



Review

Targeting hepatitis B virus cccDNA using CRISPR/Cas9



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ABSTRACT

Despite the existence of an excellent prophylactic vaccine and the development of highly effective inhibitors of the viral polymerase, chronic hepatitis B virus (HBV) infection remains a major source of morbidity and mortality, especially in Africa and Asia. A significant problem is that, while polymerase inhibitors can effectively prevent the production of viral genomic DNA from pre-genomic RNA transcripts, they do not prevent the transcription and translation of viral mRNAs from the covalently closed circular DNA (cccDNA) templates present in the nuclei of infected cells. Moreover, because these cccDNAs are highly stable, chronic HBV infections are only very rarely cured by the use of polymerase inhibitors and these drugs clearly cannot entirely prevent the subsequent development of HBV-related morbidities such as cirrhosis and hepatocellular carcinoma. As a result, there has been considerable interest in the possibility of developing treatment approaches that directly target cccDNA for elimination. Here, we discuss recent publications that analyze the ability of the bacterial CRISPR/Cas DNA editing machinery to be repurposed as a tool for the specific cleavage and destruction of HBV cccDNAs in the nuclei of infected cells and consider which steps will be necessary to make CRISPR/Cas targeting of HBV DNA a clinically feasible approach to the treatment of chronic infections in humans. This article forms part of a symposium in *Antiviral Research* on “An unfinished story: from the discovery of the Australia antigen to the development of new curative therapies for hepatitis B.”

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1. Introduction

Although nucleoside reverse transcriptase inhibitors (NRTIs) can effectively inhibit the hepatitis B virus (HBV) polymerase protein, resulting in a dramatic drop in circulating HBV DNA levels, NRTIs do not affect the pre-existing pool of covalently closed circular HBV DNA molecules (cccDNAs), which are very stable

and continue to produce substantial levels of HBV mRNAs and proteins (Gish et al., 2015; Guo and Guo, 2015). As a result, NRTI treatment, while able to repress viral replication, only rarely leads to a complete cure and patients on long-term NRTI therapy still have a greatly increased risk of severe disease related to cirrhosis or hepatocellular carcinoma (Block et al., 2015). This indicates that the complete cure of HBV disease will likely require the development of treatments that lead to the elimination of HBV cccDNA from the liver, leading to the concomitant clearance of HBV proteins,

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such as HBV surface antigen (HBsAg), from the circulation (Gish et al., 2015). One potential approach, that has recently received extensive interest, is the potential use of the bacterial CRISPR/Cas DNA editing machinery to specifically cleave and destroy HBV cccDNA in patients. Here, we discuss how CRISPR/Cas systems function, what has been achieved so far using this system to block HBV replication, and finally potential future approaches to the effective delivery of CRISPR/Cas to HBV-infected hepatocytes *in vivo*.

2. Molecular biology of CRISPR/Cas

CRISPR (clustered regularly interspaced short palindromic repeats) loci, which are found in a wide range of bacteria, are transcribed to generate targeting RNAs specific for a range of different DNA bacteriophages (Barrangou and Marraffini, 2014; Hsu et al., 2014). In bacteria that express a type II CRISPR/Cas system, these phage-derived sequences are transcribed along with sequences from the adjacent constant region to give a CRISPR RNA (crRNA) which forms a complex with the invariant trans-activating RNA (tracrRNA), using sequence complementarity between the tracrRNA and the invariant part of the crRNA. This heterodimer is then bound by the effector protein of the type II CRISPR/Cas systems, called Cas9. Cas9 has the ability to directly recognize a short DNA sequence, 5'-NGG-3' for the commonly used *Streptococcus pyogenes* (Spy) Cas9 protein, called the protospacer adjacent motif (PAM) (Barrangou and Marraffini, 2014; Cong et al., 2013; Hsu et al., 2014; Mali et al., 2013). The Cas9 protein scans a target genome for the PAM sequence and then binds and queries the DNA for the level of sequence complementarity to the variable part of the crRNA. If extensive complementarity is detected, the Cas9 protein directly cleaves both strands of the target phage DNA 3 bp 5' to the PAM, using two distinct protein domains: the Cas9 RuvC-like domain cleaves the non-complementary strand, while the Cas9 HNH nuclease domain cleaves the complementary strand (Gasiunas et al., 2012; Jinek et al., 2012). This dsDNA break then induces the degradation of the phage DNA genome and blocks infection (Garneau et al., 2010; Gasiunas et al., 2012; Sapranaukas et al., 2011).

