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Virus–host interplay in hepatitis B virus infection and epigenetic treatment strategies

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Worldwide, chronic hepatitis B virus (HBV) infection is a major health problem and no cure exists. Importantly, hepatocyte intrusion by HBV particles results in a complex deregulation of both viral and host cellular genetic and epigenetic processes. Among the attempts to develop novel therapeutic approaches against HBV infection, several options targeting the epigenomic regulation of HBV replication are gaining attention. These include the experimental treatment with 'epidrugs'. Moreover, as a targeted approach, the principle of 'epigenetic editing' recently is being exploited to control viral replication. Silencing of HBV by specific rewriting of epigenetic marks might diminish viral replication, viremia, and infectivity, eventually controlling the disease and its complications. Additionally, epigenetic editing can be used as an experimental tool to increase our limited understanding regarding the role of epigenetic modifications in viral infections. Aiming for permanent epigenetic reprogramming of the viral genome without unspecific side effects, this breakthrough may pave the roads for an ambitious technological pursuit: to start designing a curative approach utilizing manipulative molecular therapies for viral infections *in vivo*.

Abbreviations

5-caC, 5-carboxylcytosine; 5-fC, 5-formylcytosine; 5hmeC, 5-hydroxymethylcytosin; 5meC, 5-methylcytosin; BCP, basal core promoter; BP, base pair; cccDNA, covalently closed circular DNA; CGI, CpG island; CHB, chronic HBV infection; CRISPR, clustered regularly interspaced short palindromic repeat; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HBsAb, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; KDM, histone lysine demethylase; KMT, histone lysine methyltransferase; NTCP, sodium-taurochlorate cotransporting polypeptide; PTM, post-translational covalent modification; rcDNA, relaxed circular DNA; TALE, transcription activator-like effectors; TBP, TATA-binding protein; TET, ten-eleven translocation; TSS, transcriptional start sites; URR, upper regulatory region; ZF, zinc finger protein.

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Principles of epigenome orchestration between pathogens and hosts in viral infections

Epigenetic mechanisms provide a series of regulatory principles that define changes in gene expression, without altering the genetic information encoded in the DNA itself (Fig. 1). Importantly, these chemical modifications of DNA or its associated proteins are stable and heritable, and hence determine the cell phenotype. During mitosis, gene expression patterns become inherited to daughter cells. Several observations suggest that probably—to a limited extent—this inheritance also takes place transgenerationally by to date largely unknown mechanisms [1]. There is a consensus regarding the fact that developmental programs, external environmental triggers, pathogens (including viruses), and a variety of inflammatory and malignant disease-causing agents can have influence on the epigenome. However, recently evidence is growing indicating that particular DNA sequence motifs can directly alter the overlying epigenetic signatures [2]. This gives rise to a new concept; other than previously thought, a clear separation between the genome and the epigenome cannot be made. Gene regulation and transcriptional activity is rather an interactive functional interplay of the genomic base and its associated epigenomic orchestra.

At the DNA sequence level, there are several mechanisms considered as being 'epigenetic'. They influence the regulation of gene expression and may or may not be involved in transgenerational inheritance of acquired transcriptional character states. These mechanisms involve covalent modifications of cytosines, mostly in the context of CpG dinucleotides (CpG signaling), such as methylation [3] or hydroxymethylation [4] at the C5 atom in the 6-atom ring of cytosine (5meC or 5hmeC, respectively). However, the ultimate substrate for gene expression in the cell nucleus is the DNA–protein complex (chromatin) rather than naked DNA itself. Nucleosomes—the fundamental unit of DNA packaging—consist of a stretch of DNA wound around an octamer composed of each two molecules of the histone protein types H2A, H2B, H3, and H4. The degree of local chromatin compaction can be altered through numerous post-translational covalent modifications (PTMs) to histones, with acetylation, methylation, phosphorylation, and ubiquitination being only few of many possible modifications at various specific residues [5]. Moreover, variants of all histone protein types, except for H4, have evolved in all eukaryotic supergroups [6,7]. Both histone modifications and variants occur in a dynamic fashion and create chromatin structures either being permissive or suppressive for gene expression, depending on the genomic context and the combination of modifications at a given site. The theoretical number of combinatorial PTMs and their resulting functional consequences are still hardly comprehensible and understandable, but at least two very basic principles have been recognized: The introduction of generally activating PTMs, such as H3 acetylation, can directly weaken DNA–nucleosome interactions, and thus lead to chromatin decompaction and better accessibility of the DNA. Alternatively, PTMs can create binding sites allowing the recruitment of effector proteins, which can thus perform their localized function.

Importantly, epigenetic modifications are reversible. Many enzymes that introduce or remove specific PTMs have been characterized [5,8], including the well-described histone acetyltransferases (HATs) and histone deacetylases (HDACs), histone lysine methyltransferases (KMTs), histone lysine demethylases (KDMs), DNA methyltransferases (DNMTs), and the ten-eleven translocation (TET) enzymes [4], which are involved in active DNA demethylation via conversion of methylated DNA from 5meC to 5hmeC and further via 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) intermediates.

In many virus-borne diseases, such as hepatitis B virus (HBV) infection, the spatiotemporal epigenetic state of both the viral and host genomes are critical for the course of transcriptional programs, which allow the production of new virus particles [9]. For this purpose, all viruses—as molecular parasites—modify gene expression programs of their hosts in order to exploit the cellular machineries for transcription, RNA editing, and translation of structural and modulating viral proteins (Fig. 2). Obligatorily for this purpose, viral and host gene expression programs must be orchestrated accordingly. Research focusing on the regulation of epigenome plasticity of both virus and host aims to uncover key molecular principles that are crucial for a detailed understanding of pathogenesis, diagnosis, and treatment of infectious human diseases. In the case of chronic HBV infection, this is of particular importance as it includes the development of complex secondary diseases, such as cirrhosis and hepatocellular carcinoma (HCC) [10].

This is of major clinical relevance as worldwide approximately 240 million people suffer from chronic HBV infection (CHB). This accounts for a death burden of approximately 680 000 annual cases due to HBV-related cirrhosis and liver cancer [11]. Most frequently, the mode of HBV transmission is vertically from mother to child. Strikingly, up to 90% of the

