HBV was rapidly cleared from 2 other macaques. These macaques could be a useful, immune-competent primate for studies of HBV infection.[61,62]

Application for Drug Development

Systems to transfect cells with HBV DNA and study HBV replication have been available for more than 3 decades, and to transfect mice for studies of HBV replication have been available for 2 decades, so many agents have been identified that target the later stages of the HBV life cycle.[8,10,64] These include inhibitors of HBV DNA transcription, RNA stability, capsid assembly, RNaseH digestion, virion secretion, HBsAg secretion, and reverse transcription (reference: article in this issue about Drug Development). Drugs approved by the Food and Drug Administration for treatment of chronic HBV infection inhibit HBV reverse transcriptase. Agents such as GLS4, JNJ-379, and NVR3–778, have been developed to target core proteins, capsid assembly, and other steps of virus replication.[134–136] Immune-modulating drugs have been studied in animal models, including an agonist of the toll-like receptor 7 (GS-9620 and RO6864018), therapeutic vaccines (GS4774 and NASVAC), and the activator of retinoic acid-inducible gene-I and nucleotide binding oligo-merization domain containing 2 (NOD2) (SB9200).[137–140]

Cell models of HBV infection can be screened to identify agents that disrupt the early stages of the HBV life cycle, especially the entry process. Myrcludex B, which was characterized in HepaRG cells and uPA SCID mice with humanized livers, is being tested in a phase 2

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trial.[132,141,142] Myrcludex B is a lipopeptide that mimics the HBV preS1 2–48 aa region and disrupts interaction between NTCP and HBV.[3,28,132,142,143] Studies of HepG2-NTCP cells and other cells that express an NTCP transgene have led to identification of other inhibitors of HBV entry (see Table 3). Bile acids, the endogenous substrates of NTCP, block entry of HBV into cells. Tauroursodeoxycholic acid blocks binding of HBV to NTCP at a half maximal inhibitory concentration of approximately 1 μM.[28,144–146]

Some drugs approved by the Food and Drug Administration impair NTCP transporter function and inhibit HBV entry into cells. This class of drugs includes cyclosporin A, ezetimibe, irbesartan, proscillaridin A, rapamycin, fasiglifam, and zafirlukast.[144,147–155] Small molecules that interact with NTCP have been identified from natural products or other sources using NTCP-reconstituted cell lines. This class includes vanitaracin A (a tricyclic polyketide), NTI-007 (an imidazopyridine derivative), NPD8716 (a coumarin derivative), SCY995 (a cyclosporin derivative), and WD1 (a macrocyclic peptide).[35,156–160] Some small molecules identified in cell culture experiments have been shown to reduce expression of NTCP, including Ro41–5253 (a synthetic retinoid), epigallocatechin gallate (a flavonoid), and glabridin (a licorice-derived polyphenol).[161,162] Bexarotene, an anti-neoplastic agent that activates retinoid X receptor, inhibits HBV infection of cells.[163] Expression of the phospholipase A2 group IIA gene (PLA2G2A), which is regulated by retinoid X receptor and activates arachidonic acid and eicosanoid biosynthesis pathways, reduces HBV infection by unclear mechanisms. Some compounds target HBV particles themselves, such as proanthocyanidin and oolong homobisflavan (derived from extracts of grape seed and Chinese tea). These inhibit virus entry without modifying NTCP-mediated bile acid metabolism.[164] Anti-HBV compounds identified from screens of iHep cells include genistein and PA452, which have unclear mechanisms.[165] Cells that are susceptible to HBV infection have facilitated the identification of anti-HBV drugs. In addition to entry inhibitors, these cells might be used to identify agents that block cccDNA formation or maintenance or HBV integration into the genome.

Reporter Systems for Evaluating the HBV Infection Process

Anti-HBV agents have been primarily identified in screens (not necessarily high-throughput) to identify agents that reduce HBV DNA or antigens in infected cells, quantified by real-time polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay. However, expression of reporter genes (such as those encoding luciferase or green fluorescent protein) in infected cells has been used in high-throughput screens for identifying inhibitors of HIV and HCV infection.[166–169] What Are the Examples of Using Reporters With HBV Infection?

The HCV entry process has been extensively analyzed using HCV pseudoparticles: retroviral- or lentiviral-based virions that display HCV envelope proteins on the surface and carry a luciferase gene as part of the incorporated viral genome. Meredith et al[38] prepared lentiviral-based HBV pseudoparticles that carried HBs antigens on the viral envelope. These pseudoparticles entered cells in an NTCP-dependent manner, and this system is expected for using a high-throughput screen to identify HBV inhibitors.

