Unexpected Host Range of Hepatitis C Virus Replicons

Zhu Q, Guo J-T, Seeger C. Replications of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. J Virol 2003;77:9204–9210. (Reprinted with permission from the American Society for Microbiology (ASM).)

Abstract

The hepatitis C virus (HCV) pandemic affects the health of more than 170 million people and is the major indication for orthotopic liver transplantations. Although the human liver is the primary site for HCV replication, it is not known if extrahepatic tissues are also infected by the virus nor if nonprimate cells are permissive for RNA replication. Because HCV exists as a quasispecies, it is conceivable that a viral population may include variants that can replicate in different cell types and other species. We have tested this hypothesis and found that subgenomic HCV RNA can replicate in mouse hepatoma and nonhepatic human epithelial cells. Replicons isolated from these cell lines carry new mutations that could be involved in the control of tropism of the virus. Our results demonstrated that translation and RNA-directed RNA replication of HCV do not depend on hepatocyte or primate-specific factors. Moreover, our results could open the path for the development of animal models for HCV infection.

Comments

Viruses are obligate intracellular parasites that absolutely depend on a functional host cell machinery for their multiplication. Consequently, studies of the viral life cycle require systems that support virus propagation in cultured cells; unfortunately, these systems are not always available. The hepatitis C virus (HCV) is a prominent example how the lack of a suitable cell culture system can impede scientific progress for many years.[1,2] A breakthrough was the development of the so-called "replicon system," which is based on the autonomous replication of HCV subgenomes in the human hepatoma cell line Huh-7.[3] These replicons are composed of (1) the HCV 5′ nontranslated region, (2) the selectable marker neomycin phosphotransferase, which confers resistance against the cytotoxic drug G418, (3) a heterologous internal ribosome entry site (IRES) that allows efficient expression of the HCV NS3 to NS5B proteins, and (4) the HCV 3′ nontranslated region (Fig. 1). Upon transfection of Huh-7 cells with replicon RNA molecules and subsequent selection, G418-resistant cell clones could be established that carry large amounts of HCV RNA and express substantial levels of viral proteins. Studies performed in the past few years have revealed two major determinants for efficient RNA replication in Huh-7 cells: cell culture adaptive mutations in the HCV coding region of the replicons and host cell permissiveness.[4–14]

Most adaptive mutations have been identified in NS3, NS4B, and NS5A and have been shown to cluster in certain regions of these proteins. The most prominent hot spots reside at two distinct positions in NS4B and in the center of NS5A, where serine residues that are involved in phosphorylation of this protein are often replaced by nonphosphorylatable amino acid residues.[4,9] The most potent effect on RNA replication is mediated by combinations of mutations in NS3 (*e.g.*, E1202G) with single substitutions in NS4B or NS5A (*e.g.*, S2204I).[9,15] The second determinant influencing HCV RNA replication is the permissiveness of the host cell; that is, the extent to which a given cell supports replication of this RNA. Only a low number of cells in a culture appear to be sufficiently permissive, and these cells are enriched during G418 selection. This conclusion is based on the observation that removal of the replicon from replicon-harboring cells (*e.g.*, by treatment with interferon-α [IFN-α] or a selective inhibitor) results in a population of "cured" cells that support higher levels of HCV RNA replication compared with naïve cells.[7–9]

Since the original description in 1999, the replicon system has been improved substantially. Transient replication assays as well as full length genomes replicating transiently and stably in Huh-7 cells have become available.[11,12] However, two major limitations existed: the lack of virion production and the restriction of HCV replicons to Huh-7 cells. The latter limitation has now been overcome by Zhu and coworkers, who reported the successful propagation of HCV replicons in HeLa cells and the mouse hepatoma cell line Hepa1-6.[16] Their experimental approach was based on the assumption that the replication-enhancing mutations observed in Huh-7 cells (*e.g.*, S2204I) reflect an adaptation to a particular host cell environment and that further mutations might be required to adapt replicons to other cell lines. Thus, when using complex mixtures of replicon RNAs with a high genetic heterogeneity, the chances to select for an adapted variant should be much higher compared with the use of rather