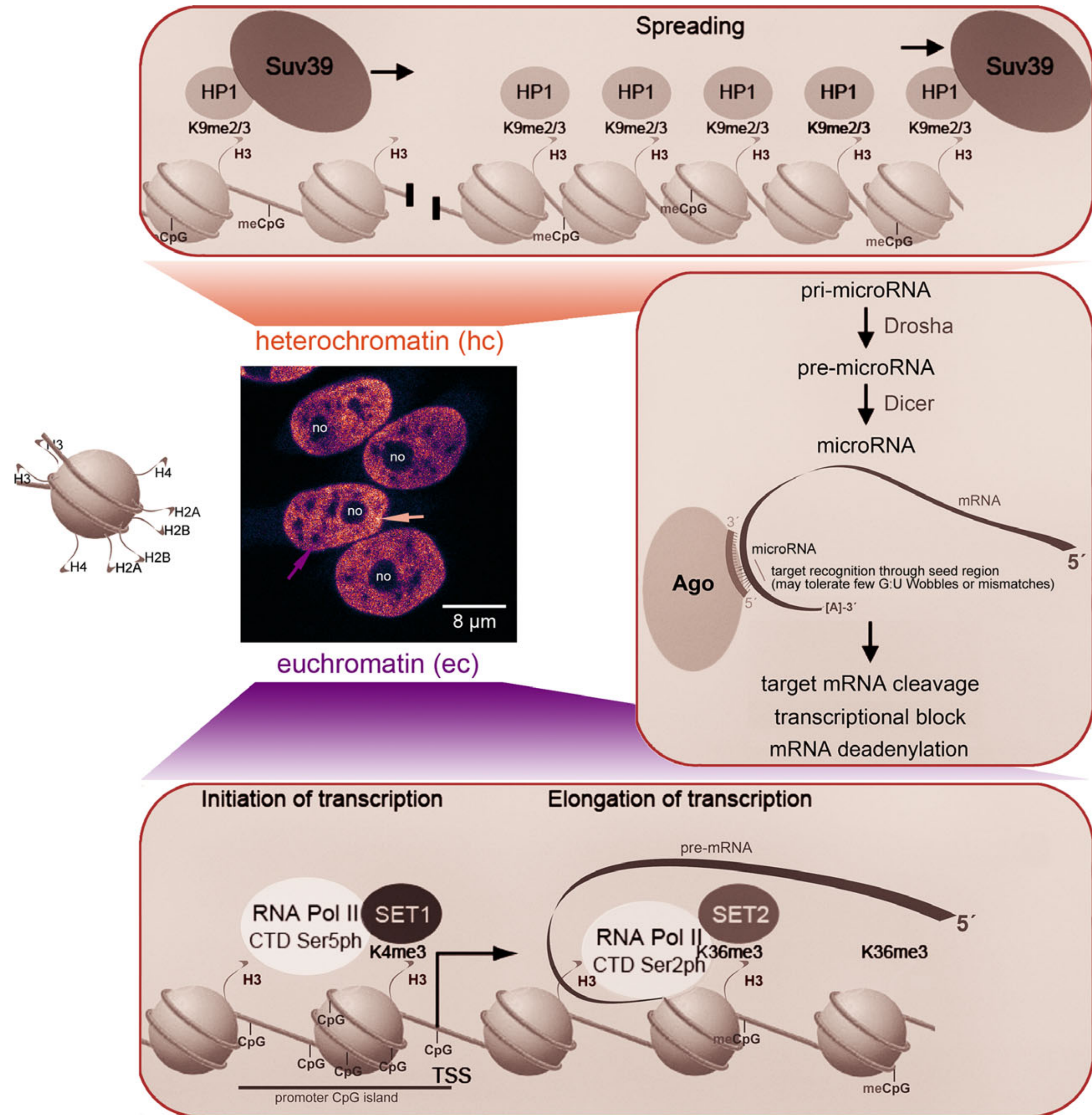

Fig. 1. Mechanisms of chromatin structure regulation and the control of gene expression. The central immunofluorescence microscopic image shows Chinese hamster ovary cell nuclei, whose DNA was stained with To-Pro-3. Densely stained areas with condensed heterochromatin can clearly be distinguished from areas with less intensely stained chromatin comprising of decondensed euchromatin. Frequently repressed genes are associated with heterochromatin, which possesses histone H3 methylation at lysine 9 as a hallmark. Heterochromatin protein 1 (HP1) reads out H3K9me and its binding results in the spreading of heterochromatin arrays. DNA segments surrounding transcription start sites (TSS) with enriched 5-methylcytosine are associated with repressed gene activity (top). Other post-translational histone modifications, such as H3K4me3 or H3K36me3, occur with actively transcribed genes (bottom), whereby RNA polymerase II phosphorylated at serine 2 of the carboxy terminal domain (CTD) repeat becomes often enriched in the proximity of gene promoters, and RNA polymerase II phosphorylated at serine 5 of the CTD repeat is associated with transcriptional elongation within the gene body. Ago, agonaute protein; CpG, demethylated CpG; CTD, carboxy terminal domain; HP1, heterochromatin protein 1; K9me2/3, lysine 9 di-/tri-methylation; meCpG, methylated CpG; RNA Pol II, RNA polymerase II; SET I, Su(var)3-9, Enhancer-of-zeste and Trithorax 1; SET 2, Su(var)3-9, Enhancer-of-zeste and Trithorax 2; Suv39, suppressor of variegation 3-9; TSS, transcription start site, K36me3, lysine 36 tri-methylation.

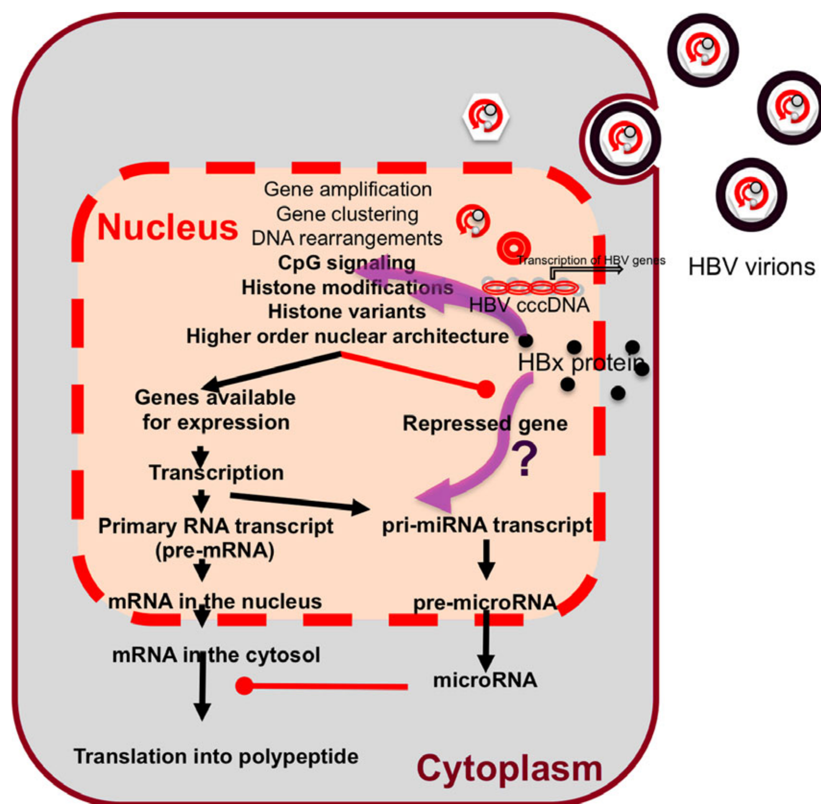

Fig. 2. Host–pathogen functional interplay during HBV replication. Schematic illustration of host–pathogen interactions and localization specificity during HBV replication. The hepatocyte nucleus provides the environment for HBV replication. At various stages of HBV transcription, host and virus epigenetic modifications interact and alter the viral replication process. cccDNA = covalently closed circular DNA, HBx = hepatitis B virus X protein.

neonates born from hepatitis B surface antigen (HBsAg)-positive mothers run the risk of becoming infected [12]. While CHB occurs in only 5–10% when the infection takes place at adult age, chronic infection rates are markedly higher in children. In fact, chronification rates exceed 90% when infants become infected during the first 6 months of life. Initial symptoms of HBV-induced hepatitis usually remain mild or even subclinical. The devastating sequels cirrhosis and malignancy occur decades later. CHB is associated with a 37-fold elevated probability of developing HCC [13]. Currently, a wide variety of experimental and translational research is directed to illuminate the underlying pathophysiology of age-specific chronification and HBV-induced malignant transformation. However, the exact molecular mechanisms remain unknown to date.

HBV genetic variation

Hepatitis B virus is a highly species-specific hepatotropic virus. The viral genome of HBV contained in the viral particles is a relaxed circular, partially double-stranded DNA (rcDNA) molecule of around 3.2 kb. While in the cellular nucleus, the transcriptionally active form of the viral genome is a covalently closed circular DNA (cccDNA). During the replication cycle of HBV, one of the genomic mRNAs named pgRNA becomes associated with the viral polymerase to be retrotranscribed to DNA. As HBV polymerase lacks proof reading activity, an average frequency of nucleotide misinsertion of 6.28×10^{-4} (range from 3.59×10^{-4} to 1.51×10^{-3}) has been calculated [14]. The particular organization of the HBV ORFs implies that 61% of the viral genome is shared by two independent ORFs coding viral proteins in different frames. This peculiar genomic organization, in addition to the error rate of the viral polymerase, involves a particular evolution rate of the different regions of the viral genome and a global evolutionary rate of 7.9×10^{-5} nucleotide substitutions/site/year [15]. The high variability of HBV genomes allows identification of 10 genotypes (A-J), which feature mostly a geographical distribution [16]. HBV genotypes and subgenotypes are classified based on 8% and 4% divergence of the complete genome, respectively. HBV genotypes B, C, F, H, I, and J have a genome size of 3215 nucleotide , while genotypes A, D, E, and G have different genomic sizes: 3221, 3182, 3212, and 3248, respectively [16].

