Regulation of hepatitis B virus replication by epigenetic mechanisms and microRNAs

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem that causes a wide spectrum of liver diseases, including acute or chronic HBV infection. Acute HBV infections either resolve or progress to chronicity. Chronic hepatitis B (CHB) is associated with chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC; McMahon, 2009). It is estimated that more than 350 million patients worldwide are chronically infected with HBV, with the majority of these patients living in the Asia-Pacific region. More than one million deaths occur each year as a direct consequence of CHB (McMahon, 2009). Medical intervention using antiviral nucleoside/nucleotide analogs and interferon (IFN) was established to treat chronically infected patients (Pardo et al., 2007). However, currently available therapies do not lead to the termi-nation of HBV infection in the majority of patients (Mailliard et al., 2013). The entry of HBV virions is likely initiated through a non-specific interaction with negatively charged glycans at the surface of hepatocytes (Schulze et al., 2007; Bremer et al., 2009) followed by specific binding to the sodium-taurocholate cotransporting polypeptide (NTCP) receptor by a specific sequence (2-48aa) located in the preS domain of the LHBsAg protein (Yan et al., 2012). After uncoating, the HBV capsid is transported by the cellular machinery to the nuclear pore. The open circular form of HBV genomic DNA is then converted to a covalently closed circular DNA (cccDNA) molecule in the nucleus. This process requires that the covalently attached viral polymerase is removed from the negative DNA strand by a proteinase and that the positive strand DNA is completed by the cellular replicative machinery so that it matches the negative strand to covalently join the two ends to form a circular, supercoiled molecule (Guo and Hu, 2007).

In the nucleus, HBV cccDNA is incorporated into the host chromatin and exists as an individual minichromosome with a “beads-on-a-string” structure, which is revealed by electron microscopy (Bock et al., 1994; Newbold et al., 1995). This minichromosome has been shown to consist of both histone and non-histone proteins. By immunoblotting with HBcAg, the histone proteins H3 and H2B were the most prominent species, while lower levels of H4, H2A, and H1 were also detectable (Bock et al., 2001). Using the cccDNA-ChIP assay, the group of Massimo Leverro has confirmed the recruitment of the H3 and H4 histones along with the approximately 3.2 kb in length. The viral genome harbors seven open reading frames, coding for the viral polymerase, HBc core, and e antigens (HBcAg and HBeAg); the regulatory HBx protein; and the preS/S gene encoding the three surface antigens (LHBsAg, MHBsAg, and SHBsAg). The genome also contains a number of regulatory elements (Seeger and Mason, 2000). The entry of HBV virions is likely initiated through a non-specific interaction with negatively charged glycans at the surface of hepatocytes (Schulze et al., 2007; Bremer et al., 2009) followed by specific binding to the sodium-taurocholate cotransporting polypeptide (NTCP) receptor by a specific sequence (2-48aa) located in the preS domain of the LHBsAg protein (Yan et al., 2012). After uncoating, the HBV capsid is transported by the cellular machinery to the nuclear pore. The open circular form of HBV genomic DNA is then converted to a covalently closed circular DNA (cccDNA) molecule in the nucleus. This process requires that the covalently attached viral polymerase is removed from the negative DNA strand by a proteinase and that the positive strand DNA is completed by the cellular replicative machinery so that it matches the negative strand to covalently join the two ends to form a circular, supercoiled molecule (Guo and Hu, 2007).

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The hepatitis B virus (HBV) genome forms a covalently closed circular DNA (cccDNA) minichromosome that persists in the nucleus of virus-infected hepatocytes. HBV cccDNA serves as the template for viral mRNA synthesis and is subject to epigenetic regulation by several mechanisms, including DNA methylation and histone acetylation. Recently, microRNAs (miRNAs), a class of small non-coding RNAs, were also directly connected to the epigenetic machinery through a regulatory loop. Epigenetic modifications have been shown to affect miRNA expression, and a sub-group of miRNAs (defined as epi-miRNAs) can directly target effectors of the epigenetic machinery. In this review, we will summarize recent findings on the epigenetic mechanisms controlling HBV cccDNA function, primarily focusing on the epi-miRNA functions operating in HBV replication. Investigation of the epigenetic regulation of HBV replication may help to discover novel potential therapeutic targets for drug development with the goal to eradicate the HBV cccDNA pool in hepatocytes.