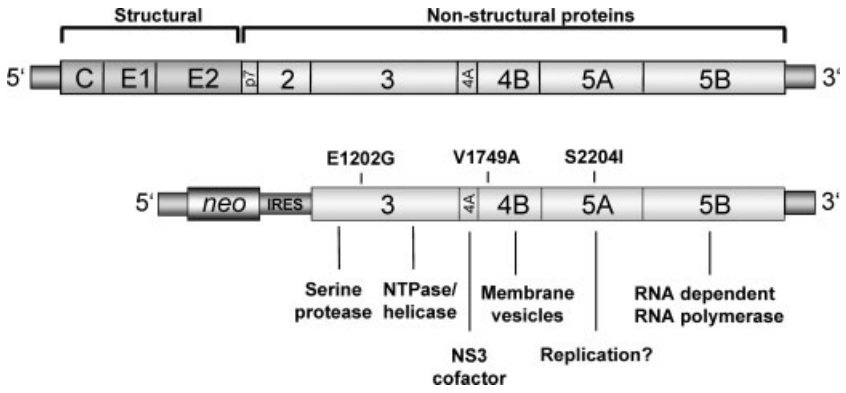

Fig. 1. Comparison of the HCV genome organization (**upper panel**) with the structure of a subgenomic selectable replicon (**lower panel**). The 5′ and 3′ nontranslated regions are indicated with small bars and sequences encoding the neomycin phosphotransferase (neo) or the HCV polyprotein with thick bars. Translation of the neo gene is directed by the HCV IRES, whereas the proteins required for RNA replication (NS3 to NS5B) are translated under control of the IRES from another virus (the encephalomyocarditis virus). Functions ascribed to HCV proteins that are required for RNA replication are indicated below the replicon scheme. The positions of amino acid substitutions mentioned in the text are indicated above. The role of NS5A in RNA replication is as yet unknown. C, core protein; E1 and E2, envelope glycoproteins; NTPase, nucleoside triphosphatase.

uniform replicon RNA that is generated by *in vitro* transcription from cloned HCV templates.

Zhu and coworkers reasoned that because of the high error rate of the viral replicase, HCV replicons exist within a single cell as a swarm of sequence variants in which (on a statistical basis) each RNA molecule differs from the other at least at one position. Therefore, they isolated total RNA from Huh-7 cell clones that carried stably replicating HCV replicons. These total RNA preparations were used to screen various cell lines, including BHK (baby hamster kidney cells), Vero (kidney epithelial cells from the African green monkey), HeLa (human cervix carcinoma cells), and Hepa1-6 (hepatoma cells from the mouse) by means of RNA transfection and subsequent G418 selection. Only in HeLa cells was a low number of G418-resistant colonies obtained. Analysis of seven stable cell clones revealed that 90% of the cells expressed viral proteins at a given time point, with replicon RNA copy numbers in the range of 3,000 molecules per cell.

Although these properties are comparable to Huh-7 replicon cell clones, distinct differences have been described in this and a subsequent study by the same group.[17] First, although replicon RNA copy numbers in Huh-7 cells very much depend on host cell growth, this does not appear to be the case for HeLa cells. This observation argues for some fundamental differences in host cell requirements of HCV replicons in HeLa versus Huh-7 cells; however, the discrepancy could also be explained by a much less pronounced inhibition of HeLa cell growth by cell-to-cell contact. Second, the IC_{50} observed for IFN-α was approximately tenfold lower in HeLa cells compared with Huh-7 cells (0.1 IU/ml vs. $\approx$1 IU/ml, respectively).[17] Third, treatment of HeLa cells carrying HCV replicons with IFN-α led to apoptosis in more than 30% of cells. However, the induction of cell death could be prevented when replicons were blocked with an NS5B-specific inhibitor prior to addition of IFN-α, suggesting that viral RNA or protein stimulated an innate cellular response that sensitized cells towards apoptosis.[17] In contrast, Huh-7 replicon cells treated in a similar fashion did not undergo programmed cell death.