Particular characteristics of HBV genotypes include differences in natural history and pathogenesis.

Although additional studies must be conducted to comprehensively investigate the clinical implications of all HBV genotype variations, some differences have already been established. In this regard, the comparison of genotypes C and B, both highly prevalent in Asia, suggests the association of genotype C with more severe liver disease, cirrhosis, and hepatocellular carcinoma. Similarly, comparison of genotypes A and D suggests a strongest association of genotype A with more severe liver disease than genotype D [17,18]. These observations clearly suggest a direct connection of the clinical and molecular characteristics of viral genotype differences. However, general studies including all genotypes must be carried out in order to investigate the particular outcome related to each HBV genotype.

HBV episome genome structure and regulation

Covalently closed circular DNA—the transcriptionally active HBV episome

In the course of the infection, the HBV cccDNA localizes in the hepatocyte nucleus, associates with both viral and cellular proteins and becomes organized as chromatin via nucleosome deposition, forming an HBV minichromosome [19,20] (Fig. 3). The viral proteins HBx and HBc are additional parts of the HBV episome. In particular, HBc plays a role in the structural episome organization, altering the nucleosome number, and leads to elevated episome copy numbers: HBc preferentially binds to the CpG-rich regions of the HBV genome thus promoting a chromatin structure permissive for transcription and replication [21]. However, the exact functional relevance and biological consequence of these observations remain to be determined [20,22].

HBV transcription is a complex multistep process despite its comparatively small genome. All known HBV mRNAs are expressed from the negative strand only in direction 1 to 3200. Solely the preS1 promoter features a canonical TATA box. Instead, cryptic TATA-like sequence motifs occur in other promoters, whose mRNA transcripts exhibit some 5′-heterogenity [23]. This is indicative of nondiscrete transcriptional start sites (TSS), and it is well possible that the cryptic TATA-like elements induce positional variability for the binding of promoter nucleosomes/transcription factors, which contribute to the definition of the TSS. A greater degree of freedom for such binding would allow the expression of mRNAs with variable 5′ ends

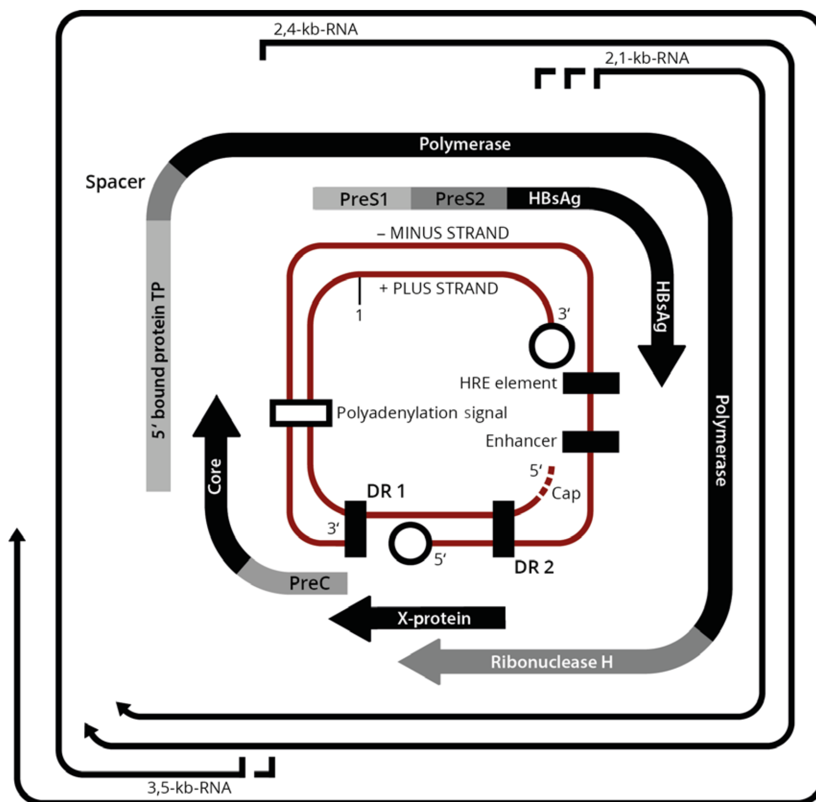

Fig. 3. Hepatitis B virus genomics. The HBV genome of infectious virions is a relaxed circular, partially double-stranded DNA (rcDNA) molecule of 3.2 kb, containing four open reading frames (ORFs) and associated promoters (Core, preS1, preS2/S, and X) controlling the expression of five mRNAs (pgRNA, pcRNA, 2.4 kb, 2.1 kb, and 0.7 kb). pgRNA and 2.1 kb are polycistronic mRNAs expressing two proteins each. In total, seven proteins are encoded: E antigen (HBeAg), Core (HBc), polymerase (P), small, middle, and large S antigen (HBsAg S, M, L), and X (HBx). Functionally, HBsAg and HBc are structural proteins. In chronic infections, the HBV genome forms the covalently closed circular DNA (cccDNA) which persists as a stable episome within hepatocyte nuclei serving as a template for transcription and viral protein expression [22].

[24–26], and thus to synthesize more than one mRNA from a single promoter (e.g., core proteins).

Numerous host regulatory proteins such as transcription factors and chromatin modifiers physically interact with the HBV minichomosome, e.g., CREB, ATF, STAT1, STAT2, and STAT3; HATs (CBP, p300, PCAF/GCN5); HDACs (HDAC1 and hSirtl); and DNMTs (Table 1 and Fig. 4) [22,27–31].

Interestingly, the HBV genome contains several regions rich in CpG dinucleotides. Depending on the genotype, this formally fulfills the definition of 3–6 CpG islands (CGI) (Fig. 4). As the largest CGI, CGI I is located partially overlapping both Enh1 and Enh2, spanning the HBx TSS. The shorter CGI II (genotypes B, F, and H) is downstream of Enh2 and the HBe/HBc TSSs. The CGI III (conserved in all genotypes) overlaps with the TSS of the HBV polymerase protein. CGIs IV to VI are less conserved in different HBV genotypes, whereby CGI IV (genotypes A, B, D, and E) overlaps with the small S TSS and CGI VI with the large S TSS. CGI V (genotypes B, C, and D) is located between CGI IV and I and represents the CGI with the lowest CG content. Figure 4 represents the first comprehensive illustration of CGI localization in all known HBV genotypes. Previous studies have used different nomenclature for HBV CGIs according to their occurrence in only one or a few HBV genotypes. However, the biological relevance of differentially methylated CGIs for the control of viral transcription is still poorly understood so far [32,33].

Regulatory sequences within the HBV genome that are associated with transcription factors have been described in more detail. Two enhancer sequences, EnhI and EnhII, potentiating the activity of the HBV promoters independently, appear to be of superior importance [34,35]. It was demonstrated that their activity is increased in hepatic cell lines when compared to nonhepatic cells. The 270-nucleotide-sized Enh1 (position 970 to 1250) can bind transcription factors and transcription regulators such as HNF-3, RFX1, EF-C, NF1, HNF-4, RXRa ⁄PPAR, and COUP- TF via its central domain [36–38]. The adjacent 5′- and 3′-ends interact with C⁄EBP and HNF-1 augmenting the activity of the central domain [39]. EnhII is 120 nucleotides in size and interacts with multiple transcription factors including HLF, FTF, E4BP4, and Sp1 [38,40]. Subregions of EnhII apparently influence specific HBV promoters, e.g., subregion α activates the Core promoter, while other subregions act on the preCore promoter (γ and δ) [41].