A key step forward in making the Spy Cas9 system more user-friendly for genetic engineering in human cells was the demonstration that the crRNA and tracrRNA could be linked by an artificial loop sequence to generate a fully functional small guide RNA (sgRNA) ~100 nt in length (Cong et al., 2013; Mali et al., 2013). Further work, including mutational analysis of DNA targets, has revealed that sequence specificity for Spy Cas9 relies both on the PAM and on full complementarity to the 3' ~13 nt of the ~20 nt variable region of the sgRNA, with more 5' sequences making only a minor contribution. Spy Cas9 therefore has an ~15 bp (13 bp in the guide and 2 bp in the PAM) sequence specificity which, while high, is generally not sufficient to entirely avoid a small number of potential off-target cleavage sites in the large human genome. Nevertheless, this is a high level of specificity and a small number of off-targets in non-transcribed regions of the human genome may not be highly problematic. Moreover, this concern can be dealt with by mutating the Cas9 protein to inactivate one of the two independent HNH and RuvC nuclease sites, to generate a so-called "nickase" (Cong et al., 2013; Ran et al., 2013). It is then possible to target two nickase Cas9s to two closely proximal (<20 bp) sites on the two strands of the DNA target. Once nicked on both strands, the DNA will fall apart to give a staggered dsDNA break, analogous to what is obtained upon cleavage at a single recognition sequence using wild-type Cas9, except that the DNA target specificity is now ~30 bp, amply sufficient to ensure complete specificity even in a large genome, such as that present in human cells. This dsRNA

Table 1

Overview of published studies using CRISPR/Cas9 to inactivate HBV DNA. This table provides a brief overview of the experimental systems used, the major HBV DNA targets for Cas9 cleavage reported, the HBV strain analyzed and the HBV DNAs quantified by PCR analysis.

Manuscript	HBV system	Major targets	Genotype	DNA measured
<i>In vitro studies</i>				
Seeger and Sohn (2014)	HepAD38 HBV DNA model and <i>de novo</i> infection of HepG2 cells	X and Core ORFs	Ayw Strain	Total HBV DNA
Kennedy et al. (2015)	HepAD38 tet system of chronic infection and <i>de novo</i> infection of HepaRG cells	Core, Surface and Polymerase ORFs	Ayw Strain	Total HBV and cccDNA
Ramanan et al. (2015)	Transfection of HBV expression plasmid, <i>de novo</i> infection of HepG2 cells, and HepG2.2.15 HBV replication model	Core, Polymerase, and X ORFs (Enhancer overlap)	Ayw Strain	Total HBV and cccDNA
Dong et al. (2015), Wang et al. (2015), Liu et al. (2015), Lin et al. (2014), Zhen et al. (2015)	Transfection of HBV expression plasmids, and <i>in vitro</i> HBV cccDNA replication models (HepG2.2.15, HepG2-H1.3, HepAD38)	X, Core, Polymerase, and Surface ORFs	Genotype A, B, C, D	Total HBV and cccDNA
Karimova et al. (2015) (Nickase)	HepG2.2.15, HepG2-H1.3 HBV replication models, and <i>de novo</i> infection of HepG2 cells	Surface and X ORFs	Ayw strain, considered cross genotype conservation	Total HBV DNA
<i>In vivo studies</i>				
Lin et al. (2014), Liu et al. (2015), Ramanan et al. (2015), Dong et al. (2015), Zhen et al. (2015)	Hydrodynamic tail vein injection of HBV expression plasmid	Polymerase (+Enhancer), X, Surface, and Core ORFs	Ayw and Adr strains	Total HBV DNA

break is repaired by the error-prone non-homologous end joining (NHEJ) pathway, resulting either in perfect repair, in which case the target is again cleaved by Cas9, or resulting in an indel, which blocks further cleavage and introduces a mutation that often disrupts the underlying open reading frame (ORF).

3. Using CRISPR/Cas to target HBV cccDNA

The promise of CRISPR/Cas as a tool for the cleavage and elimination, or at least inactivation, of HBV cccDNAs *in vivo* has prompted a considerable number of studies, all published over the last year, that provide a clear proof of principle that this approach indeed has the potential to actually result in a full cure of chronic HBV infections (Table 1). A number of related