Keywords: hepatitis B virus, microRNA, epigenetic regulation, histone deacetylases, DNA methyltransferases

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HBeAg and HBx proteins to the cccDNA minichromosome. Using the same approach, several cellular transcription factors (CREB, ATF, YY1, STAT1, and STAT2) and chromatin modifying enzymes (PCAF, p300/CRP, HDAC1, SIRT1, and EZH2) have been shown to bind to the cccDNA in human hepatoma cells containing replicating HBV (Pollicino et al., 2006; Belloni et al., 2009, 2012). The histone deacetylases (HDACs) p300/CRP and PCAF and the histone deacetylases (HDMCs) HDAC1 and SIRT1 were shown to be recruited with different kinetics onto HBV cccDNA, implying that HBV cccDNA-bound histones may be subjected to regulatory post-translational modifications (Levrevo et al., 2009).

Because cccDNA is the transcriptional template of the virus (Quasdorff and Pforte, 2010), it is required for the maintenance of HBV infection. Unlike HBV transcripts and replicative intermediates, cccDNA is very stable in quiescent hepatocytes and is responsible for the persistence of infection during the natural course of chronic HBV infection and during prolonged antiviral therapy (Wiele-Lapostolle et al., 2004). The cccDNA may persist for many years in the liver of patients, even after successful antiviral treatment and reinforcement of immunologic control (Zoulim, 2005). Currently, little is known about the mechanism of HBV cccDNA maintenance in the nuclei of hepatocytes. However, it has been shown that the cccDNA can be eliminated when infected hepatocytes are removed by immune cell-mediated killing or other non-cytopathic mechanisms (Murray et al., 2005) and replaced by cell turnover (Lutgehetmann et al., 2010).

REGULATION OF HBV cccDNA TRANSCRIPTION BY EPIGENETIC MODIFICATION

HISTONE ACETYLLATION AND METHYLATION

Recently, it was proposed that the functionality of HBV cccDNA might be controlled by epigenetic mechanisms, regulating its transcriptional activity and HBV replication. Histones and non-histone proteins either bind directly to the cccDNA or are indirectly recruited to viral minichromosomes through protein–protein interactions. Thereby, the acetylation and deacetylation of cccDNA-bound histones may regulate HBV transcription. Exploring a ChIP assay using anti-acetylated-H3 or -H4 antibodies, Pollicino et al. (2006) found that HBV replication is indeed regulated by the acetylation status of H3/H4 histones bound to the viral cccDNA, both in cell-based replication systems and in the liver of chronically HBV infected patients. The co-recruitment of PCAF and p300/CRP parallels viral replication in vitro, whereas HDMC1 recruitment onto the HBV cccDNA correlates with low HBV replication in vitro and with low viremia in vivo. The importance of epigenetic modifications of cccDNA-bound histones in the regulation of HBV replication is further confirmed by experiments exploring the class I and class II HDMC inhibitors trichostatin A (TSA), valproate, and nicotinamide (NAM). These HDMC inhibitors induce an evident increase of both cccDNA-bound acetylated H4 and HBV replication. Another study demonstrated a similar role for the acetylation of cccDNA-bound histones, as well as a role for methylation and phosphorylation of these proteins (Gong et al., 2011).

A recent study demonstrated that in cultured hepatoma cells with HBV replication and in mouse models with repopulated human hepatocytes, administration of IFN-α resulted in the active recruitment of the transcriptional co-repressors HDAC1, SIRT1, and polycomb repressor complexes 2 (EZH2 and YY1) to HBV cccDNA as well as the hypoacetylation/hypermethylation of cccDNA-bound histones. IFN-α treatment also reduced the binding of the transcription factors STAT1 and STAT2 to the IFN-sensitive response element on active cccDNA (Belloni et al., 2012). These observations suggested that IFN-α could epigenetically regulate HBV replication, and the hypoacetylation/hypomethylation of histones was associated with decreased replication of HBV. Furthermore, it was shown that small molecules that inhibit p300 and PCAF or activation of SIRT1/2 and EZH2 could induce an “active epigenetic suppression” of the HBV cccDNA minichromosome to suppress HBV replication (Palumbo et al., 2013).