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HBV-carrying NanoLuc particles were prepared by transfecting HepG2 cells with a plasmid encoding HBV in which the core region was replaced by a gene encoding NLuc and a helper plasmid that carried an HBV genome that was defective in packaging.[170] The resulting HBV/NLuc particles entered target HepG2-NTCP cells and primary human hepatocytes in an NTCP-dependent manner and produced NLuc for at least 12 days after infection.[171] NLuc quantification theoretically evaluates the virus life cycle from the entry and cccDNA formation through core promoter-mediated transcription. In this system KX2–391 was identified as an inhibitor of the transcription mediated by a HBV precore promoter.[172] These HBV infection–based reporter systems are expected to be useful for large-scale screening to identify anti-HBV agents that target early processes of the HBV life cycle.

Systems for Identifying cccDNA-targeting Agents

Agents that target cccDNA are of the highest priority to be desired aiming for curing HBV infection. The most reliable method for detection of cccDNA is Southern blot analysis, which requires larger amounts of samples than those obtained from 96- or 384-well plates. Southern blotting is therefore not useful in high-throughput screens. Real-time PCR with a cccDNA-specific primer probe set is a practical alternative for cccDNA detection; however, it is not clear whether real-time PCR detects cccDNA with a high level of specificity in the presence of excess amounts of rcDNA. In addition to the demand of a standard protocol to specifically detect cccDNA, techniques that mimic or could be used to monitor cccDNA at high specificity, sensitivity, and throughput, along with decreased cost and time, would facilitate drug development. Several studies have described plasmid-based systems that mimic cccDNA and can be used to evaluate its dynamics.

Several groups have developed recombinant cccDNA produced from a precursor plasmid converted by either Cre/*loxP*-mediated or ϕC31 integrase-mediated recombination technology.[29,165,173,174] Following transfection of cells or hydrodynamic injection of mice, these recombinant cccDNAs supported sustained viral replication for extended periods. One system provided viral replication beyond 70 days in mice.[165] Li et al[175] used a recombination minicircle system to transfer into cells a luciferase-encoding reporter that generates a precursor plasmid containing the entire HBV sequence, in which the core region was fused with a split *Gaussia* luciferase (GLuc)-encoding gene. This precursor plasmid could be converted (through DNA recombination in *Escherichia coli*) to eliminate the backbone vector and yield a minicircle HBV-reporter carrying the entire HBV genome fused with the full *GLuc* gene and a splicing site.[175] This minicircle HBV cccDNA-reporter, which generated a *GLuc*-containing pgRNA transcript after splicing and eventually yielded nucleocapsids, was maintained for a minimum of 23 days in HepG2 cells. In this system, luciferase activity serves as a surrogate reporter for evaluating cccDNA stability. Constructs of this type are expected to be of use in high-throughput screening.

HepDE19 cells, which can induce HBV replication and subsequent recycling and cccDNA formation upon tetracycline depletion, permit monitoring of cccDNA levels based on measurement of HBeAg; HBeAg production in this cell line depends on cccDNA level.[176] However, the use of this cell line is limited by the cross-reactivity of HBeAg with virus core protein in enzyme-linked immunosorbent assay analysis. To address this limitation, Cai et

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al[16] produced a second-generation version of this cell line, HepBHAe82, which encodes an HA-tagged HBe. An assay using this improved cell line detected cccDNA with high sensitivity and low background, so it could be of great value for identifying cccDNA-targeting inhibitors.

Future Directions

No system is optimal for HBV research or drug development; efforts are needed to develop better models. Cell culture systems that most closely resemble normal hepatocytes in the human body but are more convenient, less expensive, unlimited in supply or more readily available, and allow more efficient amplification of infection and spread would be highly desirable. Further improvement of the primary human hepatocyte culture conditions and optimization of iHep cells are of particular interest.

Animal models of HBV infection have increased our understanding of the antiviral immune response and liver disease progression. However, we urgently need mice that are fully permissive to HBV infection, including the establishment and amplification of cccDNA, perhaps starting with mice that express the NTCP transgene in liver. Further discoveries of factors required for virus entry or replication and spread could lead to such a model. This knowledge can be applied to macaque or *Tupaia* models, to generate alternative outbred nonprimate or primate models for drug development.

These systems can be used not only to study the process of chronic HBV infection, but also in drug discovery. Reporter systems that quickly identify changes in specific features of the HBV life cycle would facilitate discovery of new antiviral agents. Systems for discovery of agents that target cccDNA could facilitate efforts to find a cure for chronic HBV infection.