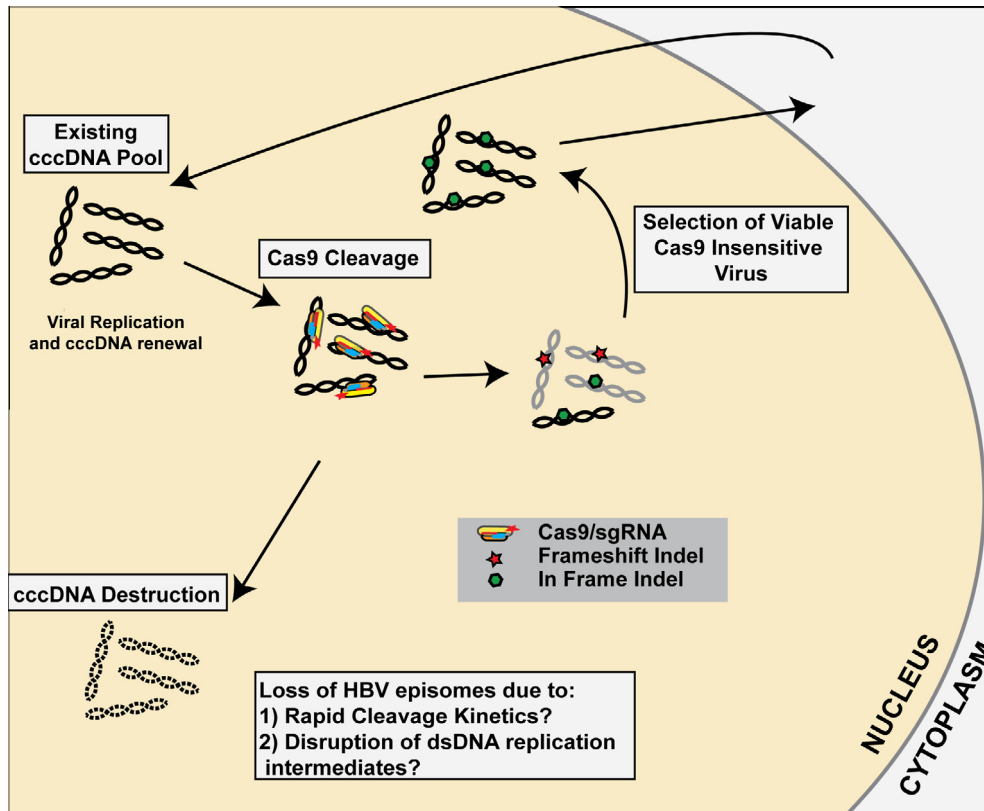


Fig. 1. Overview of the fate of Cas9-cleaved HBV cccDNAs. Once an HBV-specific Cas9/sgRNA combination is expressed in an HBV-infected hepatocyte, site-specific cleavage of the cccDNA occurs. This induces the elimination of the majority of the cccDNA molecules by mechanisms that remain unclear. Alternatively, cleaved HBV cccDNA can be repaired by NHEJ. Molecules that are restored to the wild-type sequence are again cleaved, resulting in the accumulation of mutated HBV DNA molecules, generally bearing small indels, that are refractory to further cleavage due to the mismatch with the sgRNA. If the indel causes a frame-shift, the mutated ORF is inactivated. Conversely, indels that are in-frame can either result in a functional ORF, if the targeted region is not essential, or a defective ORF, if it is. In either case, pre-genomic RNA transcripts, as well as other viral mRNAs, will still be made and exported to the cytoplasm where the pre-genomic RNA can be used as a template to generate defective viral DNA molecules.

