

REVIEW

Hepatitis B virus reverse transcriptase: diverse functions as classical and emerging targets for antiviral intervention

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Hepatitis B virus (HBV) infection remains a global health problem with over 350 million chronically infected, causing an increased risk of cirrhosis and hepatocellular carcinoma. Current antiviral chemotherapy for HBV infection include five nucleos(t)ide analog reverse transcriptase inhibitors (NRTIs) that all target one enzymatic activity, DNA strand elongation, of the HBV polymerase (HP), a specialized reverse transcriptase (RT). NRTIs are not curative and long-term treatment is associated with toxicity and the emergence of drug resistant viral mutations, which can also result in vaccine escape. Recent studies on the multiple functions of HP have provided important mechanistic insights into its diverse roles during different stages of viral replication, including interactions with viral pregenomic RNA, RNA packaging into nucleocapsids, protein priming, minus- and plus-strand viral DNA synthesis, RNase H-mediated degradation of viral RNA, as well as critical host interactions that regulate the multiple HP functions. These diverse functions provide ample opportunities to develop novel HP-targeted antiviral treatments that should contribute to curing chronic HBV infection.

Emerging Microbes and Infections (2013) 2, e56; doi:10.1038/emi.2013.56; published online 4 September 2013

Keywords: Hepadnavirus; hepatitis B virus; reverse transcriptase; protein priming; review; RNA packaging; RNase H

INTRODUCTION

Hepatitis B virus (HBV) chronically infects over 350 million people and remains a global health threat.^{1,2} HBV is a member of the *hepadnaviridae* family, which includes members that can infect mammalian or avian species such as duck hepatitis B virus (DHBV).² The ca. 3.2-kb genome of HBV is replicated by a virally encoded polymerase (HP), a specialized reverse transcriptase (RT). HP uses the viral pregenomic RNA (pgRNA) as the template to synthesize minus-strand viral DNA via its RNA-dependent DNA polymerization activity (Figure 1).^{2,3} The minus-strand DNA is then used by HP as the template for plus-strand DNA synthesis. HP also has RNase H activity that is required to degrade the pgRNA template during minus-strand DNA synthesis. Minus-strand DNA synthesis is initiated by a novel protein priming mechanism in which HP itself serves a protein primer as well as the catalyst.^{4–9}

In order to become competent for protein-primed initiation of reverse transcription, HP must first bind to a short RNA structure termed epsilon (HBV ϵ or H ϵ) on the 5' end of pgRNA in a host chaperone-dependent reaction.^{6,10–14} HP–H ϵ interaction is required not only for protein-primed DNA synthesis by HP, but also for pgRNA packaging into viral nucleocapsids where viral DNA synthesis takes place.^{15,16} Currently, HP is the only viral target successfully tapped for anti-HBV chemotherapy through the use of one class of chemical compounds, nucleos(t)ide analog RT inhibitors (NRTIs), which primarily target HP DNA strand elongation activity.^{17,18} However, these treatments are not curative and long-term therapy is associated with toxicity and emergence of drug resistant HP mutations.^{18,19} Furthermore, antiviral drug-resistant HP mutants can also acquire resistance to the current HBV vaccine due to the compact nature of the HBV genome and the overlap of the viral genes encoding HP and

the viral envelope proteins, which are targeted by the vaccine.^{19–22} These 'vaccine escape' mutants may pose a serious threat to the success of the global HBV vaccine campaign.

HEPADNAVIRAL POLYMERASE DOMAIN STRUCTURE AND INTERDOMAIN INTERACTIONS

Hepadnaviral polymerases are composed of four domains that include an N-terminal terminal protein (TP) domain followed by a spacer region, an RT domain and a C-terminal RNase H domain (Figure 2A).^{3,23–25} Although the RT and RNase H domains are conserved with other RTs, the TP domain is only found in hepadnaviruses and not in any other RT.^{23,24,26–28} Efforts to obtain high-resolution structural information about hepadnaviral polymerases have been hampered by the difficulty in obtaining sufficient amounts of highly purified and active proteins, but important motifs and residues critical for various polymerase activities have been identified by genetic and biochemical studies (Figure 2A).

The TP domain

The TP domain was originally identified by its attachment to the 5' end of viral minus-strand DNA.²⁶ TP, unique to hepadnaviral RTs, is required for ϵ binding, RNA packaging, and protein priming.^{23,24,29–35} Genetic screens in DHBV have identified a short sequence near the C-terminus of TP, the T3 motif (Figure 2A), which is important for all the TP functions identified so far.^{29,36,37} Mutations of the corresponding residues in the HP T3 motif also disrupt HBV DNA synthesis, although there is some dispute regarding the importance of particular HP T3 residues in RNA packaging and genome replication.^{32,36,38} The T3 motif in DHBV polymerase (DP) is part of a larger C-terminal region of TP that is transiently surface exposed following chaperone- and adenosine