To determine whether or not HCV replicons in HeLa cells had acquired cell type–specific adaptive mutations, Zhu and coworkers compared the number of G418-resistant colonies obtained after transfection of HeLa cells with total RNAs isolated from HeLa or Huh-7 cells harboring selectable replicons.[16] As summarized in Table 1, replicon RNA isolated from HeLa cells yielded significantly more colonies upon transfection of naïve HeLa cells compared with naïve Huh-7 cells. In contrast, replicon RNAs isolated from Huh-7 cells were more efficient when introduced into naïve Huh-7 cells as compared with naïve HeLa cells. Because the number of G418-resistant colonies is a measure of the efficiency of RNA replication, these data indicated the selection for cell type–specific replicon variants that multiplied more efficiently in HeLa

Table 1. Increase of the Number of G418-Resistant Replicon Cell Clones by Prior Passage of Replicon RNA in the Same Cell Line

Source of Replicon RNA	Transfected Into		
	Huh-7	HeLa	Hepa1-6
Huh-7	166*	4	<1
HeLa	20	160	<1
Hepa1-6	40	132	3

*Number of G418-resistant colonies obtained per ng replicon RNA that was present in total RNA of a given replicon-harboring cell clone and used for transfection. Data are taken from Table 2 of Zhu and coworkers.[16]

cells. Nucleotide sequence analysis revealed that the major cell culture adaptive mutations present in Huh-7 cells (E1202G and S2204I) were maintained in replicons isolated from HeLa cells, but several additional mutations were found that so far had not been observed in Huh-7 cells. Most notable was an amino acid substitution in the N-terminus of NS4B (V1749A), because it was conserved in replicon RNAs isolated from two independent HeLa replicon cell clones.

Encouraged by these observations, Zhu and coworkers investigated whether or not this or other additional mutations would expand the tropism of such replicons beyond Huh-7 and HeLa cells. A panel of 10 different cell lines of human, monkey, rat, hamster, and mouse origin was transfected with total RNA isolated from HeLa replicon cells. However, transfection of only the mouse cell line Hepa1-6 yielded G418-resistant colonies. Sequence analysis revealed that replicons isolated from these mouse cells had preserved the majority of mutations found in HeLa cells that were used as the replicon RNA source, and only a few additional mutations were detected.

In an attempt to generate cloned versions of cell type–adapted replicons, a panel of selectable replicon constructs was made that carried various combinations of mutations identified by sequence analysis of HeLa cell–derived replicons. Two of these constructs yielded a low number of G418-resistant colonies in HeLa cells but not in Hepa1-6 cells. Interestingly, in both cases the replicons contained the novel NS4B mutation (V1749A) in different combinations with other mutations. Unfortunately, no replicon was tested that carried this NS4B mutation alone or only in combination with the highly adaptive ones (E1202G and S2204I). Nevertheless, these data suggest that the NS4B mutation contributes to the expanded tissue tropism. However, the fact that no colonies were obtained in the mouse liver cell line Hepa1-6 indicates that this mutation is HeLa cell–specific.

Given the low number of colonies obtained with HeLa cells and the negative results obtained with Hepa1-6 cells, Zhu and coworkers wondered if they had missed replicon variants that carry more efficient cell type–specific adaptive mutations but represent a very minor species in the replicon RNA population present in these cells. Therefore, they constructed replicon complementary DNA libraries derived from Huh-7, HeLa, and Hepa1-6 replicon cells and transfected replicon RNA pools with a complexity of 2,000 variants into the different cell lines. This approach enabled them to establish replicon RNA-containing clones of Hepa1-6 cells. It will be interesting to see which replicon variant was able to establish sustained RNA replication in these mouse cells.