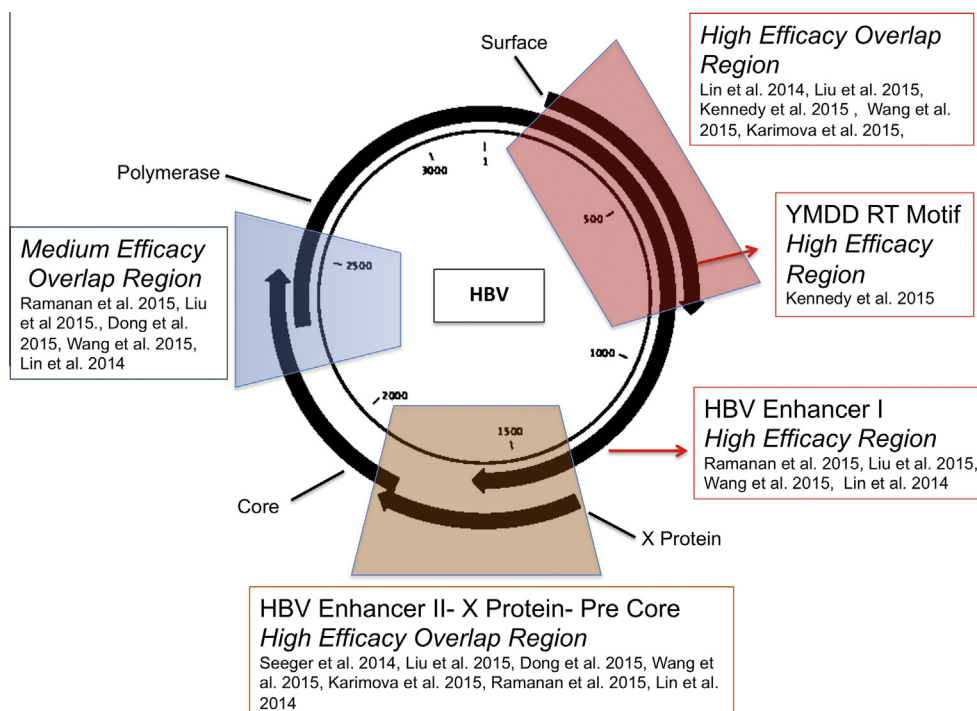


Fig. 2. Location of optimal target sites in the HBV genome. This figure summarizes data, derived from the nine relevant publications listed in Table 1, that identify highly active, effective targets for Cas9/sgRNA cleavage and inactivation of the HBV genome. In general, these fall into regions of overlap between two viral ORFs and hence have the potential to inactivate two viral genes simultaneously.

experimental systems that address aspects of the HBV replication cycle have been used to demonstrate CRISPR/Cas cleavage of the HBV genome and a resultant drop in HBV DNA levels and/or antigen production. These include:

- (A) The HepAD38 system, in which an integrated HBV DNA genome is under the control of a tet-regulated promoter, which is repressed by the presence of tetracycline. Removal of tetracycline induces HBV transcription, the production of HBV cccDNA and the release of infectious HBV (Kennedy et al., 2015).
- (B) *De novo* infection of hepatoma cell lines, such as HepG2 or HepaRG cells, with HBV derived from HepAD38 cells (Dong et al., 2015; Karimova et al., 2015; Kennedy et al., 2015; Lin et al., 2014; Liu et al., 2015; Ramanan et al., 2015; Seeger and Sohn, 2014; Wang et al., 2015; Zhen et al., 2015).
- (C) Transfection of HBV expression plasmids into hepatoma cell lines, such as Huh7 (Dong et al., 2015; Karimova et al., 2015; Lin et al., 2014; Liu et al., 2015; Ramanan et al., 2015; Wang et al., 2015; Zhen et al., 2015).
- (D) Hydrodynamic injection of mice with HBV DNA, as well as CRISPR/Cas expression plasmids, resulting in *in vivo* co-transfection of the mouse liver (Dong et al., 2015; Lin et al., 2014; Liu et al., 2015; Ramanan et al., 2015; Zhen et al., 2015).

All these model systems detected a substantial decline in the level of total HBV DNA, as well as cccDNA where measured, and also detected a drop in the secretion of HBV antigens, such as HBsAg, into the supernatant media. An overall summary of what has been observed is provided in Fig. 1. Briefly, the existing cccDNA pool, generally ~5–50 cccDNA copies per nucleus, can be efficiently cleaved by Cas9 resulting in one of two fates. A percentage of cccDNA appears to be totally lost, possibly due to rapid cleavage efficiency resulting in a high percentage of linear DNAs that is not repaired but rather destroyed, perhaps after escaping into the cytoplasm during mitosis, or lost due to cleavage of DNA replication intermediates. A second cccDNA population is repaired by NHEJ, resulting in wildtype DNAs, that can be recleaved by Cas9, and mutated cccDNAs bearing small indels at the Cas9 cleavage site. If the cleavage site is in an essential gene, frameshift mutations will be lethal and in-frame indels will also be lethal, if the site is critical for protein function such as the “YMDD” motif in the polymerase (Kennedy et al., 2015), but may remain viable if this is not the case. Regardless, mutated HBV DNAs can be transcribed to yield pre-genomic RNAs that will be exported to the cytoplasm, packaged into virions (assuming that sufficient core protein is available) and then reverse transcribed prior to release from the cell or re-import into the nucleus, where the biogenesis of the HBV cccDNA is completed with the help of cellular enzymes. Of course, if the cccDNA is defective, it will then be unable to serve as a template for the production of functional viral proteins (Fig. 1).

The ability of CRISPR/Cas to serve as an effective tool for the elimination of cccDNA therefore depends in significant part on the efficiency of cccDNA cleavage and on the mutational inactivation of all HBV mutants that arise as a result of NHEJ repair of Cas9 cleavage sites. As summarized in Fig. 2, evidence from published reports (Table 1) suggests three key regions of the HBV DNA genome as particularly favorable targets for Cas9 cleavage and mutational inactivation. One is the region of overlap between the HBV polymerase and surface Ag ORFs, the second is a region containing the HBV enhancer II element as well as the viral X protein ORF and the pre-core region, while a perhaps slightly less effective region is defined by the overlap between the HBV polymerase and Core ORFs. Each of these regions has been shown to result, when cleaved, in a marked drop in viral DNA and protein levels. It is also

worth noting that Cas9 cleavage sites that target regions of overlap between two HBV ORFs have the potential to generate frameshift mutations in both, likely resulting in an exacerbated inhibitory effect on HBV replication.