The Core promoter contains multiple transcription factor-binding sites (including Sp1, HNF-4, HNF3, COUP-TF1, and the liver-specific factors HNF-3 and HNF-4) and controls the expression of both pcRNA and pgRNA, whereby the combinatorial composition of associated transcription factors determines their reciprocal expression [25,42,43]. The Core promoter is subdivided into the basal core promoter (BCP) and the upper regulatory region (URR), which contains both reinforcing and attenuating functional elements [44]. BCP itself is a weak promoter, which becomes fully active only in proliferative hepatocytes where the upstream regulatory regions along with EnhI and EnhII can be stimulated by specific transcription factors (Table 1) enhancing the expression of pcRNA and pgRNA [44,45].

The preS1 promoter controls the expression of a 2.4 kb mRNA encoding the large surface protein and depends on the activity of EnhII, the presence of HNF-1, and the TATA-binding protein (TBP). Moreover, Sp1, NF1, Oct1, and HNF-3 can interact with the preS1 promoter [46–49].

The preS2/S promoter regulates the expression of a 2.1 kb mRNA with variable 5′-ends encoding both the medium and the small form of the surface protein [23]. Its activity does not depend on the enhancer sequences, and it contains a noncanonical TATA box-like motif and an initiator element [25,50] where this last element is mandatory for its activation [51].

The 140-nucleotide X promoter regulates the transcription of a 0.7 kb mRNA. It is under control of EnhI, which is localized upstream of the X promoter. The X promoter is activated by EnhI in the very early steps of the infection [52], interacting with ubiquitous transcription factors, such as NF1, C⁄EBP, ATF, and AP1⁄Jun-Fos. This rather low selectivity allows its activation in any metabolic condition of hepatocytes eventually favoring the establishment of the infection [53,54]. HBx inhabits a key function in HBV transcriptional regulation and was proven indispensable during the early steps of HBV infection [55].

The expression of HBV mRNAs is a highly regulated process, where two main steps can be differentiated—each one controlled by one of the two enhancers Enh1 and Enh2. In the first step, EnhI is active and allows the expression of the 0.7-kb mRNA in order to increase the levels of HBx, which thereafter induces the activation of EnhII and thus exerts its action over the preC/C, preS1, and preS2 promoters [56]. HBx acts in a positive feedback loop to increase its own expression rate, leading to elevated HBx levels in both the nucleus and the cytoplasm. Nuclear HBx promotes the activation of EnhI, while preventing HBV inactivation through DNA methylation by cellular defense mechanisms [22]. Elevated cytoplasmic HBx promotes a G1-arrest of hepatocytes via

Table 1. Mechanisms of interaction of HBV with host transcription factors.

Name	Target in cccDNA	Effect	Metabolic involvement	References
Ubiquitous transcription factors				
Nuclear factor 1 (NF1)	PreS1 EnhI	Activation		[137,138]
Specificity protein 1 (SP1)	PreS1, PreS2/2, PreC/C	Activation		[42,47,139,140]
Activator protein 1 (AP-1)	PreS1, X, PreC/C	Activation		[30,141]
TATA-binding protein (TBP)	PreS1, PreS2/2	Activation		[51,142]
Prospero-related homeobox protein (Prox1)	PreS1, X, PreC/C EnhII	Activation		[143]
cAMP response element-binding protein (CREB)	PreS2/S EnhII	Activation		[27,28]
Nuclear factor kappa-beta (NF-κβ)		Inhibition		[144]
Octamer transcription factor (Oct1)	PreS1	Activation		[145]
Nuclear respiratory factor 1 (NRF1)	X	Activation		[146]
Hepatic-enriched transcription factors				
Hepatocyte nuclear factor 3 α, β, and γ (HNF3)	Enh1 (HNF3α, HNF3β, HNF3γ) PreS1, EnhII (HNF3α, HNF3β)	Chromatin remodeler	Carbohydrate metabolism β-oxidation of lipids	[36,37,46, 147–149]
Hepatocyte nuclear factor 1 α and β (HNF1)	EnhII	Activation of preS1 through Oct1. Inhibition of pcRNA favoring pgRNA-transcription stimulated by HBx.	Differentiation Hepatocyte polarization Glucose and amino acid regulation	[145,150,151]
CAAT enhancer-binding protein α and ζ (C/EBP)	PreS2/S EnhII	Activation of PREC/C (in low concentrations), inhibition of PREC/C (in high concentrations)	Lipid and glucose homeostasis	[152,153]
Nuclear receptors in hepatocytes				
Hepatocyte nuclear factor 4 (HNF4)	PreS1, PreS2/S, PreC/C EnhI, EnhII	Activation of preS1, preS2/S, PREC/C promoters. Inhibition of pcRNA and increase pgRNA expression.	Differentiation Lipid, cholesterol, and acids homeostasis	[52,151,154]
Peroxisome proliferator-activated receptors (PPAR) and retinoid X receptors (RXRα)	PreS1, X, PreC/C EnhI		Expressed in response to lipid-derived endogenous ligands as fatty acids. PPAR/RXRα activate expression of certain genes related with fatty acid oxidation, gluconeogenesis, and ketone body synthesis.	[155–157]
Farnesoid acid receptor (FXR)	PreC/C EnhII	Enhancement of pgRNA expression.	FXR heterodimerizes with RXR to activate genes related with cholesterol, lipid, and bile metabolism	[157,158]
Small heterodimer partner (SHP)		Inhibition of FXR interaction.	Expressed in response to bile acids.	[158,159]
Testicular orphan receptor 4 (TR4)		Inhibition of TR4/TR2 and PPAR/RXRα interaction with cccDNA.		[160,161]