In conclusion, the study by Zhu and coworkers represents an important extension of the HCV replicon system. Although transient replication assays are not yet possible with HeLa and Hepa1-6 cells, and the number of G418-resistant colonies obtained even with cell culture–adapted replicons is low, the availability of such alternative replicon cell clones is critical for a better understanding of the HCV life cycle. Because cell lines can deviate quite substantially in their morphologic and physiologic state from cells *in vivo*, the availability of multiple replicon cell lines avoids possible pitfalls that can be encountered when working with only one particular cell line. Moreover, this study clearly shows that HCV RNA replication does not depend on liver cell–specific factors but rather can replicate in nonliver tissues. This observation corroborates earlier studies describing low-level HCV replication in infected nonhepatic cell lines like Daudi (B cell) or Molt-4 and MT-2 (T cell). Finally, the finding that HCV replicons can replicate in mouse cells and that replication in such nonhuman cells may be achieved by certain adaptive mutations offers some hope for the development of a mouse model for HCV infection. Although this is probably still a long way to go, the first step has been made.

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References

1. Pietschmann T, Bartenschlager R. Tissue culture and animal models for hepatitis C virus. Clin Liver Dis 2003;7:23–43.
2. Kato N, Shimotohno K. Systems to culture hepatitis C virus. Curr Top Microbiol Immunol 2000;242:261–278.
3. Lohmann V, Körner F, Koch JO, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–113.
4. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.
5. Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol 2001;75: 4614–4624.
6. Lohmann V, Körner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol 2001; 75:1437–1449.
7. Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 2002;76: 13001–13014.
8. Murray EM, Grobler JA, Markel EJ, Pagnoni MF, Paonessa G, Simon AJ, Flores OA. Persistent replication of hepatitis C virus replicons expressing the beta-lactamase reporter in subpopulations of highly permissive Huh7 cells. J Virol 2003;77:2928–2935.
9. Lohmann V, Hoffmann S, Herian U, Penin F, Bartenschlager R. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J Virol 2003;77:3007–3019.
10. Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. J Virol 2001;75:8516–8523.

11. Ikeda M, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J Virol 2002;76:2997–3006.
12. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, Strand D, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002;76:4008–4021.
13. Kishine H, Sugiyama K, Hijikata M, Kato N, Takahashi H, Noshi T, Nio Y, et al. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. Biochem Biophys Res Commun 2002;293:993–999.
14. Gu B, Gates AT, Isken O, Behrens SE, Sarisky RT. Replication studies using genotype 1a subgenomic hepatitis C virus replicons. J Virol 2003; 77:5352–5359.
15. Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol 2003;77: 3181–3190.
16. Zhu Q, Guo J-T, Seeger C. Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. J Virol 2003;77:9204–9210.
17. Guo JT, Zhu Q, Seeger C. Cytopathic and noncytopathic interferon responses in cells expressing hepatitis C virus subgenomic replicons. J Virol 2003;77:10769–10779.

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/hep.20122

Interfering With Capsid Formation: A Practicable Antiviral Strategy Against Hepatitis B Virus?

Deres K, Schroder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, Kramer T, et al. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science 2003;299:893–896. (Reprinted with permission from the American Association for the Advancement of Science.)

Abstract

Chronic hepatitis B virus (HBV) infection is a major cause of liver disease. Only interferon-α and the nucleosidic inhibitors of the viral polymerase, 3TC and adefovir, are approved for therapy. However, these therapies are limited by the side effects of interferon and the substantial resistance of the virus to nucleosidic inhibitors. Potent new antiviral compounds suitable for monotherapy or combination therapy are highly desired. We describe nonnucleosidic inhibitors of HBV nucleocapsid maturation that possess *in vitro* and *in vivo* antiviral activity. These inhibitors have potential for future therapeutic regimens to combat chronic HBV infection.