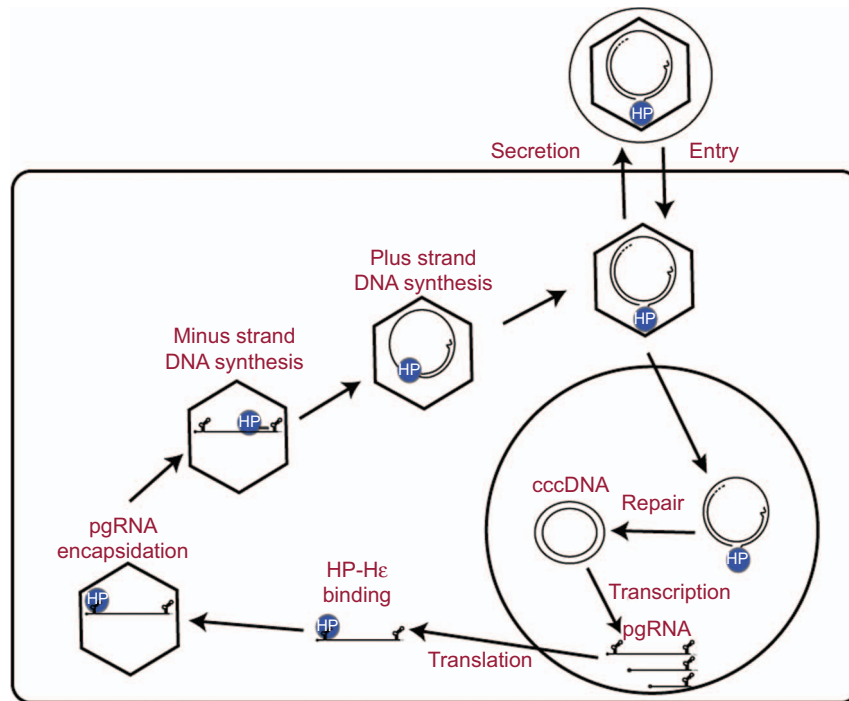


Figure 1 HBV replication cycle. Upon entry into the cytosol, the viral capsid with its encapsidated viral genome, a partially DS, RC DNA covalently attached to HP, traffics toward the nucleus where the genomic DNA is repaired to form cccDNA. cccDNA is transcribed by host RNA polymerase II to produce all viral transcripts including the overlength pgRNA. pgRNA acts as the template for translation of HP (as well as the core or capsid protein). HP binds to an RNA structural element called epsilon (He) on the 5' end of pgRNA. HP–He binding triggers pgRNA encapsidation followed by minus- and plus-strand DNA synthesis inside the capsid. The mature capsid, containing RC DNA, can then be enveloped and secreted or alternatively deliver RC DNA to the nucleus to amplify the cccDNA pool. cccDNA, covalently closed circular DNA; DS, double-stranded; RC, relaxed circular.

triphosphate (ATP)-dependent activation, which likely contributes directly to DHBV ϵ (D ϵ) RNA binding³⁹ (see the section on ‘Polymerase–host interactions’).

As the RT domain is also important for ϵ binding, the T3 motif in TP is thought to interact with the RT domain at a region called RT-1 (Figure 2A) (for more information, see the section on ‘The RT domain’), forming a composite RNA-binding site, although there is no direct proof yet for this interaction.³⁷ These data together led to a model in which the polymerase is activated by host chaperones to expose the C-terminal region of TP, including the T3 motif, which interacts with the RT1 motif in the RT domain, allowing for ϵ binding and subsequent RNA packaging and protein priming.

Mutagenesis of charged and hydrophobic residues of the HBV TP domain identified several important residues that contribute to RNA packaging and genome replication.^{38,40} In particular, R105 in TP was found to be important for pgRNA packaging (Figure 2A).³⁸ While Y173 is required for RNA packaging, both W74 and Y147 are important for genome replication but not for RNA packaging (Figure 2A).⁴⁰ These hydrophobic residues are hypothesized to be important for either intra- or intermolecular protein interactions,⁴¹ although further analysis will be needed to verify this prediction.

The spacer region

Although much of the spacer region of HP can be mutagenized without disrupting HP function,²⁴ three cysteine residues located in the C-terminal region of the spacer, as well as one additional cysteine residue in the N-terminus of the RT domain, are required for RNA packaging (Figure 2A).⁴² Although their exact function is unknown, these cysteines could be part of a zinc finger that coordinates He binding,

or could play an important structural role in HP function such as the formation of disulfide bonds.

The RT domain

The RT domain of the hepadnaviral polymerase shows significant homology to retroviral RTs, including the human immunodeficiency virus (HIV) RT.²⁸ Because of this similarity, the RT domain of HP has been structurally modeled using HIV RT as a template.^{43–46} HP shares homologous short motifs with retroviral RTs that include boxes A through E along with boxes F (or box II) and G (or box I) (Figure 2A).^{47–49} These motifs are known to form a well-defined catalytic core in retroviral RTs based on crystal structural analyses.^{50,51} No high-resolution structure for any hepadnaviral polymerase is available yet, but three lines of evidence support structural homology with retroviral RTs: first, amino-acid sequence homology; second, resistance to NRTIs occur in similar RT regions of HP as HIV RT; third, mutational analysis of these motifs show similar phenotypes to retroviral RTs.^{48,52–54}

A conserved F residue in box A of DP, F451, plays a crucial role in deoxy-ribonucleoside triphosphate *versus* nucleoside triphosphate discrimination, as in retroviral RTs.⁵³ Fundamental for polymerase activity, the conserved tyrosine–methionine–aspartate–aspartate active site in box C is required to catalyze DNA synthesis in both HBV and DHBV including protein priming (Figure 2A).^{4,6,23,24} The box E motif of the polymerase, which contains the proposed DNA primer grip found in retroviruses, is important for DHBV and HBV replication and specifically plays a role in the polymerase– ϵ interaction and DNA polymerization.⁵⁴ Remarkably, HIV RT sequences (*ca.* 60 amino acids) from box C to box E can substitute the homologous DP