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Abbreviations used in this paper:

AdV	adenovirus
alb-uPA	urokinase-type plasminogen activator under control of an albumin promoter
cccDNA	covalently closed circular DNA

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DMSO	dimethyl sulfoxide
GLuc	*Gaussia* luciferase
HBeAg	HB e surface antigen
HBsAg	HB surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
iHep	induced human hepatocyte-like
NC	nucleocapsid
NTCP	solute carrier family 10 member 1
PCR	polymerase chain reaction
PEG	polyethylene glycol
pgRNA	pregenomic RNA
rcDNA	relaxed circular DNA
siRNA	small interfering RNA.

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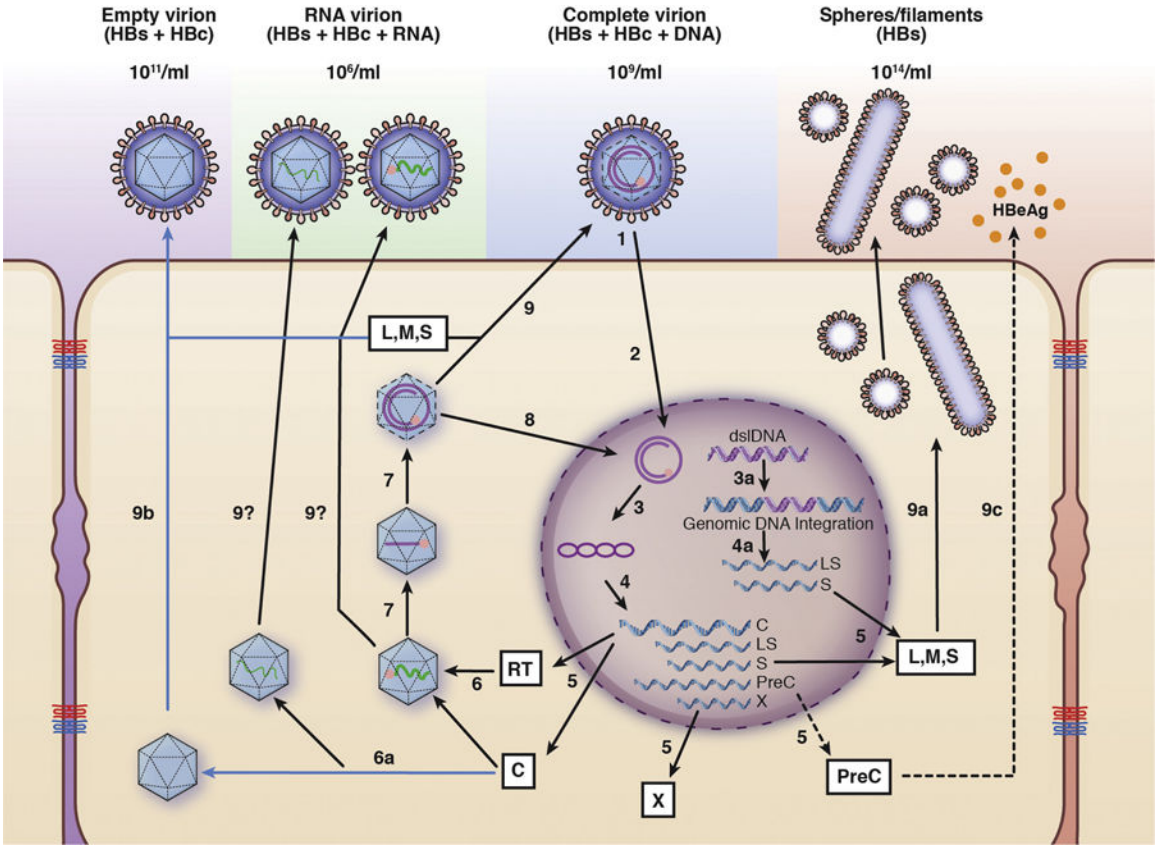

Figure 1.