Comments

Persistent hepatitis B is still the major cause of liver cell carcinoma worldwide. Public perception of first-world hepatitis is dominated by hepatitis C virus (HCV), while infection with the hepatitis B virus (HBV) is thought to be predominantly restricted to Asia and Africa. However, even in the first world, despite the availability of a safe and effective vaccine and various therapeutic modalities, prevalence of hepatitis B is still between 0.2 and 0.5 %.[1]

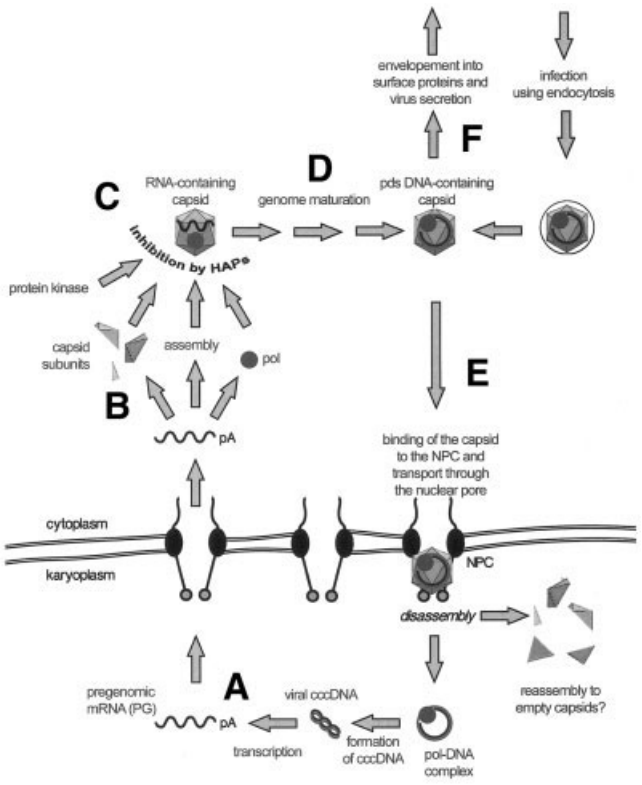

Fig. 1. Intracellular life cycle of the hepatitis B virus. After infection, the covalently closed circular DNA form of the virus within the nucleus encodes for the viral messenger RNAs. (A) Multiplication of genomic information is achieved by synthesis of the pregenomic messenger RNA (PG). (B) After being exported to the cytoplasm, the PG serves as the template for translation of the viral pol and the capsid proteins. (C) PG, pol, and a cellular protein kinase assemble to an immature capsid. (D) Inside the capsid, pol converts the encapsidated RNA into a partially double-stranded (pds) DNA genome. (E) These DNA-containing capsids can be transported through the nuclear pore complex (NPC) into the nucleus, which leads to amplification of the nuclear viral DNA early in infection. (F) Later, when viral surface proteins are synthesized, these capsids can be enveloped and secreted as progeny virus. The HAPs inhibit the assembly of the capsids (C). Thus all steps downstream should be inhibited, as it was shown for genome maturation (D). According to the HBV life cycle, HAP treatment should additionally block nuclear transport of the viral genome (E) and virus secretion (F).

HBV shows some homology to retroviruses in that it contains a DNA genome but replicates by way of an RNA intermediate known as a *pregenome* (PG). The PG is synthesized within the nucleus of the infected hepatocyte by cellular RNA polymerase II[2] using the viral DNA genome as the template (Fig. 1A; for review, see Seeger and Mason[3]). After being exported into the cytoplasm, the PG encodes for the viral capsid protein (also termed *core protein*) and the viral polymerase (pol) (Fig. 1B). Next, pol specifically interacts with the PG[4] while the capsid proteins interact with each other, resulting in spontaneous assembly to capsids (core particles).[5] Capsid assembly is

combined with the encapsidation of a pol–PG complex[6] and a cellular protein kinase,[7] which has not yet been unequivocally identified (Fig. 1C). Inside the lumen of the capsids, pol first converts the PG into minus-strand DNA by reverse transcription, followed by synthesis of an incomplete plus-strand DNA (Fig. 1D). The DNA-containing capsids can either mediate the transport of the viral genome into the nucleus[8] (Fig. 1E) or they can be enveloped with the viral surface proteins, resulting in secretion of progeny virus[9] (Fig. 1F). At least the nuclear transport involves phosphorylation of the capsid proteins.[10,11]