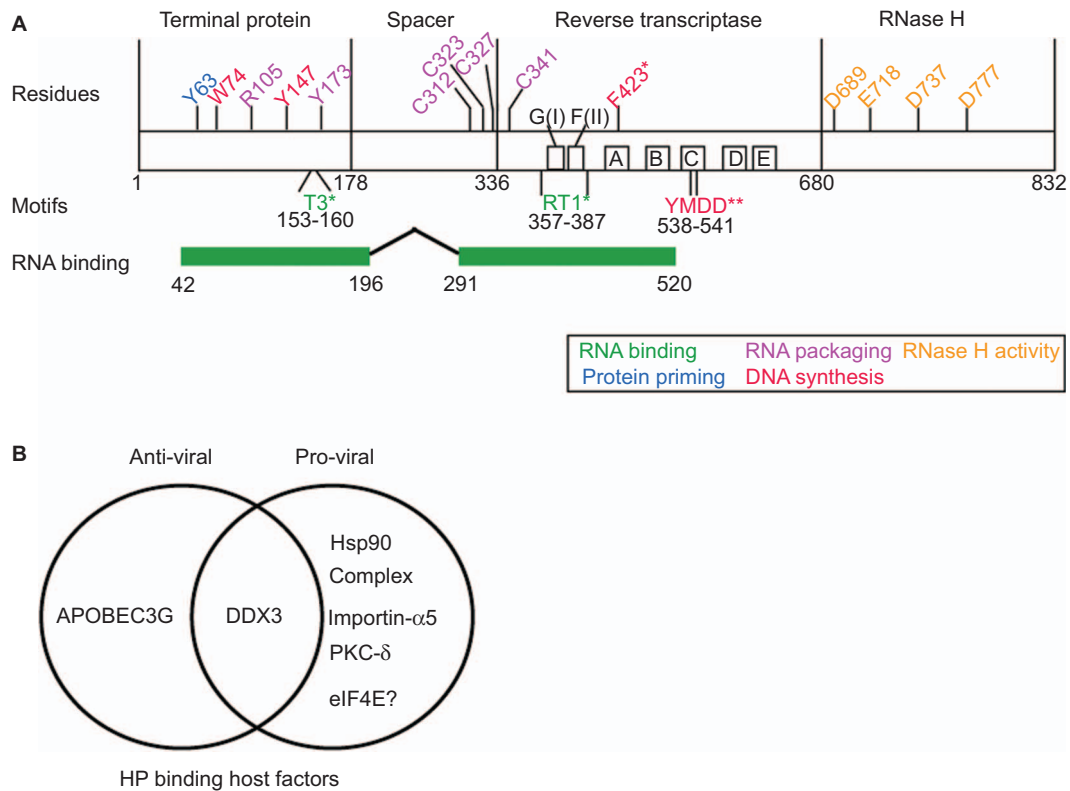


Figure 2 HP domain structure and host interactions. **(A)** HP is schematically depicted with its domains, important motifs and critical residues indicated. Motifs and residues are color-coded to denote the known steps of HBV replication for which they are important, as outlined in the box in the lower right corner. Small boxes A–G denote the conserved regions across reverse transcriptases. Minimal regions of HP required for RNA binding are represented as green boxes. *, verified function in DHBV but not HBV. ** denotes the fact that the YMDD polymerase active site is required for both protein priming and all subsequent DNA synthesis. **(B)** Reported antiviral and proviral HP-binding factors. eIF4E is listed with a “?” because it is anticipated, but not yet verified, to promote viral replication. YMDD, tyrosine–methionine–aspartate–aspartate.

sequences and this chimeric DP can produce mature viral relaxed circular-DNA.⁵⁴ Mutations downstream of box E around a hypothesized nucleic acid binding region (based on HIV) of HP reduce pgRNA packaging, although these mutations might affect the global DP structure.^{55–57} Boxes F and G are within the aforementioned RT1 motif (Figure 2A). Mutations in RT1 inhibit DP–De binding and protein priming and RT1 is thought to interface with the T3 motif in TP to form a composite RNA binding site,³⁷ as mentioned above.

The RNase H domain

The RNase H domain of both DHBV and HBV contains a stretch of *ca.* 100 amino acids in which a ‘DEDD’ motif is located (Figure 2A), which is important for RNase H activity by coordinating metal ion binding.⁵⁸ The negatively charged residues in the DEDD motif are important for both DHBV and HBV replication and specifically for RNase H activity (Figure 2A).^{23,24,59,60} Additionally, purified recombinant RNase H from HBV genotype D and H shows *in vitro* RNase H activity. Aside from its RNase H activity, the RNase H domain is also important for pgRNA packaging.^{15,27,30}

TP–RT domain interactions

For both HP and DP, the TP as well as the RT domains are required for ϵ binding and protein priming.^{10,33–35,61–63} In order for protein priming to occur, proper TP and RT domain interactions, including the possible T3–RT1 interaction discussed above, must occur

such that the priming Y residue in TP is positioned correctly at the polymerase active site in RT, with the additional constraint that the ϵ internal bulge must also be positioned appropriately relative to the polymerase active site and the primer Y residue to serve as the template for the priming reaction. As only one polymerase molecule may be packaged per nucleocapsid,^{16,64} TP–RT domain interactions normally occurs *in cis*, i.e., intramolecularly. However, for both DP and HP, independently expressed TP and RT domains can *trans*-complement one another and reconstitute ϵ binding and protein priming *in vitro*.^{4,33,61,65} Furthermore, either the TP or RT domain, even when linked *in cis* to an RT or TP domain, can also interact, *in trans*, with another RT or TP domain productively to carry out *trans*-priming (see ‘protein priming’ section below).³³ The strong TP–RT domain interactions are weakened following protein priming, which induces a structural alteration in the polymerase resulting in TP–RT dissociation, which, in turn, may induce the dissociation of the polymerase from ϵ and facilitate the transfer of the polymerase–nascent minus-strand DNA complex to DR1.³³

POLYMERASE–HOST INTERACTIONS

A number of host factors bind and modulate the activities of hepadnaviral polymerases (Figure 2B). Considering the diverse functions attributed to the polymerase and the multiple conformational states it has to adopt to carry out these functions, it is perhaps of little surprise that polymerase functions are subject to modulation by a multitude of host factors.