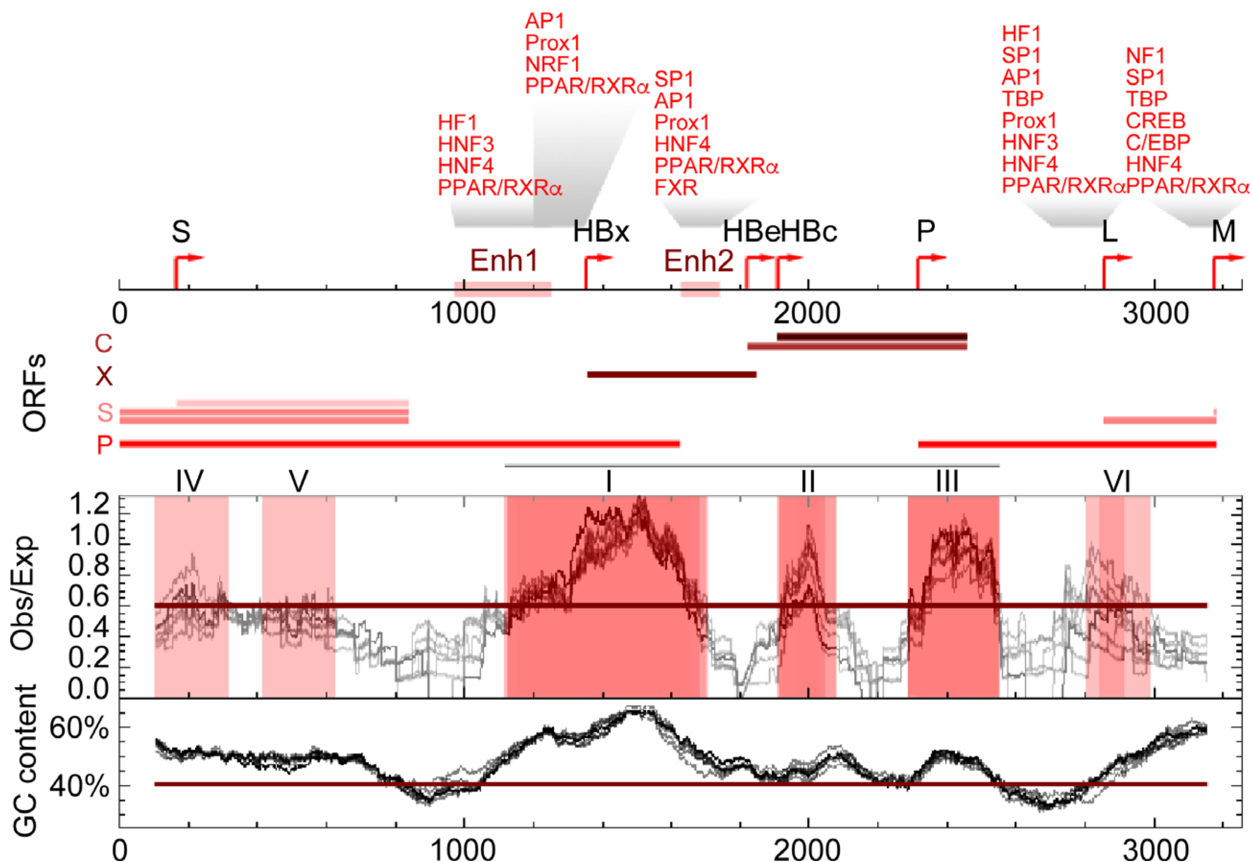

Fig. 4. Localization of CpG islands (I-VI) and GC distribution in the HBV genome and respective variations between genotypes A-H. Transcription factors associated with the HBV genome are presented with their localization in relation to viral open reading frames (ORF). Regional GC content is illustrated at the bottom. As a reference, the red line represents the average GC content in humans and mice. The ratio of observed-to-expected CpG content is shown above. The red line indicates the threshold for a significant amount of CpGs.

induction of P21 and P27 proteins thus creating favorable conditions for viral replication [56–58]. Upon this G1-arrest of infected hepatocytes, their metabolic activity increases. This provides a sufficient pool of transcription factors and other cellular proteins needed for viral replication, resulting in the accumulation of cccDNA. Eventually, cell division reduces the cccDNA pool—not only by diluting the cccDNA density but also through the reduced availability of transcription factors. This contributes to limited cccDNA availability, which in turn leads to a reduced viral transcription rate [59,60].

Altogether, HBx is involved in the establishment of a transcriptionally active state of the viral episome [61]. Therefore, HBx may contribute to a stable gene activation and expression from the cccDNA [62]. While the exact underlying mechanism has not been illuminated well to date, the above-described genomic elements clearly play a central pathophysiological role through their interplay with both the HBV and the host cell epigenomes. The mechanisms through which HBV makes use of numerous host transcription factors for running its gene expression programs are outlined in Table 1.

Transcription factors modulate chromatin structures and this modulation may in turn open or restrict the chromatin accessibility for other transcription factors, respectively. In order to understand these regulatory networks, it will be crucial to analyze in depth the plastic chromatin structure changes in HBV as well as host genomes of infected hepatocytes. The first steps in this understanding have already been made. We here summarize current achievements and discuss their relevance in the next paragraphs. Moreover, we discuss novel epigenetic HBV treatment strategies.

Epigenomics of HBV and HBV-induced hepatocyte deregulation—the told and the untold

Upon HBV infection of the hepatocyte, the viral relaxed circular DNA (rcDNA) enters the nucleus,

becomes converted to the cccDNA, and forms a highly stable minichromosome. The cccDNA associates with both histones and viral proteins to form an episome, which itself is susceptible for epigenetic modifications and also causes alterations of host cell gene regulation. Solid data on correlations of epigenetic changes in chronic HBV infections and clinical outcome parameters are still scarce. Most studies focus on either DNA methylation of the virus itself or assess epigenetic marks in hepatocyte genes in the light of HBV-induced hepatocarcinogenesis.

The dynamic epigenome of HBV—molecular backgrounds and clinical implications

Transcriptionally active cccDNA is associated with histone modifiers such as histone acetyltransferase proteins (PCAF, P300/CBP) [22,63,64]. Such activating epigenetic enzymes provide novel therapeutic targets that may successfully interfere with replication. In this respect, Tropberger *et al.* [65] demonstrated that histone PTMs occur in well-defined patterns across the HBV genome. This suggests that the same mechanisms underlie cccDNA expression regulation as they are known for host cellular chromatin. Importantly, these mechanisms can be targeted by epigenetic drugs (as outlined in detail later).

Although epigenetic modifications in cccDNA are mediated by cellular proteins, the activity of histone PTMs is highly dependent on the interaction with HBx. Indeed, HBx enhances HBV replication by recruitment of histone modifiers (PCAF, P300/CBP), thus favoring episome remodeling into a replicative active status [22,66,67].

Furthermore, a decrease of viral transcription and viral particle production has been shown in the presence of histone deacetylases (HDAC1) SIRT1, and polycomb repressor complexes 2 (EZH2). Indeed, interferon-α antiviral treatment induced similar epigenetic effects on cccDNA [22,68,69].

In addition to histone modifications, DNA CpG signaling seems to play a key role in transcription regulation of HBV and, depending on the HBV genotype, 3–5 CpG islands have been described (Fig. 4). The impact of each specific CpG island on viral transcription regulation is still largely unknown, e.g., the degree of CGI I methylation could not be correlated to the serum viral load [32,70,71]. Despite this finding, differential methylation has been demonstrated depending on the state of viral infection, suggesting a relevant role of CpG islands in viral replication control and eventually in the outcome of the infection [70–72]. Moreover, human liver biopsy specimens indeed revealed different levels of cccDNA methylation dependent on the stage of the disease. A low level of methylation has been described in cases of hepatitis, while in cirrhotic tissues, the cccDNA was often unmethylated. However, an increase in methylation was demonstrated in HCC samples [71]. Therefore, both the underlying mechanism and biological relevance of CpG methylation in transcription control of HBV mRNA need to be further addressed [32,73]. In this respect, methylome clustering analyses confirmed characteristically lower DNA methylation levels for chronic active hepatitis and liver cirrhosis compared with HCC and hepatoma cell lines [74].

The chicken-and-egg question currently remains unanswered: It is not clear whether the changes in viral DNA methylation are driving malignant transformation or whether the differential methylation is a side effect of neoplasia. This suggests cancer-specific changes in the methylome of the HBV cccDNA and pinpoints to a functional relevance of these changes in the long-term regulation of HBV replication.

Besides influencing histone PTMs on the HBV cccDNA, HBx seems to affect also viral DNA methylation by impairing the association of DNA methyltransferases (DNMTs) with the cccDNA [22,69]. In order to establish and maintain active HBV infection and episomal stability of the cccDNA, it is assumed that hypomethylated genomes (especially observed for CGI I and III) [32] acquire activating histone PTMs at associated nucleosomes in the presence of HBx [75]. This allows high levels of transcription, replication, and continuous nuclear entry of viral DNA. We assume that inside the hepatocyte nucleus, viral genomes can continuously become methylated and eventually be silenced and lost during subsequent mitosis in the course of prolonged infections. This results in a heterogeneous population of differentially activated so-called minichromosomes [76]. In an equilibrium state, the continuous entry of rcDNA acts as a source of genome templates. Similarly, it was shown that efficient establishment and mitotic stability of the episomal gene therapy vector pEPI-1 requires a nuclear localization within transcriptionally competent chromatin domains. On the other hand, its association with heterochromatin domains is correlated with its loss after a few cell cycles [77]. Hence, an approach targeting cccDNA for CpG hypermethylation could thus lead to a breakdown of the equilibrium state, leading to efficient viral silencing and elimination.

In both, occult HBV infection and in HCC, this equilibrium can be lost—probably by new mutations, downregulation of HBx, or cellular changes related with malignancy—and high levels of DNA methylation

and probably changes in histone marks can occur in the HBV episome [78]. Those changes can downregulate viral mRNA expression, leading to low viral protein and particle production [73]—a desirable aim for novel epigenetic treatment approaches.

Epigenome plasticity of the host genome in HBV-infected hepatocytes—from bench to bedside

Reprogramming of host hepatocytes for virus particle production requires that an infection with HBV leads to alterations in the expression profiles of selected genes. Obligatorily, the deregulation of gene expression is a phenotypic consequence of epigenome plasticity modulated by HBV. However, analyses of putative HBV-induced promoter DNA methylation patterns in various models or human specimens yielded conflicting results [31,79–81]. Only a few studies, which focus on a limited number of selected genes, are available to date describing HBV-induced changes in the epigenome of hepatocytes.