HBV—in contrast to HIV and HCV, which, among others, harbor an essential protease—shows only the polymerase as its genuine enzymatic activity. Thus, nucleos(t)ide analogues (NAs), which affect either the reverse transcriptase or the DNA-dependent polymerase activity of pol, are successfully used to interrupt HBV multiplication. A similar reduction of the viral load can be achieved by using interferon-α, a treatment that has been established for more than 20 years. Both treatments, however, show only limited success. In only 30% of all patients—approximately—can sustained response be achieved if virologic parameters such as a significant reduction of viral load and HBeAg elimination and/or disease-related parameters such as normalized alanine aminotransferase levels and improved liver histology are chosen as markers. Although successful interferon-α therapy improves survival and quality of life, virus eradication occurs only in rare cases.[12,13] The same is true for NAs, although long-term experience with NAs is very limited. In addition, resistance is likely to occur with all NAs, as has been documented for lamivudine and adefovir dipivoxil.

Various experimental strategies have therefore been investigated to block the hepadnaviral life cycle at points other than viral polymerase. In addition to numerous investigations using antisense oligonucleotides or ribozymes, these approaches include the generation of capsid mutants that contain nuclease digesting the encapsidated PG[14] or dominant negative capsid protein mutants,[15,16] which are unable to support PG packaging and genome maturation, as well as peptides interfering with capsid assembly.[17] However, all of these studies are still at the very early experimental level.

Based on a previous work of this group[18] in which the authors identified the nonnucleosidic compound Bay 41-4109 to be effective in the HBV transgenic mouse model, Deres and colleagues[19] next analyzed the mode of action. In addition to Bay 41-4109, they included the congeners Bay 38-7690 and Bay 39-5493 in their investigation and compared them with the established NA lamivudine. They found that all three nonnucleosidic inhibitors, also referred to as *heteroaryldihydropyrimidines* (HAPs), showed a dramatic inhibition of HBV replication in HBV-producing cell lines. By titrating the HAPs to the stably HBV-producing cell line HepG.2.2.15[20] and measuring the amount of HBV DNA, the authors showed that the HAPs had a twofold- to tenfold-lower IC_{50} than lamivudine (20–150 vs. 300 nM), which is currently the most frequently used NA in hepatitis B treatment. Only the (−)-enantiomers and not the (+)-enantiomers showed this antiviral effect, implying a very specific interaction between drug and target.

Exploring the mode of inhibition, they confirmed that the decreased number of synthesized HBV genomes corresponded with a decrease in viral polymerase activity, as would be expected. In contrast, the amount of viral RNAs remained unchanged. Up to this point, the results could be explained by classical NA activity; however, in contrast to NA, HAP treatment decreased the number of capsids and the amount of capsid protein in stably HBV expressing cells. This observation implied an inhibition of translation, yet there was no inhibition of capsid protein synthesis. Pulse chase experiments showed that the amount of newly synthesized capsid protein was similar in Bay 39-5493–treated and untreated cells. In addition, this assay showed that unassembled capsid proteins have a much shorter half-life (only 3 hours) than the assembled particles, which was calculated to be more than 24 hours. Although no answer is provided in this article, it appears that the difference in half-life is not caused by protease-mediated degradation of the non-assembled capsid protein, which may have been induced by HAPs treatment. More likely, the different turnovers are intrinsic properties of assembled capsid and unassembled capsid protein; this explains why intranuclear capsids show such a strong accumulation in persistently HBV-infected human liver cells. A conclusive explanation for these observations is that the HAPs prevented capsid formation and that the nonassembled capsid proteins were subjected to rapid degradation.

An obvious next step was to ask whether or not the HAPs directly interact with the capsid protein. To address this question, the authors expressed the core protein in *Escherichia coli* and performed *in vitro* capsid assembly assays. Using this experimental strategy, they could show that the block of capsid formation correlated with reversible *in vitro* binding of the HAPs to the capsid protein.

In conclusion, Deres et al. showed consistent and exciting evidence that the inhibition of capsid assembly becomes a real and reachable target in HBV drug treatment, although it is difficult to fully assess whether or not all three HAPs have the same effect given the limited information provided in the article.