HBV DNA METHYLATION

In addition to post-translational modifications of histones, methylation of the CпG islands on HBV genomic DNA also contributes to the regulation of HBV gene expression (Mogal et al., 2011; Riverbark et al., 2012). It has been shown that early integrated HBV DNA is methylated in HCC cells (Miller and Robinson, 1983; Chen et al., 1988). The non-integrated HBV DNA (Vivekanandan et al., 2008b) and cccDNA (Guo et al., 2009) could also be methylated in liver tissues from patients. Currently, at least six CпG islands have been identified in the HBV genome, including three conventional regions overlapping the start site of the HBV S gene (island 1), the region encompassing enhancer 1 and the X gene promoter (island 2), and the Sp1 promoter and start codon of the G gene (island 3; Zhang et al., 2013b). Methylation of CпG islands 1 and 2 was found in HBV DNA extracted from liver biopsies from CHB patients, suggesting that increased methylation of HBV DNA may decrease the production of viral proteins (Vivekanandan et al., 2008b). The hypermethylation of island 2 was correlated with low levels or absence of HBeAg production (Vivekanandan et al., 2008a), as well as reduced HBeAg expression (Guo et al., 2009). It was shown that individuals with occult HBV infection, which is characterized by the persistence of HBV DNA in the liver of individuals who test negative for the HBeAg, had a higher degree of methylation in island 2 compared to non-occult CHB patients (Vivekanandan et al., 2008a). Another study with a cohort of cirrhotic patients did not find an association between the methylation status of HBV cccDNA and HBeAg expression in liver tissues, but confirmed that a higher methylation density was associated with lower viral load, lower RNA copies per cccDNA, and lower virusron productivity (Kim et al., 2011).

Consistent with these findings, transfection of methylated HBV DNA in HepG2 cells resulted in reduced HBV mRNA levels, decreased intracellular HBeAg and core HBeAg expression, and decreased secretion of HBV viral proteins into cell supernatants. Furthermore, an in vitro equivalent of cccDNA showed decreased viral protein production in HepG2 cells after DNA methylation (Vivekanandan et al., 2009). After transfection of HBV DNA into HepG2 cells, an inverse relationship between methylated HBV DNA and viral mRNA levels was observed in dependence on the upregulation of host DNA methyltransferase (DNMT). Cotransfection with DNMT3a and HBV DNA was associated with decreased production of HBeAg and HBeAg, as well as host proteins implicated in carcinogenesis (Vivekanandan et al., 2010).
Although the viral miRNAs encoded by HBV have not been verified, the products of these miRNAs play a pivotal role in the epigenetic regulation network. Many studies have shown that a set of miRNAs play a pivotal role in the epigenetic regulation network (Chuang and Jones, 2013). Along with the epigenetic regulation of miRNA expression, many miRNAs themselves can regulate the expression of components of the epigenetic machinery, creating a highly controlled feedback mechanism. A number of the miRNAs related to epigenetic regulation were defined as so-called “epi-miRNAs.” For example, DNMT1 overexpression was responsible for the hypermethylation of the miR-148a and miR-152 promoters. As a direct effect of these two miRNAs on HBV RNA transcripts was validated by GFP reporter assay (Zhang et al., 2013a). In addition, HBx was shown to activate HBV transcription through opposition to the protein phosphatase 1 and HDAC1 complex on the HBV cccDNA (Cougot et al., 2012), or down-regulate DNMT3A expression through miR-101 induction (Wei et al., 2013b). Loss of HBx reduced recruitment of p300, caused rapid hypoacetylation of the cccDNA-bound histones and increased early recruitment of SIRT1 and HDAC1, accompanied by lower HBV replication (Belloni et al., 2009).