These epigenetic alterations were mostly observed in HCC samples or hepatoma cell lines representing advanced stages of malignant transformation. Deregulated genes include CDKN2A, GSTP1, CDKN1A, and several others [82–85], whereby these genes were reportedly downregulated by promoter CpG island hypermethylation in HCC. Similarly, another study reported hypermethylation of the CDKN2A promoter associated with in preneoplastic lesions, which was interpreted as that CDKN2A hypermethylation could be an early event during HCC development [86]. More recently obtained data, however, do not support this conclusion, as DNA methylation pattern differences at any of the same sites examined could not be detected in earlier stages of malignant transformation [79]. Notably, the latter study examined mouse homologous sites for CDKN2A and GSTP1 that were mentioned in the respective literature. Moreover, no CDKN2A hypermethylaion was seen; its DNA methylation level rather resembled that of hypomethylated GAPDH. Two sites examined in GSTP1 reached intermediate DNA methylation levels, when compared to a hypermethylated site in TSH2B. Here, in contrast to all relevant studies cited utilizing techniques indiscriminative for DNA methylation or hydroxymethylation, specific antibodies against either 5meC or 5hmeC were used for immunoprecipitation. Interestingly, with respect to the hydroxymethylated control gene SF1, 5hmeC enrichment at the same site in CDKN2A was observed, which was found hypomethylated when interrogated by 5meC antibody immunoprecipitation. Additionally, several other sites were discovered, at which 5meC levels were low or moderately elevated, but at the same time 5hmeC was enriched (sites in CDH13, CDKN1A, MCL1, CDKN2A, and GSTP1). This might explain some of the discrepancies described above, as a lack of discriminative techniques for 5meC and 5hmeC in the previous studies might have led to biased quantification of actual DNA methylation. Furthermore, the few studies available mostly focused on already transformed hepatocytes. Taking this into account, earlier results and the results from Jenke and coworkers might not be mutually exclusive, but rather represent different stages in the course of malignant transformation with DNA hypermethylation not being an initial but rather a late event for HBV-induced gene repression (e.g., CDKN2A). These discrepancies highlight the need for novel approaches, in order to assess the plasticity of the epigenome landscape in hepatocytes infected with HBV, which will help to understand how hepatocytes become reprogrammed and how this might contribute to the chronic course of the disease.

Future hepatitis B treatment options

Epigenetic drugs

Hepatitis B virus-induced epigenetic alterations represent novel therapeutic targets for the prevention of HBV-associated HCC development and progression. DNA methylation and post-translational histone modifications are the most studied epigenetic marks altered by viral infections [87] that can be medically modified using so-called epidrugs. To date, malignancy remains to be the pivotal domain for the clinical implication of epidrug treatments in humans [88]. Data on epidrug use in HBV are still limited to cell culture and a few *ex vivo* investigations. An overview of the studies utilizing epidrugs in HBV infection models is outlined in Table 2.

Given the wide heterogeneity of HBV-induced epigenetic host cell nuclei alterations and the multistep cascade ultimately resulting in hepatocarcinogenesis, current knowledge regarding epidrug use for HBV can only be regarded as a spotlight in the dark. Nevertheless, some promising observations have been made in cell culture and mouse model experiments. Several HDAC inhibitors have been shown to decrease cell proliferation [89], increase apoptosis [90], and to decrease both lipogenesis [91] and gluconeogenesis [92]. These effects could be beneficial for anti-HCC treatment in HBV-positive patients. 5-Azacytidine was

Table 2. Biological effects of epidrugs on host cells and HBV in *in vitro* and *in vivo* (HBV-)HCC models

Epidrug/ Compound	Target	Study	Model system	Host-related effects	HBV-related effects	Conclusion
5-Azacytidine (decitabine)	DNA	[162,163]	HB611 cell line, 14p3HB transgenic mice	–	• HBV DNA methylation ↓ • HBsAg production ↑	5-Azacytidine can be used to activate silenced hypermethylated (trans-) genes
		[93,94]	9 HBV positive HCC cell lines*, HBV-HCC patients' liver biopsies	• host PCDH10 and GSTP1 promoter methylation ↓ → restoration of PCDH10 and GSTP1 mRNA expression	–	
5-AZA-CdR, (decitabine) + TSA	DNA, HDAC	[93,164]	Huh7, HepG2; (effect not shown in 7721 cells)	• rescue of HBV-HCC-induced ↓ in PADI4 • effect not shown when treated with 5-Aza-CdR or TSA alone	–	Reversal of PADI4-associated global hypomethylation in HBV-HCC
Nicotinamide	Sirt1	[165]	HBx transgenic mice	• Sirt1 ↓ • Β-catenin ↑ • oxidation-induced apoptosis ↑	–	HBx-induced Sirt1-sequestration as a potential cause of anticancer drug resistance
Resveratrol	Sirt1	[91]	HBx transgenic mice	• antioxidant activity ↓ • hepatocyte proliferation ↑ • lipogenesis ↓ • hepatocarcinogenesis ↓	–	Potential chemopreventive agent for HBV-associated HCC
		[165,166]		• Sirt1 ↑ • Β-catenin ↓ • JNK phosphorylation ↓ • oxidative stress-induced apoptosis ↓		HBx-induced Sirt1-sequestration as a potential cause of anticancer drug resistance
		[167]	BALB/c nude mice bearing HepG2 cells (HBV negative)	• HCC growth ↓ • NF-κB ↓ • VEGF ↓		Resveratrol may prevent and slow down the progression of HCC
		[168–170]	HepG2	• apoptosis ↑, p53 ↑ p21 ↑, cell cycle arrest in G1 phase • Reactive oxygen species ↓ • VEGF ↓		
		[171]	HuH7	• JNK1/2 ↓, SP-1 DNA-binding activities ↓ • urokinase-type plasminogen activator ↓ • cell migration and invasion ↓		
Romidepsin	HDAC	[68]	Clinical study (peripheral t-cell lymphoma)	-	• reactivation of HBV infection	Possible promotion of viral replication
SAHA (vorinostat)	HDAC	[90]	HepG2.2.15	• cell proliferation ↓ • apoptosis ↑	• replication ↑	Potential cautious use for HCC treatment
		[89]	HuH7, WRL-68	• cell proliferation ↓ • nuclear/cytoplasmic ratio ↓ • proliferating cell nuclear antigen expression ↓	–	Potential chemoprevention for chronic HBV

Table 2. (Continued).

Epidrug/ Compound	Target	Study	Model system	Host-related effects	HBV-related effects	Conclusion
				• pre-S2 mutant-induced oncogenic phenotype ↓		patients at high risk for HCC
Curcumin	HDAC p300	[172–177]	Huh7, Hep3B, HepG2, SK-Hep-1, QGY-7703, HA22T/VGH Bel7402, SGC7901, SNU449 (HBV negative) cells	• cell cycle arrest in G2/M • apoptosis ↑ • telomerase activity ↑ • HCC nuclear and mitochondrial DNA damage	–	Curcumin as a host targeted therapy for HBV infection to complement virus-specific therapies and to hamper HCC development
		[178]	HEP3B, SK-Hep-1 and SNU449 cell lines;human HCC and normal hepatocytes; nude mouse xenograft injected with SK-Hep-1 cells	• Notch1 signaling ↓ • PARP ↑ • cyclin D1 ↓ • p21 ↑ • HCC tumor growth *in vivo* ↓		
		[92]	HepG2, HepG2.2.15, and HEK293 cells	• PGC-1a downregulation • gluconeogenesis ↓	• HBV replication ↓	
		[174, 179–185]	Various (HBV negative) *in vivo* HCC mouse models§	• tumor incidence and growth ↓ • apoptosis ↑ • telomerase expression ↓ • tumor angioneogenesis ↓ • intrahepatic metastases ↓	–	

shown to restore tumor suppressor gene expression by means of DNA hypomethylation in the gene promotor region of genes that had previously become hypermethylated under the influence of HBV in several cell culture experiments [93,94].