Molecular chaperones

The first host factors shown to interact with the hepadnaviral polymerase are components of the 90 kDa heat shock protein (Hsp90) complex, a multicomponent regulatory chaperone consisting of Hsp90 itself and several cochaperones, including Hsp70, Hsp40, Hop/p60 and p23 (Figure 2B).^{10,12–14,39,63,66,67} The host chaperone complex associates with DP and HP and is required to establish and maintain the polymerase in a conformation competent for ϵ interaction in a dynamic process driven by ATP hydrolysis. The exact details of polymerase activation by chaperones remain to be defined, but as alluded to earlier, the chaperones transiently expose a C-proximal region of TP (including the T3 motif), which may, in turn, bind ϵ directly.³⁹ Components of the Hsp90 complex remain bound to the polymerase and are packaged into viral nucleocapsids.^{10,14} For in-depth reviews of the interactions and effects of the Hsp90 complex on hepadnaviral polymerases, refer to the following references.^{66,68}

Eukaryotic translation initiation factor 4E (eIF4E)

As HBV pgRNA encapsidation, which requires HP, is dependent on close proximity of H ϵ to the 5' cap,⁶⁹ it was postulated that a cap-binding protein(s) might associate with HP and play a role in pgRNA packaging. Indeed, the eukaryotic translation initiation factor 4E (eIF4E) binds to HP independently of pgRNA, although the presence of pgRNA enhances the HP–eIF4E interaction.⁷⁰ eIF4E is also packaged into viral nucleocapsids. The functional consequence of eIF4E binding to HP, if any, remains unclear.

Apobec3G

Apobec3G, a human cytidine deaminase that deaminates retroviral DNA leading to hypermutation,^{71–73} binds to HP in an RNA independent manner and is packaged into nucleocapsids in an HP- and H ϵ -dependent manner.⁷⁴ Apobec3G can block HBV replication independent of its deaminase activity by a strong inhibitory effect on a very early stage of viral minus-strand DNA synthesis.^{75,76}

Immune modulatory factors

The interplay between HBV and the host immune system is complex and remains the subject of intense investigation. An immune modulatory factor, DDX3, was recently identified as a host factor that interacts with HP independently of pgRNA and is incorporated into nucleocapsids.⁷⁷ Expression of DDX3 in cells with replicating HBV reduces HBV genome replication without affecting pgRNA encapsidation, and was initially thought to be primarily an antiviral (or restriction) factor. However, this interaction was later linked to HP-dependent disruption of DDX3 binding to an effector kinase complex involved in interferon (IFN) signaling and suppression of IFN- β promoter activity.⁷⁸ As DDX3 is a required component of both hepatitis C virus and HIV replication,^{79–81} further studies may be warranted to elucidate the physiological role of DDX3 during HBV replication.

Other work has also connected HP to immune suppression as HP expression alone can suppress IFN- α -mediated response through HP-mediated repression of signal transducer and activator of transcription (STAT) translocation to the nucleus.^{82,83} HP is reported to bind both protein kinase C- δ and importin- α 5, two proteins that modulate activation of STAT1 by phosphorylation and STAT1/2 nuclear translocation respectively.⁸⁴ HP possibly binds and subsequently inactivates these factors, ultimately repressing STAT activation, although more work will be needed to characterize the direct effects of HP binding on protein kinase C- δ and importin- α 5. Additionally, it will be important to determine the significance of the HP-induced

immune suppression phenomenon during HBV infection at physiological levels of HP expression *in vivo*.

POLYMERASE- ϵ INTERACTION AND pgRNA PACKAGING

As hepadnaviral polymerase- ϵ binding is required for RNA encapsidation and protein priming, extensive work has been carried out to decipher the determinants of both the polymerase and ϵ required for ribonucleoprotein (RNP) formation.^{4,33,61,63,65,85,86} For a recent review on hepadnaviral RNP formation.⁶⁶ HP–H ϵ RNP formation shows fairly limited requirements of H ϵ and does not require the H ϵ apical loop (Figure 3).^{6,63} The HP requirements for H ϵ binding are also minimal in that the entire RNase H domain, the C-terminal part of the RT domain, the majority of the spacer and the N-terminal part of TP can be deleted without affecting HP–H ϵ binding (Figure 2A).⁶³ This is in contrast to the HP requirements for RNA packaging, which include nearly the full-length of HP.³⁰ Similarly, the entire H ϵ structure, plus a closely spaced (no more than 65 nucleotide away) 5' cap, is required to direct pgRNA packaging.^{15,16,69}

Mapping of the important H ϵ and HP determinants for HP–H ϵ interaction was mostly performed *in vitro* using truncated HP constructs purified from bacterial expression systems and reconstituted with the Hsp90 chaperone complex.⁶³ Recently, full-length HP was expressed and purified using a human cell line and was shown to display H ϵ binding activity *in vitro* and in cells.⁶ This simplified system has verified previous findings of H ϵ requirements for HP–H ϵ interaction and will facilitate further characterization of important H ϵ and HP regions for HP–H ϵ interaction *in vitro* and in cells.