**CELLULAR miRNAs INHIBIT HBV REPLICATION BY DIRECT BINDING**

As HBV produces different transcripts during its life cycle, the transcripts are proposed to be targeted by cellular miRNAs. In a screen for cellular miRNAs affecting HBV replication, Zhang et al. (2010) employed a loss-of-function approach by transfecting antagonirs targeting 328 human miRNAs into HepG2 cells. Two miRNAs, miR-199a-3p and miR-210, were shown to suppress HBsAg expression. The direct effect of these two miRNAs on HBV RNA transcripts was validated by GFP reporter assay (Zhang et al., 2010). In addition, Russian group found that miR-125a-5p is able to interfere with HBsAg expression, thus reducing the amount of secreted HBsAg (Potenza et al., 2011). Recently, many cancer-related miRNAs, including miR-15a/miR-16-1 (Wang et al., 2013a), the miR-17-92 cluster (Bung et al., 2013), and miR-224 (Sciutti et al., 2013), were shown to target HBV miRNAs directly by luciferase reporter assay and inhibit HBV replication (summarized in Figure 1). Notably, the expression of these miRNAs was also linked to epigenetic regulation, as well as to promoter methylation (Dakhallah et al., 2013) and histone acetylation (Zhang et al., 2013a; Wang et al., 2013b).

**CELLULAR miRNAs REGULATE HBV REPLICATION INDIRECTLY**

In addition to direct targeting, some cellular miRNAs, including epi-miRNAs, were found to be capable of inhibiting or stimulating HBV replication by indirectly regulating cellular transcription factors. It was shown that the transcription of HBV cccDNA was tightly regulated by a number of liver-enriched transcription factors and nuclear receptors through the recognition of HBV promoter/enhancer elements (Quasdorf and...
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FIGURE 1 | Summary of cellular miRNAs effect on HBV replication. HBV DNA could be integrated into host genome or form cccDNA minichromosome in the hepatocytes nucleus. It serves as the template for viral transcription, viral DNA, and antigen production. The miRNAs which regulate HBV replication through epigenetic mechanism, transcription factors regulation, immune regulation, and direct targeting viral transcripts were indicated.

Protzer, 2010). miR-122 may exert its effect on HBV indirectly via downregulation of its target cyclin G1, thus interrupting the interaction between cyclin G1 and p53 and abrogating p53-mediated inhibition of HBV replication (Wang et al., 2012a). miR-372 and -373 are upregulated in HBV-infected liver tissues and promote HBV gene expression through a pathway involving the transcription factor nuclear factor I/B (Guo et al., 2011). The higher expression of miR-501 in HCC tissues could enhance HBV replication partially by targeting HBXIP (Jin et al., 2013). In contrast, miR-141 significantly suppresses HBV expression and replication in HepG2 cells. Bioinformatic analysis and experimental assays indicate that peroxisome proliferator-activated receptor alpha is a relevant target of miR-141 during this process (Hu et al., 2013). For immune-related miRNAs, miR-155 enhances innate antiviral immunity by promoting the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway through the targeting of SOCS1, mildly inhibiting HBV infection in human hepatoma cells (Su et al., 2011).

By screening a set of cellular miRNAs, our group found that epigenetically regulated miR-1 over-expression resulted in a marked increase in HBV replication, accompanied with upregulated HBV transcription, antigen expression, and progeny secretion. HDAC4, the cellular target of miR-1, was able to suppress HBV replication. The expression of nuclear receptor farnesoid X receptor alpha (FXRA) was increased by miR-1, leading to the enhanced transcriptional activity of the HBV core promoter (Zhang et al., 2011). Furthermore, another epi-miRNA that targets HDAC1, miR-449a, had an even higher capacity for enhancing HBV replication but a lower level of induction of FXRA (Zhang et al., unpublished data). Additionally, both of these two defined epi-miRNAs could inhibit the G1/S cell cycle transition and promote cell differentiation by increasing the expression of hepatocyte-specific factors, which may be beneficial for HBV replication (Zhang et al., 2011). Collectively, host epi-miRNAs can modulate HBV replication by regulating cellular epigenetic factors or specific transcription factors that directly bind to the HBV cccDNA minichromosome (summarized in Figure 1).
HBV replication and gene expression, likely in the response to the changing hepatic microenvironment. Considerably, many cellular mRNAs indirectly influence the HBV life cycle by regulating the expression of relevant cellular proteins and may play important roles in hepatitis B pathogenesis. Further studies need to be performed to elucidate the regulatory loop involving miRNAs and the ccDNA epigenetic machinery and certainly to investigate how to translate these findings into clinical applications.

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