HBV replication. 1. Virus binding and entry into the host cell (large rectangle). 2. Intracellular trafficking and delivery of rcDNA to the nucleus (large circle). 3. Conversion of rcDNA to cccDNA, or integration of the double-stranded linear (DSL) DNA into cell DNA (3a). 4. and 4a. Transcription to synthesize viral RNAs (wavy lines), including the core (C) mRNA for both the core and the reverse transcriptase (RT) proteins; large surface (LS) mRNA for the large (L) surface or envelope protein; small (S) mRNA for the middle (M) and small (S) envelope proteins; X mRNA for the X protein; and PreC mRNA for the PreCore protein. The C mRNA is also the pgRNA: the template for producing progeny viral DNA via reverse transcription. 5. Translation to synthesize viral proteins. 6. Assembly of the pgRNA- (and RT-) containing NC, or alternatively, empty capsids (6a). 7. Reverse transcription of pgRNA to synthesize the (–) strand single-stranded (SS) DNA and then rcDNA. 8. Nuclear recycling of progeny rcDNA to form more cccDNA (intracellular cccDNA amplification). 9. Envelopment of the rcDNA-containing NC and secretion of complete virions, or alternatively, secretion of empty virions (9b) or HBsAg spheres and filaments (9a). Processing of the PreCore protein and secretion of HBeAg are shown in 9c. The secretion of putative RNA virions is not yet resolved (9?). The different viral particles outside the cell are shown with their approximate concentrations in the blood of infected persons indicated: the complete, empty, or RNA virions as large circles (outer envelope) with an inner diamond shell (capsid), with or without rcDNA (gapped, double concentric circle) or RNA (wavy line) inside the capsid, respectively; HBsAg spheres and filament as small circles and cylinders. Approximate concentrations of the different viral particles in the

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blood of infected patients are indicated but can vary among patients and over time in the same patient. Intracellular capsids, diamonds, with either viral pgRNA, SS ([–] strand) DNA (straight line), rcDNA, or empty. Dashed lines of the diamond in the rcDNA-containing mature NC and the complete virion, destabilization of the mature NC. Boxed letters, viral proteins translated from the mRNAs. Filled small circle inside the capsids, the RT protein binding to the 5′ end of the pgRNA and covalently attached to the 5′ end of the (–) strand DNA. Twisted circle, cccDNA. The thin dashed line and arrow indicate that HBeAg (small orange circles outside the cell) is frequently decreased or lost late in infection. For simplicity, synthesis of the minor DSL form of the genomic DNA in the mature NC, its secretion in virions, and infection of DSL DNA-containing virions are not depicted here, as are the functions of X. Adapted from Hu and Liu,[177] with the Creative Commons Public License, https://creativecommons.org/licenses/by/4.0/legalcode.

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Table 1.

Advantages and Limitations of Cell Culture Systems for Studying HBV

Systems	Advantages	Limitations
Human hepatoma cell lines (HepG2, Huh7)	• Readily available • Robust in supporting steps in replication after transcription	• Fail to support infection • Oncogenic transformation: only partially mimic normal hepatocytes
HepaRG	• Support infection • Mimic normal hepatocytes better than other hepatoma cell lines	• Low efficiency of infection • Complicated conditions for differentiation
HepG2-NTCP	• Readily available	• Oncogenic transformation: only partially mimic normal hepatocytes
Primary human hepatocytes	• Support HBV infection • Mimic normal hepatocytes the best • Support complete HBV life cycle	• Low efficiency of infection • Limited in supply • High cost • Donor variability
iHep cells	• Unlimited in supply • Support complete HBV life cycle • Mimic normal hepatocytes better than hepatoma cell lines	• Complicated conditions for differentiation • Induced hepatocytes may not fully mimic mature hepatocytes

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Table 2.

Animal Models of Chronic HBV Infection

		Immune response						Pathogenesis			Preclinical studies	
		HBV infection				Adaptive immunity						
						B cell	T cell					
Animal	**System**	**cccDNA**	**Infectability**	**Persistence**	**Innate immunity**	**Seroconversion to HBeAg and HBsAg seroconversion**	**Tolerance and clearance**	**Hepatitis**	**Cirrhosis**	**HCC**	**Antiviral**	**Immune modulator**
Transgenic mice	Expression of HBV transgenes	−	−	+	−	−/−	Tolerance	−	−	+/−	+	+
	Expression of human NTCP	−	+/−	−	−	−/−	NA	−	−	−	−	−
Transfected mice	Hydro-dynamic injection	−	−	+/−	+	+/+ or +/−	Tolerance and clearance	−	−	−	+	+
	AAV injection	−	−	+	+	−/−	Tolerance	−	−	+	+	+
	AdV injection	−	−	+/− (dose–dependent)	+	−/− or +/+	Tolerance or clearance	−	−	−	+	+
Mice with humanized liver	human hepatocytes	+	+	+	+/−	−/−	tolerance	+	−	−	+	−
	Human hepatocytes and human hematopoietic cells	+	+	+	+	−/−	Tolerance	+	+	−	+	+
Other animals	Woodchuck	+	+	+	−	+/+	Tolerance and age-dependence	+	+	+	+	+
	Tupaia	+	+	+/−	+/−	+/−	Tolerance and clearance	+/−	+/−	+/−	?	?
	Macaque	+/−	+/−	+/−	−	−/−	Tolerance	−	−	−	−	−
	Macaque expressing human NTCP transgene	+	+	+	+	+/+	Tolerance and clearance	?	?	−	?	?