One approach adopted for characterizing ϵ RNA structural requirements for DP and HP binding is the systematic evolution of ligands by exponential enrichment (SELEX) screen.^{87,88} With DP, a SELEX screen identified RNA aptamers that contain less predicted base pairing in the upper stem region, but remain active in DP binding and priming *in vitro* and active in replication in cell culture and in animals.^{88,89} For HP, a recent SELEX screen randomized the apical loop and/or upper stem sequences of H ϵ and identified H ϵ variants that retain HP binding *in vitro*.⁸⁷ Interestingly, some strong HP-binding RNA aptamers are predicted to have a less stable upper stem, similar to DHBV and other avian hepadnaviruses.

SUPPRESSION OF pgRNA TRANSLATION

HP directly regulates viral translation by repressing the translation of pgRNA through interaction with its 5' H ϵ .⁹⁰ This translational repression is dependent on H ϵ being located within *ca.* 60 nucleotides from the 5' end cap, very similar to pgRNA packaging requirement.^{69,91} The mechanism by which HP suppresses translation is not entirely clear,

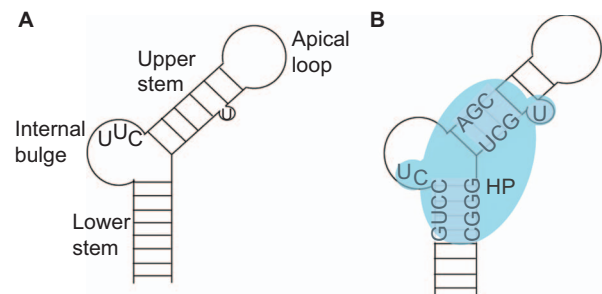


Figure 3 H ϵ requirements for HP interaction. (A) H ϵ is depicted with the conserved lower stem, internal bulge, upper stem and apical loop elements. Additionally, residues involved in priming are indicated in the internal bulge region. (B) H ϵ residues important for interaction with HP (blue oval) are indicated.

but it is thought that HP binding to the 5' Hε could prevent ribosomal binding and subsequent translation. It is also unresolved why the position of the 5' Hε is critical for both RNA packaging and translational repression, although it could be related to cap binding factors that require a short enough distance to interact with and regulate the HP–Hε complex for both activities.^{91,92}

PROTEIN PRIMING

Hepadnaviral DNA synthesis can be divided into at least three distinct stages that are associated with major polymerase conformational changes: (i) initiation of protein priming (deoxyguanylation of the TP Y priming residue); (ii) the DNA polymerization stage of protein priming (the addition of 2–3 residues to the initiating deoxy-guanosine monophosphate (dGMP)), and (iii) the subsequent DNA elongation following minus-strand template switch and during plus-strand DNA synthesis (Figures 1 and 4).^{6,7,9,62}

DHBV

Protein priming in hepadnaviruses was initially discovered using DHBV and was later found to require DP, Dε and components of the Hsp90 chaperone complex.^{9,10,13,14,67} For an in-depth review of DHBV priming, the reader is referred to the following reviews.^{3,66,68} Although viral replication is dependent on priming at a conserved tyrosine residue (Y96 for DHBV and Y63 for HBV),^{4,8,93} the structural determinants for selecting the priming residue remain poorly understood and primer specificity is not absolute. Priming at other residues including other Y and S residues on both the TP and RT domains can occur *in vitro*.^{94,95} Also, full-length as well as truncated DP, in addition to utilizing *cis*-linked TP for priming of DNA synthesis (*cis*-priming), can also use separate TP or RT domain fragments *in trans* as primers (*trans*-priming). That priming can occur at multiple residues on DP and also *in trans* raises the possibility that host factors might also be modified by nucleotidylation and potentially regulated by the viral polymerase.³³ Indeed, nucleotidylation is increasingly being recognized as an important protein modification with functional consequences.⁹⁶ As *trans* TP and RT fragments can complement each other to allow for ε binding and priming, truncation analysis of both

the TP and RT domains has helped to narrow down the minimal boundaries for both the TP and RT domains for priming activity.³³ Identification of these minimal sequences not only provides insights into the requirements for protein priming, but should also facilitates ongoing efforts to obtain high-resolution structures of this most important yet difficult enzyme.

HBV

Studies on HBV protein priming have been difficult due to the lack of suitable *in vitro* systems. HP expressed in *Escherichia coli* and reconstituted with chaperone proteins shows Hε binding and but no protein priming activity, in contrast to DHBV.^{10,12} HP expressed by *in vitro* translation in rabbit reticulocyte lysate does not show any priming activity even though the same translation system was the first used to produce a priming active DP.⁹ HP expressed in insect cells using a recombinant baculovirus system does show a low level of *in vitro* priming (with a specific activity less than 1%), using the authentic Y63 priming residue, but protein priming in this system is independent of Hε,^{4,5} raising the possibility that the activity detected may represent the recently discovered, protein-primed terminal transferase activity of HP.⁷

We have recently expressed and purified HP using a human cell system.⁶ HP purified using this system recapitulates authentic Hε binding and Hε-dependent protein priming *in vitro*, including both initiation (dGMP attachment to Y63 in TP) and polymerization (addition of two deoxy-adenosine monophosphate (dAMP) residues to the initiating dGMP) in the presence of physiologically relevant Mg²⁺ (Figure 4). In order for HP to show protein priming activity *in vitro*, HP has to be isolated bound to Hε as an RNP complex formed in cells. The Hε determinates required for HBV priming in this system are strict and similar to the RNA packaging requirements, including the need for both the Hε bulge and apical loop and a short distance between the 5' cap and Hε (Figure 5). Furthermore, we have developed an assay to directly visualize the nascent minus-strand DNA products formed during protein priming by releasing them from HP using tyrosyl-DNA phosphodiesterase 2,⁶ an enzyme able to specifically break the linkage between a tyrosine residues of a protein and