However, the problem of tissue-specific drug delivery and systemic off-target effects together with genome-wide effects substantially limit the clinical implication of epidrugs in HBV patients especially when other treatment options still exist. Thus, further research is needed to elucidate the favorable effects and biological drawbacks of anti-HBV epidrug treatment regimens *in vivo* and *in vitro*.

Future hepatitis B treatment options

Targeting the cccDNA by nucleases

In order to more effectively control HBV infection, new approaches are currently being developed including the targeting of the viral receptor (NTCP) [95], the viral RNase H activity of the P protein [96], or capsid formation. Despite progress in this area to reduce the viral load to almost undetectable levels, the viral genome remains in the infected cells, able to reactivate when conditions allow [97,98]. Promising new options against HBV infection are provided by techniques allowing to directly target the cccDNA, the source of viral protein production. For such DNA targeting, several systems are available which include zinc finger proteins, transcription activator-like effectors (TALEs), and Type II bacterial clustered regularly interspaced short palindromic repeat (CRISPR)/ CRISPR-associated (Cas) system [99].

The last two decades, zinc finger proteins (ZFs) have been successfully re-engineered for targeting DNA. ZFs represent the most common class of transcription factors in the human body, characterized by the coordination of one or more zinc ions to stabilize the protein folding. ZFs generally consist of an array of fingers where each finger is a modular protein of ~30 amino acids which recognize three base pairs (bps). Computational biology approaches can be used to determine the appropriate amino acid sequence to be grafted into the finger's DNA recognition site in order to switch recognition sites and to bind to almost any three bps of interest. [100–102] This way, new proteins can be designed to bind a given stretch of 18 bps, which by mathematics refers to a specific sequence in the human genome. Upon fusion of nucleases to these engineered ZFs, double-strand cleavage of the target DNA is achieved. The cellular repair machinery will

subsequently try to repair this damage by nonhomologous end joining, inducing insertions/deletions/mutations in the target sites. Such repair thus alters the ORFs and prevents the expression of normal proteins, as has also been demonstrated for HBV [99,100] (Table 3).

Applications of genome editing are numerous, and importantly, engineered ZFs have been tested in clinical trials without signs of adverse effects (www.sangamo.com). Despite such reported successes by designer ZFs, or the more recently introduced TALEs [103,104], every genomic position to be targeted required a cumbersome project to produce and validate the fusion proteins. The recent introduction of the CRISPR/Cas systems facilitated such gene targeting efforts. Originally being a bacterial immune system against foreign DNA, the CRISPR/Cas system is currently rapidly developing as a biotech tool. It represents a cheap and versatile way of DNA recognition via RNA–DNA complementarity [105,106]. Short RNA molecules (referred to as single-guide RNAs, sgRNA) are designed to bind the genomic locus of interest on one hand and the Cas9 protein on the other hand. Cas9 is the effector protein of the type II CRISPR/Cas system, which cleaves the dsDNA it recognizes. Several cccDNA interference strategies have taken advantage of this flexible DNA targeting tool (Table 3).

Intriguingly, the therapeutic potency of disrupting of the viral genome utilizing CRISPR/Cas has been demonstrated as the expression of viral mRNA and proteins could effectively be reduced *in vitro* (Table 3). Importantly, animal models validated that targeting HBV indeed results in reduced viral mRNA and protein expression, a decrease in nuclear cccDNA abundance, and blood stream virion presence. Altogether, these targeted nucleases open novel avenues for effective treatment against the infection [107–109]. Despite these promising data, the possibility of undesired off-targeting effects must be taken into account [110–112], whenever nuclease-based approaches are used. In this sense, off-target binding might affect the host genome at nonintended locations causing unwanted effects on the cell.

Table 3. List of works targeting the HBV genome.

Author/Year	DNA-binding domain	Effector domain	Targets	Reported effect
Cradick *et al.* 2010 [186]	Zinc finger	Nuclease	5′ end ORF Core	Downregulation of mRNA and Indel induction
Weber *et al.* 2014 [187]	Zinc finger	Nuclease	RNaseH, 5′ end ORF X, 3′ end ORF Core	Decreased levels of cccDNA and Indel induction
Bloom *et al.* 2013 [103]	TALE	Nuclease	Surface, ORF P (RNaseH), 3′ end ORF Core	Downregulation of mRNA, decreased levels of cccDNA and Indel induction
Chen *et al.* 2014 [104]	TALE	Nuclease	X promoter, 5′ end ORF Core	Downregulation of mRNA and decreased levels of cccDNA
Lin *et al.* 2014 [108]	CRISPR/Cas	Nuclease	ORF Core, ORF e, ORF HBs (L, M, and S), ORF P (RH), ORF X	mRNA and protein downregulation
Seeger and Sohn 2014 [188]	CRISPR/Cas	Nuclease	EnhII, preC	Protein downregulation, Indel induction
Dong *et al.* 2015 [107]	CRISPR/Cas	Nuclease	5′ end ORF X, EnhII, 3′ end ORF Core, 5′ end ORF P	Protein downregulation, decreased levels of cccDNA, Indel induction
Liu *et al.* 2015 [189]	CRISPR/Cas	Nuclease	ORFS, ORF Core, and ORF X	Protein downregulation, decreased levels of cccDNA
Ramanan *et al.* 2015 [109]	CRISPR/Cas	Nuclease	ORF Core, ORF e, ORF X, and ORF P (RNaseH)	Protein downregulation, decreased levels of cccDNA
Karimova *et al.* 2015 [190]	CRISPR/Cas9	Nickase	ORF X, ORF HBs (S) episomal and integrated	Downregulation of mRNA, decreased levels of cccDNA and Indel induction
Wang *et al.* 2015 [191]	CRISPR/Cas9	Nuclease	ORF X, ORF Core, ORF HBs (S and M), ORF P (RT)	Downregulation of mRNA and Indel induction
Kennedy *et al.* 2015 [192,193]	CRISPR/Cas9	Nuclease	ORF P (RT), surface and Core	Decreased levels of cccDNA and Indel induction
Zhu *et al.* 2016 [194]	CRISPR/Cas9	Nuclease	5′ end ORF S, 3′ end ORF X	Downregulation of mRNA and proteins and Indel induction
Xirong *et al.* 2014 [118]	Zinc finger	Dnmt3a	HBx promoter	Downregulation of mRNA, decreased levels of cccDNA
Zhao *et al.* 2013 [119]	Zinc finger	KRAB	ORF X	mRNA and protein downregulation

An additional unintended consequence of gene targeting, which must be considered when using nucleases in an HBV model, is that double-strand breaks of the viral episome might increase the integration events of the viral genome in the host genome [113]. In this regard, Hepadnaviruses, which do not carry any integration machinery so their integration is dependent on double-strand breaks, act along with the cell repair machinery to integrate viral genomes in fragile sites of the host cell genome [113–115]. In order to overcome these possible off-targeting effect and the likely increase of potentially disturbing integration events, new approaches using obligate heterodimeric nucleases coupled to two different targeting domains [116,117] and the targeting of non-nuclease effector domains, like epigenetic modulators (DNMTs, histone modifiers) [118] or transcription downregulators (KRAB) [119] are of considerable interest.

Future hepatitis B treatment options

Epigenetic editing of the cccDNA

Taking advantage of DNA targeting systems, less invasive approaches have been suggested, including epigenetic editing where the nuclease activity is replaced by an epigenetic modifier [120–122] (Fig. 5).