NOTE. +/− signs indicate the presence/absence or occurrence/non-occurrence of the corresponding characteristics, whereas question marks (?) indicate the status of the specific characteristics are currently not sure unclear.

AAV, adeno-associated virus; NA, not applicable.

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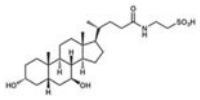
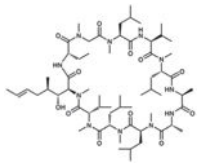
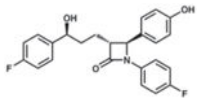
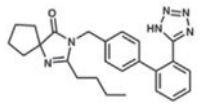
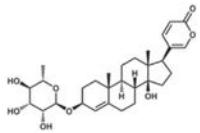
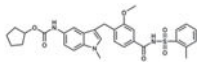
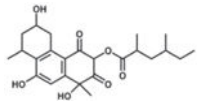
Table 3.

Identified Anti-HBV Entry Inhibitors by Application of New Models

Name	Structure	Model system for identification	Reference
bile acids tauroursodeoxycholic acid (TUDCA) etc.		HepaRG Huh7-NTCP HepG2-NTCP PHHs PTHs	28, 144, 146, 153
Cyclosporin A		HepaRG Huh7-NTCP HepG2-NTCP PHHs	150, 153
Ezetimibe		HepaRG HepG2-NTCP PHHs PTHs	144, 149
Irbesartan		HepG2-NTCP	148, 152
Proscillaridin A		HepG2-NTCP	151
Zafirlukast		HepaRG	147
Vanitaracin A		HepG2-NTCP PHHs	157, 158

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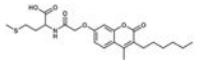
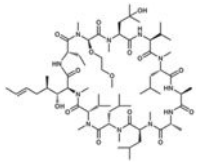
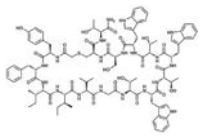
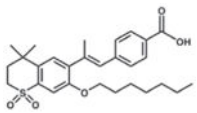
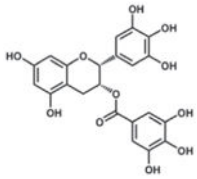
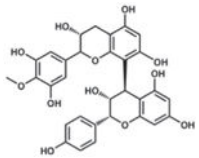
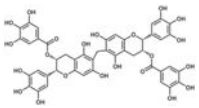
Name	Structure	Model system for identification	Reference
NPD8716		HepG2-NTCP PHHs	156
SCY995		HepG2-NTCP PHHs	35
WD1		HepG2-NTCP PHHs	159
Ro41–5253		HepaRG PHHs	162
Epigallocatechin gallate		HuS-E/2 Huh7-NTCP	161
Proanthocyanidin		HepG2-NTCP PHHs	164
Oolonghomobisflavan		HepG2-NTCP PHHs	164

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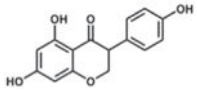
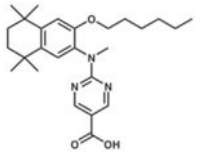
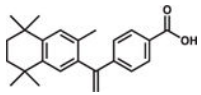
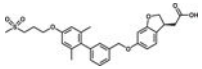
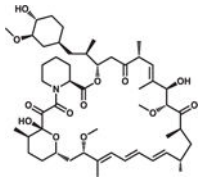
Name	Structure	Model system for identification	Reference
Genistein		iHeps PHHs	49
PA452		iHeps PHHs HepG2-NTCP	49
Bexarotene		HepG2-NTCP HepaRG PTHs	163
Fasiglifam		HepG2-NTCP	155
Rapamycin		HepG2-NTCP	154
Myrcludex-B (lipopeptide)	Myristoyl-GTNLSVPNPLGFFPDHQLDPAFGANSNNPDWDF NPNKDHWPEANQVG	HepaRG PTHs Humanized mouse	118, 142

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