One possible approach to increasing the effectiveness of CRISPR/Cas as a tool for the elimination of viable HBV genomes is to use two sgRNAs simultaneously, resulting in the cleavage of the HBV at two sites, for example in the polymerase and X ORFs. The resultant linear DNAs, even if repaired by NHEJ, would be totally non-viable and, indeed, the use of two sgRNAs has been shown to enhance the inhibition of HBV DNA replication (Wang et al., 2015).

Finally, there has been considerable discussion of the question of whether “off-target” cleavage by CRISPR/Cas, in this case of the cell genome, could result in deleterious mutations. While off-target cleavages clearly can occur, these appear to be rare, and bioinformatic analysis of potential human target sites can certainly be used to avoid any cleavage of transcribed genes. Nevertheless, should this be a concern, it is possible to use “nickase” mutants of Cas9, as described above, together with two sgRNA targeting two closely proximal sites on the HBV DNA genome (Cong et al., 2013; Ran et al., 2015). Indeed, Karimova et al. (2015) have reported the design of sgRNA pairs targeting the HBV surface and X ORFs that were able to efficiently block HBV replications in chronically and *de novo* infected hepatoma cell lines.

4. Future directions

All the experiments reported thus far to examine the ability of CRISPR/Cas to disrupt the HBV DNA genome utilized either tissue culture models of chronic or *de novo* HBV infection or used *in vivo* transfection of the mouse liver using hydrodynamic injection. Moreover, all studies reported so far used the Spy Cas9 protein. We believe that the application of this technology in a clinical setting will require the development of viral vector systems that can effectively transduce the large majority of hepatocytes in an HBV-infected liver and express Cas9 and at least two sgRNAs in these infected cells (Kennedy and Cullen, 2015; Ran et al., 2015).

In our view, the only feasible vector modality relies on adeno-associated virus (AAV)-based vectors as these can be produced at very high titers, up to 10^{14} genome equivalents per ml. Moreover, several AAV serotypes, such as AAV8, show a marked hepatotropism and even more highly hepatotropic AAV variants have recently been described (Lisowski et al., 2014). However, AAV vectors have a strictly limited packaging capacity of ~4.8 kb of which ~0.3 kb is required to accommodate the *cis*-acting AAV inverted terminal repeats (ITRs). AAV is therefore too small to accommodate the ~4.2 kb Spy Cas9 gene together with two sgRNAs and the required transcriptional regulatory sequences. However, this problem, as we and others have discussed elsewhere, can be solved by using a smaller Cas9 gene, such as the one encoded by *Staphylococcus aureus* (Sau), which is ~3.2 kb in size. The combination of the Sau Cas9 gene together with two small polymerase III (pol III)-dependent promoters, such as ~70 bp tRNA-derived promoters (Mefferd et al., 2015), as well as two sgRNAs (~100 bp), a pol II promoter to drive Cas9 transcription (~600 bp) and a short, synthetic poly(A) addition site (~100 bp) gives a total size well below the 4.8 kb AAV packaging limit, and we have indeed now successfully constructed and packaged AAV vectors expressing the Sau Cas9 gene that are designed to simultaneously target the HBV DNA genome for cleavage at two distinct locations.

While this represents an important step forward, the key is clearly to demonstrate efficacy *in vivo* in a relevant animal model. Perhaps ideal would be the immunodeficient humanized liver mouse model, which can be readily infected with HBV to establish

a chronic HBV infection, characterized by readily detectable levels of HBV cccDNA, that closely mirrors chronic HBV infection in humans (Dandri et al., 2013, 2006). An alternative would be to use a transgenic mouse model in which the complete HBV genome is integrated into the mouse genome (Cheng et al., 2015). These mice produce infectious HBV and secrete viral antigens, such as HBsAg, into their serum (Larkin et al., 1999). Importantly, HBV expression is almost completely restricted to the liver. Either mouse model could be used to test the ability of the AAV vectors described above to effectively clear and/or mutationally inactivate HBV *in vivo*. If this goal can be achieved, then the eventual clinical application of comparable HBV-specific, AAV-delivered Cas9/sgRNA combinations must certainly be considered as a possible approach to the cure of chronic HBV infections in humans.

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