We and others extensively published on the potential of engineering ZFs to target genes for induction or repression of gene expression. Actually, the very first indication on the feasibility of epigenetic editing to modulate gene expression was derived using the ZF-platform to write histone methylation (H3K9) already back in 2002 [123]. Only in 2013 was this followed up with a demonstration of effective silencing of the oncogene *her2/neu* by writing H3K9me [124]. Simultaneously, the first examples of writing DNA methylation on expressed genes to successfully inhibit gene expression were published [125,126]. Importantly, it was shown that even epigenetically silenced heterochromatin genes can be accessed for re-expression by engineered ZF-fusions [127,128]. Furthermore, the targeting of TET enzymes to hypermethylated CpGs using engineered ZFs lowered the methylation level resulting in gene re-expression [129,130]. The power of epigenetic editing has been confirmed by many researchers who joined the field [131,132] using TALEs, a plant pathogen-based designer DNA-binding platform. Excitingly, CRISPR/Cas can also be repurposed for epigenetic editing by blocking the endonuclease activity of Cas9 by introducing two mutations in the catalytic domain, which results in a catalytically dead Cas9 (dCas9). Fusions of dCas9 with epigenetic effector domains induced rewriting of epigenetic marks at multiple given locations in the genome [133].

Experimental evidence about the direct causal effect of DNA methylation on HBV functioning is scarce. Initially, Vivekanandan *et al.* [134] reported methylation-dependent downregulation of viral mRNA expression. The only actual epigenetic editing study targeting HBV so far was reported by Xirong *et al.* [118]. In this report, the authors engineered a zinc finger protein targeting the HBx promoter (overlapping CGI 1 and enhancer 1). Upon fusion of this protein to the C-terminal catalytic domain of Dnmt3a (Dnmt3aC) both *in vitro* and *in vivo* methylation of CpG sites upstream of the targeting region could be demonstrated. This targeted methylation approach did not only downregulate the expression of viral mRNAs and proteins (Core, HBsAg and HBx) but also led to a substantial decrease in viral particle production [118].

Although long-term efficacy of HBV-targeting epigenetic editors have not yet been reported, sustained epigenetic effects in cancer cell lines reveal the potential toward permanent gene expression modulation by epigenetic editing strategies [135,136]. As epigenetic maintenance mechanisms most likely apply to HBV episomes present in the cell nucleus, epigenetic editing provides a promising way to actual attack the virus directly at its source. In this regard, the intended sustained inhibition of viral transcription potentially will contribute to the ‘dilution’ of the cccDNA with subsequent cellular divisions or at least will result in silencing of the viral genome until cell death.

Silencing of HBV expression could have an effect on the spread of the disease and the HCC development of the infected patients. Additionally, epigenetic editing can be used as a useful research tool to understand the role of the epigenetic modifications in HBV transcription control, which can also be extrapolated to many other DNA viruses. If indeed permanent reprogramming of the viral genome without unspecific effects can be achieved, novel avenues are opened to start designing an actual cure for HBV-infected patients.

In summary, epigenetic editing represents a valuable methodology to remove or to introduce epigenetic modifications in the viral genome. This approach thus is expected to shed light on yet unknown aspects regarding transcriptional control of the HBV genome. Importantly, it likely provides alternative tools for controlling the viral transcription and subsequent replication in clinical cases [133]. As established for host chromatin, where DNA methylation and/or histone PTM can be written or erased, we here reviewed that the HBV episome is indeed susceptible of being epigenetically modified [118]. Epigenetic editing tools

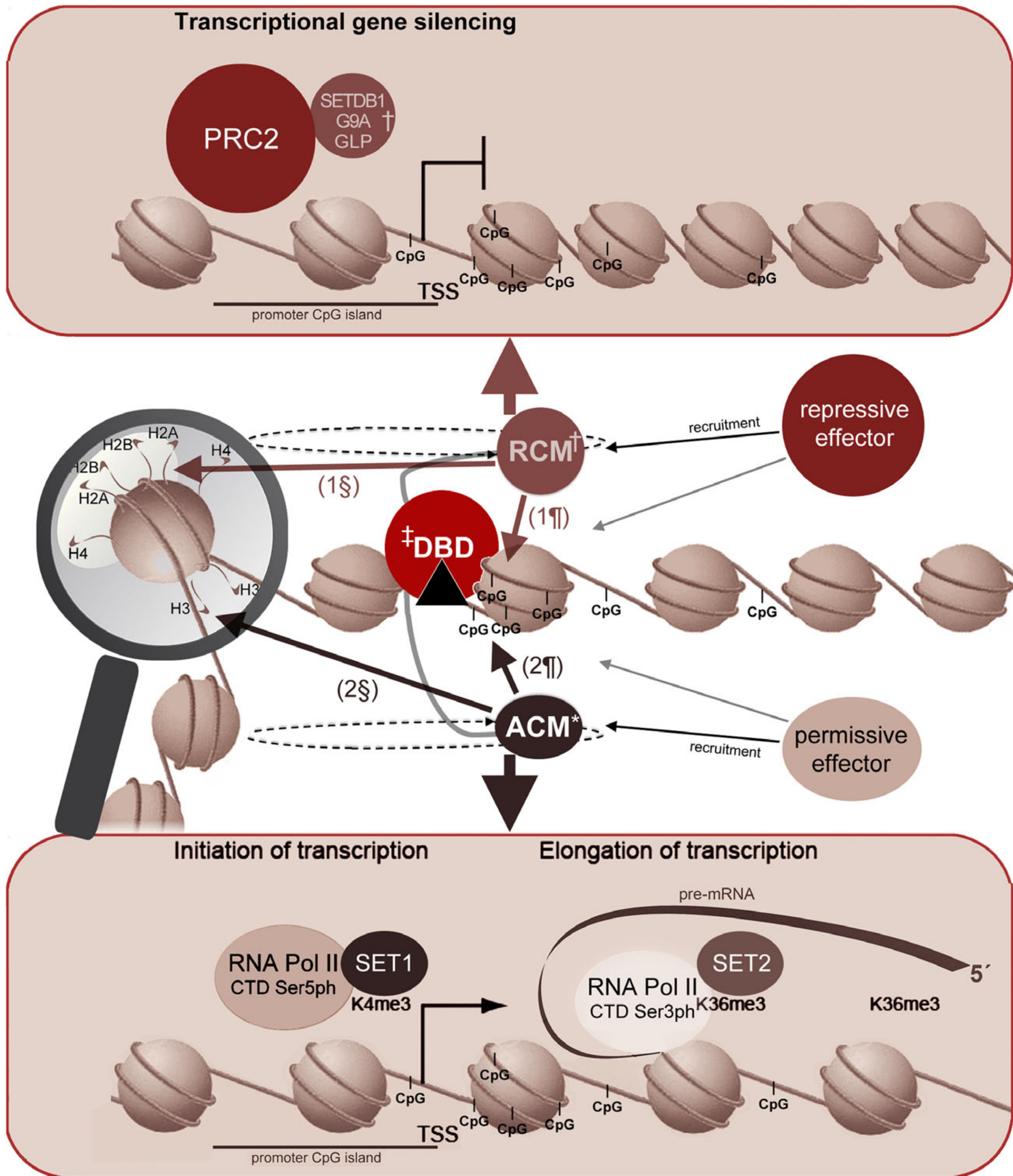

Fig. 5. Epigenetic editing. Targeted rewriting of epigenetic marks: the illustration shows the general concept of targeting epigenetic enzymes, which can modify either DNA methylation or histone modifications. Targeted sequences are recognized by a DNA-binding domain (ʃDBD) fused to an epigenetic enzyme, thus inducing changes in the local epigenetic composition (histone modifications [1a, 2a] or DNA methylation [1b, 2b]), thereby causing gene repression (top, †RCM, repressing chromatin modifier) or activation (bottom, *ACM, activating chromatin modifier.

thus provide unique instruments to elucidate and exploit hidden aspects of HBV biology. For example, the exact role played by each CpG island in HBV transcription control or the role of the viral epigenome in the outcome of the infection and development of liver diseases as cirrhosis and HCC is still largely unknown, but finding answers will offer new targets for therapeutic interventions [71,72].

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Conflict of interest

All authors declare that there is no conflict of interest regarding the publication of this paper.

Author Contributions

JCR and KOH wrote sections 1-4 and created the tables. JCR and MGR wrote the section on epigenetic editing. JP contributed to sections 1 and 2 and created the figures. KOH wrote the section on epidrugs. MCN contributed to sections 1 and 2. All authors critically reviewed the manuscript.

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