The major question remains as to whether or not the HAPs will "do their job" in an *in vivo* model of hepatitis B and ultimately in patients with hepatitis B. The previous work of Weber and colleagues[18] has already showed that at least Bay 41-4109 works in HBV-transgenic mice; however, in these mice no reduction of viral covalently closed circular DNA—the key problem in HBV treatment—can be studied. Since the HAPs (or at least Bay 39-5493) were shown not to interact with the duck hepatitis B virus (DHBV) capsid, it is unlikely that they would work in the commonly used DHBV duck model system. However, the DHBV capsid is not closely related to the HBV capsid. Consequently, one may ask why the authors have not tested the woodchuck hepatitis virus capsid, which shows a much closer homology to the HBV capsid sequence and would allow evaluation in an experimental animal system.

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References

1. World Health Organization. Prevalence of hepatitis B in various areas. Available at: http://www.who.int/emc- documents/hepatitis/docs/whocd-scsrlyo20022/disease/prevalence_various_areas.html.
2. Köck J, Schlicht HJ. Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity. J Virol 1993;67:4867–4874.
3. Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev 2000;64:51–68.
4. Bartenschlager R, Schaller H. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J 1992;11:3413–3420.
5. Birnbaum F, Nassal M. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. J Virol 1990;64:3319–3330.
6. Junker Niepmann M, Bartenschlager R, Schaller H. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J 1990;9:3389–3396.
7. Gerlich WH, Goldmann U, Muller R, Stibbe W, Wolff W. Specificity and localization of the hepatitis B virus-associated protein kinase. J Virol 1982; 42:761–766.
8. Rabe B, Vlachou A, Pante N, Helenius A, Kann M. Nuclear import of hepatitis B virus capsids and release of the viral genome. Proc Natl Acad Sci U S A 2003;100:9849–9854.
9. Gerelsaikhan T, Tavis JE, Bruss V. Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. J Virol 1996;70: 4269–4274.
10. Köck J, Kann M, Putz G, Blum HE, von Weizsacker F. Central role of a serine phosphorylation site within duck hepatitis B virus core protein for capsid trafficking and genome release. J Biol Chem 2003;278:28123–28129.
11. Kann M, Sodeik B, Vlachou A, Gerlich WH, Helenius A. Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. J Cell Biol 1999;145:45–55.
12. Schalm S, De Man R, Janssen H. Combination and newer therapies for chronic hepatitis B. J Gastroenterol Hepatol 2002;17:S338–S341.
13. Lau DT, Everhart J, Kleiner DE, Park Y, Vergalla J, Schmid P, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology 1997;113:1660–1667.
14. Beterams G, Bottcher B, Nassal M. Packaging of up to 240 subunits of a 17 kDa nuclease into the interior of recombinant hepatitis B virus capsids. FEBS Lett 2000;481:169–176.
15. von Weizsäcker F, Köck J, Wieland S, Offensperger WB, Blum HE. Dominant negative mutants of the duck hepatitis B virus core protein interfere with RNA pregenome packaging and viral DNA synthesis. HEPATOLOGY 1999;30:308–315.
16. Scaglioni P, Melegari M, Takahashi M, Chowdhury JR, Wands J. Use of dominant negative mutants of the hepadnaviral core protein as antiviral agents. HEPATOLOGY 1996;24:1010–1017.
17. Bottcher B, Tsuji N, Takahashi H, Dyson MR, Zhao S, Crowther RA, Murray K. Peptides that block hepatitis B virus assembly: analysis by cryomicroscopy, mutagenesis and transfection. EMBO J 1998;17:6839–6845.
18. Weber O, Schlemmer KH, Hartmann E, Hagelschuer I, Paessens A, Graef E, Deres K, et al. Inhibition of human hepatitis B virus (HBV) by a novel non-nucleosidic compound in a transgenic mouse model. Antiviral Res 2002;54:69–78.
19. Deres K, Schroder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, Kramer T, et al. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science 2003;299:893–896.
20. Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci U S A 1987;84:1005–1009.

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/hep.20144