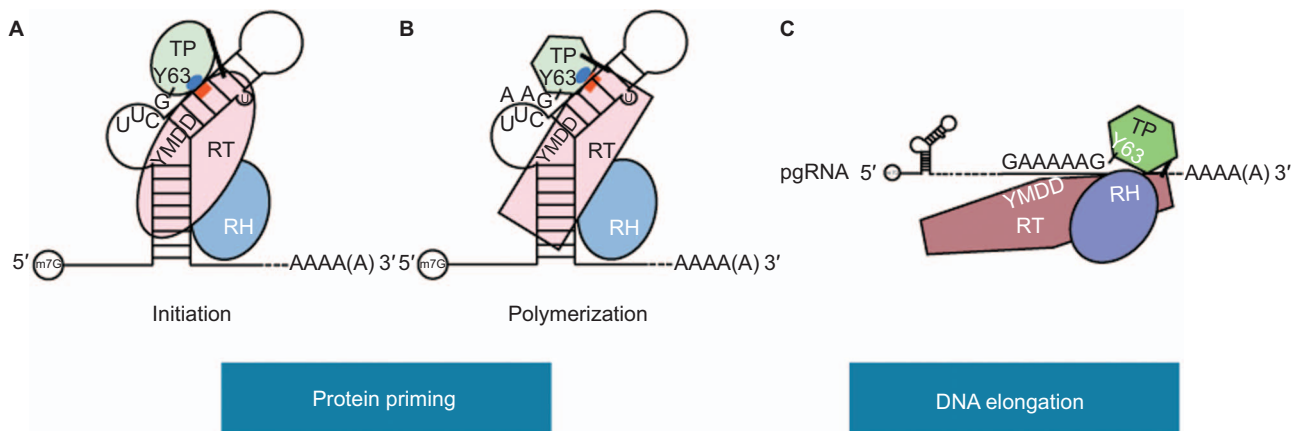


Figure 4 HP structural changes during protein priming and DNA elongation. **(A)** The initiation stage of protein priming. The HP–Hε complex initiates protein priming by adding a single dGMP residue to Y63 of the TP domain using the YMDD polymerase active site. **(B)** The DNA polymerization stage of protein priming. Following the initiation of protein priming, HP adds two dAMP residues to the dGMP that is already attached to TP. Both stages of protein priming use the internal bulge of Hε as the specific obligatory template. **(C)** DNA elongation following protein priming. The HP–dGAA covalent complex is transferred from Hε to a complementary sequence at the 3' end of pgRNA where minus-strand DNA elongation continues. During each stage, distinct HP conformations are thought to be required for HP to carry out the different steps of viral DNA synthesis, as depicted by changes in the domain shape and color. The T3 motif is represented as a blue oval located in the TP domain; the RT1 motif is represented as an orange rectangle located in the RT domain. YMDD, tyrosine–methionine–aspartate–aspartate.

the 5' end of DNA.^{97,98} The availability of this system should facilitate efforts to elucidate the viral and host requirements for HBV protein priming, and help characterize why HP expressed using this system has priming activity, while other systems that produce priming active DP are unable to produce priming active HP. Among other possibilities the presence of the capped and polyadenylated Hε that is bound to HP may allow additional RNA- or RNP-binding factors to be copurified, producing a priming active, multicomponent HP complex, while DP does not require these additional factors for protein priming.

Effects of metal ions on priming

Mn²⁺ affects the function of DP by stimulating *in vitro* protein priming activity, allowing for low level Dε-independent protein priming and inducing an alternate DP conformation.⁸⁵ Mn²⁺ also affects the activity of the insect cell-derived HP.⁹⁹ Surprisingly, HP purified from human cells displays a Mn²⁺-dependent protein-primed transferase activity, in which HP can attach to itself long stretches of DNA using all deoxy-ribonucleoside triphosphate substrates completely independent of Hε or any other template.⁷ Protein-primed transferase activity, similar to Hε-dependent DNA synthesis, shows a conformational change after production of a nascent DNA oligomer (Figure 4). Although Mn²⁺ is most likely not the divalent metal ion used for HP functions during viral replication, it is possible that Mn²⁺ could exert mutagenic effects on HBV genomic replication during long-term infection by activating the template independent transferase activity of HP. Additionally, as cryptic site priming (i.e., independent of Y63) can also occur with HP in the presence of Mn²⁺,⁷ HP might modify host proteins by covalent attachment of nucleotides and/or DNA strands and regulate their activity, as discussed above.

DNA ELONGATION

After protein priming, which produces a three nucleotide (HBV) or four nucleotide (DHBV) long DNA oligomer attached to the polymerase, minus-strand DNA synthesis continues only after transfer

of this covalent polymerase–DNA oligomer complex to the complementary, direct repeat 1 (DR1) region at the 3' end of pgRNA (Figure 4).^{11,100–104} The viral pgRNA is degraded by the polymerase RNase H activity during minus-strand DNA synthesis.^{23,24,60} Subsequently, the polymerase is able to continue with plus-strand DNA synthesis by using a small, RNase H-resistant fragment derived from the 5' end of pgRNA as the primer.¹⁰⁵ Hepadnaviral DNA replication occurs not only with the polymerase protein attached to the DNA product, but also within the constraints of the nucleocapsid. The capsid environment directly controls viral DNA synthesis within it, possibly through modulating the polymerase activities.^{106,107} In DHBV, mutations mimicking the capsid in a non-phosphorylated state completely block reverse transcription at an early stage, while mutations mimicking constitutive capsid phosphorylation allow for minus-strand DNA synthesis, but cause a severe defect in plus-strand DNA synthesis and accumulation. It is currently of great interest to characterize how the capsid and polymerase proteins work together to carry out pgRNA packaging and reverse transcription.

TARGETING HP FOR ANTIVIRAL THERAPY

There are currently seven Food and Drug Administration-approved treatments for chronic HBV infection, including IFN-α and its pegylated form and five NRTIs, which inhibit the DNA strand elongation activity of HP.^{17,18} NRTIs are highly effective in inhibiting HBV replication. However, these treatments are not curative and require life-long therapy with a high risk of drug resistance and toxicity.^{17,48} In the search for a cure of chronic HBV infection, it will be important to target all the essential functions of HP in addition to its DNA synthesis activity (Figure 6).

Targeting polymerase-ε binding

As polymerase-ε binding is prerequisite for both pgRNA packaging and protein priming, RNP formation represents an attractive early target for antiviral development. We have shown that the antibiotic

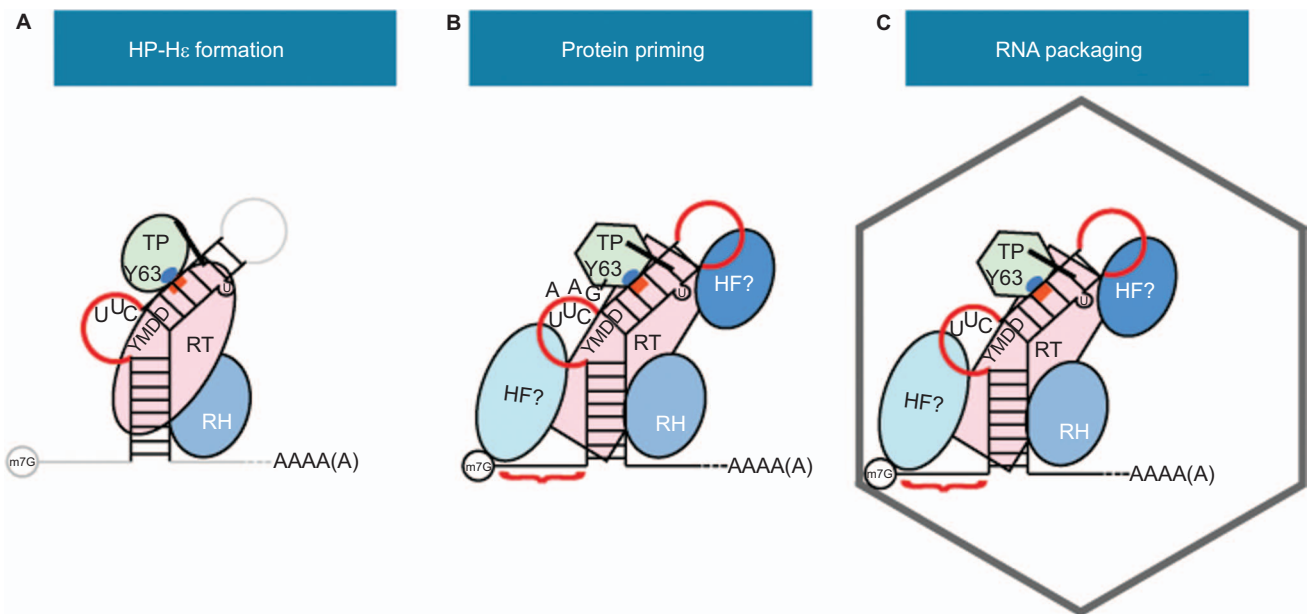


Figure 5 Hε requirements for HP–Hε formation, protein priming and RNA packaging. HP–Hε RNP formation requires the Hε bulge, but the apical loop and the distance between the 5' cap and Hε are not critical (non-critical elements are designated as light gray, while the critical elements are highlighted with red). On the other hand, protein priming and RNA packaging require both the Hε internal bulge and apical loop, as well as a short distance between the 5' cap and Hε. Modified from *J Virol* 2012; **86**(9): 5134–5150. doi: 10.1128/JVI.07137-11, copyright © 2012, with permission from American Society for Microbiology.

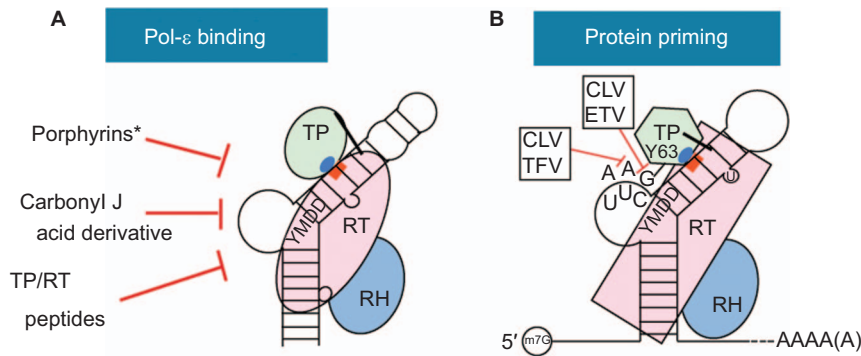


Figure 6 Antiviral compounds targeting polymerase-ε binding and protein priming. DP–Dε binding (A) and HP priming (B) are depicted along with known corresponding inhibitors. * denotes that porphyrins can also inhibit HP–Hε binding. CLV, clevudine; ETV, entecavir; TFV, tenofovir.

geldanamycin inhibits polymerase-ε association in both HBV and DHBV by blocking the chaperone function of Hsp90.^{12–14} Inhibition of host proteins as fundamental to cellular function as Hsp90 may not be ideal, and other compounds that target the polymerase-ε interaction have been identified. For example, hemin and other porphyrins block polymerase-ε interaction in both HBV and DHBV.¹⁰⁸

Another approach to identify agents that target HP–Hε binding is through SELEX selection of strong HP-binding RNA aptamers that compete with Hε for HP binding.⁸⁷ An HP-binding ‘decoy’ aptamer transfected into cells replicating HBV shows a strong inhibitory effect on pgRNA packaging and DNA synthesis. Similar in basic mechanism, peptides containing DHBV T3 or RT1 sequences inhibit priming, likely through preventing DP–Dε binding.^{36,37} Lastly, a carbonyl J acid derivative, which targets HIV polymerase–primer/template binding,^{109,110} disrupts the formation of hepadnaviral polymerase-ε complexes.¹¹¹

Targeting protein priming

It should also be advantageous to target protein priming for anti-HBV treatment as priming is the earliest stage of viral reverse transcription. Distinct conformations adopted by HP for protein priming *vs.* the subsequent DNA strand elongation afford the opportunity to design structurally distinct agents to inhibit HP priming functions that would be complementary to current NRTIs (Figure 4).^{6,7,9,62,85,112} Currently, only one Food and Drug Administration-approved NRTI, entecavir, a guanosine analog NRTI, can inhibit HP priming initiation by competing for incorporation with the initiating nucleotide, deoxy-guanosine triphosphate.^{113,114} As HP resistance to entecavir only occurs with multiple simultaneous HP mutations, it would be of great interest to determine if entecavir-mediated priming inhibition contributes to this increased barrier to viral resistance.¹⁸ Surprisingly, we found that clevudine, a thymidine analog NRTI approved for HBV treatment in South Korea, can also inhibit the initiation of protein priming, through an apparent non-competitive mechanism and without being incorporated into viral DNA.¹¹⁴ Additionally, both clevudine and tenofovir, an adenosine analog NRTI, can inhibit the DNA polymerization stage of protein priming.¹¹⁴ This was not too surprising for tenofovir as it can compete with the natural deoxy-adenosine triphosphate (dATP) substrate during the polymerization stage and can stop viral DNA synthesis via chain termination once it is incorporated. However, it was again surprising that clevudine could inhibit the DNA polymerization stage of protein priming; as for its inhibition of priming initiation, it also inhibited polymerization without being

incorporated into the DNA. Lastly, via a mechanism entirely different from NRTI inhibitors, a catalytic dead RT domain fragment derived from DP can potently inhibit DP priming activity *in vitro* when provided *in trans*, likely by ‘snatching’ the TP domain from the *cis*-linked RT domain.³³ Preventing proper TP–RT domain interactions could prove an effective way of blocking polymerase functions in ε binding, RNA packaging and DNA synthesis.

Targeting RNase H activity

Much of the focus for HBV treatment to date has been targeted at the DNA polymerization activity of HP, but the RNase H activity represents yet another essential enzymatic activity of HP with untapped potential as an antiviral target. Recently, active HBV RNase H purified from *E. coli* was used to screen a group of 21 known or predicted inhibitors of HIV RNase H and/or integrase for their effects on HBV RNase H activity.⁵⁹ Twelve of these compounds were found to have inhibitory effects on HBV RNase H activity. Although the specific inhibitory mechanism of these compounds is not yet known for HBV, structural predictions and inhibitory profiles suggest that some compounds likely compete with Mg²⁺ binding to the RNase H active site, while others appear to act by novel non-competitive mechanisms.⁵⁹

CONCLUSION

Genetic and biochemical studies to date have revealed many important hepadnaviral polymerase residues and motifs that are important for polymerase-ε binding, RNA packaging, protein priming, DNA strand elongation and RNase H activity. However, much remains to be learnt. Many of the studies to date on protein priming have been performed using the DHBV model system, while the recently developed HP protein priming assay will now allow similar studies to be performed with HBV. Additionally, there is still much to be discovered regarding the viral and host requirements for HP protein priming and the conformational dynamics of HP throughout viral replication. The mechanisms of the mutually dependent packaging of HP and pgRNA into assembling nucleocapsids and how the changing capsid environment regulates HP activities and viral DNA synthesis are also not well understood at present. In addition to further genetic and biochemical studies, intensified efforts to obtain high-resolution structures of HP and various HP complexes essential for HP functions and HBV replication will greatly inform the development of novel effective agents targeted at diverse HP functions, including the HP–Hε interaction, HP interdomain interactions, pgRNA packaging, protein priming and RNase H activity. As these treatments would target different stages in viral replication through distinct

mechanisms than current antivirals, they could contribute significantly to a cure for chronic HBV infection.

ACKNOWLEDGMENTS

Work in the authors' laboratory has been supported by a Public Health Service grant (R01 AI074982 to Jianming Hu) from the National Institutes of Health. Scott A Jones was supported by the Training Grant 'Viruses and Cancer', 2 T32 CA60395, from the National Cancer Institute.

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